An Investigation into the Biochemical Effects of Heavy Metal Exposure on Seaweeds

by

Siobhan Ryan

Supervised by

Dr Orla O' Donovan & Dr Peter McLoughlin

Submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Submitted to Waterford Institute of Technology

October 2010

Declaration

No element of the work described in this thesis, except where otherwise acknowledged, has been previously submitted for a degree at this or any other institution. The work in this thesis has been performed entirely by the author.

Signed: _____

Date: _____

Acknowledgements

3

This study could not have been completed without the help of the following people to whom I am very grateful:

Dr. Orla O'Donovan and Dr. Peter McLoughlin for their guidance and support.

Dr. Brian Murphy and Dr. Karen Tambling for all their help and advice.

The Irish Research Council for Science, Engineering and Technology, without whose funding this research would not have taken place.

I would like to thank my friends, colleagues and fellow postgraduates for their help, encouragement and reassuring words.

Finally, I would like to express my sincere gratitude to my Family, especially my parents who were always there for me, without whom I could not have made it this far.

Abstract

An investigation into the biochemical effects of heavy metal exposure on seaweeds (*Siobhan Ryan*).

Optimised methods, developed in this novel study, were utilised throughout the research. In environmental studies of Newfoundland and Ireland, interspecies and spatial variations in the total protein content and polyphenol levels of seaweeds were demonstrated. A positive correlation between total protein and temperature was established. A downstream increase in polyphenol levels of seaweeds was observed and correlated with a downstream increase in seawater salinity. *P. lanosa* contained the highest levels of all metals. Levels of Pb^{2+}, Cr^{3+} and Cu^{2+} in seaweed were highest for sites in Waterford City. The highest proportions of Pb^{2+}, As^{3+}, Cr^{3+}, Cu^{2+}, Ni^{2+} and Al^{3+} were intracellular.

Baseline levels of total protein, extracted protein and polyphenols of four different seaweed species varied significantly. *P. lanosa* contained the highest total protein. *F. vesiculosus* and *A. nodosum* yielded the highest levels of polyphenols. Significant interspecies variations in total and intracellular metals were observed. Zn^{2+}, As^{3+} and Mn^{2+} were dominant for all species. *P. lanosa* demonstrated the best overall metal bioaccumulation potential. Regression analysis demonstrated correlations between total protein and Pb^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+}.

Seasonal variations in levels of Cd^{2+}, total protein, extracted protein and polyphenols of *P. lanosa* were observed. The highest levels of total protein were yielded in May. February demonstrated the highest extracted protein and November the lowest polyphenols. Increases in protein concentration following Cd^{2+} exposure were observed for *P. lanosa* sampled in February. Increases in polyphenol levels following Cd^{2+} exposure were observed in the November samples. Potentiometric titrations and FTIR analysis demonstrated seasonal variations in the binding potential of *P. lanosa*.

Gel filtration chromatography, HPLC analysis and SDS PAGE demonstrated changes to the molecular weights of protein derived from *P. lanosa* following heavy metal exposure.

Table of Contents

List of Figures

List of Tables

22

Chapter 1

Introduction

1.   Introduction

1.1   Seaweeds

Seaweeds are evolutionarily varied, macroscopic, multicellular, red, green and brown algae.   Rocky shores are abundantly covered with a macro-vegetation that is predominantly seaweeds (Lobban & Harrison, 1997).   Brown and red seaweeds are almost completely marine organisms (Hashim & Chu, 2004).   Green seaweeds such as *Ulva* sp which flourish throughout the year are found attached to stones and rocks (Nicholson & Brightman, 1979).

1.1.1   Classification

The science of classification of living organisms is called Taxonomy (Lee, 1986) and the largest and most comprehensive taxonomic group within plants is called the Division (Bold & Wynne, 1985; Stuessy, 1990).   Numerous traits can be used to classify algae, these include the nature of the chlorophyll(s), cell wall composition and flagellation.   All types of algae however contain chlorophyll a.   The presence of phytopigments other than chlorophyll a is a feature of a specific algal division. Difference in the reserve polymers, produced as a result of photosynthesis, is an important variable used in algal classification (Davis *et al*., 2003).   The brown colour associated with the Phaeophyta results from the presence of the xanthophyll pigment fucoxanthin.   The red colour of the Rhodophyta is caused by the presence of the pigment phycoerythrin which reflects red light and absorbs blue light. The green colour of the Chlorophyta derives from chlorophyll a and b (Hashim & Chu, 2004).

It is important to point out, however, that there have been many classification schemes employed to date.   According to Bold & Wynne (1985) and Davis *et al.* (2003), divisions include Cyanophyta, Prochlorophyta, Phaeophyceae, Chlorophyta, Charophyta, Euglenophyta, Chrysophyta, Pyrrhophyta, Cryptophyta and Rhodophyta.

Seaweeds in this study fall under the following categories: Phaeophyceae (brown algae), Chlorophyta (green algae) and Rhodophyta (red algae).

1.1.1.1 Phaeophyceae

There are about 265 genera and 1500-2000 of brown algae. Most brown species are marine algae and survive attached to rocks along sea coasts. Along North Atlantic coasts, large brown algae can grow abundantly both in the intertidal zone, where belts of *Fucus* can be found and below low water, where the rocky sea floor is frequently covered by *Lamanaria* (Van Den Hoek *et al*., 1995).

1.1.1.2 Chlorophyta

There are around 500 genera (~8000 species) in this division. Many live in freshwater but there are also several marine and terrestrial species. Green algae are particularly abundant on rocky coasts and dominate the upper part of the intertidal zone. In this area, rocks are often completely covered with green algae such as *Ulva.* This species can form thick growths on sandy shores, particularly in calm environments (Van Den Hoek *et al*., 1995).

1.1.1.3 Rhodophyta

There are 5000-5500 species of red algae, which are dispersed among 500-600 genres. Few of which occur in freshwater (~150 species). Most red algae are marine and live attached to rocks or other algae along the coast. Along West European coasts, red algae generally do not grow below a few metres beneath the low-water mark, whereas, red species have been observed at depths of 268m off the coast of the Bahamas (Van Den Hoek *et al*., 1995).

1.1.2   Ecology

Various ecological factors determine where seaweeds as a group and also particular species may be located. None of these factors act alone, however their combined effects cause a predictable and specific distribution of seaweeds. Seaweeds are affected by their adjacent ecological conditions. The environmental distribution of specific seaweeds is controlled by factors such as water temperature, salinity, nutrients, currents/wave action and light intensity (Lee, 1986).

1.1.3 Structure

The seaweed structure is the result of millions of years of evolutionary change (Lee, 1986). Seaweeds are generally more simple in structure than plants of terrestrial habitats (Martínez, 2003). Several types of seaweed assume the form of very thin threads, or filaments. These are essentially chains of cells, attached end to end like a string of beads (Lee, 1986). Macroalgae such as *Polysiphonia* are delicately branched, filamentous seaweed which have a soft texture. Other seaweeds can be sheet-like or tubular and can be one to numerous cells thick, an example of this is *Ulva* (Littler *et al.,* 1983). Seaweeds such as *Fucus* and *Laminaria* have thick cell walls and contain thick blades and branches. They can also be described as being of a leather or rubber-like texture (Lobban & Harrison, 1997). The shape assumed by the seaweeds is reflection of their biological needs. Plants growing in water do not need the ordinary roots, stems and leaves of land plants. The latter need roots for attachment and absorption of water and mineral nutrients from the soil. Seaweeds are immersed in their own water and nutrient supply and therefore can absorb the materials directly into their cells. They maintain a secure attachment to rocks by a primitive root-like device, the 'holdfast' (Lee, 1986). Land plants need a firm stem to hold itself upright and transfer the material absorbed through the roots to the leaves. Seaweed is buoyed up by water, and has no need of elaborate conducting tissue although some larger genera have a primitive conduction system. It could be a disadvantage for a seaweed to have a rigid stem, since the sometimes violent movement of the sea could break such a structure. Certain species do have a stipe, a cylindrical flexible shaft rising from the holdfast supporting the remainder of the plant (Lee, 1986). Many species of brown algae have air bladders to help keep the thallus floating near the surface, where it can get the light required for photosynthesis (Martínez, 2003). Photosynthesis in land plants is carried out in leaves. In seaweeds, photosynthesis is performed by a complex range of various types of blades, membranes and filaments. The reactions of photosynthesis are maintained extremely efficiently using resources extracted directly from the surrounding watery medium (Lee, 1986). See Figure 1.1 for a comparison of land plant and seaweed structures.
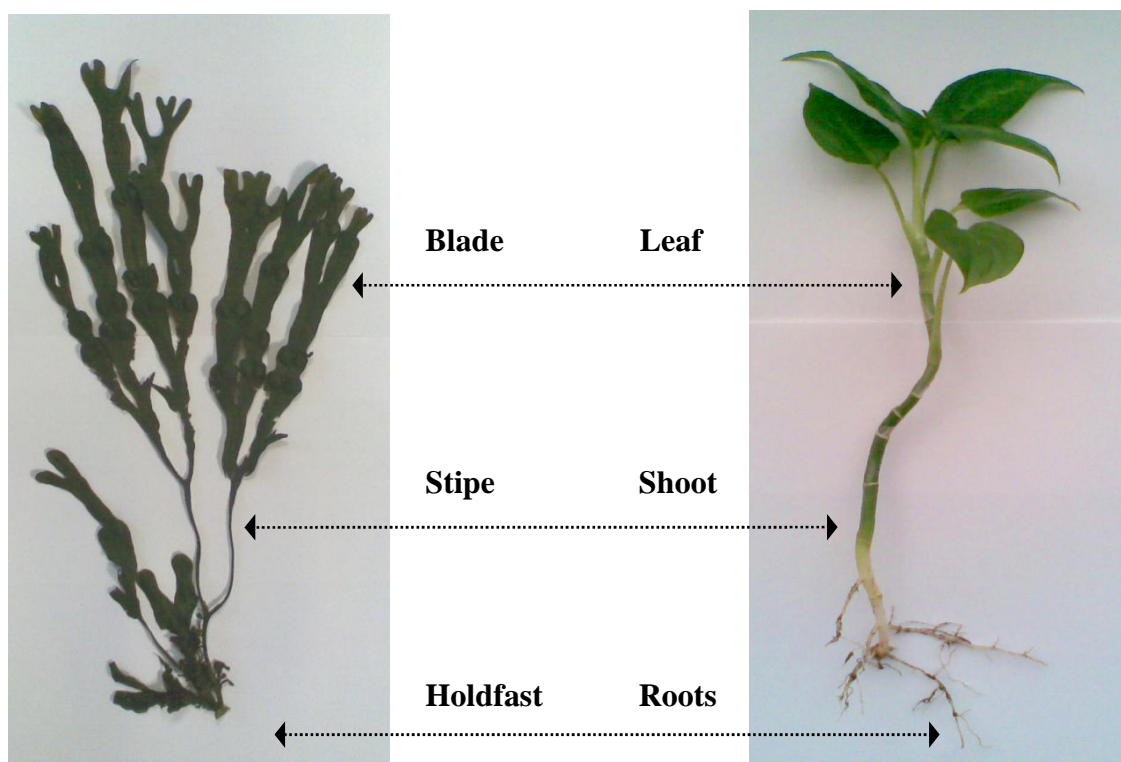
Figure 1.1. Comparison of land plant and seaweed structures.

1.1.4   Seaweed Composition

Seaweed cells are enveloped by walls and membranes and contain membrane-bound organelles. Interactions with the environment occur through these membranes and walls. Cell walls provide rigidity, they are also crucial to cell growth and development. They are also vital in mating and in the release of reproductive cells (Lobban & Harrison, 1997).

Significant variations between Phaeophyceae (brown algae) and other algal divisions such as the Chlorophyta (green algae) can be seen when comparing the storage products they utilise as well as in their cell wall chemistry. In the Phaeophyceae, laminaran is the main storage product, whereas the Rhodophyta produces and stores floridean starch. Flagella are absent in the Rhodophyta but they are found in the Chlorophyta and Phaeophyceae (Davis *et al*., 2003).

Biosorption is a term that describes the removal of heavy metals by the passive binding to non-living biomass from an aqueous solution. Biosorption by algae has largely been attributed to the cell wall where both electrostatic attraction and complexation are factors. Algal cell walls of Phaeophyceae, Rhodophyta, and many Chlorophyta are

typically composed of a fibrillar skeleton and an amorphous embedding matrix (Davis *et al*., 2003) (Figure 1.2).

Outside

Outer cell wall amorphous embedding matrix

Inner cell wall fibrillar skeleton

Cellulose Fibres

Phospholipid

Protein

Alginate & Fucoidan Matrix

Inside
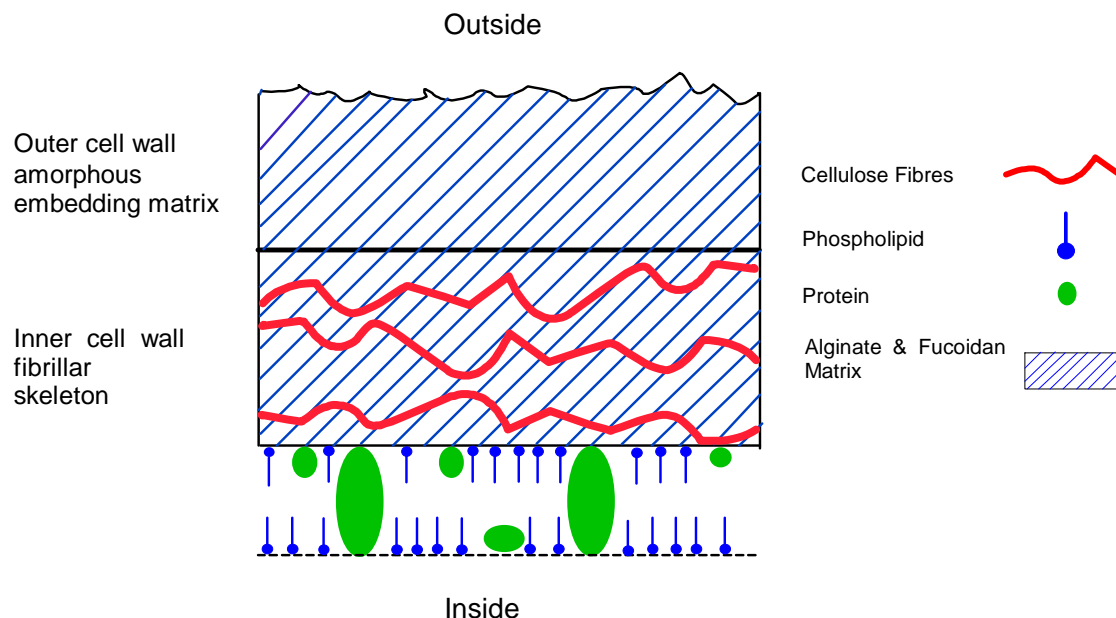
Figure 1.2. Cell wall structure of brown algae (Schiewer & Volesky, 2000).

The inner layer constructed of a microfibrillar skeleton that gives rigidity to the wall while the outer layer is an amorphous embedding matrix (Lee, 1986). The latter layer is usually perceived as forming a non- or para-crystalline matrix in which the inner layer is embedded. There is some proof however that the matrix does not penetrate the fibres, but rather is attached to this layer via hydrogen bonds (Mackie & Preston, 1974; Basha & Murthy, 2007). The inner, rigid fibrillar layer of brown algae is predominantly composed of the uncharged cellulose polymer (a β(1-4)-linked unbranched glucan). Two other fibrillar molecules, xylan (principally a β(1-3)-linked D-xylose) and mannan (a β(1-4)-linked D-mannose) occur in the red and green algae (Smidrød & Draget, 1996).

The major organic constituents of the cell walls of algae are carbohydrates. These can be generally divided into water soluble or water insoluble carbohydrates. The water insoluble carbohydrates associated with algal cell walls include cellulose, mannan, xylan, alginic acid and fucinic acid. The occurrence of chitin has also been reported (Kreger, 1962; Davis *et al*., 2003).

The most widespread fibrillar skeleton material is cellulose which can represent 2–20% of the dry weight. It can be replaced by xylan in the Chlorophyta and Rhodophyta in addition to mannan in the Chlorophyta (Davis *et al*., 2003), (Figure 1.3).
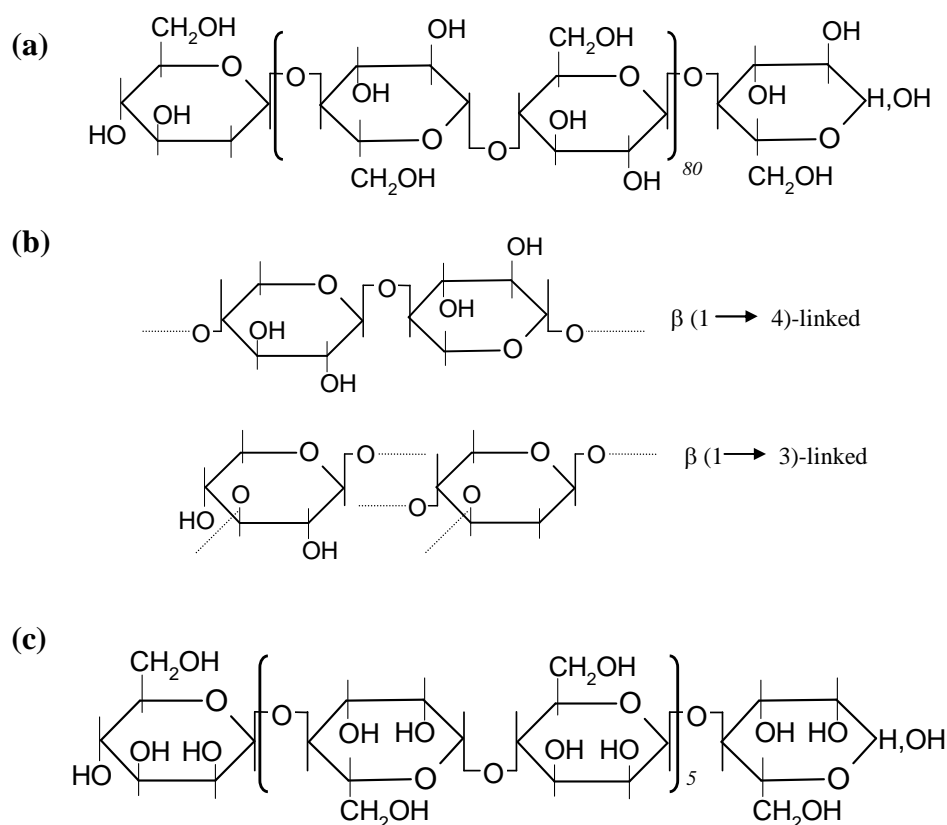
Figure 1.3. Carbohydrates associated with brown, red and green seaweeds. (a) Algal cellulose, a β(1-4)-linked unbranched glucan, of the brown algae; (b) structural units present in xylan from red algae, both β(1-3) and β(1-4)-linked forms have been isolated; (c) mannan, a β(1-4)-linked d-mannose from red algae (Percival & McDowell, 1967).

The most common types of seaweeds (Chlorophyta, Phaeophyceae and Rhodophyta) demonstrate different biosorption characteristics, due to the different structures of the cell wall polysaccharides. The function that any given group of the biomass plays depends on factors such as: the number of sites in the biosorbent material, its accessibility, its chemical state (i.e. availability), and the affinity between site and metal (i.e. binding strength) (Herrero *et al*., 2008).

The embedding matrix associated with brown seaweeds is predominately alginic acid with a smaller amount of the sulphated polysaccharide, fucoidan. The Rhodophyta contains several sulphated galactans such as agar, carrageenan, porphyran, etc. The brown and red seaweeds contain the largest number of amorphous embedding matrix polysaccharides. This quality, combined with their renowned ability to bind metals, makes them potentially exceptional heavy metal biosorbents (Davis *et al*., 2003).

Alginic acid (alginate) is the common name given to a family of linear polysaccharides containing 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues arranged in a non-regular, blockwise order along the chain. The residues characteristically occur as (–M–)n, (–G–)n, and (–MG–)n sequences or blocks (Haug, 1961), (Figure1.4). Alginic acid (alginate) is associated with brown algae and contributes to both the strength and flexibility of the cell wall (Smidrød & Draget, 1996).

(a)

β-D-Mannuronic Acid   α-L-Guluronic Acid

(M)   (G)

(b)

(c)

GMMMMGGGGGGGMGMGMGMGMMMMG

M-Block   G-Block   MG-Block   M-Block
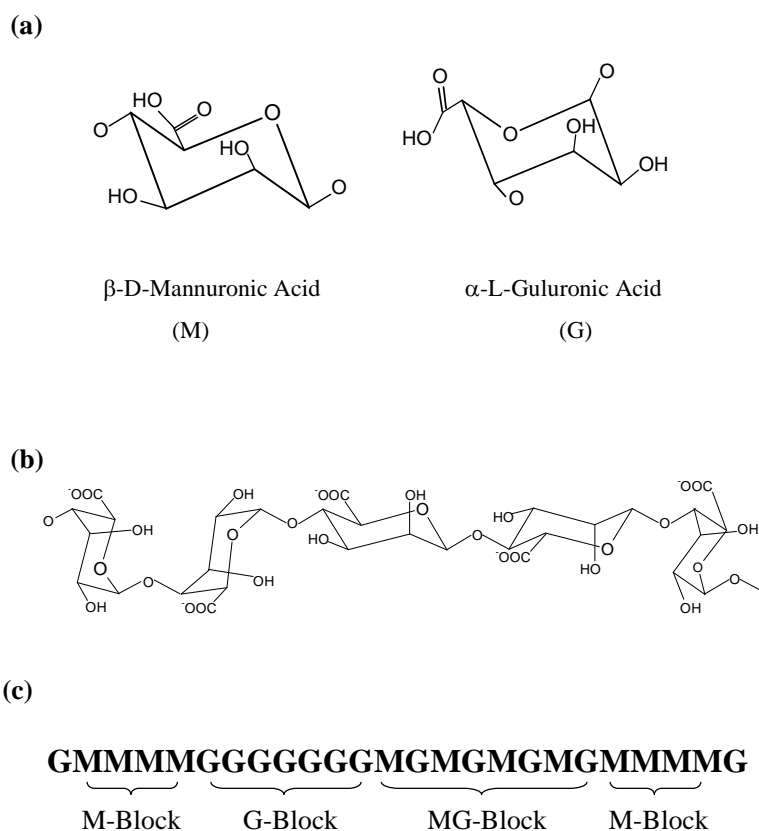
Figure 1.4. Alginate structural data. (a) alginate monomers (M vs. G); (b) the alginate polymer; (c) chain sequences of the alginate polymer (Smidsrød & Draget, 1996).

Alginate usually constitute around 10–40% of the total dry weight, and fucoidans (between 5 and 20% dw) (Davis *et al*., 2003). They are most likely to be the main groups involved in pollutant binding reactions. Brown algae cell walls are very porous and therefore easily permeable to small ionic species. Furthermore, seaweeds possess rigid physical shapes and structures which make their function in biosorption processes particularly suitable. Additionally, the seaweed thallus generally has flat chips which facilitate rapid metal ion mass transfer and effective metal binding (Davis *et al*., 2000).

Haug *et al.* (1967) were the first to investigate the variations of the uronic acid sequence in alginates from different sources. They uncovered the mannuronic acid to guluronic acid ratios (M:G) of alginates. Marked differences among the M:G ratios of alginates from different brown algae were observed (Haug *et al*., 1967). Variation in the affinity of some divalent metals to alginates with different M:G ratios was also demonstrated. Additionally, the affinity of alginates for divalent cations such as Pb^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}, Ca^{2+}, etc. increased with the guluronic acid content (Haug, 1961). It was also established that M- and G-block sequences exhibit significantly different structures and their proportions in the alginate determine the physical properties and reactivity of the polysaccharide (Haug *et al*., 1967). Alginic acid may be present in the cell wall matrix and in the mucilage or intercellular material. Its abundance depends on the depth at which the algae grow and it also displays seasonal variation. The latter may be indicative of changes associated with growth stages (Davis *et al.,* 2003). The alginate of *Sargassum fluitans* has been reported to account for 45% of its dry weight (Fourest & Volesky, 1996). Davis *et al.* (2003b) also reported similar alginate yields for protonated *Sargassum fluitans* and *Sargassum oligocystum* of approximately 45% and 37%, respectively.

Fucoidan, the extracellular polysaccharide, has been found in several members of the Laminariaceae family, with dry mass between 5 and 20 % (Black, 1953). Fucoidan is a branched polysaccharide sulphate ester. The major building blocks associated with fucoidan is l-fucose 4-sulphate (Davis *et al.,* 2003) and these are predominantly α(1-2)-linked (Mackie & Preston, 1974), (Figure 1.5).
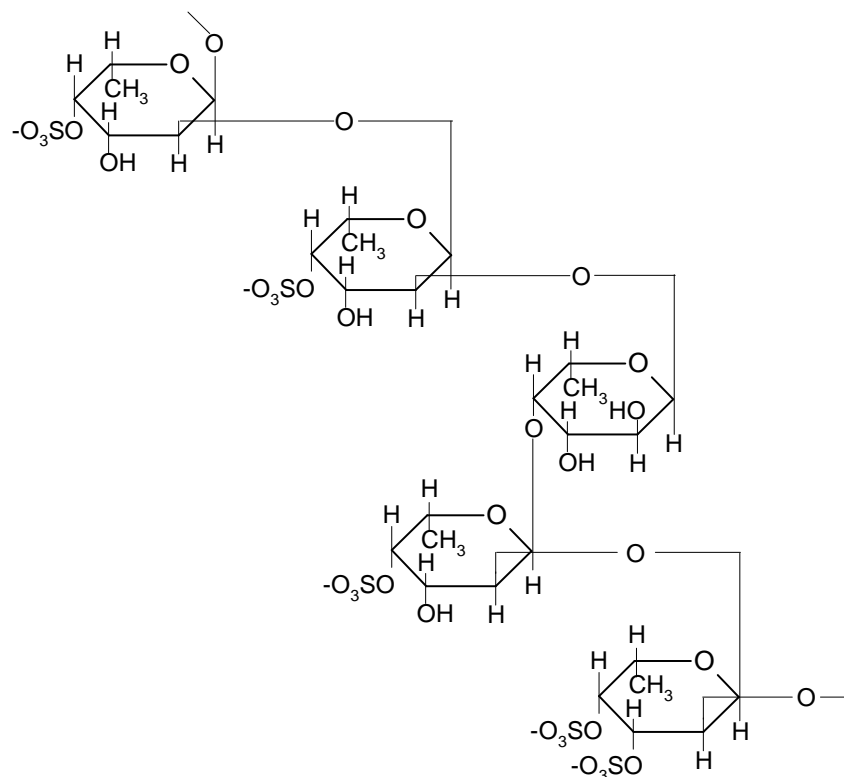
Figure 1.5. The structure of fucoidan. A branched polysaccharide sulphate ester with l-fucose building blocks as the major component with predominantly α(1-2)-linkages.

In red seaweed the cell wall is largely composed of D-mannan and D-xylans (Castro *et al.*, 2004) or sulphated galactans (carrageenans and agar) of different types. Variations depend on the species, age, season and origin of the alga (Herrero *et al.*, 2008). Carrageenans and agar are the main polysaccharides in red algae (Herrero *et al.*, 2008). These polysaccharides are galactans exclusively composed of galactose or modified galactose units (Percival, 1979). The major difference between the agars (Figure 1.6) and carrageenans is that the former contains D- and L-galactose units whereas the latter consists entirely of the D-sugar (Percival, 1979).
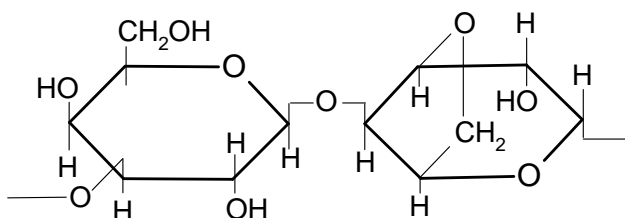
Figure 1.6. Repeating disaccharide unit of agar.

Carrageenans are linear polymers of about 25,000 galactose units. They consist of alternating 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units (Figure 1.7), whereas the gelling hydrocolloid agar is primarily composed of the L-rather than the D-3,6-anhydro-α-galactopyranose units (Herrero *et al*., 2008).
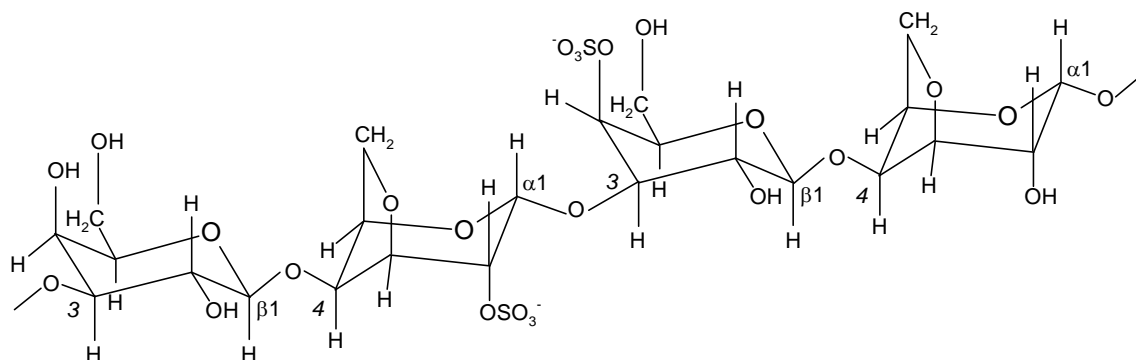
Figure 1.7. Structure of Carrageenan. Carrageenan consists of alternating 3-linked-β-D-galactopyranose and 4-linked-α-D-galactopyranose units.

Furthermore, other minor polysaccharides can be associated with the cell wall of seaweeds: ulvans (Figure 1.8) in green seaweeds fucoidans (brown seaweeds), xylans (some red and green seaweeds) (Burtin, 2003).
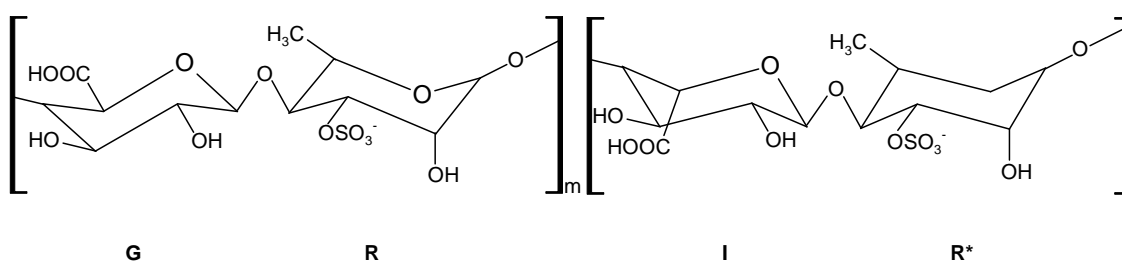
Figure 1.8. The structure of ulvan, the main disaccharide units [β-D-Glcp A-(1→4)-α-L-Rhap 3s] and [α-L-Idop A-(1→4)-α-L-Rhap 3s], G: (1→4)-linked β-D-glucuronic acid; R: (1→4)-linked α-L-rhamnose-3-sulphate (linked with β-D-glucuronic acid); I: (1→4)-linked α-Liduronic acid; R*: (1→4)-linked α-L-rhamnose-3-sulphate (linked with α-L-iduronic acid), (Qi *et al*., 2006).

Algal carbon can be stored in monomers (e.g., mannitol), it can also be stored in polymers (Davis *et al*., 2003). The first storage product of photosynthesis is mannitol and is derived from the six-carbon sugar, d-mannose (Figure 1.9). Mannitol is present in all brown algae and can comprise up to 30% of their dry weight (Davis *et al*., 2003). The second major storage product in brown algae is laminaran (Figure 1.9). This glucan is made up of a mixture of polysaccharides (Davis *et al*., 2003). The linkages are mainly β(1-3) (Figure 1.9), but a small amount of β(1-6) -links can also be present. Two forms of laminaran chains exist—M, with mannitol attached to the reducing end and G, with glucose attached to the reducing end (Percival & McDowell, 1967; Rioux *et al*., 2010).
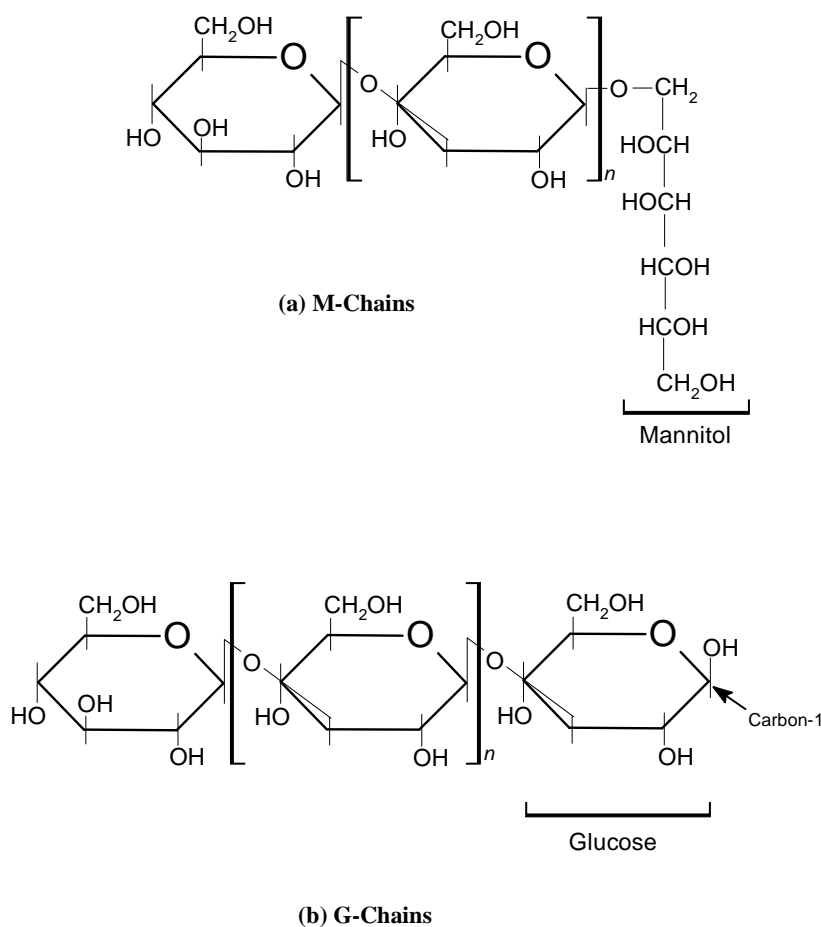
Figure 1.9. The structure of laminaran (principally a β(1-3)-linked unbranched glucan) consists of two types of chains. In (a) mannitol is attached to the reducing end (M-chains), whereas in (b) glucose is attached to the reducing end (G-chains).

1.1.5   Seaweeds Under Investigation

1.1.5.1 Fucus ceranoides

This species is located in estuaries, where it is attached via a small holdfast, to rocks (Figure 1.10). The seaweed is green-olive in colour with repeating forked fronds and has a prominent mid-rib (Nicholson & Brightman, 1979).

Figure 1.10. *Fucus ceranoides* (www.algaebase.org, 2010).

1.1.5.2 Polysiphonia lanosa

There are around 200 species of the red seaweed *Polysiphonia* worldwide. Variations among species are generally based on microscopic features. *P. lanosa* is easily recognised by its very fine abundantly branched plants (Lee, 1986). The seaweed is comprised of a series of tubes wrapped around a central axis to form cylindrical axes and branches. The tubes are elongated cylindrical cells surrounding a large, cylindrical *'central'* cell. *P. lanosa* (Figure 1.11) is easy to identify as it is a small, wiry,

tufted seaweed which can grow attached to a host seaweed in the intertidal zone (Lee, 1986).
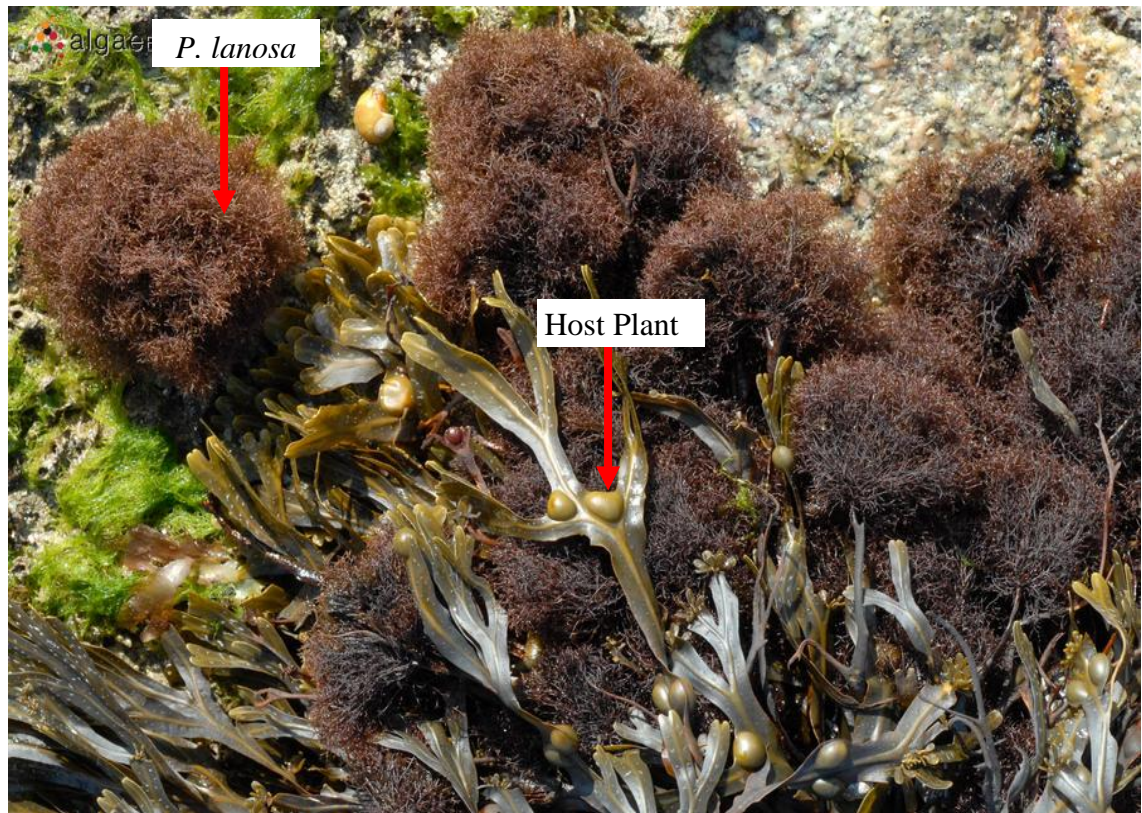
Figure 1.11. *Polysiphonia lanosa* (www.algaebase.org, 2009).

1.1.5.3 Ascophyllum nodosum

A. nodosum (Figure 1.12) is a dark, olive green seaweed which has long, flat, strap-shaped branches that are normally attached by a short stalk to a disc-shaped holdfast. The branches are forked and produce large air bladders at intervals. The branches are notched from which, short branches grow in Spring and early Summer. These have swollen, greenish-yellow tips, in which the reproductive structures are produced. *A. nodosum* grows optimally on sheltered rocky shores and it usually occupies a zone immediately below *F. vesiculosus* (Nicholson & Brightman, 1979).

Figure 1.12. *Ascophyllum nodosum* (www.algaebase.org, 2009).

1.1.5.4 *Fucus vesiculosus*

This *Fucus* species usually grows best in the middle of the intertidal zone. It can grow up to three feet long and has a prominent midrib in its flattened blades (Lee, 1986). The repeatedly-forked dark olive-brown fronds of *F. vesiculosus* are attached to a disc-shaped holdfast by a cylindrical stalk (Nicholson & Brightman, 1979), (Figure 1.13). It also has paired air bladders which keep the seaweeed afloat, thereby exhibiting its photosynthetic tissues to the sun's rays. In sheltered areas, the air bladders can be plentiful and the fronds flat and broad whereas more exposed plants are inclined to be narrower and bear fewer bladders (Lee, 1986).

Figure 1.13. *Fucus vesiculosus*. (www.algaebase.org, 2009).

1.1.5.5 Ulva sp

Ulva sp (Figure 1.14) is a green seaweed that exists at all times during the year. It grows attached to rock, on all types of shoreline. Plants which come adrift can continue to flourish in a free-floating state in sheltered environments. The fronds have an irregular outline, they are wavy (especially at the margins), and are translucent. *Ulva* sp can grow in environments where fresh-water runs into the sea and also where there is a moderate amount of pollution (Nicholson & Brightman, 1979)
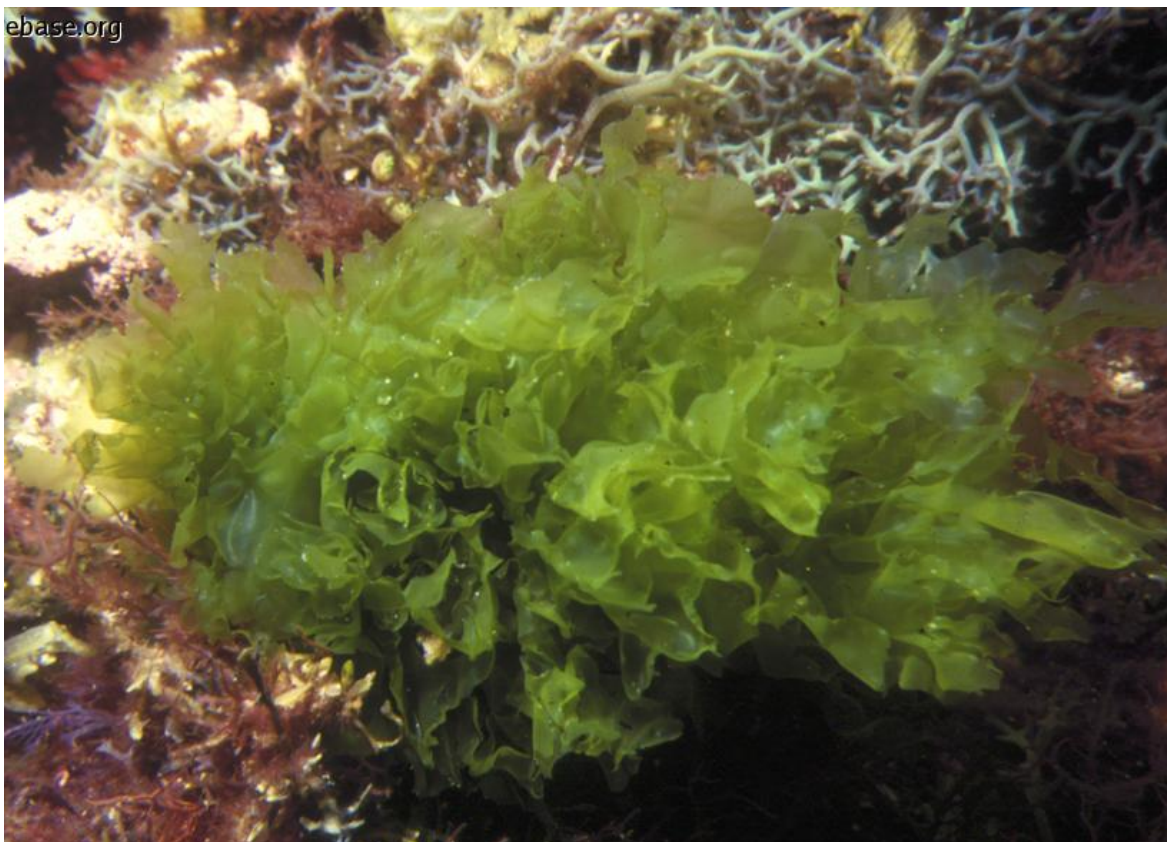
Figure 1.14. *Ulva* sp (www.algaebase.org, 2009).

1.1.6   Use of Seaweed and Seaweed Products

The worldwide exploitation of macroalgae is a multi-billion dollar industry. A great deal of this is associated with the farming of edible seaweeds and on the production of agar, carrageenan and alginate (Smit, 2004). When all seaweed products are taken into account, hydrocolloids have the largest influence on modern Western world. Their commercial significance is a consequence of their use in various industries which take advantage of their physical properties such as gelling, water-retention and their ability to emulsify (Smit, 2004). In recent times however, pharmaceutical companies have started to turn to seaweeds in the search for new drugs from natural resources. Furthermore, these commodities are also being increasingly used in medical and biochemical research. Before the 1950s, the therapeutic properties of seaweeds were restricted to traditional and folk medicines. In the 1980s and 1990s, compounds possessing biological activities (bioactivities) were discovered in algae (Smit, 2004).

China and Japan are the major seaweed cultivators, producers and consumers in the world. Seaweed products are utilised daily in the form of processed food items like processed dairy, meat and fruit products and domestic products such as paint, toothpaste, solid air fresheners, cosmetics, etc (Dhargalkar & Pereira, 2005). Seaweeds are an exceptional source of vitamins A, Bl, B12, C, D & E, riboflavin, niacin, pantothanic acid and folic acid as well as minerals such as Ca, P, Na, K. The amino acid content of seaweeds is well balanced and contains most, if not all, of the essential amino acids needed for life and good health. They contain over 54 trace elements needed for human body's physiological functions in quantities significantly higher than vegetables and other land plants. These elements are in chelated, colloidal, optimally balanced forms and are thus bio-available (Dhargalkar & Pereira, 2005) The main commercial hydrocolloids extracted from seaweeds are agar and carrageenan which are derived from red seaweeds and alginate from brown seaweeds. These commodities are difficult to synthesise therefore enormous commercially important products depend on seaweed resources (Dhargalkar & Pereira, 2005). Large quantities of seaweed are consumed in Southeast Asia, whereas algal hydrocolloids (alginate, carrageenan and agar), used as thickeners and stabilising or emulsifying agents, are the main use in the Western world (in products such as desserts, dairy, sauces, beer, fruit drinks, extruded foods) (Warrand, 2006).

Heavy metal poisoning can be successfully treated with the use of sodium alginates. Additionally, seaweed alginates have a soothing and cleansing effect on the digestive tract in humans. Furthermore, sulphated polysaccharides from seaweeds have been used in films that are positioned between bones to be grafted in order to speed up the growth of the connective tissue (Dhargalkar & Pereira, 2005). Residual, unused seaweed biomass can be used in the production of biogas (methane) by means of anaerobic digestion. Furthermore, seaweeds have become a key ingredient in cosmetic products such as soaps, shampoos, powders, creams and sprays (Dhargalkar & Pereira, 2005).

Chemicals responsible for antibiotic activities are prevalent in macroalgae. Compounds of particular interest are the halogenated compounds such as haloforms, halogenated alkanes and alkenes, alcohols, aldehydes, hydroquinones and ketones (Lincoln *et al.*, 1991). The catalogue of terpenoids with antibiotic qualities is especially widespread, and many of these are also halogenated. Compounds such as sterols and heterocyclic

and phenolic compounds sometimes have antibiotic properties. Many of these could potentially be developed into antiseptics and cleansing agents (Smit, 2004).

The polyphenols in seaweeds differ chemically to those in terrestrial plants. Algal phenols are derived from the simple C6 precursor phloroglucinol (phlorotannins – as opposed to tannins associated with terrestrial plants), (Hay & Fenical, 1988). In a previous investigation by Salvador *et al*., (2007), the antibacterial and antifungal activity of 82 marine macroalgae (18 green, 25 brown and 39 red), collected from Mediterranean and Atlantic coasts of the Iberian Peninsula, was studied to assess their potential to be used as natural preservatives in the cosmetic industry. Bioactivity was analysed from crude extracts of fresh and lyophilised samples against three Gram-positive bacteria, two Gram-negative bacteria and one yeast by means of the agar diffusion technique. Out of the macroalgae tested, 67% were active against at least one of the six test microorganisms. The highest percentage of active taxa was found in the brown species (84%), followed by red (67%) and green (44%). Nevertheless, the red algae had the broadest spectrum of bioactivity. *Bacillus cereus* was found to be the most sensitive test microorganism and *Pseudomonas aeruginosa* the most resistant (Salvador *et al*., 2007). Additionally, in a study by Bansemir & co-workers (2006), dichloromethane, methanol and water extracts from 26 seaweed species were screened for their antibacterial activities against five fish pathogenic bacteria strains. The most active species were found to be the red seaweeds, *Asparagopsis armata, Ceramium rubrum, Drachiella minuta, Falkenbergia rufolanosa, Gracilaria cornea* and *Halopitys incurvus.* Furthermore, results also demonstrated that significant inhibition zones were only observed for the dichloromethane extracts. It was concluded therefore that the compounds responsible for antimicrobial activity in seaweeds were lipophilic. The overall results from this study confirmed the use of seaweeds as a possible source of antimicrobial compounds or as a health-promoting food for the aquaculture industry (Bansemir *et al*., 2006).

1.2   Metals

1.2.1   Metals in the Environment

Metals in minerals and rocks are ordinarily harmless, only becoming potentially toxic when they dissolve in water. They can enter the environment in various ways such as,

natural weathering of rocks, leaching of soils and vegetation and volcanic activity. Some of the highest mercury levels are found, not in coastal waters, but in the deep sea, near the mid-ocean ridges, as a result of submarine volcanic activity.   In assessing marine pollution therefore, distinctions must be made between natural anthropogenic activities. Activities such as mining and smelting ores, burning of fossil fuels, disposal of industrial waste, and the processing of raw materials for manufacturing have added to metal levels in the environment (Lobban & Harrison, 1997). The majority of the metals in the environment are transported by water in their dissolved or particulate state, and most of them reach the oceans by means of rivers or land runoff.   Furthermore, rainwater carries vast amounts of cadmium (Cd), copper (Cu), Zn and especially Pb from the atmosphere to the oceans. Such metals in the atmosphere originate from the burning of fossil fuels. Metals in sediments may be in the reduced or oxidised state and can be released into the overlying water (Lobban & Harrison, 1997).

Metals in an aquatic environment may be present in dissolved or particulate forms. They may be dissolved as either free hydrated ions or as complex ions (chelated with inorganic ligands such as OH^-, Cl^-, or CO^{2-}_3) or they may be complexed with organic ligands such as amines, humic and fulvic acids and proteins. Particulate forms may be found in a range of states; as colloids or aggregates (e.g., hydrated oxides); precipitated as metal coatings onto particles; adsorbed onto particles; incorporated into organic particles such as algae; held in the structural lattice in crystalline detrital particles (Lobban & Harrison, 1997).   The physical and chemical forms of metals in seawater are controlled by a number of environmental variables such as pH, redox potential, ionic strength, salinity, alkalinity, the presence of organic and particulate matter, and biological activity, as well as by the intrinsic properties of the metal. Modifications to these variables can result in the alteration of the metals' chemical forms and can therefore contribute to the availability, accumulation and toxicity of these elements to aquatic organisms (Lobban & Harrison, 1997).   In coastal waters, the concentrations of heavy metals decrease with distance from river mouths. This is due to the dilution effect and also as a result of the salting-out process of high-molecular-weight fractions and flocculation of inorganic matter as salinity increases in a downstream direction. Metals may adsorb to these newly formed particles and sink to the sediments. Conversely, some metals previously attached to particles in the river water may be displaced by chloride ions and become available for uptake by algae (Lobban & Harrison, 1997).

1.2.2   Heavy Metals

In general, the term 'heavy metal' has been used to describe metals that have atomic numbers higher than that of iron (59), or having densities greater than 5 g/mL. From an environmental point of view, metals may be classified into the following three groups: (1) noncritical; (2) toxic, but very insoluble or very rare; (3) very toxic and relatively accessible. Some heavy metals however, in category 3, such as manganese, iron, Cu and Zn are essential micronutrients and are often referred to as trace metals. These metals may limit algal growth if their concentrations are too low, however they can become toxic at higher concentrations; frequently the optimum concentration range for growth is narrow. Other heavy metals in category 3 such as Hg and Pb are not necessary for growth and they can be toxic to algae at very low concentrations (e.g., 10 – 50 μg/L) (Lobban & Harrison, 1997). Contamination caused by metal ions is a major environmental problem. Aqueous effluents originating from many industries contain dissolved heavy metals. If these discharges are released without treatment, they may have an undesirable impact on the environment (Herrero *et al.*, 2006). Heavy metals are not biodegradable and are likely to accumulate in living organisms, causing various diseases and disorders (Bailey *et al.*, 1999).

Greater awareness of the ecological effects of toxic metals and their accumulation through food chains has provoked a demand for purification of industrial wastewaters prior to their discharge into the natural water bodies and increasing interest has been shown in the removal of heavy metals (Herrero *et al.*, 2006). The use of inexpensive biological materials (including algae), for the removal and recovery of heavy metals from contaminated industrial wastes has emerged as a possible alternative method to conventional techniques such as chemical precipitation, filtration, ion exchange, electrochemical treatment or membrane technology (Herrero *et al.*, 2006).

Heavy metals such as Cd, Cr, Cu and Pb are significant environmental pollutants. These metals are toxic to all organisms at varying concentrations (Bayçu, 2002). As a result of increased industrialisation throughout Europe, the reduction of heavy metal pollution of water sources has become a major focal point in EU policy since the mid 1970s (Thévenot *et al.*, 2007). For example, the 80/778/EEC Directive relates to the quality of water intended for human consumption (Table 1.1). This directive however was amended by the Drinking Water Directive (Directive 98/83/EU) (Table 1.1). The key aim of the directive is to protect human health from the adverse effects of water

contamination and is considered as a predecessor to the Water Framework Directive (2000/60/EC).

Table 1.1. EU directive 80/778/EEC relating to the quality of water intended for human consumption. (*Directive (98/83/EC) indicator parameters).

Parameter	Guide Level (GL)	Maximum admissible concentration (MAC)
Calcium (mg/L)	100.0	———
Magnesium (mg/L)	30.0	50.0
Sodium (mg/L)	20.0	150.0
Potassium (mg/L)	10.0	12.0
Aluminium (mg/L)	0.05	0.2
Boron (µg/L)	1,000.0	1,000.0 *
Iron (µg/L)	50.0	200.0
Manganese (µg/L)	20.0	50.0
Copper (µg/L)	100.0	2,000.0 *
Zinc (µg/L)	100.0	———
Phosphorus (µg/L)	400.0	5000.0
Cobalt (µg/L)	———	———
Arsenic (µg/L)	———	10.0 *
Berylium (µg/L)	———	———
Cadmium (µg/L)	———	5.0
Chromium (µg/L)	———	50.0
Mercury (µg/L)	———	1.0
Nickel (µg/L)	———	20.0 *
Lead (µg/L)	———	10.0 *

1.2.3 Metals Under Investigation

1.2.3.1 Cadmium

Exposure to Cd is implicated in a number of medical complications, mainly kidney dysfunction and bone disease, as well as some cancers. Battery factories, Zn smelters, pigment plants and soldering activities are sources of occupational exposure to Cd. Currently however, the most significant source results from the production of nickel-Cd batteries. Occupational exposure to Cd mainly occurs as a result of respiration, but may also involve the gastrointestinal system (Järup, 2002). Environmentally however, the main source of Cd exposure is tobacco smoke for smokers, followed by diet for the non-smoker. Adsorption of Cd in the lungs is 10-50% whereas gastrointestinal adsorption is less than a few %. Consequently, blood Cd concentrations of smokers (~1-4 mg/L) are ~ 4 to 5 fold higher than those of non-smokers. Furthermore, Cd is absorbed into soil

from the use of fertilisers and as a result of Zn-mining processes, where Cd is a discarded impurity. Cd concentrations are also high in certain species of shellfish and mushrooms. It accumulates predominately in the kidney and liver but is also found in other tissues including bone and placenta (Järup, 2002). The Irish EPA guidelines for Cd in drinking water is 0.005 mg/L (Irish EPA, 2010).

Cd is a by-product of the Zn mining industry. Several Zn mines operate in Ireland, however, the closest one to Waterford is located near Moyne Co. Tipperary ($52^o45'38.18''$ N, $7^o40'47.39''$W). The Lisheen Mine which commenced operations in 1998 is located in the Suir river catchment, between the Rossesstown River and Drish River. Both rivers flow into the Suir river (Dillon *et al*., 2004), (Figure 1.15).

Figure 1.15. Sampling sites along the Suir Estuary (Sites 1-4) and Fethard-on-Sea (Site 5) and sources of heavy metals (Google Earth, 2010).

1.2.3.2 Chromium

Cr is a naturally occurring metal found in rocks, animals, plants, and soil. It can be present in several different forms including, Cr(0), Cr(III), and Cr(VI). The Cr(0) form, is used for making steel. Cr(VI) and Cr(III) are used for chrome plating, dyes and pigments, leather tanning, and wood preserving. Cr(III) is an essential nutrient that helps the body to metabolise sugar, protein, and fat (ATSDR, 2008). Inhaling high levels of Cr(VI) can cause irritation to the lining of the nose, nose ulcers, runny nose, and breathing problems, such as asthma, cough, shortness of breath, or wheezing. The concentrations of Cr in the atmosphere that can cause these symptoms may vary according to the different types of Cr compounds, with effects occurring at much lower concentrations for Cr(VI) compared to Cr(III) (ATSDR, 2008). Cr(VI) compounds are known human carcinogens. Inhalation of Cr(VI) has been shown to cause lung cancer. Additionally, an increase in stomach tumours was observed in humans and animals exposed to Cr(VI) in drinking water (ATSDR, 2008). The EU Directive (98/83/EC) guidelines for Cr in drinking water are 0.05 mg/L.

Cr also has historical importance to Co. Waterford, as it has traditionally been used in industry. A tannery using Cr in its leather processing was located in Portlaw (52°17'6.02"N, 7°18'53.17"W) on the Clodiagh River (a tributary of the River Suir). The company, which was set up in 1934 by Irish Tanners Ltd, was at one point, the largest in Europe. The tannery was closed in 1985 however, reports by the Irish EPA in 1995 showed that, although Cr pollution had decreased after the closure, some traces were still evident in the Clodiagh River. Michell Ireland Ltd also operated a tannery in Killowen Portlaw (near the site of the former tannery) from 1993-2003) (Figure 1.15).

1.2.3.3 Lead

Short-term exposure to high levels of Pb can result in brain damage, paralysis (lead palsy), anaemia and gastrointestinal symptoms. Longer-term exposure can cause damage to the kidneys, reproductive and immune systems and nervous system (Food Safety Authority of Ireland, Toxicology Factsheet, Issue 1, 2009). A major consequence of low-level Pb exposure is on intellectual development in young children. Pb traverses the placental barrier and accumulates in the foetus. Infants and young children are more susceptible than adults to the toxic effects of Pb, and they also absorb Pb more readily. Consumption of food containing Pb is the main source of exposure for

the general public (Food Safety Authority of Ireland, Toxicology Factsheet, Issue 1, 2009).

Pb is present in drinking water primarily from its dissolution from lead pipes or lead-containing solder. The concentration of Pb in drinking water therefore depends on a number of factors including pH, temperature, water hardness and standing time of the water. Therefore, the method of Pb sampling is critical and depending on the method used results can vary significantly (World Health Organization, 1996). The EU Directive (98/83/EC) requires Pb in drinking water not to exceed 0.025 mg/L. Pb has also historical importance in this geographic region. Pb crystal has been synonymous with Waterford Crystal Limited since it began operations in 1783. The factory was located in Waterford City ($52^o15'24.43"$ N, $7^o7'45.14"$) until its closure in 2009. A key component in crystal production is Pb oxide (red lead) (Figure 1.15).

1.2.3.4 Copper

Cu is an essential nutrient, although at increased levels it can become a contaminant (EPA Ireland, 2009). Inhaling high levels of Cu can cause irritation of the nose and throat. Ingesting high levels of Cu can cause nausea, vomiting, and diarrhoea. Very-high doses of Cu can result in liver and kidney damage and even death (US Department of Public Health and Human Services, 2008).

The primary source of Cu in drinking water is from corrosion of internal Cu plumbing. The levels of Cu in drinking water depend on the length of time the water has been stagnant in the Cu piping and thus fully flushed water generally has low levels of Cu (Irish EPA, 2009). The EU Directive (98/83/EC) guideline for Cu in drinking water is 2.0 mg/L. The incidence of Cu in the environment is expected to increase due to the amplified use of Cu piping during the recent expansion of the Irish construction industry. As well as its industrial significance, Cu is also important historically in this region ($52^o8'17.06"$ N, $7^o22'22.83"$W) due to intensive mining of the metal in the 19th century (Figure 1.15).

1.3   Biomonitoring

1.3.1   The Use of Seaweeds as Biomonitors

The extraordinary ability of several seaweeds to accumulate metals is renowned, with some (e.g. *Fucus* spp*., Ulva* spp*.*) able to thrive in estuaries and coastal waters impacted by high metal concentrations (Pawlik-Skowrónska *et al.*, 2007). Their local abundance, widespread distribution and sedentary nature (Brown *et al.*, 1999) has led to the use of seaweeds as biomonitors of pollution by metals (Pawlik-Skowrónska *et al.*, 2007)

Biomonitoring has a distinct advantage over the direct analysis of seawater for metal levels, as it indicates the concentrations of metals that are biologically available, thereby providing a more accurate indication of the environmental impact of a metal (Rice & Lapointe, 1981). Another advantage of seaweed is that it is capable of concentrating trace metal levels several more times than in the surrounding seawater (Lobban & Harrison, 1997).

In order to become a suitable biomonitor, seaweeds should meet certain conditions. For example, the seaweed must be a good accumulator of trace metals and be available all year. Other requirements include a widespread distribution for large area analysis and they should also be easy to identify (Kramer, 1994).

A major difficulty with the use of seaweed as biomonitors is that the level of metal present in the seaweed is related to the plants growth rate. As a consequence, the concentrations measured may be due to more enhanced growth conditions than actual metal levels in the environment. This predicament is not limited to seaweed as described by Fitzgerald *et al.* (2003) who found that several factors affected uptake of heavy metals in salt marsh plants, these included, differences in age and growth stages, seasonal variations, presence of iron plaques on the roots, level of metal contamination in the locality, soil properties, tides and salinity. In addition, concentration factors for metals in different algae have been reported to vary significantly between species (Stengel *et al.,* 2004). It is thought that the variation in metal concentration in algae from the same site may be attributed to variability in growth rates, the analysis of different plant parts, environmental and physico-chemical parameters, or plant age and habitat (e.g. shore height at which the algae were collected). Studies have confirmed that all the factors that affect algal growth will also affect metal uptake (Stengel *et al.,*

2004). Furthermore, variation in the ability of different seaweeds to bioaccumulate metals has previously been reported. For example, the concentration factors (CF) for *F. vesiculosus* in relation to Pb and Zn were 2,400 and 23,000 respectively (Preston *et al.*, 1972).

1.4 Metal Accumulation by Living Seaweeds (Bioaccumulation)

1.4.1 Biosorption

The occurrence of heavy metals in the environment is of major concern due to their toxicity and threat to plant and animal life. Furthermore, the recovery of heavy metals from industrial waste has become increasingly important as the public realises the necessity for recycling and conservation of essential metals. Anthropogenic sources of heavy metals include process waste streams from metal plating, mining operations, and semi-conductor manufacturing operations (Hashim & Chu, 2004). Furthermore, increasingly severe discharge restrictions on heavy metals have accelerated the exploration of highly efficient and economically attractive treatment methods for their removal (Hashim & Chu, 2004). Biosorption is an emerging technology that has attracted increased attention in recent times. Biosorption utilises the ability of microbial and plant biomass to sequester heavy metal ions from aqueous solution (Hashim & Chu, 2004). The biological mode of biosorption can be explained by the different kinds of chemical and physical interactions among the functional groups present on the cell wall and the heavy metals in solution (Esposito *et al.*, 2002). Charged groups such as carboxylate and hydroxyl that are present in biomass cell wall are thought to be responsible for the binding of metal ions (Hashim & Chu, 2004).

Biosorption by dead biomass has resulted in the investigation of materials of biological origin as potential metal biosorbents. The various materials tested include bark, chitin, lignin, modified wool, bacteria, fungi and algae, among others. Among these materials, algal biomass has gained significant importance (Herrero *et al.*, 2008) due to the cost saving, low sensitivity to environmental and impurity factors, the possible contaminant recovery from the biomaterial and its elevated adsorption capacity (Lodeiro *et al.*, 2006). There is an increasing amount of work being focused on heavy metal biosorption by brown seaweed. Murphy & co-workers (2007) carried out a study on Cu binding by dried biomass of red, green and brown macroalgae. It was found that, of the

seaweeds investigated, *F. vesiculosus* (brown) contained the greatest number of acidic surface binding sites while *P. palmata* (red) contained the least. Since it is thought that carboxyl groups (weak acidic groups) are chiefly responsible for metal sorption, particularly in brown seaweeds, it was expected that *F. vesiculosus* would exhibit superior biosorption performance over *P. lanosa*. Refer to section 1.1.4 for seaweed composition and biosorption potential.

Heavy-metal ions can become trapped in the structure of the cell and consequently biosorbed onto the binding sites available in the cellular structure. This mode of uptake does not require the action of a biological metabolic cycle and is known as biosorption or passive uptake. The heavy metal can then pass across the cell membrane into the cell via the cell metabolic cycle. This method of metal uptake is referred to as active uptake. The metal uptake by both active and passive methods is termed as bioaccumulation (Iyer *et al.*, 2004). Most of the studies dealing with microbial metal remediation via growing cells describe the biphasic uptake of metals, i.e., initial rapid phase of biosorption followed by slower, metabolism-dependent active uptake of metals (Malik, 2004 and Garnham *et al.*, 1992). Reports employing growing cultures of marine microalgae indicate that intracellular Cd levels are often higher than the biosorbed ones (Perez-Rama *et al.*, 2002).

In studies using biomass (dead/pretreated), it was found that metal was not taken up into the cells, instead it was just adsorbed at the cell surface and, therefore, only a small fraction of bioaccumulation capacity was exploited (Malik, 2004). Current methods of biosorption are sensitive to background conditions such as pH, ionic strength and the existence of organic or inorganic ligands. Furthermore, biosorption also lacks specificity in metal binding. The ability to reuse the biomass after desorption is only possible if reasonably weak chemicals are used for desorption (Malik, 2004). As previously mentioned, biosorption alone may not be sufficient for effective metal remediation. The use of active and growing cells may be a better option as these living cells can continuously uptake metals following physical adsorption (Malik, 2004). Additionally, when the metals diffuse into the cells during detoxification, they get bound to intracellular proteins before being transported into vacuoles and other intracellular sites. These processes are frequently irreversible and reduce the risk of the metal being released back to the environment (Gekeler *at al.*, 1988). With the exception of this, the use of growing cells in bioremoval could avoid the need for a separate

biomass production process, e.g. cultivation, harvesting, drying, processing and storage prior to the use (Malik, 2004).

Considerable limitations to bio-accumulate by living cell systems exist, these include, sensitivity of the system to extremes of pH, high metal/salt concentration and the requirement of external metabolic energy. However, in toxic metal removal, it is necessary to ensure that the growing cells can maintain a constant removal capacity after multiple bioaccumulation–desorption cycles (Malik, 2004).

Biosorption occur when a metal is captured by binding sites naturally present and functional even when the biomass is dead. The main advantage of biosorption is in the use of biomass raw materials which are usually abundant seaweeds or waste from other industrial operations (e.g., fermentation wastes), (Volesky, 2001).

The exceptional potential of certain types of biomass to concentrate and immobilise particular heavy metals depends to a certain degree on (Volesky, 2001):

- the type of biomass
- the mixture in the solution
- the type of biomass preparation
- the chemico-physical process environment

Broad-range biosorbents can accumulate all the heavy metals from solution with a small degree of selectivity among them. By manipulating the characteristics of a biosorbent, the biosorption of a specific metal can be accomplished for both sorption uptake or desorption regeneration (Volesky, 2001).

Biosorption is based on several mechanisms that quantitatively and qualitatively vary according to the type of biomass, its origin and its processing. Metal sequestration can include complex mechanisms, primarily ion exchange, chelation, adsorption by physical forces and ion entrapment in inter- and intrafibrilar capillaries and spaces of the structural polysaccharide cell wall network. Due to the widespread occurrence of the raw biomass material and to its high metal uptake capacity, current studies have concentrated on marine algae. The major constituent responsible for metal sorption is the alginate which is present in a gel form in their cell walls (Burtin, 2003).

It is thought that carboxyl groups (weak) are primarily responsible for metal sorption, especially in brown seaweeds (Murphy *et al.*, 2007). Other cell wall functional groups, such as the strongly acidic sulphate groups ($R–OSO_3^-$) of fucoidan and carrageenan in seaweeds, and the amino groups of chitin ($R_2–NH$) and chitosan ($R–NH_2$) in fungi has also been explored (Kratochvil & Volesky, 1998). A study by Murphy *et al.,* 2008 compared the Cr biosorption by red, green and brown seaweed biomass. Results following Fourier transform infrared analysis revealed interactions of amino, carboxyl, sulphonate and hydroxyl groups in Cr binding to the green seaweed *Ulva* spp. The remaining seaweeds showed involvement of these groups to varying degrees as well as ether group participation in the brown seaweeds and for Cr(VI) binding to the red seaweeds.

The utilisation of sorbents in industrial fixed-bed sorption columns for the removal of a wide range of metals has been shown to be practically and economically viable due to its cost efficiency and high metal removal capacity. For example, Bakir and co-workers (2009) investigated the removal of heavy metals from aqueous metal solutions using a seaweed based fixed-bed sorption column. *P. lanosa* and a seaweed-waste material resulting from the processing of *A. nodosum* (referred to as waste *Ascophyllum* product (WAP)) were the chosen biosorbents. Results demonstrated that WAP was very efficient in the removal of Zn(II), Ni(II) and Al(III) in both single and combined solutions. *P. lanosa* however was more efficient for the removal of Sb(III). Furthermore, when combined with other metals, Sb(III) reduced the removal capacity of the other metals by WAP (Bakir *et al*., 2009). Additionally, the metal removal properties of a large variety of macroalgae have previously been investigated. In a study by Bakir *et al*., 2010, the regeneration of a WAP based biosorbent using a 0.1M HCl wash resulted in the release of high metal concentrations during multi-desorption cycles. It was also demonstrated that regeneration of the sorbent coincided with minimal loss in metal removal efficiency.

1.4.2   Intracellular Uptake

1.4.2.1 Glutathione

A common effect following exposure to Cd pollution is the overproduction of reactive oxygen species (ROS), potentially causing oxidative damage in plant cells and therefore requiring the involvement of antioxidant defence systems (Sanita di Toppi *et al*., 2007). Glutathione (GSH) is recognised as an antioxidant that plays a major role in the defence against free radicals in plants (Figure 1.16). Under conditions of environmental stress, organisms are capable of altering their metabolism to minimise the strain imposed by such adverse conditions. This involves changes of different biochemical pathways (Okamoto & Colepicolo, 1998).
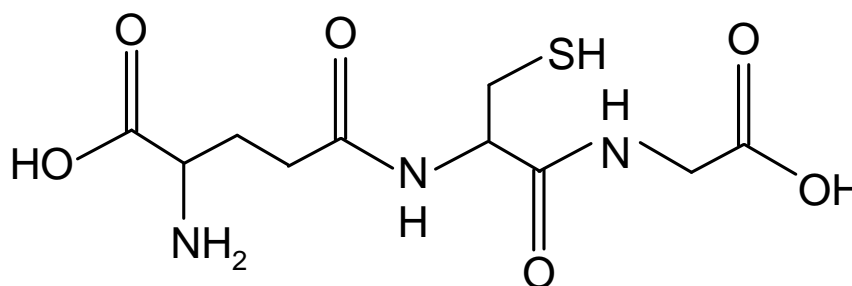
Figure 1.16. Structure of glutathione.

GSH has a number of roles in all organisms, with its major function directed towards protecting them from environmental hazards. GSH-dependent reactions are vital in protecting plants from oxidative stress, xenobiotic compounds, and heavy metals. In the latter case, GSH serves as a substrate for the synthesis of phytochelatins (PC) (Figure 1.17). Mutations that hinder GSH synthesis, or its conversion into PC, result in increased Cd^{2+} sensitivity in plants (Wang & Oliver, 1997).
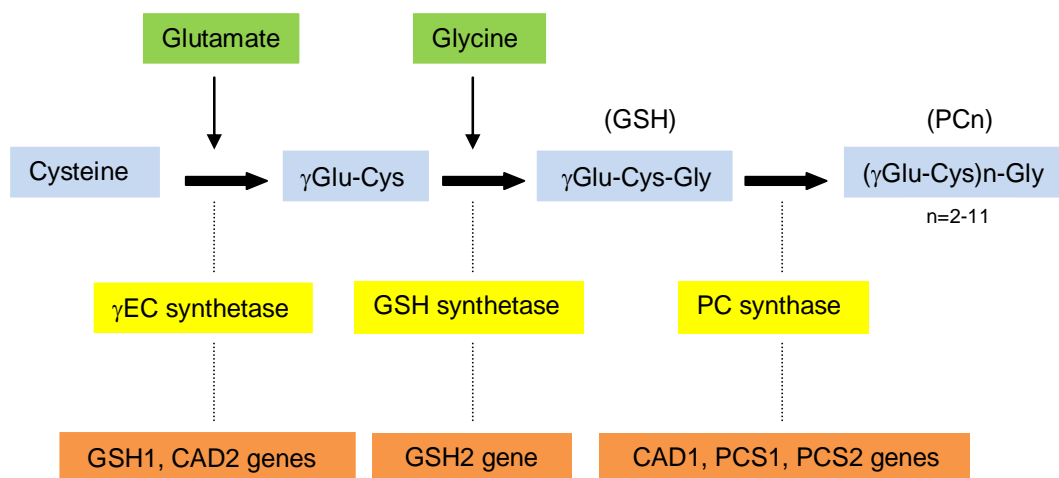
Figure 1.17. Biosynthesis of phytochelatins (Inouhe, 2005).

GSH is produced from the amino acids - cysteine, glutamic acid and glycine, (Wang & Oliver, 1997) with synthesis in plants occurring in two steps (Rüegsegger & Brunold, 1993). In the first step, γ-Glutamylcysteine synthetase (γ-ECS) catalyses the ATP-dependent ligation of glutamate and cysteine to produce γ-glutamylcysteine, (Wang & Oliver, 1997), (Figure 1.18).
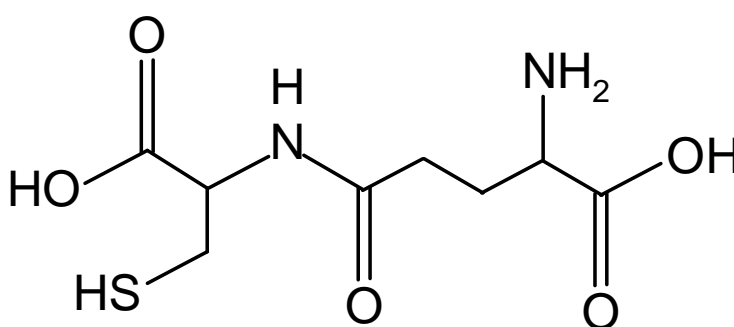
Figure 1.18. Structure of γ-glutamylcysteine.

In a second stage, γ-glutamylcysteine is condensed with glycine in an ATP-dependent reaction, catalysed by the enzyme GSH synthetase (GS) to create GSH, (Wang & Oliver, 1997), (Figure 1.17). The first step is a rate limiting reaction for GSH synthesis. The γEC synthetase activity is enhanced by Cd^{2+} ions and exclusively inhibited by the treatment with L-buthionine-[S,R]-sulphoximine (BSO). In previous studies, it has

been shown that salicylic acid activates the enzyme Ser-acetyltransferase (Hernandez-Allica *et al*., 2006). This enzyme catalyses O-acetyl-ser from L-Ser and acetyl-CoA resulting in the synthesis of cysteine (Kawashima *et al.,* 2005), which therefore causes accumulation of GSH and activation of GSH reductase to maintain an enhanced pool of reduced GSH (Hernandez-Allica *et al*., 2006). Additionally, it has been demonstrated that salicylic acid potentially obstructs phytochelatin synthase (PCS) activity (thereby inhibiting PC biosynthesis in response to Ni) and conserving GSH to operate as an antioxidant, thus preventing Ni-induced oxidative stress in *Thlaspi* hyperaccumulators (Hernandez-Allica *et al*., 2006).

Following Cd exposure, GSH pools of *Enteromorpha linza* increased but were prone to oxidation which indicates Cd-induced oxidative stress (Malea *et al*., 2006). Metal-induced depletion of GSH as a result of PC synthesis may therefore increase the susceptibility of cells to oxidative stress (De Vos *et al*., 1992). In *E. prolifera* however, Cd only induced PC synthesis (active detoxification) (Malea *et al*., 2006). Additionally, Cu is known to catalyse not only the oxidation of GSH but also that of other intracellular thiols, therefore Cu may also be capable of catalysing the oxidation of PC-SH groups. This may therefore explain a sharp decline in the concentration of PC of sensitive plants exposed to more than 20μM of Cu^+. Moreover, oxidation of the Cu^+ - thiolate compound will cause the release the Cu ion, resulting in an amplified toxicity (De Vos *et al*., 1992).

Tsuji *et al.* (2003) demonstrated that Cd increases PC synthesis in the marine green alga, *Dunaliella tertiolecta* from GSH by the activation of PCS and promotes the synthesis of GSH not only through transcriptional activation of the GSH biosynthetic pathway, but also by means of the stimulation of endogenous generation of reactive oxygen species (ROS) such as H_2O_2. Exposure to heavy metal Cd^{2+} results in oxidative stress, as indicated by the creation of H_2O_2. GSH synthesis is regulated by oxidative stress and H_2O_2 amplifies GSH levels (Tsuji *et al*., 2003).

1.4.2.2 Phytochelatin Synthase

PCS (γ -glutamylcysteine dipeptidyl transpeptidase) was originally isolated from the flowering plant *Silene cucubalus* (Skowroński *et al*., 1998). The enzyme which uses GSH as a substrate (Grill *et al*., 1989; De Vos *et al*., 1992; Schat *et al*., 2002), cleaves

the terminal glycine from one GSH and links the γ-glu-cys to the amino terminus of another GSH to form the n=2 oligomer (Ahner *et al.*, 1995), (Figure 1.17). PC biosynthesis advances by a stepwise addition of the dipeptidyl units (Grill *et al.*, 1989). Structural similarities between GSH and PC imply the latter are synthesised from the former or its precursors (Grill *et al.*, 1985). PCS requires a metal ion for activation and Cd is the most effective activator, followed generally by Ag, Bi, Pb, Zn, Cu, Hg and Au, depending on the species of test organism used in the study (Perez-Rama *et al.*, 2001).

The PCS isoelectric point is near pH 4.8. It has a temperature optimum at 35°C (Grill *et al.*, 1989 and Skowroński *et al.*, 1998) and pH optimum at 7.0. The Michaelis–Menten kinetics (Km) for GSH is 6.7 mM. The purified enzyme demonstrates specific activity of 463 pkat/mg of protein (1 kat = the amount of enzyme that catalyzes a reaction rate of 1 mol of substrate per s). Under denaturing conditions, the molecular weight (mw) of enzyme is 25,000. The mw determination of the enzyme under non denaturing conditions yields enzyme activity of Mr 95,000 and Mr 50,000. However, enzyme preparations of an early purification step have demonstrated a single broad peak of activity at Mr 95,000. These observations propose that the enzyme is an oligomeric protein consisting of four Mr 25,000 subunits. During purification the tetrameric structure of the enzyme seems to dissociate into an active dimeric form (Grill *et al.*, 1989).

The degree of activation of the PCS upon exposure to a metal depends not only on the cellular metal concentration but also on the nature of the particular metal and the fraction that is sequestered in the membrane. For example, different metals partition between the cytoplasm and the membrane of the cell in different proportions. Reinfelder & Fisher (1994) reported that 80% of the Ag in I*sochrysis galbana* is bound to membranes (Ahner & Morel, 1995). PC peptides, heavy metal ions, and PCS create a self-regulating loop (Loeffler *et al.*, 1989). The enzyme is activated and forms PC in the presence of 'free*'* heavy metal ions that are consequently complexed by the peptides and are thus no longer able to provoke PC synthesis (Grill *et al.*, 1989). The enzymatic reaction is therefore terminated when PC chelate the metal (Ahner *et al.*, 1995).

1.4.2.3 Phytochelatins

Because tolerance is one of the most important mechanisms for survival, many organisms (Torres *et al*., 1997), from higher plants (Grill *et al*., 1989) to macroalgae (Wu, 1998) respond to the cytotoxic effects of heavy metals by synthesising metal binding proteins or peptides (Torres *et al*., 1997).

Metallothioneins are low molecular weight, cysteine rich polypeptides that complex metal ions in thiol groups (Robinson, 1989; Merrifield *et al*., 2004). They are expressed in several vertebrates, fungi, plants, algae and metal-resistant bacteria. Some macroalgae synthesise these peptides which chelate metals and store them in compartments within the cell or segregate them from the surrounding environment (Merrifield *et al*., 2004) hence preventing free heavy metal circulation inside the cytosol (Grill *et al*., 1985).

Two distinctive forms of metallothioneins are thought to be involved in the macroalgae response to heavy metal exposure (i) The gene encoded Class I and Class II metallothioneins and (ii) the inducible enzymatically synthesized Class III metallothioneins, known as phytochelatins (Morris *et al*., 1999; Malea *et al*., 2006). PC and metallothioneins are entirely different in structure but have similar roles (Grill *et al*., 1985; Pickering *et al*., 1999; Inouhe, 2005). In general, gene encoded metallothioneins consist of a single polypeptide chain with 61 amino acid residues (Grill *et al*., 1985). PC however are not primary gene products and consist of linear peptides of up to 23 amino acid units (Gekeler *et al*., 1988).

In animals, surplus heavy metal ions are counteracted by the induction of the metallothionein gene. Heavy metal detoxification in plants however, is associated with the synthesis of PC (Gekeler *et al*., 1988), (Figure 1.19).

PC can bind Cd and are essential for Cd detoxification thus playing crucial roles in the mechanisms of phytoremediation (Pickering *et al*., 1999). These heavy metal complexing peptides have the general structure (γ-glutamic acid-cysteine)n glycine (n = 2-11) (Gekeler *et al*., 1988; Schat *et al*., 2002; Hernandez-Allica *et al*., 2006). PC bind metals through coordination with the sulphydryl group of cysteine (Ahner *et al*., 1995).
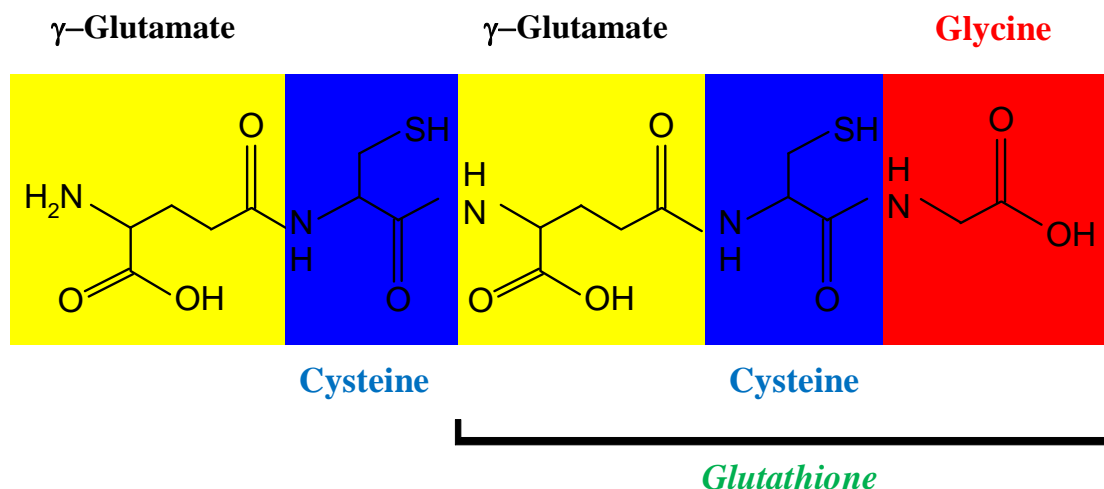
Figure 1.19. Structure of a phytochelatin unit (Cobbett, 2000).

PC synthesis induced by Cd is connected to a rapid depletion of total GSH in plant cell suspensions and plants (De Vos *et al*., 1992). However, in their study Tsuji *et al.* (2003), discovered that in Cd treated cells, the levels of GSH stayed constant whereas PC increased linearly, indicating that GSH biosynthesis was also promoted by Cd exposure. The concentration of PC synthesised and length of individual peptides depends on the organism (Gekeler *et al*., 1988). For example, each Cd ion is coordinated by 4 cysteine sulphurs, therefore, if n=2 or n=3 oligmers are synthesised, then more than one peptide must bind a single metal ion. In addition, following exposure to Cd, increases in intracellular Cd concentration of algae was matched by an increase in PC concentration (Ahner *et al*., 1995). There was also a rapid decrease in PC when Cd exposure was alleviated, thereby suggesting that the intracellular PC pool is closely regulated (Ahner & Morel, 1995). Algae therefore appear to possess a tightly regulated control mechanism that synthesises the precise concentration of PC necessary to bind intracellular Cd (Ahner *et al*., 1995).

PC synthesis is inhibited by the γ-ECS inhibitor, buthionine sulphonximine (BSO) (Schat *et al*., 2002). A great deal of evidence also indicates that PC are not involved in Cd detoxification in plants displaying enhanced tolerance to Cd. For example, the presence of BSO did not affect Cd tolerance levels in Cd-tolerant ecotypes of *S. vulgaris* and *T. caerulescens*. In their study of *T. caerulescens*, Hernandez-Allica *et al.* (2006), found that Cd tolerance in ecotypes displaying enhanced tolerance to Cd were not affected by BSO, suggesting that PC synthesis is not a key factor of the Cd tolerance

observed in this ecotype. In contrast however, BSO addition considerably increased Cd toxicity to a less tolerant ecotype, suggesting that PC mediated Cd detoxification may play a certain role in this less tolerant ecotype. For some plants however not all metal species, capable of inducing PC, are able to form stable metal-PC complexes. The formation of stable complexes is just as important for metal tolerance as the ability of plants to produce PC. In a previous study by Scarano & Morelli (2002), the formation of Cd-PCn and Pb-PCn complexes with n-values from 3 to 6 was demonstrated. The metal – PCn compounds formed with Cd appeared to be different from those produced with Pb in relation to the number of molecules of peptide involved in the complex and for the amount of the metal ion bound. As well as being involved in heavy metal detoxification (Grill *et al*., 1989) PC have been assumed to work in the cellular homeostasis of essential heavy metal ions, particularly Cu and Zn (Schat *et al*., 2002).

1.4.2.4 Cysteine and Acid Labile Sulphur

Several reports have previously suggested the possible role of cysteine in cellular Cd detoxification. Dominguez-Solis *et al.* (2001), investigated the expression of the gene encoding the cytosolic O-acetylserine(thiol)lyase (OASTL), which catalyses the last step of cysteine synthesis through the incorporation of sulphide into the O-acetyl-L-Ser molecule, under Cd stress conditions. They found a sevenfold induction of this gene after 18h exposure to 50μM $CdCl_2$. Their results are consistent with a high cysteine biosynthesis rate under heavy metal stress required for the synthesis of GSH and PC which are involved in plant detoxification mechanism. This perhaps suggests that increased cysteine availability is responsible for Cd tolerance (Dominguez-Solis *et al*., 2001).

OASTL activities in Zn and Cd treated cells decreased slightly but were still shown to be much higher than γ-Glutamylcysteine synthetase (γ-ECS) and Glutathione synthetase (GS) activities. It would appear that OASTL therefore is not related to the regulation of GSH synthesis in *D. tertiolecta* (Tsuji *et al*., 2003). Results from a study by Howarth *et al.* (2003) suggest that specific serine acetyltransferase (SAT) isoforms have a function in increasing cysteine production under conditions of heavy-metal stress when increased biosynthesis of GSH and PC is essential for detoxification purposes (Howarth *et al*., 2003).

PC is synthesised by the constitutive, metal-activated enzyme phytochelatin synthase (PCS). An additional component of the complex however is acid-labile sulphur. Cd has been known to increase sulphate reduction in plants. Integration of acid-labile sulphur into Cd-peptide complexes enhances the metal binding affinity of the peptides. Sulphide synthesis in tomatoes and other organisms that produce (γEC)nG peptide complexes appear to be a response to exposure to high concentrations of Cd.

Previous studies involving the fission yeast, *Schizosaccharomyces pombe* demonstrated that exposure to higher concentrations of Cd caused incorporation of sulphide *in vivo* (Reese *et al*., 1992). With low metal levels, tomatoes formed predominantly the low sulphide complex. Increases in the Cd concentrations resulted in an elevation in the percentage of the high sulphide complex. This resulted in CdS crystallite particles covered with (γEC)nG peptides in response to Cd (Reese *et al*., 1992). Increased activities of these alternative PC-independent sequestration mechanisms may lead to decreased cytoplasmic metal availabilities for PCS activation (de Knecht *et al*., 1995).

1.4.2.5 Polyphenols

Marine macroalgae are known to contain high levels of polyphenols (Devi *et al.,* 2008). Varieties of polyphenols however differ from species to species. For example, the polyphenols associated with the brown seaweeds are phlorotannins (Ragan & Glombitza, 1986), while brominated phenols are connected with red species (Lundgren *et al*., 1979) and coumarins with green seaweeds (Pérez-Rodríguez *et al*., 2003). Two mechanisms have been associated with the capacity of some brown algae to accumulate high metal levels: the presence of cell wall-specific polyanionic polysaccharides that provide the sequestration and precipitation of metals in the cell walls and the attraction between metals ions and algal polyphenols (Amado Filho *et al*., 1999). Heavy metal defence mechanisms in algae may involve compounds such as polyphenols that chelate free metal ions, either intracellularly or in the external medium, resulting in metalloid complexes that are less toxic to the algae. Investigations involving the metal chelation of polyphenols originating from higher plants (e.g. chestnut and pine tannins) suggest that the metal ions bind to the hydroxyphenyl- and carboxyl-groups of the tannin molecules (McDonald *et al*., 1996). It is thought that brown algal phlorotannins may chelate metal ions in a similar fashion (Ragan *et al*., 1979).

Phenolic complexes occur as secondary metabolites in all plants. They include a substantial range of substances containing an aromatic ring bearing one or more hydroxyl groups, although a more accurate definition however is based on metabolic origin as those substances derived from the shikimate pathway (Figure 1.20) and phenylpropanoid metabolism (Antolovich *et al*., 2000).
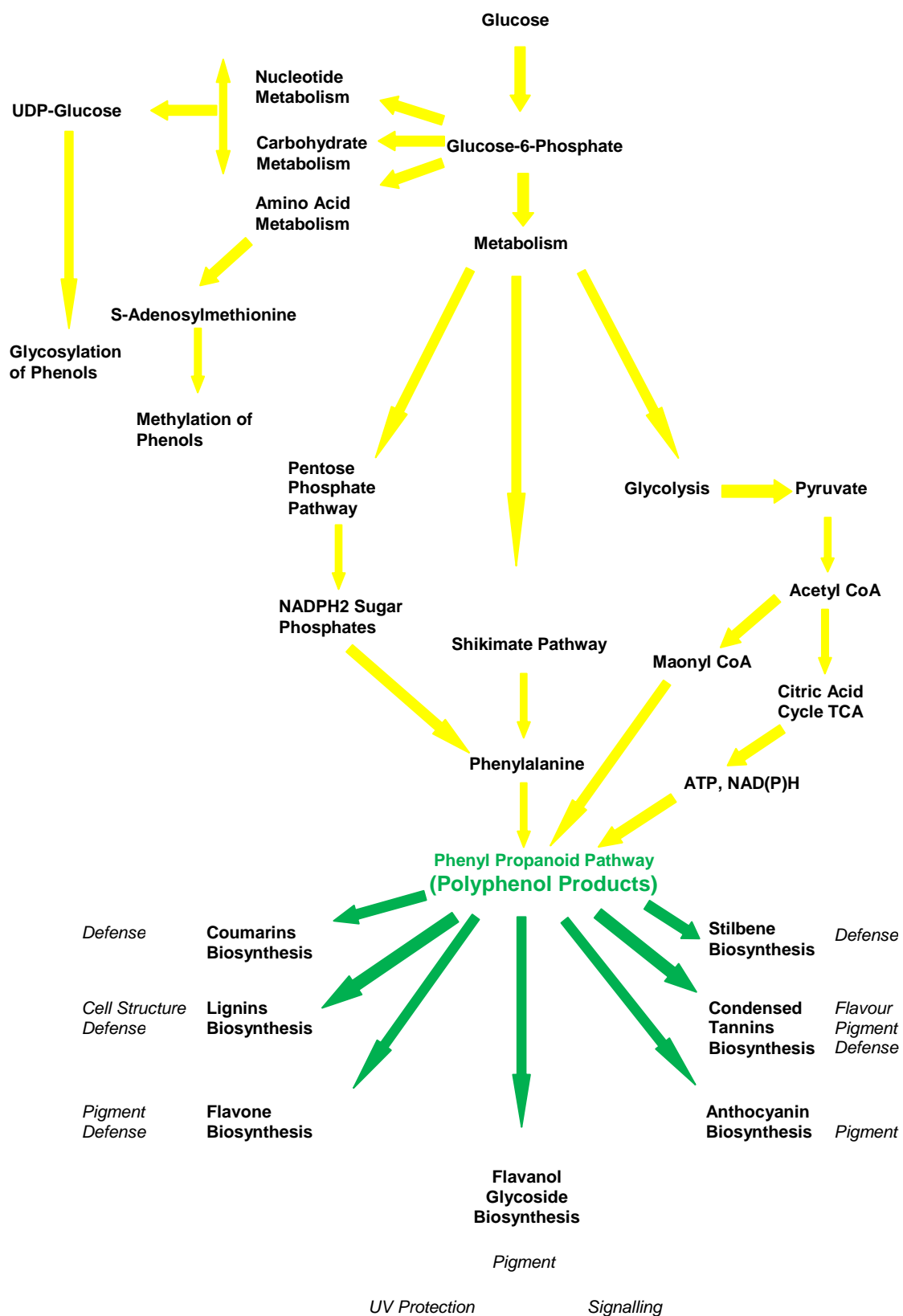
Figure 1.20. Biosynthesis of phenolic phytochemicals (Vattem *et al.*, 2005).

A schematic diagram of the shikimate pathway illustrates the biosynthesis of coumarins, flavone, anthocyanin, condensed tannins, stilbene, flavonol glycoside, and lignins (Figures 1.21 - 1.27 respectively).
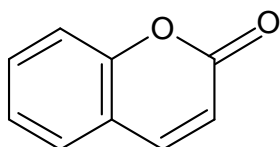
Figure 1.21. Structure of coumarins.
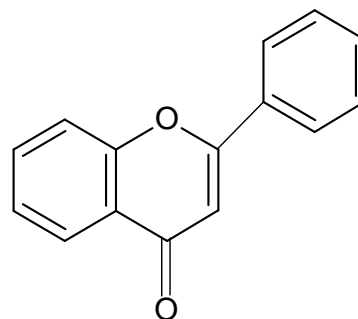
Figure 1.22. Structure of flavone.
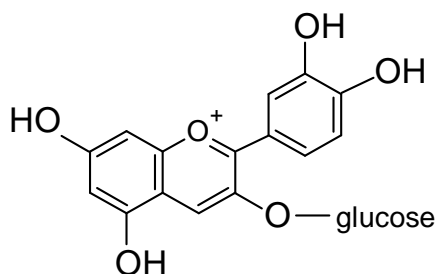
Figure 1.23. Structure of anthocyanin (with sugar)
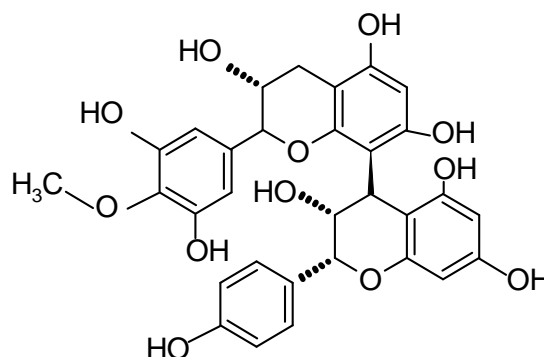
Figure 1.24. Structure of condensed tannins (also known as proanthocanidins).
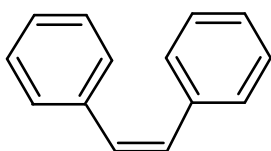
(Z) Stilbene
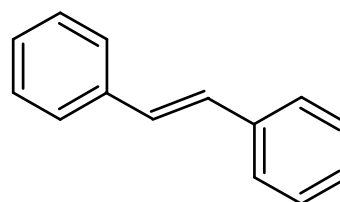
(E) Stilbene

Figure 1.25. Structure of Z-Stilbene and E-Stilbene.
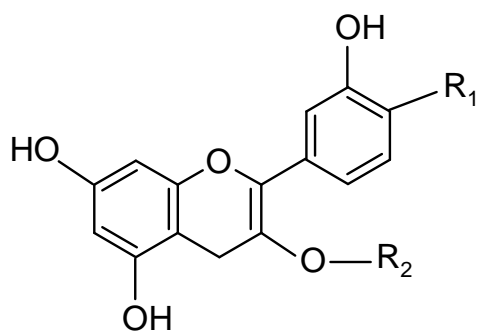
Figure 1.26. Structure of flavonol glycoside.
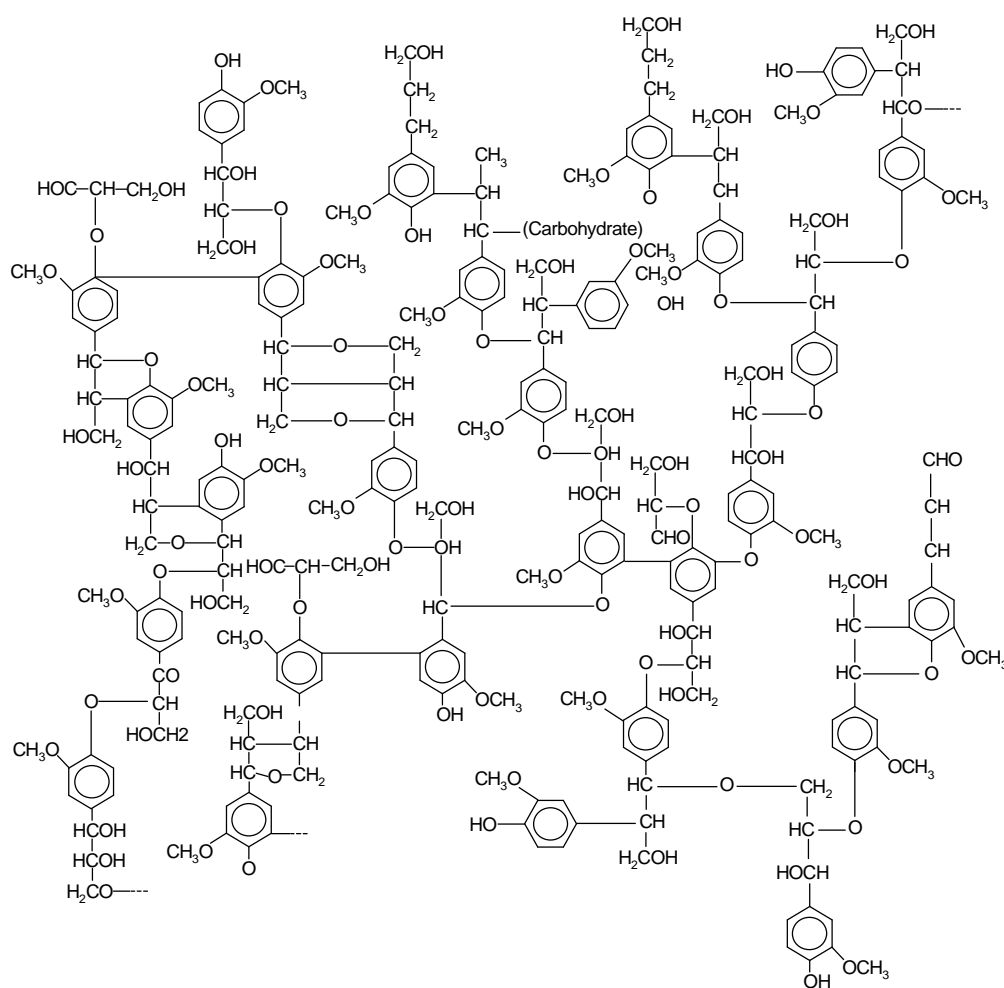
Figure 1.27. The structure of a lignin–carbohydrate complex (Glazer & Nikaido, 1995; Sakagami *et al.*, 2010).

The major active compounds in brown seaweed extracts have been reported to be phlorotannins (Ganesan *et al.*, 2008). Examples of these include, fucols, phlorethols, fucophlorethols, fuhalols, isofuhalols and eckols (Ragan & Glombitza, 1986), (Figures 1.28 – 1.33).
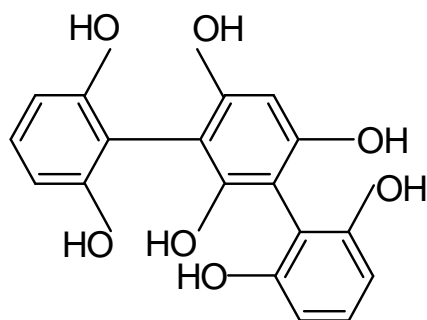
Figure 1.28. Structure of fucols.
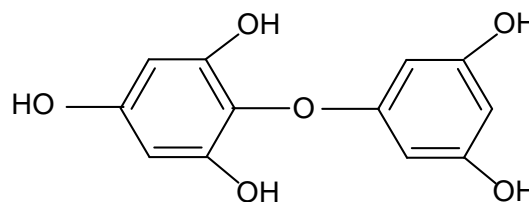
Figure 1.29. Structure of phlorethols.
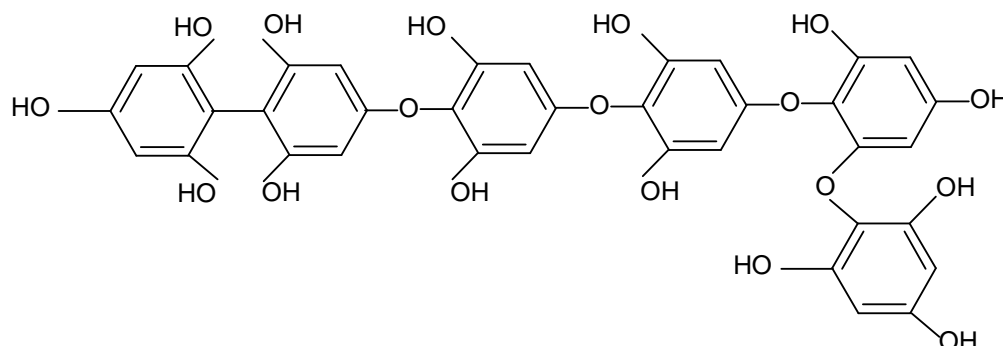
Figure 1.30. Structure of fucophlorethols.
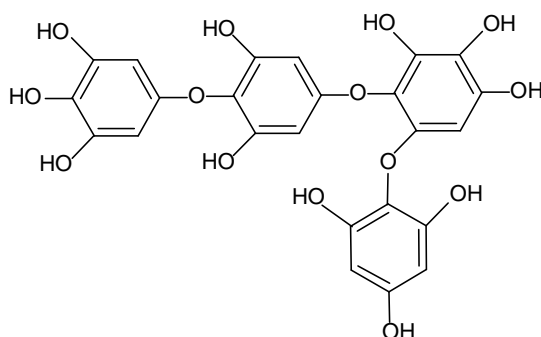
Figure 1.31. Structure of fuhalols.
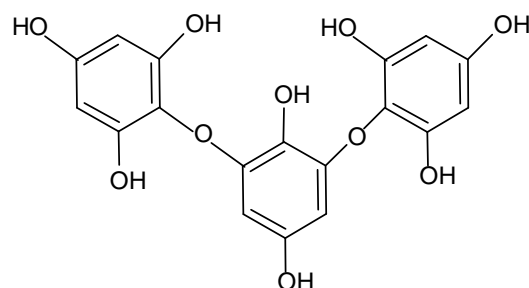
Figure 1.32. Structure of isofuhalols.
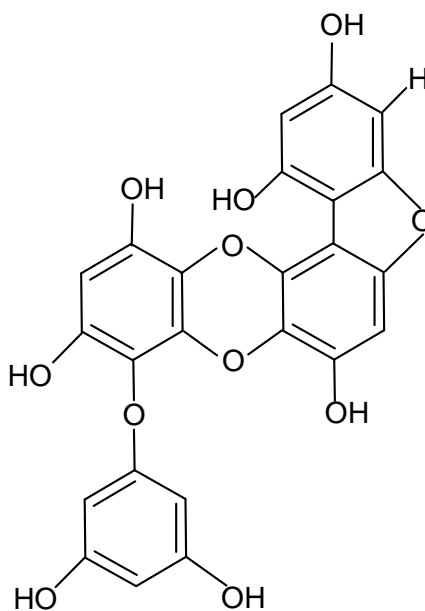
Figure 1.33. Structure of eckols.

Phlorotannin concentrations can be affected by plant size, age, tissue type, salinity, season, nutrient levels, herbivory and light intensity (Targett & Arnold, 2001). Additionally, flavonoids (Figure 1.34) are polyphenolic compounds that occur ubiquitously in plants (Holiman *et al.*, 1996).
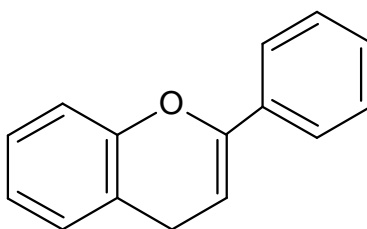
Figure 1.34. Structure of flavonoids.

Over 4,000 different flavonoids have been described. Examples of seaweed flavonoids include, catechol, caffeic acid, rutin, hesperidin, quercetin, myricetin and morin (Yoshie-Stark *et al.*, 2003) (Figures 1.35 – 1.41). Since flavonoids contain several hydroxyl groups, they are expected to express radical scavenging effects (Yoshie-Stark *et al.*, 2003).
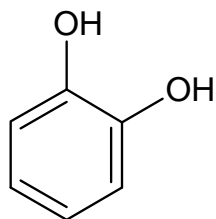
Figure 1.35. Structure of catechol.   **Figure 1.36.** Structure of caffeic acid
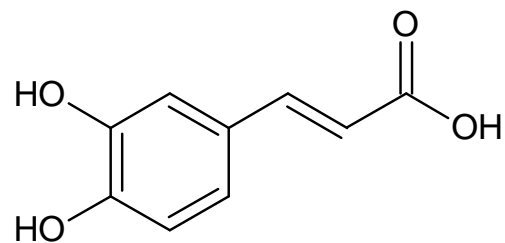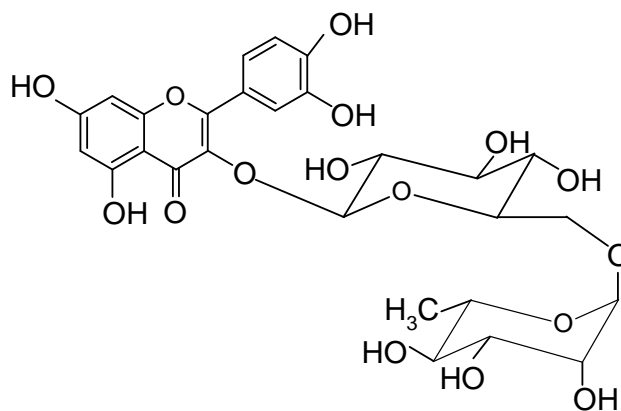
Figure 1.37. Structure of rutin.
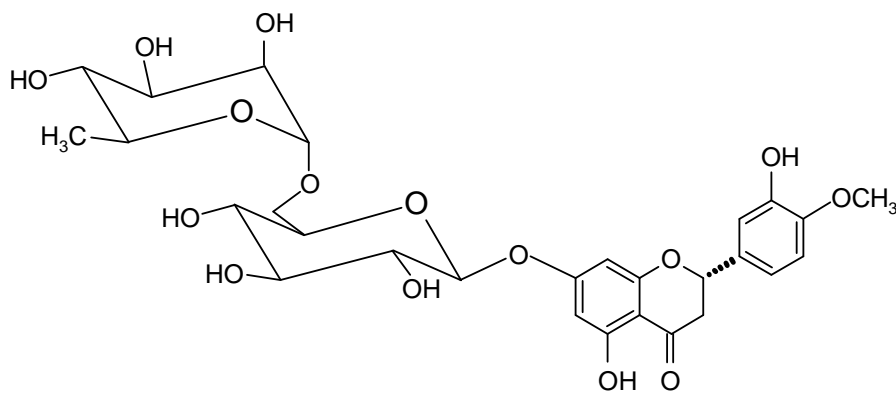
Figure 1.38. Structure of hesperidin.
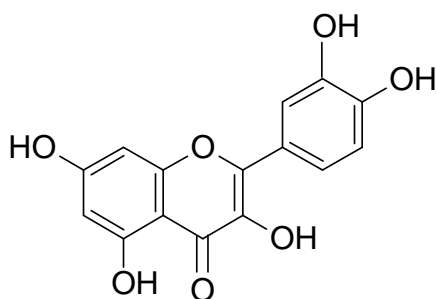
Figure 1.39. Structure of quercetin.
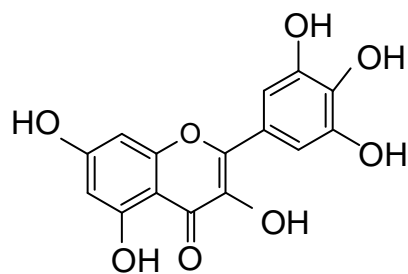
Figure 1.40. Structure of myricetin.
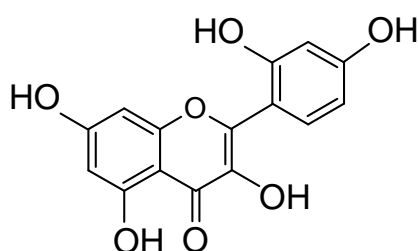
Figure 1.41. Structure of morin.

The qualitative and quantitative distribution of polyphenols varies among tissues and is influenced by growth conditions and the physiological state of the plants (Ferrat *et al.*, 2003). Seaweeds contain high amounts of polyphenols such as gallic acid, catechin, epicatechin and epigallocatechin gallate (Devi *et al.*, 2008) (Figures 1.42 - 1.45 respectively).
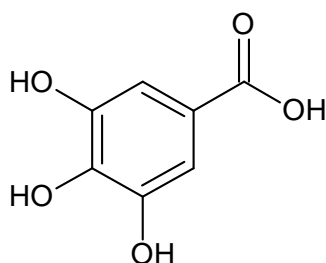
Figure 1.42. Structure of gallic acid.
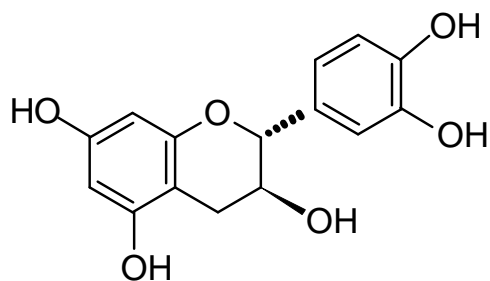
Figure 1.43. Structure of catechin.
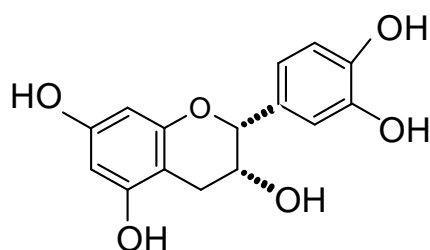
Figure 1.44. Structure of epicatechin.
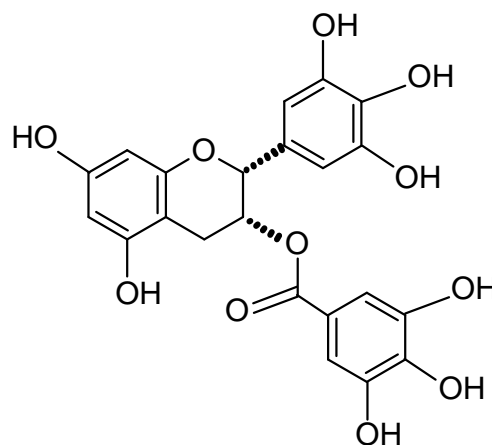
Figure 1.45. Structure of epigallocatechin gallate.

Polyphenolics in marine brown algae occur as a single structural class, the phlorotannins, which are produced by the polymerisation of phloroglucinol (1,3,5 trihydroxybenzene) (Ragan & Glombitza, 1986) and synthesised via the acetate–malonate pathway. They have a broad range of molecular sizes (126 Da-650 kDa) and are analogous to the terrestrial condensed tannins as they do not contain a carbohydrate core. Based on the type of structural linkages that result from the polymerisation process and on the number of hydroxyl groups, they can be divided into six categories (Targett & Arnold, 1998): fucols, phlorethols, fucophlorethols, fuhalols, isofuhalols, and eckols. Vesicles associated with brown algal cells, the physodes, contain most, if not all, of the phenolics found in brown algae (Ragan & Glombitza, 1986).

Phlorotannins have principally been considered as defence chemicals against herbivory (Targett & Arnold, 1998). However, since phlorotannins have antimicrobial properties, absorb light and chelate heavy metal ions, protective and defensive roles other than defence against herbivory have also been suggested, e.g. defence against bacterial infections and fouling organisms and protection against UV-B radiation and toxic heavy metal ions (Toth & Pavia, 2000). Generation of increased phlorotannin levels has been observed as a response to natural herbivory, mechanical simulations of herbivory and increased intensities of UV-B radiation (Toth & Pavia, 2000).

The ability of brown algae (Phaeophyceae) to concentrate ions of heavy metals from the environment may make them useful indicators of marine heavy-metal pollution.

Although a small portion of the metal uptake is due to cell wall polysaccharides, including alginate, the major portion of metal (Zn) uptake is a gradual, long-term process probably localised within, polyphenol-rich vacuoles called physodes. There is evidence to suggest that normal, healthy brown algae exude these polyphenols into the surrounding seawater (Ragan *et al.*, 1979, Abdala-Díaz *et al*., 2006).

Previous research has already observed that yellow-coloured organic compounds exuded from several algae, including *A. nodosum* and *F. serratus*, can form complexes with Cu(II) ions in seawater. Exuded polyphenols may therefore act as natural organic chelators, increasing or decreasing the availability of metal ions to phytoplankton and other marine organisms (Ragan *et al.,* 1979).

Ragan *et al.* (1979) demonstrated that high-molecular-weight polyphloroglucinols extracted from the brown algae *A. nodosum* and *F. vesiculosus* (and thought to be very similar to the naturally exuded polyphenols), can chelate a broad range of divalent cations in weakly acidic aqueous solution.

Several investigators have subsequently demonstrated that brown and red algae exude polyphenols which are able to form complexes with a wide range of divalent cations. For example, a previous study by Seeliger & Edwards (1979) showed that two benthic red algae (*Ceramium pedicellatum* and *Neogardhiella baileyi*) store Cu in a Cu-enriched medium, and release it in an organic form in a Cu-poor medium. Furthermore, in a study by Sueur *et al.* (1982), it was shown that various amounts of complexing ligands were released by three macroalgae tested: *Ectocarpus siliculosus* (brown algae), *Antithamnion spirographidis* (red algae), and *Audouinella purpurea* (red algae). The presence of these strong chelating compounds may well explain the obvious tolerance of *Ectocarpus* and the red seaweeds to metal toxicity as well as their well known ship-fouling potentialities (Sueur *et al.,* 1982).

Conversely other studies have shown a low percentage association of Zn and Cd with the polyphenolic fraction of *P. gymnospora* from Sepetiba Bay, a coastal area contaminated by heavy metals in Rio de Janeiro State (Brazil) (Amado Filho *et al*., 1999). Subsequently it was suggested that the high Zn and Cd found in algae from polluted areas (like Sepetiba Bay) might thus be greater than the capability of polyphenols to bind these metals (Amado Filho *et al*., 1999). A study by Lavid *et al.* (2001) demonstrated the involvement of phenols in Cd accumulation in aquatic plants.

Polyphenols in *Nymphaea* chelate Pb, Cr and Hg *in vitro*. Ragan *et al.* (1979) found that brown algal phlorotannins have a large capacity to chelate divalent metal ions (especially Cu^{2+} and Pb^{2+}), implying that they may be key chelators to heavy metals in seawater and *in vivo* in the physodes of the seaweeds. Toth & Pavia (2000), reported that, metal-sensitive phytoplankton species demonstrated an increased resistance to elevated concentrations of Zn when phlorotannins were in the medium, signifying the ability of phlorotannins to reduce the toxicity effect of metal ions in seawater (Toth & Pavia, 2000). It was demonstrated that the major part of the Zn ions accumulated in *A. nodosum (L.) Le Jol.*, was transferred through one or more membranes to vacuoles within the cells. The metal ions were irreversibly chelated to metal binding compounds. It was suggested by the authors that these substances may well be polyphenols located in physodes (Toth & Pavia, 2000). High concentrations of Cd and Cu have also been found in the physodes of *F. vesiculosus* and in extracted phlorotannin fractions from *P. gynjnospora*. This endorses the idea that phlorotannins can function as an internal detoxification mechanism in these species (Toth & Pavia, 2000).

Toth *et al.* (2000) studied the potential role of phlorotannins as an inducible and internal detoxification mechanism in the brown seaweed *A. nodosum (L.) Le Jol*. The brown seaweed was exposed to increasing concentrations of Cu in the surrounding water. Results indicated that the phlorotannin concentration and growth of the *A. nodosum* was not affected by high Cu concentrations. However, *A. nodosum* exposed to high concentrations of Cu accumulated high levels of Cu in their tissues and furthermore, Cu appeared to be irreversibly bound in the tissues. The accumulated Cu however, was not specifically connected with phlorotannins in the physodes, given that only a small portion of the total tissue Cu concentration was found in the extracted phlorotannin fractions (Toth *et al.,* 2000). Previous studies however have found high concentrations of Cd and Cu in the physodes of *F. vesiculosus* (Lignell *et al.,* 1982). Results implied that phlorotannins bound to Cu ions, but whether the chelation of Cu to phlorotannins took place in the living cells of the seaweeds or in the solution of the extracted phlorotannins is unknown. The macroalgae exposed to the high Cu treatment had high tissue Cu contents and it is possible that part of the accumulated Cu was reversibly bound in the intercellular space of the seaweeds (Lignell *et al.,* 1982). Skipnes *et al.* (1975) suggested that a small fraction of the Zn uptake in *A. nodosum* was reversibly bound in the intercellular space of the seaweeds, and if this also applies to Cu, the reversibly-bound Cu may have chelated with the phlorotannins in solution

during the process of phlorotannin extraction. Moreover, there is a possibility that the seaweeds exude phlorotannins with bound Cu to the surrounding water as a way of detoxifying the tissues. Exudation of polyphenolic substances has been thought in earlier studies to play an important ecological role (Toth & Pavia, 2000). Results from Toth & Pavia (2000) suggested that phlorotannin exudation, is an inadequate method for Cu detoxification in *A. nodosum*, given that the Cu concentration in algal tissue increased when the seaweeds were exposed to elevated Cu levels in the surrounding water. Conversely, seaweed growth was unaffected throughout the experiments, suggesting that the elevated Cu concentration in the surrounding medium (10 and 50 pg L^{-1}, i.e., low, non-toxic concentrations) was not directly harmful to the macroalgae. This and the fact that the macroalgae accumulated high levels of Cu without synthesising higher levels of phlorotannins, indicates that other substances are more vital for metal resistance mechanisms in *A. nodosum*. Furthermore, a study by Plouguerné *et al.* (2006) demonstrated that both site and season significantly affected the phenolic contents in *Sargassum muticum*.

The environmental impact of organic substances released by marine macroalgae may not be restricted to one specific effect. For example, their strong chelating capacities play an important role by increasing or decreasing the availability of trace metals for other species and themselves. Furthermore, exuded material may also be responsible for toxic effects (Sueur *et al.*, 1982). Additionally, phenolic compounds are also thought to avert oxidative damage by scavenging active oxygen species and by breaking the radical chain reactions during lipid peroxidation. These antioxidative effects require the reduced form of phenolic compounds (Olivares, 2003).

Several species of marine algae are known to contain brominated compounds such as bromophenols (Whitfield *et al.*, 1999). Examples of these include the green alga *Codium fragile* and the red alga *Pterocladiella capillacea* (Whitfield *et al.,* 1999). Bromophenols have been shown to have antioxidative properties (Dummermuth *et al., 2003*). In a study by Whitfield *et al.* (1999) the mono-, di- and tri-bromophenol levels in macroalgae from East Australia were analysed. They demonstrated that tribromophenol was found in all seaweeds under investigation and was generally present in the highest concentrations. Furthermore, levels of the compound varied from species to species, for example levels were 0.9 ng/g ww in the green alga *Codium fragile* and 2590 ng/g ww in the red alga *Pterocladiella capillacea*.

1.5 Biochemical Impacts of Metal Exposure on Live Macroalgae (Algal Proteins and Polyphenols)

- *Heat Shock Proteins (HSPs)*

Metals and other environmental factors such as temperature and oxygen generate changes in the transcript levels of several gene encoding proteins (Burdon, 1986). Heat shock proteins are a collection of chaperones, which exclusively deliver metal ions to cell organelles and metal-requiring proteins. HSPs are extremely conserved and involved in maintenance of protein homeostasis within cells. Ubiquitous in nature, HSPs are found at constitutive levels and conserved in various species from bacteria to humans (Burdon, 1986). Four major stress protein families have been discovered and are divided according to their molecular weight, (HSP 90, 70, 60, and small HSP [SHSP] with 16–24 kDa) (Feder & Hofmann, 1999). HSPs can increase tolerance to heavy metal stress by preventing membrane damage (Spijkerman *et al.*, 2007). HSP 60 specifically, responds to redox-stress caused by metal toxicity (Lewis *et al.*, 2001). Heavy metals can impede the photosynthetic activity by increased photoinhibition from excess of light (Heckathorn *et al.*, 2004). Furthermore, the expression of HSP 70 in the marine macroalga *F. serratus* increased in response to Cd stress (Ireland *et al.*, 2004).

- *Glutathione, Phytochelatins and Metallothioneins*

Formation of PC-essential heavy metal complexes may be a component of a metal homeostasis mechanism, with PC acting as a chelator. Little is known however about PC synthesis in the presence of non-essential heavy metals for marine macroalgae even though these species have been used as ecological biomonitoring organisms for some time (Jervis *et al.,* 1997). Hu & Wu (1998) however identified PC as the major intracellular Cd chelators in the marine macroalga *Kappaphycus alvarezii*. PC isolated from this seaweed demonstrated a less complex thiol profile than that found in yeast and higher plants. In the general structure of (Gly-Cys)n-Gly for PC, the n value was less than three for this seaweed (Hu & Wu, 1998).

Additionally, in a study by Rijstenbil *et al.* (1998b), defence mechanisms against Cu toxicity were examined in two dominant *Enteromorpha* species. The macroalgae were collected at three locations in the Scheldt Estuary (Netherlands, Belgium) and the

Thermaikos Gulf (Greece). For 10 days *E. prolifera* (Scheldt) and *E. linza* (Thermaikos) were incubated in seawater media at different salinities: 6, 9, 23 psu and 25, 30, 35 psu, respectively. In one experiment, media were enriched with 100 μg Cu/L and responses were compared with those of controls with no extra Cu added. *Enteromorpha*, which is regularly used as a monitor species for heavy metal contamination, were found to have relatively high Cu tissue levels (0.5-3.8 μmol/g dw, Cu). Cu levels in *E. prolifera* controls (Scheldt) decreased with salinity. This however was not the case with Cu levels in *E. linza* controls (Thermaikos). During the 10-d incubation the seaweed protein contents and tissue Cu were relatively stable. In *E. linza* (Thermaikos) seaweed protein contents were significantly lower than those of *E. prolifera* (Scheldt), although there was no indication for N limitation in *E. linza*. *E. linza* also had much lower glutathione pools than *E. prolifera*. Only under acute Cu stress (metal addition) did *E. prolifera* synthesise phytochelatins. Results from this study demonstrated that phytochelatin pools are not suitable as an indicator of the Cu levels in these algae. The GSH redox ratio GSH: (GSH + 0.5GSSG) was used as an indicator of Cu-induced oxidative stress. In *E. prolifera* (Scheldt) the ratio decreased with algal Cu content, from ~0.5 to ~0.2. The average GSH ratios in *Enteromorpha* from the Scheldt and Thermaikos demonstrated some oxidative stress induction with increasing algal Cu contents. The GSH redox ratio may be affected by environmental factors such as irradiance and desiccation, it may therefore not be a useful indicator for Cu-induced oxidative stress in situ (Rijstenbil *et al.,* 1998b).

Furthermore, Malea *et al.* (2006) studied the effects of Cd, Zn and nitrogen (N) status on the pools of metal-binding non-protein thiols: glutathione and phytochelatins of the macroalgae *E prolifera* and *E. linza*. The seaweeds were incubated at three salinities with 100 and 200 μg/L Cd and Zn. In *E. linza*, ammonium pools were found to be higher, but amino acid pools, total N and protein concentrations were lower than in *E. prolifera*. Reduced glutathione (GSH) pools correlated positively with free glutamate and protein contents. In *E. linza* GSH pools increased and the ratio of reduced to oxidised glutathione (an indicator of oxidative stress), decreased with Cd content, indicating Cd-induced glutathione oxidation. Cadmium stimulated PC synthesis in *E. prolifera* which implies that in N-rich algae, GSH pools were sufficiently high for PC synthesis. In both seaweeds the GSH and protein concentration increased with Zn contents, whereas GSH:(GSH + 0.5GSSG) decreased. This would indicate Zn-induced oxidative stress. PCs were not synthesised in response to Zn exposure and this may

have resulted in Zn-induced GSH oxidation. The presence of both oxidative effects (Cd, Zn) and detoxification (Cd) were identified by observing the responses of GSH and PC pools to metal stress. The overall conclusions from this study demonstrated that *E. linza* stored high ammonium reserves, *E. prolifera* had lower inorganic-N reserves, higher cellular amino-acid pools and higher protein concentrations. Following Cd exposure, GSH pools of *E. linza* (with low protein contents) increased, but were prone to oxidation which indicates Cd-induced oxidative stress. In *E. prolifera*, Cd only induced PC-synthesis (active detoxification). Zn did not induce PCs, but caused an increase of glutathione pools, in particular of GSSG, causing a decrease of its redox ratio, i.e., Zn caused oxidative stress in both *Enteropmorpha* species (Malea *et al.,* 2006).

Pawlik-Skowrónska *et al.* (2007) studied the amount of phytochelatins (PC) in natural populations of brown, red and green macroalgae. Concentrations of PCs and GSH were measured in seaweeds collected from locations in south-west England affected by various levels of trace metals. The purpose of the study was to assess their role of PCs under natural ecological conditions. The ability of seaweeds to produce PCs following exposure to Cd and Zn was also assessed experimentally. Furthermore, the concentrations of various metals (As, Cu, Cd, Pb and Zn) in seaweeds were also determined. Results demonstrated the presence of PCs, in native Phaeophyceae (*Fucus* spp.), Rhodophyceae (*Solieria chordalis*) and Chlorophyceae (*Rhizoclonium tortuosum*) but not in thalli of *Ulva* spp. and *Codium fragile* (Chlorophyceae). The concentrations of PCs in brown and red seaweeds corresponded to the total metal concentration of seaweed thalli. The highest concentrations of metals, PCs and GSH and the longest PC chain lengths (PC2–4) were associated with *Fucus* spp. collected from the most polluted site. It was found that a combination of PC-synthesis and maintenance of high GSH levels allowed the *Fucus* spp. and *R. tortuosum* to flourish in highly polluted environments, whereas high GSH levels, together with thick cells walls and a high polysaccharide concentration seem to be associated with metal-resistance in *Ulva* spp. The lack of PC production in the green seaweeds suggests lower intracellular metal accumulation rather than an inability to synthesise PCs. Higher concentrations of Cu found in thallus of *S. chordalis*, compared with those of *Fucus* spp. from the same site, may provoke stronger oxidative stress and result in lower concentrations of reduced GSH. As a result, *S. chordalis* at this site may have an inferior resistance to metals and a more restricted distribution than the brown species. Both fucoid species and the red

seaweed *Gracilaria gracilis* (but not *Ulva* spp. or *C. fragile)*, from low contaminated sites synthesised PCs under experimental conditions when exposed to very high concentration of Cd. Results from this study therefore demonstrate that natural assemblages of seaweeds, belonging to contrasting phylogenetic groups synthesise PCs when exposed to a mixture of metals in their environment. However, the involvement of thiol peptides in metal homeostasis, detoxification and resistance varies between species of seaweed that are growing under the same environmental conditions (Pawlik-Skowrónska *et al.,* 2007).

The metallothioneins (MTs) are a family of small, cysteine-rich proteins with the unique ability to generate stable metal-thiolate clusters surrounded by a protein backbone. The true role of MTs has not yet been fully established, but involvement in the homoeostasis of essential metal ions and/or the detoxification of heavy metals is suggested by the exceptional metal-binding properties of these proteins. The highly conserved nature of MTs suggests an important function which is essential to biological systems. MTs have been identified in a range of species, including invertebrates, plants, fungi and yeast (Morris *et al.,* 1999). In a study by Morris *et al.* (1999), a cDNA library was constructed from *F. vesiculosus* in order to examine the potential existence of a metallothionein (MT) gene in this species. Results demonstrated the presence of a gene (1229 bp) with an open reading frame (204 bp), which encoded a 67-amino-acid protein. This protein displayed several characteristic features of MT proteins, including 16 cysteine residues (24%) and one aromatic residue. The protein sequence was similar to that of plant and invertebrate MTs although it contained a unique ` linker ' region (14 amino acid residues) between the two supposedly metal-binding domains which contained no cysteine residues. This extended linker was different to those found typical vertebrate and plant MTs. Overall results demonstrated however, the presence of a MT gene, induced by copper exposure in *F. vesiculosus*.

o *Reactive Oxygen Species* (*ROS) Defense Enzymes*

The macroalga *Enteromorpha compressa* is the main species in coastal areas of Northern Chile receiving copper mine wastes. Copper is the main heavy metal in these coastal waters and is accumulated in *E. compressa* growing at the impacted sites. Algae from these sites demonstrated higher levels of lipoperoxides than from non-impacted sites. This suggests the occurrence of cellular damage resulting from oxidative stress.

A study by Ratkevicius *et al.* (2003) demonstrated that the green seaweed *E. compressa* capable of combating the effect of oxidative stress associated with a metal-enhanced environment by the activation of its antioxidant defences. The key evidence to support this finding comes from the enhanced levels of ascorbate peroxidise (AP) found in algal material from mine-impacted sites, in comparison with virtually undetectable levels in *E. compressa* from the non-impacted sites. Metals in general, are notorious for causing oxidative stress through the production of ROS. The enzyme AP is part of the ascorbate–glutathione pathway, which is thought to play a vital role in preventing cell damage caused by ROS (Ratkevicius *et al.,* 2003)**.**

Kumar *et al.* (2010) established the various biochemical processes involved in the alleviation of Cd toxicity in green alga *Ulva* spp. The seaweeds, when exposed to 0.4 mM $CdCl_2$ for 4 days, demonstrated a two fold increase in lipoperoxides and H_2O_2 content. Furthermore a decrease in growth and photosynthetic pigments by almost 30% over the control was also demonstrated. The activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione peroxidase (GPX) enhanced by two to threefold and that of catalase (CAT) diminished. Further, the isoforms of these enzymes, (Mn-SOD, ~85 kDa, GR, ~180 kDa and GPX, ~50 kDa) responded specifically to Cd^{2+} exposure. Additionally, the levels of reduced GSH and ascorbate increased significantly. Lipoxygenase (LOX) activity increased by two fold coupled with the induction of two new isoforms upon Cd^{2+} exposure. Overall results established an induction of antioxidant enzymes in Cd^{2+}exposed thallus and demonstrated their potential role in Cd^{2+} induced oxidative stress in *Ulva* spp (Kumar *et al*., 2010).

o *Polyphenols*

Metals are naturally present in seawater at low concentrations and some act as essential micronutrients (e.g., Cu, Zn and Fe) for marine biota but may become toxic when absorbed at high concentrations (Connan & Stengel, 2009). The metal binding capacity and especially cation binding, of brown algae is linked to polysaccharide contents in the cell wall (Davis *et al*., 2003) and to polyphenol (phlorotannin) contents (Ragan *et al*., 1979). Phlorotannins can be compartmentalised either in the physodes, free in the cytoplasm (Ragan *et al*., 1979; Ragan & Glombitza, 1986), or in the cell wall (Schoenwaelder, 2002; Koivikko *et al.,* 2005; Salgado *et al*., 2009). In situ, macroalgae

are exposed to a range of environmental conditions and phenolic contents have been previously reported to vary according to environmental factors. Some previous laboratory studies have shown that phenolic induction in brown algae can be stimulated by changes in irradiance, temperature and salinity (Ragan & Jensen, 1978; Ragan & Glombitza, 1986).

The physiological responses in brown seaweeds (*F. vesiculosus and A. nodosum*) and phenolic induction, composition, exudation and the potential of phenolics to chelate metals under natural environmental conditions were studied by Connan & Stengel (2009). The effect of copper enrichment and its interaction with salinity, temperature and desiccation and the effect of iron and zinc exposure, were also previously investigated (Connan & Stengel, 2009). Results demonstrated an increase in polyphenols at elevated salinity concentrations and only phenolics in *A. nodosum* showed seasonal variations. Following salinity experiments, phenolic exudation of *F. vesiculosus* was higher than that of *A. nodosum*. Salinity treatment had an effect on *F. vesiculosus* exudation only. Results for temperature treatments demonstrated that phenolic exudation was higher for *F. vesiculosus* and exudation increased at higher temperatures for both species. Combined temperature, salinity and desiccation experiments showed variations in the within-cell phenolic levels. Additionally, Cu concentration and salinity both affected the total phenolic contents with a decrease in the phenolic concentration at increased Cu concentration and decreased salinity. During the experiment the phenolic composition also changed, cell wall phenolics increased and intracellular phenolics decreased as a result of and increase in Cu concentration at a decrease in salinity levels. Overall results demonstrated that phenolic exudation was considerably effected by Cu concentration and salinity (Connan & Stengel, 2009).

1.6   Important Nitrogen-Containing Compounds Associated With Seaweeds

There are three varieties of pigments involved in algal photosynthesis, these are: chlorophylls, phycobiliproteins and carotenoids (carotenoids being C_{40} tetraterpenes). Chlorophyll *a* ($C_{55}H_{72}O_5N_4Mg$) is necessary in the reaction centre and is associated with all algae. Three extra chlorophylls are connected with seaweeds. Chlorophyll *b* ($C_{55}H_{70}O_6N_4Mg$) occurs in Ulvophyceae and in other Chlorophyta. Chlorophylls c_1 ($C_{35}H_{30}O_5N_4Mg$) and c_2 ($C_{35}H_{28}O_5N_4Mg$) is found in Phaeophyceae and other brown algae. These chlorophylls are tetrapyrrole rings with Mg^{2+} bound in the middle. Chlorophylls *a* and *b* each have a lengthy fatty-acid appendage. Besides being chemically different chlorophylls, especially chlorophyll *a*, bind to proteins to generate an even wider variety of pigments (Lobban & Harrison, 1997).

Approximately 30% of worldwide photosynthesis is performed by marine algae (Barrett & Anderson, 1977). Chlorophyll *a*, has the twofold purpose of primary photochemistry and light-harvesting in Photosystem I and II. In addition to Chlorophyll *a*, brown algae also contain the photo-accessory pigments, chlorophylls c_1 and c_2. Differences between Chlorophyll *c* and other chlorophylls are associated with the chlorophyll side chains (Barrett & Anderson, 1977). The greater fraction of chlorophyll is attached to proteins and these chlorophyll-protein complexes are the main fundamental proteins of inner chloroplast membranes (Thornber, 1975). The two major complexes that have been isolated are the P700-chlorophyll *a*-protein complex which is associated with PS I and the light-harvesting chlorophyll *a/b* protein complex which is photochemically inactive but transports light energy to PS II and PS I(Barrett & Anderson, 1977).

Phycobiliproteins are important light harvesting pigments associated with the chloroplasts of red macroalgae (Galland-Irmouli *et al.,* 2000). They are divided into three classes according to their absorption properties as follows: phycoerythrins, phycocyanins and allophycocyanins. These proteins are arranged into an organised cellular structure known as phycobilisome. The structure is made up of a core with a set of rod structures which emit from the core. Phycoerythrin is situated at the pinnacle of the rod, phycocyanin is found in the middle and allophycocyanin forms the core of the structure (Glazer, 1984). Phycobiliproteins absorb light from $450 - 650$ nm (an area where chlorophyll *a* absorbs poorly) and transfer the energy to Photosystem II (Glazer,

1984; Galland-Irmouli *et al.,* 2000). Energy is transferred from phycoerythrin to phycocyanin, then to allophycocyanin and subsequently to chlorophyll *a* (Glazer, 1984). This mechanism allows some red seaweeds to exist in moderately deep water (MacArtain *et al.,* 2007). Phycoerythrin is an important light-harvesting pigment found in red algae (Galland-Irmouli *et al*., 2000; MacArtain *et al*., 2007). They are highly water-soluble and can be divided into three major classes: B-phycoerythrin, R-phycoerythrin and C-phycoerythrin (variations are based on their absorption spectra). R-phycoerythrin is found most abundantly in red algae (Galland-Irmouli *et al.,* 2000). In a study by Galland-Irmouli *et al.* (2000) the phycoerythrin content of *P. palmata* corresponded to 12.2% dw of the total protein content.

1.7   Factors Affecting Bioaccumulation

Metal uptake may be influenced by several factors, including temperature (Fritioff *et al*., 2005), salinity (Wang & Dei, 1999), pH (Murphy *et al.,* 2008), as well as metal concentrations (Villares *et al*., 2002) and light and dark (Rice & Lapointe, 1981).

1.7.1   Temperature

Water temperature may influence water chemistry, metal solubility, and metal uptake by plants, and plant growth. Variations in temperature further change the composition of the plasma membrane lipids. This alters the plant membrane fluidity, resulting in lower membrane permeability at low temperatures and lower metal uptake. In the aquatic environment, the Cu adsorption to the alga *Dunaliella tertilecta* increases with increasing temperature. Therefore, a general increase of metal uptake with increasing temperature seems likely (Fritioff *et al*., 2005).

1.7.2   Salinity

In seawater, chloride commonly forms complexes with Zn, Cd, and, to a lesser extent, Cu, but not with Pb, and thus the free ion concentration of the former metals will be reduced. Fewer free Cd ions in water of higher salinity correlated with a lower uptake of Cd by the pondweed *Potamogeton pectinatus* (L.) growing in higher salinity water. At a higher salinity, the increased NaCl concentration reduced both intracellular and extracellular uptake of Cd in the free-floating plant, *Lemna polyrhiza* (L.). Also, the uptake of Zn by *F. vesiculosus* decreased with increasing salinity. Thus, a general

decrease in metal uptake with increasing salinity is expected (Fritioff *et al*., 2005). A decrease in salinity from 28 to 10% was found to enhance the uptake of Cd, Cr, selenium and Zn in *Ulva* sp by 1.9, 3.0, 3.6, and 1.9 fold, respectively (Wang & Dei, 1999). In the red seaweed *Gracilaria blodgettii*, Cd uptake increased two-fold when salinity was decreased from 28 to 10%. (Wang & Dei, 1999).

1.7.3   pH

pH greatly influences metal biosorption by seaweeds. Functional groups such as sulphonate ($-OSO_3$) and carboxyl ($-COOH$) exhibit acidic characteristics and therefore, the pH at which maximum metal uptake occurs is related to the pKa of these groups. The pH of the solution is an important parameter affecting biosorption of heavy metals (Murphy *et al.,* 2008). In a study by Lodeiro *et al.* (2005), the sorption of Cd increased from pH 1.0 to pH 4.0, reaching a plateau at pH around 4.0. However, at pH lower than 2.0 it was found that the Cd uptake ability was almost negligible. At pH values greater than 9, different hydroxyl species of low solubility can be produced. The pH dependence of Cd uptake is closely related to the acid–base properties of various functional groups on the algal cell surfaces, mainly carboxyl groups, and to the metal solution chemistry (Lodeiro *et al.,* 2005).

1.7.4   Seasonal Variation in Seaweed Metal Concentrations

A study by Villares *et al.* (2002), demonstrated the pattern of temporal variation in metal concentration of two green seaweeds. Highest concentrations were recorded in the May samples. Concentrations then fell with minimum values found between June and September. Subsequently, levels increased again during Autumn and Winter. Several reasons may be responsible for the seasonal variations found, including, environmental factors, such as variations in metal concentrations in solution, interactions between metals and other elements, salinity, pH, metabolic factors, such as dilution of metal contents due to growth, or they may be due to interactions between several factors. The suggestion that metal levels decrease in macroalgae during periods of growth and increase during the dormant period in Winter has long been considered (Fuge & James, 1974). Seasonal variation in metal concentrations of *F. vesiculosus* has been previously observed. The seasonal variation may have been due to growth, as concentrations were highest in Winter when growth is minimal and decreased in Summer when it is at its maximum (Villares *et al*., 2002).

The seasonal pattern of concentrations of several metals in *U. rigida* and *E. linza* was attributed to the growth effect and other factors such as the age of the tissue examined, and abiotic factors such as salinity and temperature, as well as variation in metal concentrations in the environment (Haritonidis & Malea, 1995). Wright & Mason (1999) found higher concentrations of metals in Winter in *Enteromorpha* sp*., and *Pelvetia canaliculata*. They also noted a similar pattern in metal levels in sediments and in various invertebrates, indicating that the seasonal patterns appear to be more influenced by environmental factors, such as discharges to the estuary, pH, etc., than by factors intrinsic to the organisms themselves, such as metabolism or reproduction (Villares *et al.*, 2002).

Higher concentrations of metals have previously been found during growth periods. A greater accumulation of several metals was observed in *U. lactuca* in Summer. These changes can be explained by the higher rates of photosynthesis and respiration during this season, which would favour the absorption of metals. Furthermore, the mobilisation of metals from the sediment in sites covered by vast amounts of macroalgae could contribute to this accumulation (Villares *et al.*, 2002). In a previous study, the temporal variations in metal concentrations in seaweeds have been exclusively attributed to variations in the levels of these elements in the surrounding water. In a study of five species of macroalgae, maximum values were found in the rainy season (Summer), this was attributed to potentially higher concentrations of metals in water due to an increase in terrestrial inputs (Lacerda *et al.,* 1985). In a study by Villares *et al.* (2002), it was concluded that the seasonal pattern of metal levels in seaweed could not only be attributed to variation in the concentrations of these elements in the surrounding environment, as it was unlikely for such similar changes to occur in all the metals studied.

1.7.5   Light and dark

The role of light in the accumulation of metals is the provision of metabolic energy (ATP) (Lobban & Harrison, 1997). This is evident from studies carried out by Rice & Lapointe (1981) which show increased accumulation of metals in light conditions compared to dark, indicating that energy is required in the uptake processes.

The effect of fluctuations in light intensities on metal accumulation varies between seaweed species due to differing systems used in accumulation. For example, seaweed

that relies on passive diffusion for metal accumulation requires less energy. Consequently, such seaweeds are expected to be located in the sublittoral zone. A high level of light can cause an increased demand for essential metals (and hence greater uptake) due to the promotion of growth (Kramer, 1994). A study by Wang & Dei (1999) measured the uptake kinetics of Cd and Zn in *U. lactuca*. It was shown that Cd and Zn uptake in *U. lactuca* was significantly inhibited by dark exposure.

1.8   Objectives of This Research

This research focuses on novel investigations into the metal uptake and biochemical changes in seaweed following heavy metal exposure. Such research may have positive, economic implications for society due to the potential sustainable exploitation of our natural resources for the generation of high value added products.

The overall objective of this research was to determine the biochemical effects of heavy metal exposure on a number of different seaweed species, including *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp. This assortment of seaweeds was chosen because of their widespread distribution along the South-East coast of Ireland. The metals under investigation in this study were chosen because of their significance in the local area (as previously outlined) and also due to the threat they pose to both plant and animal life.

The main objectives of this study were:

- To develop optimised methods for the total protein determination and the extraction of soluble proteins and polyphenols from *P. lanosa*.

- To perform an extensive environmental study of estuaries along the West coast of Newfoundland and the South East coast of Ireland. A diverse range of samples, seaweed, seawater and sediment, were collected during the course of the study and compared to physico-chemical site data.

- To explore and compare baseline metal (total, intracellular and surface bound), total protein and polyphenol levels of each of the main classes of seaweed associated with the various sites and locations under investigation. The seaweeds under investigation were *F. vesiculosus, F. ceranoides, A. nodosum, P. lanosa* and *Ulva* sp.

- To establish possible links between seawater metal contents and seaweed protein and polyphenol levels.

- To analyse trends in the metal content of estuarine sediment.

- To investigate the biochemical effect of heavy metal exposure on seaweeds, a number of heavy metal exposure studies were conducted. Time course studies were performed in order to determine the effects of heavy metal exposure on the protein, polyphenol and metal levels of seaweeds over several time periods. Exposure studies, examining the effect of varying heavy metal concentrations on seaweed protein, polyphenol and metal concentrations were also performed. Additionally, temporal studies were carried out in order to determine seasonal changes in total protein, extracted protein, polyphenol and metal levels in seaweeds prior to and following heavy metal exposure.

- To perform a preliminary study involving gel filtration chromatography, HPLC analysis and SDS gel electrophoresis in order to monitor changes in soluble proteins extracted from *P. lanosa* following heavy metal exposure.

In this novel study, various techniques were used to investigate the temporal, spatial and interspecies variations in protein, polyphenols and metals in seaweeds, prior to and following heavy metal exposure. Results from this research may therefore have positive global implications by informing the sustainable development of our natural resources.

Chapter 2

Development and Optimisation of Seaweed Protein and Polyphenol Quantitation Methods

2. Development and Optimisation of Seaweed Protein and Polyphenol Quantitation Methods

2.1 Introduction

Heavy metal resistance in algae is likely to involve polyphenols that chelate free metal ions either externally or intracellularly. Furthermore, a frequent outcome from heavy metal exposure is the synthesis of peptides such as GSH and PC. GSH acts as an antioxidant and PC bind to heavy metals intracellularly (Refer to Chapter 1 – 1.4.2). In order to fully comprehend these biochemical responses to heavy metals, it is necessary to have rapid, sensitive and reliable methods of extraction and detection. In this study therefore, seaweed protein and polyphenol extraction and quantitation methods were developed and optimised methods were subsequently utilised throughout the study.

2.1.1 Protein Extraction and Bradford Assay

The Lowry and Biuret assays are common methods used in protein determination however, both methods are prone to interferences by compounds such as Tris. Additionally, the Biuret reaction is a relatively insensitive method. Modified procedures designed to eliminate the above problems can further increase complications and assay time. The Bradford assay however eliminates most of the problems involved in the Lowry and Biuret procedures (Bradford, 1976). The method involving the binding of a dye (Coomassie Brilliant Blue G-250) to protein was developed by Bradford, 1976. The binding of the dye to protein causes a shift in the absorption maximum of the reddish brown dye from 365 nm to a blue colour at 595 nm (Bradford, 1976), (Figure 2.1). The dye-binding assay is based on the interaction of certain basic amino acids residues (primarily arginine, lysine, and histidine) with dissociated groups of Coomassie brilliant blue G-250 (CBB) in an acidic environment (Lozzi *et al.,* 2008). The Bradford assay is very reproducible and rapid with the dye binding process virtually complete in about two minutes, with good colour stability for one hour. Furthermore, there is little or no interference from cations or from carbohydrates. A minute quantity of colour is developed in the presence of strongly alkaline buffers but the assay can be run accurately by the use of appropriate buffer controls. The only compounds found to give excessive interferences are relatively large amounts of detergents such as sodium dodecyl sulphate (SDS), Triton X-100 and commercial

glassware detergents. Interference by small amounts of detergent can be eliminated by the use of proper controls (Bradford, 1976).

Histidine   **Coomassie G-50**

Blue

A_{max} = **595nm**
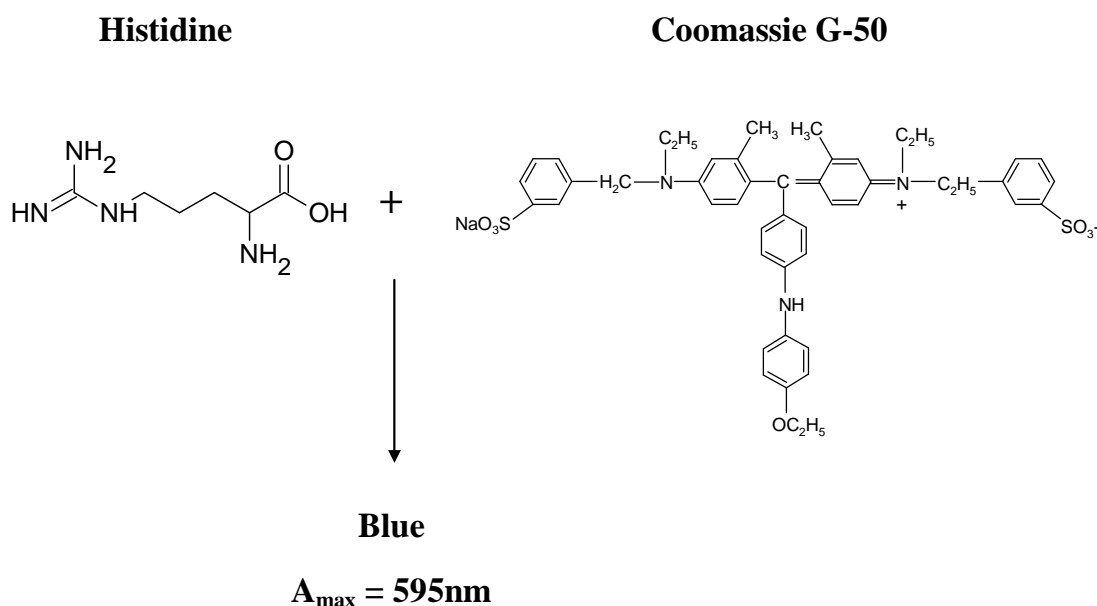
Figure 2.1. Schematic diagram of the Bradford reaction.

The main disadvantage of Bradford assay is its incompatibility with surfactants at concentrations regularly employed to solubilise membrane proteins. Ordinarily, the presence of a surfactant in a sample (even at low levels) causes precipitation of the reagent. Since the Coomassie dye reagent is extremely acidic, a small quantity of proteins cannot be assayed with this reagent due to their poor solubility in the acidic reagent (Thermo Fisher Scientific, 2009).

Protein extraction from most seaweeds is difficult as a result of the presence of large amounts of anionic cell-wall polysaccharides, including the alginates of the Phaeophyceae or the carrageenans of some Rhodophyta. The high concentration of neutral polysaccharides (e.g. xylans and cellulose) in some red or green seaweeds can also limit protein accessibility (Fleurence *et al.,* 1995a). Several protein extraction methods using a number of different extraction solvents have previously been performed (Table 2.1). Results from these previous experiments demonstrated that highest protein yields were obtained using a 0.1 M NaOH/0.5% mercaptoethanol solution (Ex. 5). The denaturing effect of NaOH and β-mercaptoethanol however, on

the tertiary structure of proteins suggests a limited and controlled use of this protocol (Fleurence *et al.,* 1995a).

Table 2.1. Influence of extraction procedures on protein yield from *U. rigida* and *U. rotundata*. Results expressed as % of total protein (Proteins extracted/Total proteins x 100), Fleurence *et al.* (1995a).

Extract. Number	*Extraction Procedure*	*Proteins extracted from total proteins of U. rigida (%)*	*Proteins extracted from total proteins of U. rotundata (%)*
1	Deionised Water	9.73±0.6	14.00±1.8
2	0.1 M Tris HCl Buffer	9.37±1.6	13.80±1.2
3	0.1 M Tris HCl Buffer with sonication	10.35±0.8	16.08±0.9
4	1st Extraction with 0.1 M Tris HCl and 2nd Extraction with 0.1 M NaOH in the presence of 0.5% β-mercaptoethanol	17.50±1.3	25.17±1.9
5	1st Extraction with deionised water 2nd Extraction with 0.1 M NaOH in the presence of 0.5% β−mercaptoethanol	26.80±1.3	36.10±1.4
6	Aqueous polymer 2-phase system (PEG/K$_2$CO$_3$)	19.10±1.1	31.56±2.1
7	Cellulase (Polysaccharidase)	<1.00	<1.00
8	Polysaccharidase mixture	18.48±2.1	22.00±1.5

An experimental method therefore allowing for the removal of algal polysaccharides in mild conditions would be helpful for the recovery of the native protein. The use of algal cell wall degradation enzymes to assist the extraction and the study of proteins has been tested on numerous algae (Fleurence, 1999b). A study performed on the red algae *Chondrus crispus, Gracilaria verrucosa* and *Palmaria palmata* suggested different behaviour of algal material according to the enzymatic procedure and the species used.

The simultaneous application of carrageenase and cellulase activities on *C. crispus* resulted in a 10-fold increase in extraction efficiency of the proteins. For *G. verrucosa*, the combined use of agarase and cellulase caused a three-fold increase in protein yield. In contrast, the use of xylanase and cellulase to *P. palmata* gave a protein yield similar to control values (extraction without enzymes) (Fleurence *et al*., 1995b). The application of cell wall enzymatic degradation resulted in different results even though the species belonged to the same phylum. Other studies performed on the *Ulva* genus demonstrated the great efficiency of a commercial enzymatic preparation, including cellulases, hemicellulases and S-glucanases, to amplify protein solubilisation. In this particular case, the recovery yield of proteins following the enzymatic procedure was similar to that of extraction in alkali medium with efficiency of the enzymatic procedures differing according to the species.

The use of polysaccharidases as extractive auxiliaries would appear, therefore, to be an effective technique facilitating access to the algal protein fraction in native or mild conditions. There is however, one main disadvantage to this method. The enzymatic approach needs to be validated and optimised for every seaweed species due to differences in the cell wall composition (Fleurence, 1999b). Because of the necessity to optimise the time consuming extraction of protein from different seaweeds using enzymes, more general, basic and less time consuming methods of algal protein extraction were undertaken during this study.

Various different methods of seaweed protein extraction have previously been performed and subsequent analysis carried out by means of the Bradford assay (Vergara *et al*., 1995; Lee *et al*., 1999; Taylor *et al*., 2002; Dere *et al.,* 2003; Gordillo *et al.,* 2006; Joubert & Fleurence, 2008).

2.1.2   Polyphenol Extraction and The Folin-Ciocalteau Assay

Factors that contribute to the efficiency of solvent extraction include: the type and volume of solvent, temperature, number of steps and particle size of sample. Methanol is the solvent most commonly employed in polyphenol extraction. It has been used to extract flavanones, flavones, flavone-glycosides, methoxyflavones and flavone dimers. Methanol 70-80% has produced good yields in extracting hydroxycinnamic derivatives, flavones, flavonols and catechins (Escribano-Bailón & Santos-Buelga, 2003). Other

extraction solvents including water and ethanol (Lapornik *et al*., 2005) and acetone (Wang *et al.,* 2009) have also been used.

A study by Lapornik *et al.* (2005) compared the effect of extraction solvent and extraction time on total polyphenols extracted from red currant, black currant and grape marc. All different materials were extracted with three solvents and extraction times (Table 2.2). The efficiency of extraction was determined by measuring the yield of extracted total polyphenols. Overall results demonstrated that the content of extracted polyphenols varied according to the using different materials, solvents and extraction times (Table 2.2). For example, in water extracts of red currant and black currant, the content of polyphenols decreased with the extraction time, whereas in water extracts made of grape, the yield of polyphenols increased considerably with time. In addition, ethanol and methanol extracts of red and black currant increased quite substantially over 12 hours.

Table 2.2. Effect of extraction solvent and extraction time on total polyphenols extracted from red currant black currant and grape marc (Lapornik *et al*., 2005).

Solvent	*Extraction time* (*h*)	*n*	*Total Polyphenols* (*mg/l*)		
			Red currant	*Black currant*	*Grape*
Water	1	3	696.4 ± 11.2	3219.2 ± 3.6	193.6 ± 2.9
Water	12	3	540.3 ± 9.3	3769.6 ± 10.5	340.2 ± 5.6
Water	24	3	401.8 ± 7.5	2426.1 ± 6.4	951.0 ± 3.5
70% Ethanol	1	3	884.1 ± 5.2	6135.7 ± 11.8	801.3 ± 2.9
70% Ethanol	12	3	1166.4 ± 8.6	7092.3 ± 5.2	1448.6 ± 5.6
70% Ethanol	24	3	1151.9 ± 8.5	7557.9 ± 12.6	5790.1 ± 5.9
70% Methanol	1	3	826.4 ± 6.3	7455.2 ± 9.1	974.4 ± 8.9
70% Methanol	12	3	1165.5 ± 6.7	6972.2 ± 13.5	2025.5 ± 8.6
70% Methanol	24	3	1145.3 ± 4.6	9721.2 ± 6.3	6782.8 ± 7.6

Several methods have previously been used for the extraction of polyphenols from seaweeds (Zhang *et al*., 2006; Connan *et al*., 2007; Chew *et al*., 2008; Wang *et al*., 2009) however, the Folin Ciocalteau assay has been widely used in quantifying the level of phlorotannin content in algal materials (Zhang *et al.,* 2006).

Among the several assays available to quantify total polyphenols, the Folin-Denis method is one of the most commonly used. The technique is based on a colour reaction between easily oxidised polyphenols or hydroxylated aromatic compounds and phosphotungsten-polymolybdic acid (Zhang *et al*., 2006). The method was later improved by Folin and Ciocalteu in 1927 with the addition of lithium sulphate to the reagent to prevent precipitation in the reaction in order to increase the sensitivity (Zhang *et al*., 2006).

The Folin-Ciocalteau reagent, a combination of phosphotungstic ($H_3PW_{12}O_{40}$) and phosphomolybdic ($H_3PMo_{12}O_{40}$) acids, is reduced to blue oxides of tungstene (W_8O_{23}) and molybdene (Mo_8O_{23}) during phenol oxidation. This reaction occurs under alkaline condition supplied by sodium carbonate. The intensity of blue colour is an indication of the quantity of phenolic compounds (Azlim Almey *et al.,* 2010). In general, the Folin-Ciocalteau reagent works by measuring the quantity of the substance being tested required to inhibit the oxidation of the reagent (Vinson *et al.,* 2005), (Figure 2.2). The reagent however, does not only measure total phenols but will react with any reducing material (Suh *et al.,* 2010). The reagent therefore measures the total reducing capability of a sample, not only the level of phenolic compounds.

This reagent forms part of the Lowry protein assay and will also react with some nitrogen-containing compounds such as hydroxylamine and guanidine (Ikawa *et al.,* 2003).
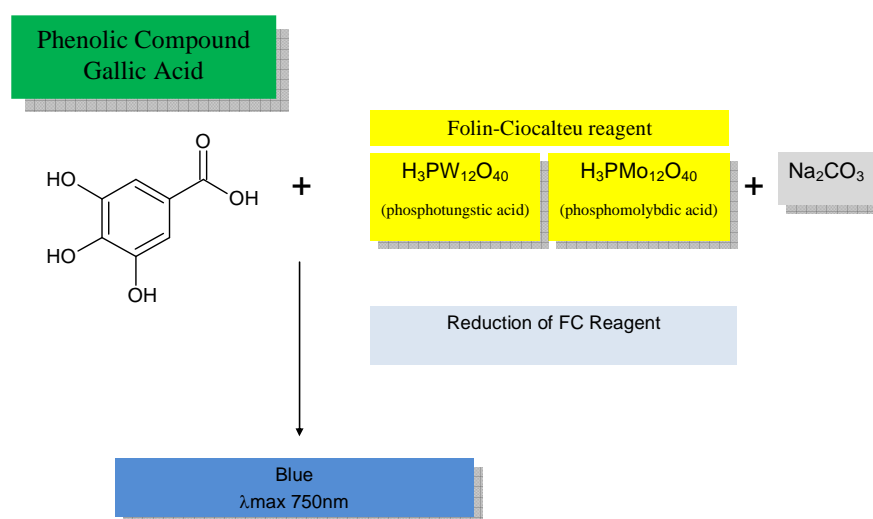
Figure 2.2. Schematic diagram of the Folin-Ciocalteau reaction.

2.1.3   Total Protein Determination and Kjeldahl Assay

One of the main problems associated with protein analysis in algae is protein extraction, executed with different degrees of success by researchers (Fleurence *et al*., 1995b; Joubert & Fleurence, 2008).   Differences in cell wall composition of algae and in procedures for protein extraction can create strong negative effects on the final results (Fleurence 1999b).   Furthermore, the most common methods used for protein determination (Lowry *et al*., 1951; Bradford, 1976) show a large amount of interference. Such interferences are a consequence of the effects of some chemical substances on specific amino acids, since the chemical reactions that produce the quantification of protein result from reactivity with side groups of the amino acids.   This means that the amino acid composition of each species is a key factor in the results obtained with different methodologies because of their distinct reactivities (Lourenco *et al*., 2002).

The utilisation of nitrogen-to-protein conversion factors to establish protein content has some important advantages over other methodologies.   Total nitrogen analysis, carried out by the Kjeldahl method, is rapid and inexpensive.   Total nitrogen content can be converted to crude protein with a high level of accuracy (Lourenço *et al.,* 2002).   In addition, there is no need for sample extraction, a major problem in protein analysis (Fleurence *et al*., 1995b).   Previous results have shown that algal proteins associated with cell membranes are barely extracted, proving the difficulty of reproducing extraction figures and increasing variations in values found with different methods. Thus, the use of Nitrogen-Protein factors also permits improved comparisons of results among researchers, since protein is estimated without a difficult extraction.   The use of the traditional factor of 6.25 (Jones, 1931), largely used for food labelling and other purposes, is based on the assumption that samples contain protein with 16% nitrogen and an insignificant quantity of non-protein nitrogen (Lourenco *et al*., 2002).

The Kjeldahl method is a way of determining the nitrogen content of organic and inorganic substances.   Although the method and apparatus have been altered significantly over the past 100 years, the basic principles introduced by Johan Kjeldahl endure today.   The Kjeldahl method is divided into three main steps:

Digestion - the breakdown of nitrogen in organic samples using a concentrated acid solution. This is achieved by boiling a homogeneous sample in concentrated sulphuric acid. This results in an ammonium sulphate solution (2.1.3.1).

Distillation – the addition of excess base to the acid digestion mixture to convert NH_4 to NH_3, followed by boiling and condensation of the NH_3 gas in a receiving solution (2.1.3.1).

Titration - to quantify the amount of ammonia in the receiving solution. The amount of nitrogen in a sample is then calculated from the amount of ammonia ions quantified in the receiving solution (2.1.3.1).

The % protein is calculated by multiplying the % N by a factor, the magnitude of the factor depends on the sample matrix (Labconco, 2009).

2.1.3.1 Chemical reactions involved in the Kjeldahl Assay

- *Digestion*

$$\text{Organic N} + H_2SO_4 \longrightarrow (NH_4)_2SO_4 + H_2O + CO_2 \quad \textbf{(Equation: 2.1)}$$
$$+$$
$$\text{other sample matrix by-products}$$

- *Distillation*

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow NH_3 \uparrow + Na_2SO_4 + 2H_2O \quad \textbf{(Equation: 2.2)}$$
(Ammonium Sulfate)

- *Titration*

$$NH_3 + H_3BO_3 \longrightarrow NH_4^+: H_2BO_3^- + H_3BO_3 \quad \textbf{(Equation: 2.3)}$$
(Ammonia Gas + Boric Acid) (Ammonium-Borate Complex) (Excess Boric Acid)

$$2NH_4H_2BO_3\text{-} + H_2SO_4 \longrightarrow (NH_4)2SO_4 + 2H_3BO_3 \quad \textbf{(Equation: 2.4)}$$
(Ammonium-Borate Complex) Ammonium-Sulphate Boric Acid
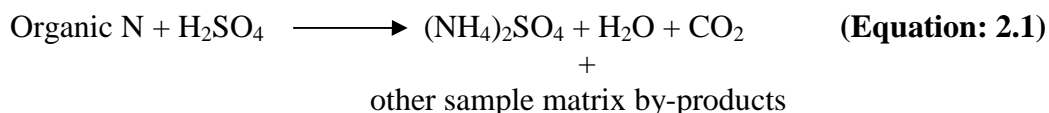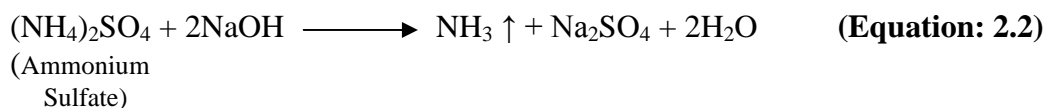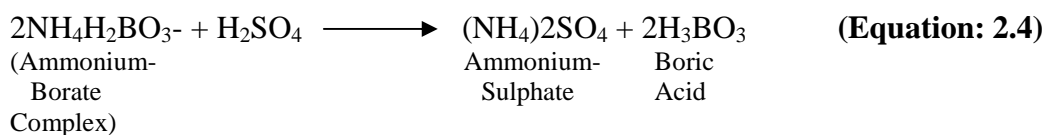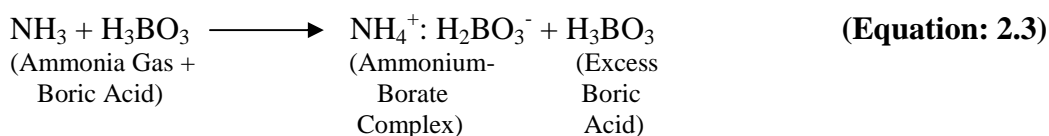
The protein content of seaweeds varies from species to species. In general, the protein fraction of brown seaweeds is low (3-15% of the dry weight) compared with that of the green or red seaweeds (10-47% of the dry weight) (Fleurence, 1999a). The use of nitrogen-to-protein conversion factors to establish protein content has some significant advantages over other methodologies. The total nitrogen analysis, of the Kjeldahl method (AOAC 1990) is fast and inexpensive. Total nitrogen from CHN analysis can subsequently be converted to crude protein with a high degree of accuracy. In addition, there is no need for the extraction of the sample to be analysed, a major problem in protein analysis. Previous results have shown that algal proteins associated with cell membranes are barely extracted, thus confirming the difficulty of reproducing extraction results and increasing differences in values found with different methods. The use therefore of nitrogen to protein factors permits superior comparisons of results among researchers (Lourenço *et al*., 2002).

The Kjeldahl method has been performed by many researchers for the determination of total protein content in seaweed samples. These include *Laminaria ochroleuca* (Sánchez-Machado *et al.,* (2004)); *Sargassum vulgare* (Marinho-Soriano *et al*., (2006)) and *Undaria pinnatifida* (Dawczynski *et al.,* (2007)).

2.2   Objectives of Research

The aim of this study was to develop optimised methods for the extraction and quantitation of soluble proteins, total protein and polyphenols in the red macroalgae, *P. lanosa*. Several studies have previously utilised different protein and polyphenol extraction methods with variable results. This unique study however specifically examines the various different extraction parameters including, extraction solvent, temperature and duration, in order to optimally quantify protein and polyphenol levels in seaweeds.

- Protein extraction optimisation methods involved the use of different extraction solvents (deionised water, methanol and NaOH in the presence of β-mercaptoethanol), durations (1, 16 and 24h) and temperatures (4°C and room temperature) with results from this study compared with data from previous work.

- Polyphenol extraction optimisation methods also involved variations in the above parameters such as, the use of different extraction solvents (deionised water, methanol, acetone), durations (1, 16 and 24h) and temperatures (4°C and room temperature) with optimised results compared with those from previous studies.

- The Kjeldahl method was used to determine the total protein content of wet and dry seaweed. Comparisons were then made between wet and dry *P. lanosa* for the total protein value in order to determine if oven drying had a negative effect on the overall protein content of seaweeds. Total protein values established in this study were then compared with those in the literature.

2.3 Experimental

2.3.1 Chemicals

- Kjeldahl Antifoam Tablets – Code: A001-10 (Lennox, Dublin, Ireland)

- Kjeldahl Catalyst Tablets (Kjeltabs S) – Code: AA08 (Lennox, Dublin, Ireland)

- Concentrated Sulphuric Acid – 'AnalaR' sp. Gr. 1.84 (AGB Scientific, Dublin, Ireland)

- Boric Acid Solution (with 4% BDH '4.5' indicator) (AGB Scientific, Dublin, Ireland)

- 30% Sodium Hydroxide – 1.33 rectapur (AGB Scientific, Dublin, Ireland)

- Hydrochloric Acid (Solid) (Ridel de Haën, Germany)

- Sodium Hydroxide (Solid) (Ridel de Haën, Germany)

- β-Mercaptoethanol – ≥ 98% (BDH / Lennox, Dublin, Ireland)

- Bradford Reagent (Sigma Aldrich Ltd., Dublin, Ireland)

- Bovine Serum Albumin Standard (Sigma Aldrich Ltd., Dublin, Ireland)

- Methanol–HiPerSolv Chromanorm HPLC Isocratic Grade (AGB Scientific, Dublin, Ireland)

- Folin-Ciocalteu's Reagent – (AGB Scientific, Dublin, Ireland)

- Gallic Acid Standard – Product No. 305124D (AGB Scientific, Dublin, Ireland)

- Acetone – min 99% (Ridel de Haën, Germany)

2.3.2 Instrumentation

- Retsch Mill – MM 301 (Retsch GmbH, Germany)

- Büchi Digestion Flasks (Mason Technology, Dublin, Ireland)

- Büchi Digestion Unit (K-424) (Mason Technology, Dublin, Ireland)

- Büchi Distillation Unit (K-350) (Mason Technology, Dublin, Ireland)

- Platform Shaker – STR 6 (Stuart Scientific)

- Refrigerated Superspeed Centrifuge – RC-5B (Dupont Instruments)

- μQuant Microplate Reader (Medical Supply Co., Ltd., (MSC), Dublin, Ireland)

2.3.3 Seaweed Collection and Preparation

P. lanosa was collected from Fethard-on-Sea Co. Wexford, Ireland (52.39°N, 6.94°W), November 2007 (Figure 2.3). The location at Fethard-on-Sea was selected because of the relatively low metal content in both seawater and seaweed (established through preliminary work by the Estuarine Research Group (ERG) at Waterford Institute of Technology) and also because of the relative abundance of the seaweed species at this site.

The samples were rinsed thoroughly on site with seawater in order to remove any adhering debris. The plants collected from various locations on the site were combined to give composite batches. Once harvested, the seaweed was placed in tubs containing seawater and transported to the laboratory within 3 hours.

On returning to the laboratory, samples were thoroughly rinsed in deionised water and blot dried. Samples were divided into two portions. Each portion was subsequently divided into triplicate samples. One set of triplicates was frozen at -20°C and the other set of triplicates oven dried (in triplicate) to a constant weight at 60°C for 24 hours (Dawes *et al.*, 1973). The weight of samples before and after drying was recorded. The oven-dried portion was ground in a mill to give a particle size ≤ 500 μM (Murphy *et al.*, 2007). These samples were then stored in screw-top polyethylene bottles, at room temperature, until required.

Figure 2.3. Seaweed collection location, Fethard-on-Sea, Co. Wexford, Ireland (Google Earth, 2010).

2.3.4 Protein Extraction

In order to optimise the protein extraction and quantitation method, several different soluble protein extractions were performed (Table 2.3).

Table 2.3. Summary of Protein Extraction Methods for *P. lanosa.*

		Protein Extraction Optimisation Summary		
Assay No.	Seaweed Condition	Solvent	Temp. (^{o}C)	Incubation Time (h)
1	Wet	Water	4	24
2	Powder	Water	RT	24
3	Wet	Water	RT	24
4	Wet	70% MeOH	RT	24
5	Powder	0.1M NaOH	RT	1
6	Powder	Water/ 0.1 M NaOH / 0.5% β−mercaptoethanol	4 & RT	16 and 1 (2 extracts)
7	Powder	Water/ 0.1 M NaOH/ 0.5% β−mercaptoethanol	4 & RT	16 and 1 (5 extracts)

2.3.4.1 Extraction 1

Frozen seaweed samples were thawed and blot dried. 1g of seaweed was transferred in triplicate to a chilled mortar. Approximately 1g of lab-grade sand was added to the sample with 30ml of deionised water and the mixture ground. Extraction of the wet samples was carried out in triplicate for 24 hours at 4°C. Samples were centrifuged at 11,000 *x g* for 20 minutes and the supernatants collected. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated at 4°C until analysis was carried out (Table 2.3, Assay 1).

2.3.4.2 Extraction 2

0.250 g of algal powder was extracted in triplicate with 30 ml of deionised water. Following incubation at room temperature (RT) for 24 hours, samples were centrifuged at 11, 000 x g for 20 minutes at 4°C. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated at 4°C until analysis was carried out (Table 2.3, Assay 2).

2.3.4.3 Extraction 3

Frozen seaweed samples were thawed and blot dried. 1g of seaweed was transferred in triplicate to a chilled mortar. Approximately 1g of lab-grade sand was added to the sample with 30ml of deionised water and the mixture ground. Extraction of the wet samples was carried out in triplicate for 24 hours at room temperature (RT). Samples were centrifuged at 11,000 x g for 20 minutes and the supernatants collected. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated at 4°C until analysis was carried out (Table 2.3, Assay 3).

2.3.4.4 Extraction 4

Frozen seaweed samples were thawed and blot dried. 1g of seaweed was transferred in triplicate to a chilled mortar. Approximately 1g of lab-grade sand was added to the sample with 30ml of 70% methanol and the mixture was ground. Extraction of the wet samples was carried out in triplicate for 24 hours at RT. Samples were centrifuged at 11,000 x g for 20 minutes and the supernatants collected. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated at 4°C until analysis was carried out (Table 2.3, Assay 4).

2.3.4.5 Extraction 5

0.250 g algal powder was extracted in triplicate with 30 ml of 0.1M NaOH solution. Following incubation at room temperature (RT) for 1 hour, samples were centrifuged at 11,000 x g for 20 minutes at 4°C. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated at 4°C until analysis was carried out (Table 2.3, Assay 5).

2.3.4.6 Extraction 6

0.200g algal-powder was suspended in deionised water (20 ml), in triplicate, to allow cell lysis by osmotic shock and facilitate protein extraction. The suspension was gently stirred overnight at 4 °C. After incubation, the suspension was centrifuged at 11,000 *x g* for 20 min and the supernatant collected for the assay of proteins. The pellet was resuspended with 20ml of 0.1 M NaOH in presence of β-mercaptoethanol (0.5% v/v). The mixture obtained was gently stirred at room temperature for 1 h before centrifugation (11,000 *x g* for 20 min). The supernatant was collected and combined with the supernatant of the first centrifugation. Samples were filtered using Whatman filter paper and stored in screw-top polyethylene bottles and refrigerated at 4°C until analysis was carried out (Fleurence *et al.,* 1995a with modifications), (Table 2.3, Assay 6).

2.3.4.7 Extraction 7

As 2.3.4.6, except, following the initial extraction with deionised water, the pellet was subsequently resuspended 4 times with 20 ml 0.1 M NaOH (in presence of mercaptoethanol (0.5% v/v)) and the supernatants combined (Fleurence *et al.*, 1995a with modifications), (Table 2.3, Assay 7).

Analysis of each triplicate sample was performed in replicates of three using the Bradford assay. Protein concentration was determined from the Bradford standard curve using Bovine Serum Albumin as a standard.

2.3.5   Protein Determination - Microplate assay

BSA standards 0.01 – 0.1 mg/ml were prepared. 50 μl of standards and samples were transferred to microplate wells in triplicate. 50 μl of buffer was used as blanks. Controls were 50 μl of sample plus 150 μl buffer. 150 μl of Bradford reagent was added to each well containing standard and sample. Samples and standards were mixed thoroughly and allowed to stand for 5 – 60 mins. Absorbance was read at 595 nm using the microplate reader (Bradford, 1976 with modifications; Sigma Aldrich – Adapted Method).

2.3.6   Polyphenol Extraction

In order to optimise the extraction and quantitation methods, several different polyphenol extractions were performed (Table 2.4).

Table 2.4. Summary of Polyphenol Extraction Methods for *P. lanosa.*

Polyphenol Extraction				
Optimisation Summary				
Assay No.	Seaweed Condition	Solvent	Temp. (^{o}C)	Incubation Time (h)
1	Wet	Water	RT	3
2	Wet	Water	4	3
3	Wet	70% MeOH	4	3
4	Powder	70% MeOH	RT	24
5	Powder	70% MeOH	4	3
6	Powder	70% MeOH	RT	16
7	Powder	Water/ 70%MeOH	4 and RT	24 and 1 (5 extracts)
8	Powder	50% MeOH/ 70% Acetone	RT	1 and 1 (2 extracts)

2.3.6.1 Extraction 1

Frozen seaweed samples were thawed and blot dried. 1g of seaweed was transferred in triplicate to a chilled mortar. Approximately 1g of lab-grade sand was added to the sample with 30ml of deionised water and the mixture was ground using a pestle and mortar. Incubation was at room temperature for 3 hours. Samples were centrifuged at 1,500 x g for 10 minutes at 4°C. The supernatant was collected and filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated in darkness, at 4°C until analysis was carried out. Extraction of samples was carried out in triplicate (Table 2.4, Assay 1).

2.3.6.2 Extraction 2

Frozen seaweed samples were thawed and blot dried. 1g of seaweed was transferred in triplicate to a chilled mortar. Approximately 1g of lab-grade sand was added to the sample with 30ml of deionised water and the mixture was ground using a pestle and mortar. Incubation was at 4°C for 3 hours and samples were centrifuged at 1,500 x g for 10 minutes at 4°C. The supernatant was collected and filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated in darkness, at 4°C until analysis was carried out. Extraction of samples was carried out in triplicate (Table 2.4, Assay 2).

2.3.6.3 Extraction 3

Frozen seaweed samples were thawed and blot dried. 1g of seaweed was transferred in triplicate to a chilled mortar. Approximately 1g of lab-grade sand was added to the sample with 30ml of 70% methanol and the mixture was ground using a pestle and mortar. Incubation was carried out at 4°C for 3 hours and samples were centrifuged at 1,500 x g for 10 minutes at 4°C. The supernatant was collected and filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated in darkness, at 4°C until analysis was carried out. Extraction of samples was carried out in triplicate (Table 2.4, Assay 2).

2.3.6.4 Extraction 4

0.200 g algal powder was extracted with 30 ml of 70% methanol in triplicate. Following incubation at room temperature for 24 hours, samples were centrifuged at 1,500 x g for 10 minutes at 4°C. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated in darkness, at 4°C until analysis was carried out (Table 2.4, Assay 4).

2.3.6.5 Extraction 5

0.200 g algal powder was extracted with 30 ml of 70% methanol in triplicate. Following incubation at 4°C for 3 hours, samples were centrifuged at 1,500 x g for 10 minutes at 4°C. Supernatants were filtered using Whatman filter paper. The filtered supernatants were stored in screw-top polyethylene bottles and refrigerated in darkness at 4°C until analysis was carried out (Table 2.4, Assay 5).

2.3.6.6 Extraction 6

0.250 g ground dried seaweed sample was placed 20ml of methanol/water (70:30), in triplicate. Samples were thoroughly shaken on a platform shaker overnight (16h) at room temperature. Samples were centrifuged at 1500 x g for 10 minutes at 4°C and the supernatant was recovered. The samples were stored in screw-top polyethylene bottles and refrigerated in darkness at 4°C until analysis was carried out (Table 2.4, Assay 6).

2.3.6.7 Extraction 7

0.250 g algal-powder (in triplicate) was suspended in deionised water (20 ml), to allow cell lysis by osmotic shock and facilitate polyphenol extraction. The suspension was gently stirred overnight at 4 °C. After incubation, the suspension was centrifuged at 1,500 x g for 10 min and the supernatant collected for the assay of polyphenols. The pellet was treated with 70% Methanol. The mixture obtained was gently stirred at RT for 1 h before centrifugation at 4 ^{o}C (1,500 x g for 10 min). The supernatant was collected and combined with the supernatant of the first centrifugation. Following the initial extraction with deionised water, the pellet was subsequently extracted 4 times with 70% methanol and supernatants combined. The samples were stored in screw-top polyethylene bottles and refrigerated in darkness at 4°C until analysis was carried out (Table 2.4, Assay 7).

2.3.6.8 Extraction 8

0.250 g algal powder was mixed (in triplicate) with 20 ml of 50% methanol plus HCl (to obtain a final pH of 2.0). The tube was thoroughly shaken, on a platform shaker, at room temperature for 1 h. The tube was centrifuged at 1,500 x g for 10 minutes at 4°C and the supernatant was recovered. 20 ml of 70% acetone was added to the residue. Shaking and centrifugation was repeated with the two extracts combined. The samples were stored in screw-top polyethylene bottles and refrigerated in darkness at 4°C until analysis was carried out (Table 2.4, Assay 8).

Analysis of each triplicate sample was performed in replicates of three by means of the Folin-Ciocalteau assay. Polyphenol concentration was determined from the Folin-Ciocalteau standard curve using Gallic Acid as standard. Polyphenol concentration was stated in terms of Gallic Acid Equivalents (GAE).

2.3.7   Polyphenol Determination - Folin-Ciocalteau Microplate Assay

Gallic Acid Standards were prepared from a 100 μg/ml Stock (Serial Dilutions) as follows: 100, 50, 25, 12.5 and 6.25 μg/ml. 20 μl of each standard and sample was loaded onto a 96-well microplate. 100 μl of Folin-Ciocalteau (40ml Folin-Ciocalteau Reagent & 600 ml deionised water) was added. Samples were mixed well and allowed to stand for 5 minutes at room temperature. 80 μl of a 7.5 % Na_2CO_3 solution was added and mixed well. The plate was covered and left in the dark at room temperature for 2 hours. The absorbance was read at 750 nm. For Sample analysis, 200 μl buffer was used as zero and 20 μl buffer was used instead of sample as a blank (Based on Zhang *et al*., 2006 with modifications).

2.3.8   Total Protein Determination

The Kjeldahl method was used to determine the total protein concentrations of both dried (1g) and fresh (1g) *P. lanosa* samples. From this, the overall effect of oven drying on total protein concentration was determined. While this method is very useful in determining the total protein content, the seaweed and its protein is digested and destroyed during the analysis which prevents further analysis of the sample. Triplicate samples were used to measure the total protein content.

2.3.8.1 Digestion procedure

The digester unit was preheated for 10 − 15 minutes. Samples and reagents were then added to clean digestion flasks as follows:

- 1 g sample
- 2 Kjeldahl tablets
- 1 antifoam tablet
- 2 glass beads
- 20 ml concentrated sulphuric acid

The flasks were fitted with an exhaust manifold and clips before being placed in the digester under a vacuum. Digestion was complete 10 − 15 minutes after the solution became clear. The tube assembly was removed from the digester and each flask was covered with a watch glass and allowed to cool for a few minutes.

2.3.8.2 Distillation procedure

75 ml Boric acid solution containing mixed indicator was added to 250 ml conical flasks. The boric acid was placed under the receiver tube of the unit. 80 ml distilled water was added to the digestion flask to dilute the acid. The digestion flask was placed in the distillation unit and 100 ml of 30% NaOH was added. Addition of 30% NaOH neutralised the acid content of the flask. As the distillate migrated across, the colour of the Boric acid changed from orange to blue. When the 4-minute distillation period was complete, the flask with the distillate was removed prior to titration.

2.3.8.3 Titration procedure

The distillate was titrated with 0.1 M HCl until the colour changed from blue back to orange.

2.3.8.4 Calculations to determine % total protein

- **% Nitrogen = (V x 0.14)/W**

    where: V = Titre of 0.1 M hydrochloric acid used in the titration (ml)
    W = Weight of sample (g)
    0.14 = Molarity of N x molecular weight of N

- **% Protein = % nitrogen x F**

    where: F = 6.25 (conversion factor)

    The magnitude of the factor depends on the sample matrix. For example, a factor of 6.25 is used for grains. This factor has also been widely used for the total protein determination of seaweeds (Sánchez-Machado *et al.,* 2004; Marinho-Soriano *et al.,* 2006; Dawczynski, 2007).

- **Actual calculation (Table 2.5)**
    where: Titre blank = 0.8 ml

Table 2.5. Example of total protein calculations.

Replicate No.	Powder Weight (g)	Titre of Sample (ml)	Titre - Blank Titre (ml)	Nitrogen (%)	Protein (%)
1	1.00	35.0	34.2	4.77	29.82
2	1.01	35.0	34.2	4.75	29.71
3	1.01	34.0	33.2	4.61	28.83

Average % Protein	Std. Dev.	95% CI
29.5	0.55	0.62

2.3.8.5 Calculation to determine % total protein dry weight to wet weight conversion factor

This calculation was performed in order to determine possible loss of seaweed protein following oven drying at 60°C for 24h (Table 2.6).

- Dry weight to wet weight conversion factor = Wet weight (g) ÷ Dry weight (g)

  where: Wet weight (g) = Original weight of seaweed prior to oven drying

  Dry weight (g) = Weight of seaweed following oven drying at 60°C for 24 h.

Table 2.6. Calculations for determining dry weight to wet weight conversion factors of *P. lanosa.*

Replicate No.	Wet Weight (g)	Dry Weight (g)	Conversion Factor
1	4.27	1.00	4.25
2	4.28	1.01	4.25
3	4.29	1.01	4.25

Conversion Factor Average	Std. Dev	95% CI
4.25	0.00	0.00

∴ 1 g of dry seaweed = 4.25 g of wet seaweed

4.25 g of wet seaweed was equal to 1 g of dry seaweed. Therefore, the conversion factor from dw to ww was 4.25.

This calculation was also used to determine the dry weight to wet weight conversion factors of *A. nodosum, F. vesiculosus* and *Ulva* spp. The conversion factors were calculated as follows: *A. nodosum* (2.8)*, F. vesiculosus* (3.6) and *Ulva* spp 5.1.

2.4   Results

2.4.1   Protein Extraction

During this study, various protein extraction methods were compared (Table 2.3). Overall results showed that the content of extracted proteins varied in response to the use of different materials (dry/fresh seaweed), solvents and extraction times. Extracted protein concentration ranged from 4.25 mg/g dry weight (dw) to 29.7 mg/g dry weight (dw) (Figure 2.4). Extraction methods using deionised water and 70% methanol saw the lowest extracted protein yield, with values ranging from 4.25 – 10.6 mg/g dw. Methods using NaOH/β-mercaptoethanol resulted in substantially higher yields (9.4 – 29.7 mg/g (dw)). A method, which utilises dry seaweed, initially extracted in deionised water at 4°C and subsequently extracted at room temperature in an NaOH/β-mercaptoethanol solution was optimised for the extraction of proteins in *P. lanosa*. It is possible that multiple pellet extractions with NaOH may have caused flocculation of proteins which resulted in particles being suspended in the extract. This gave suspected false high readings for the Bradford Assay in Extraction number 7. Therefore, the method (Extraction number 6), (Figure 2.4) which requires a single NaOH/β-mercaptoethanol extraction of the pellet was favoured for all subsequent protein extractions.
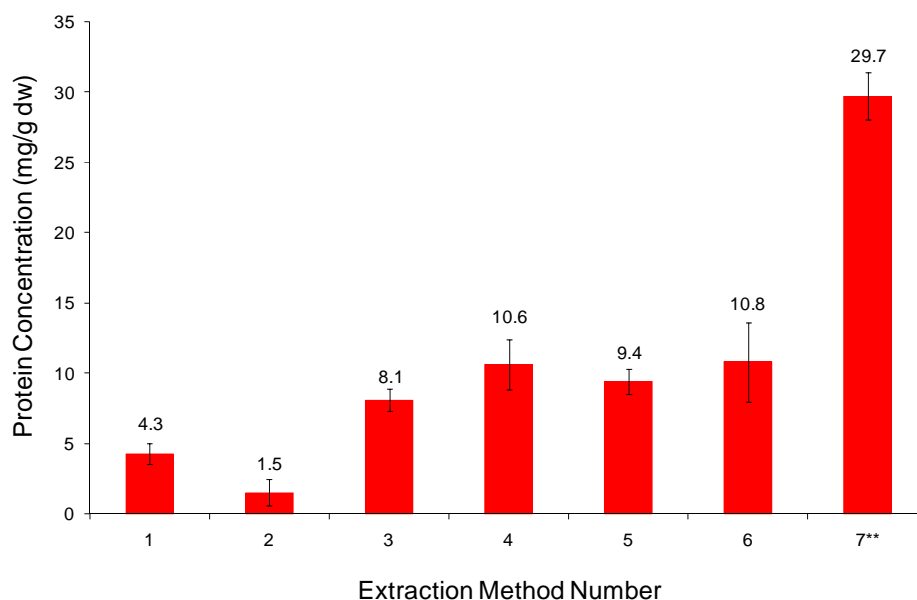
Figure 2.4. Extracted protein concentration of *P. lanosa* following several different extraction methods. Each triplicate sample was assayed in triplicate. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

** Multiple extractions with NaOH may have caused flocculation of proteins, therefore results for Assay No. 7 may be unreliable.

In this study, several protein extraction methods were tested and compared. An extraction method involving a 0.1M NaOH solution in the presence of 0.5% β-mercaptoethanol was deemed to be the optimum method. Using this method, extracted protein levels of *P. lanosa* were determined for the first time and values were consistent with those of another red seaweed *P. arctica* and were within ranges of values for other seaweeds (Table 2.7). This method was therefore considered suitable for the extraction of seaweed proteins and was therefore used in subsequent protein extraction studies.

2.4.2   Polyphenol Extraction

During this study, various polyphenol extraction methods were also tested (Table 2.4). Overall results demonstrated that the polyphenol concentration varied in response to the use of wet/dry samples, solvents and extraction times. Polyphenol concentration ranged from 5.95 mg/g dw to 14.4 mg/g dw (Figure 2.5). Water extracts of *P. lanosa* saw the lowest polyphenol yields ranging from 5.95 to 6.8 mg/g dw (Gallic Acid Equivalents (GAE)). Methods using 70% methanol further enhanced polyphenol extraction with values ranging from 7.7 to 12.9 mg/g dw (GAE). However, deionised water/methanol and water/acetone extractions yielded the highest level of polyphenols, 14.2 and 14.4 mg/g dw (GAE) respectively. Therefore, a method which uses dry seaweed, extracted at room temperature with 50% methanol and 70% acetone solvents was optimised for the extraction of polyphenols from *P. lanosa*. However, because the differences in polyphenol yields for extractions 6, 7 and 8 were minimal, it was determined, that extraction 6 is used for subsequent polyphenol analysis. This is more efficient and cleaner method than extractions 7 and 8.

It should also be noted that assays number 4 and 6 undergo similar extractions, the only difference being extraction duration. Both samples were extracted with 70% methanol however, assay 4 was extracted over 24h yielding 4.7 mg/g dw, while assay 6 was extracted over 16h, yielding 12.9 mg/g dw.
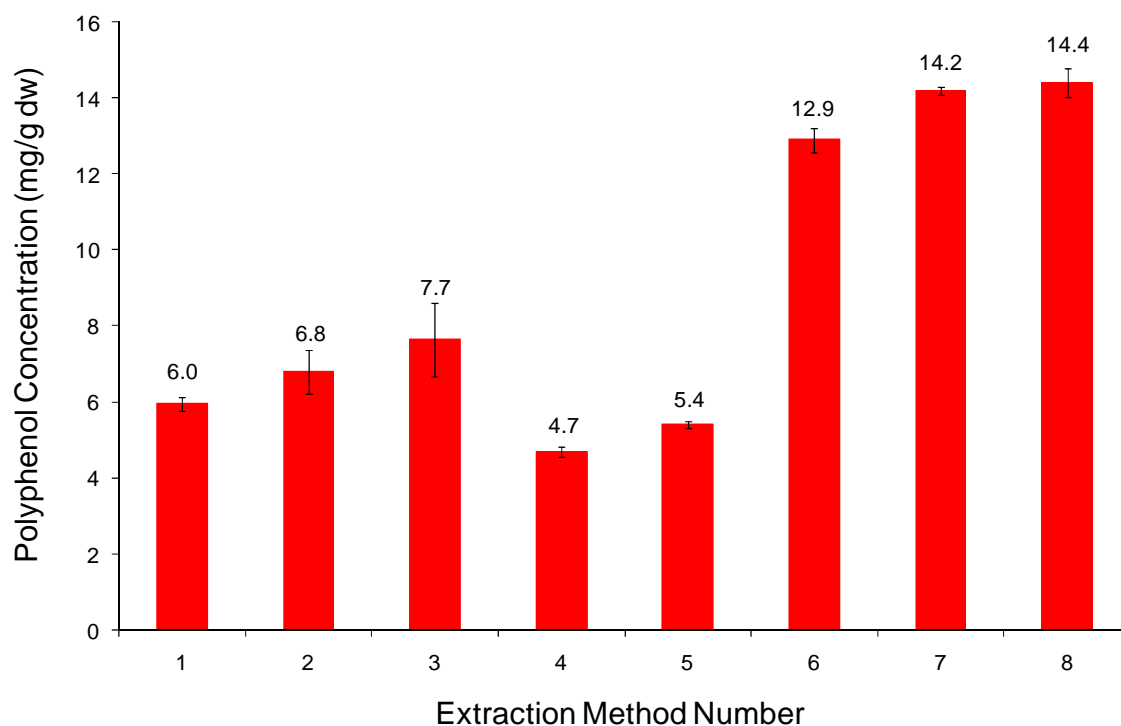
Figure 2.5. Polyphenol concentration of *P. lanosa* following several different extraction methods. Each triplicate sample was assayed in triplicate. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

In general, values obtained in this current study lie within values previously obtained for red seaweeds (Table 2.8). Various polyphenol extraction methods were tested and compared in this study. A process involving the extraction of seaweed polyphenols in a 70% methanol, at room temperature, over 16h was considered to be the optimum method. Using this method, polyphenol levels of *P. lanosa* were determined for the first time and values were consistent with those of other red seaweeds (Table 2.8). This method was therefore considered suitable for the extraction of seaweed polyphenols and was therefore used in subsequent chapters.

2.4.3 Total Protein Determination

The Kjeldahl method was used to determine the total protein content of fresh and dry *P. lanosa*. The total protein content of dry and fresh *P. lanosa* was determined to be 295 mg/g dw (29.5 % dw) and 75 mg/g dw (7.5% ww) respectively (Figure 2.6). In addition, the effect of oven-drying on seaweed protein was determined. Results demonstrated

that after drying, the total protein content of *P. lanosa* was reduced from 75 mg/g ww (7.5 % ww) to 69 mg/g ww (6.9% ww) – a loss of 6.0 mg/g (0.6%). Protein loss, as a result of oven-drying is not uncommon. In a previous study, it was concluded that the nutritional (protein) composition of the seaweed *Sargassum hemiphyllum* was affected by different drying methods (Chan *et al.,* 1997).
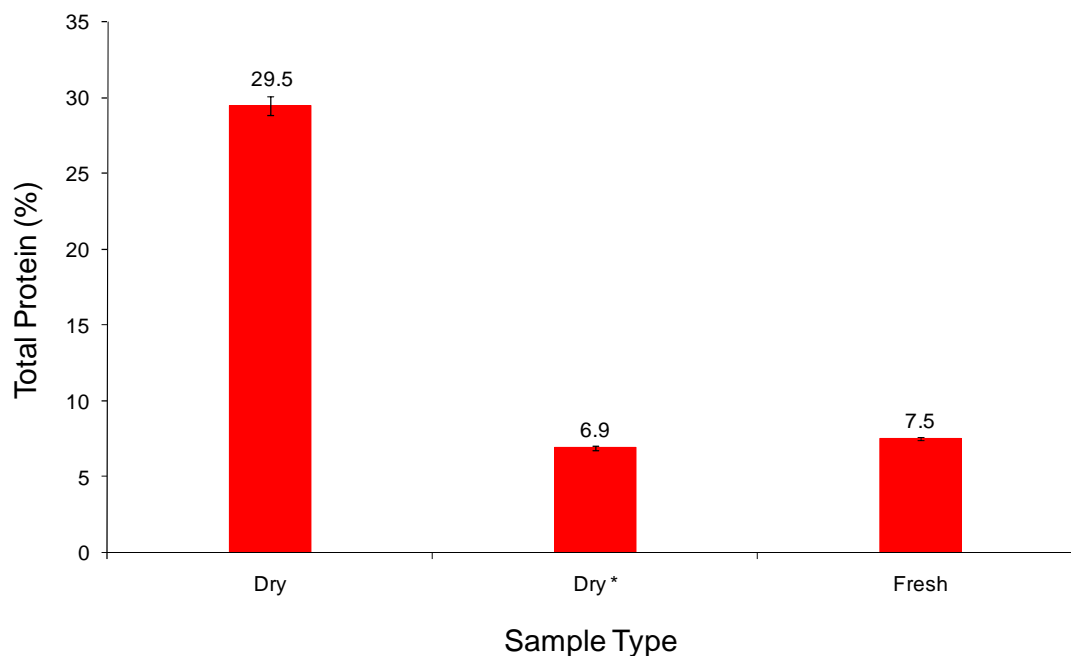
Figure 2.6. Total protein content of oven-dried versus wet *P. lanosa*. Both wet and dry samples were acquired from the same *P. lanosa* batch. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. * This value was calculated by using the dry weight to wet weight conversion factor for *P. lanosa* (4.25). For example, 1g of fresh/wet seaweed yielded 7.5% total protein ww and 1g of dry seaweed yielded 29.5% total protein dw. By using the dry weight to wet weight conversion factor for *P. lanosa* (4.25), the 29.5% as yielded by the dry sample equates to 6.9% ww (i.e., 29.5% divided by 4.25).

In this study, the effect of oven drying on the total protein content of *P. lanosa* was established. Results demonstrated a minimal protein loss of just 6.0 mg/g (0.6%). This method of drying was therefore considered appropriate for seaweed total protein determination throughout the study.

2.5   Discussion

2.5.1   Protein Extraction

Protein extraction from most seaweed is difficult due to the existence of large amounts of anionic cell-wall polysaccharides. Algal cell wall degradation enzymes have been used previously to assist the extraction of proteins on several algae. However, the efficiency of the enzymatic procedures varied according to the species of algae. This approach therefore needs to be validated and optimised for every seaweed species due to differences in cell wall composition (Fleurence, 1999b). In addition, a study by Fleurence *et al.* (1995a) compared several algal protein extraction procedures. Results revealed that the highest protein yield was observed after the application of the procedure which used NaOH/β-mercaptoethanol following extraction with deionised water. This procedure demonstrated a threefold increase in protein extraction of one seaweed species compared to a simple aqueous procedure. However, the denaturation effect of NaOH and β−mercaptoethanol on the tertiary structure of proteins suggests a more limited and controlled use of this method (Fleurence *et al.,* 1995a).

The comparison of data relating to the soluble protein content in algae is very difficult, this is mainly due to differences in the analytical methods in use (Barbarino & Lourenco, 2005). Various studies however have previously been performed on red, brown and green seaweeds in order to determine their soluble protein content (Table 2.7). Protein concentrations for the red, brown and green seaweeds range from 4.63 − 310.25, 1.50 − 43.56 and 4.56 − 280.67 mg/g dw respectively. A protein concentration of 10.8 mg/g dw obtained in this study for *P. lanosa* lies within the ranges of values in literature and is similar to the value previously achieved for *P. arctica* (7.51 mg/g dw), (Gordillo *et al.*, 2006).

Table 2.7. Global seaweed soluble protein levels determined by the Bradford Assay.

	Species	*Source of Seaweed*	*Extraction Solvent*	*Protein Concentration mg/g (dw)*
Red	*P. lanosa* [§]	**Fethard-on-Sea**	**Refer to 2.3.4.6**	**10.80 ±2.8**
	P. lanosa [d]	Turkey	N.R.	310.25 ± 168.4
	P. arctica [e]	Norwegian Arctic	Phosphate Buffer pH	7.51
	Gracilariopsis lemaneiformis[a]	North Carolina	Phosphate buffer pH	5.54
	Gracilaria tenuistipitata [b]	Tainan, Taiwan (1996 – 1997)	Sodium phosphate buffer pH7.	≤ 25.00
	G. verrucosa [d]	Turkey	N.R.	9.40
	Ceramium strictum [e]	Norwegian Arctic	Phosphate Buffer pH	24.17*
	Devaleraea ramentacea [e]	Norwegian Arctic	Phosphate Buffer pH	56.85*
	Odonthalia dentata [e]	Norwegian Arctic	Phosphate Buffer pH	8.16*
	Palmaria palmata [e]	Norwegian Arctic	Phosphate Buffer pH	53.95*
	Phycodrys rubens [e]	Norwegian Arctic	Phosphate Buffer pH	24.41*
	Ptilota plumosa [e]	Norwegian Arctic	Phosphate Buffer pH	38.90*
	Palmaria palmata [f]	Brittany, France	Enzymatic Extraction	8.26 – 13.2 *
	Palmaria palmata[f]	Brittany, France	Sodium Acetate Buffer (pH 5)	4.63 *
Brown	*Sargassum filipendula* [c]	North Carolina	1M NaOH for 20h	1.50 – 2.70 [#]
	Cystoseira barbata [d]	Turkey	N.R.	10.49 ± 6.15 – 43.11 ± 21.36
	Alaria esculenta [e]	Norwegian Arctic	Phosphate Buffer pH	8.21 *
	Chordaria flagelliformis [e]	Norwegian Arctic	Phosphate Buffer pH	28.66 *
	Fucus distichus [e]	Norwegian Arctic	Phosphate Buffer pH	43.56 *
	Laminaria saccharina [e]	Norwegian Arctic	Phosphate Buffer pH	2.48 *
	Scytosiphon lomentaria [e]	Norwegian Arctic	Phosphate Buffer pH	4.80 *
	Sphacelaria plumosa [e]	Norwegian Arctic	Phosphate Buffer pH	6.80 *
Green	*Ulva* spp [d]	Turkey	N.R.	78.57 ± 75.39 – 277.58 ± 135
	Enteromorpha linza [d]	Turkey	N.R.	75.70 ± 24.64
	Enteromorpha compressa [d]	Turkey	N.R.	82.00 ± 6.98
	Ulva rigida [d]	Turkey	N.R.	28.9 ± 33.19 – 280.67 ± 124.0
	Acrosiphonia sp [e]	Arctic	Phosphate Buffer pH	9.49*
	Chaetomorpha melagonium [e]	Arctic	Phosphate Buffer pH	49.49 *
	Monostroma arcticum [e]	Arctic	Phosphate Buffer pH	4.56*

[§] Present Study; [a)] Vergara *et al*., 1995; [b)] Lee *et al*., 1999; [c)] Taylor *et al*., 2002; [d)] Dere *et al.,* 2003; [e)] Gordillo *et al.,* 2006; [f)] Joubert & Fleurence, 2008; [#] = mg/g blotted mass; * Based on fresh weight to dry weight conversion factors (Gordillo *et al.,* 2006); N.R. = Not Reported.

2.5.2   Polyphenol Extraction

Results from previous studies showed that the content of extracted polyphenols varied in response to the use of different materials (e.g., red currant, black currant, grape marc, etc), solvents (e.g. ethanol, methanol, water, etc) and extraction times (Table 2.2) (Lapornik *et al*., 2005). A decrease in polyphenol concentrations over longer extraction periods has previously been observed (Table 2.2). For example, methanol and ethanol extractions of red currant yielded higher polyphenol levels after 12 h when compared with those at 24h (Lapornik *et al*., 2005).

Previous studies involving various extraction methods demonstrated that polyphenol concentrations from seaweeds varied according to technique (Table 2.8). In general, the brown seaweeds, particularly *F. vesiculosus*, yielded the highest polyphenols, followed by both the red and green varieties. Methanol and acetone extractions yielded the highest levels of polyphenols for the brown seaweeds. For the red seaweeds, methanol extraction of *P. stricta* yielded the highest polyphenols (124.00 mg/g dw), (Zhang *et al*., 2006), however all other methods yielded values between $1.15 - 10.50$ mg/g dw (Table 2.8). In the case of green seaweeds, polyphenol levels were generally low with *U. lactuca* yielding the highest polyphenol levels.

Table 2.8. Comparison of seaweed polyphenols as determined by the Folin-Ciocalteau Assay.

	Species	*Extraction Solvent*	*Mode of Extraction*	*Polyphenol Concentration (mg/g dw)*
Red	*P. lanosa* [§]	**70% Methanol**	**Powder extracted, 16h @ RT**	**12.90±0.31**
	P. palmata [d]	DI Water	Powder extracted, 24h @ RT	10.00 [*]
	P. palmata [d]	70% Acetone	As above	7.50 [*]
	P. stricta [a]	Methanol	Powder vortexed @ RT for 30s. Centrifuge 10 min at 12,000 x *g*	124.00 [*]
	P. stricta [a]	Cold Water	As above	10.30 [*]
	P. stricta [a]	Hot Water	As above	10.50 [*]
	Kappaphycus Alvarezzi [c]	50% Methanol	Freeze dried sample. Extracted for 1 h on orbital shaker	1.15 [#]
Brown	*F. vesiculosus* [a]	Methanol	Powder vortexed @ RT for 30s. Centrifuge 10 min at 12,000 x *g*	232.10 [*]
	F. vesiculosus [a]	Cold Water	As above	108.40 [*]
	F. vesiculosus [a]	Hot Water	As above	125.10 [*]
	A. nodosum [b]	MeOH/Water (v/v)	Dried Seaweed. Extracted @ 40°C for 3h	60.00 [*]
	Padina Antillarum [c]	50% Methanol	Freeze dried sample. Extracted for 1 h on orbital shaker	24.30 [#]
	F. vesiculosus [d]	DI Water	Powder extracted, 24h @ RT	175.00 [*]
	F. vesiculosus [d]	70% Acetone	As above	245.00 [*]
	A. nodosum [d]	DI Water	As above	140.00 [*]
	A. nodosum [d]	70% Acetone	As above	165.00 [*]
Green	*U. lactuca* [d]	DI Water	Powder extracted, 24h @ RT	10.00 [*]
	U. lactuca [d]	70% Acetone	As above	7.50 [*]
	Caulerpa Racemosa [c]	50% Methanol	Freeze dried sample. Extracted for 1 h on orbital shaker	1.44 [#]

[§] Present Study; [a)] Zhang *et al.*, 2006; [b)] Connan *et al.*, 2007; [c)] Chew *et al.*, 2008; [d)] Wang *et al.*, 2009; DI = Deionised Water; [*] = Phloroglucinol Equivalents (PGE); [#] = Gallic Acid Equivalents (GAE)

2.5.3   Total Protein Determination

Previous studies have been conducted in order to determine the total protein content of various seaweed species. Values for red seaweeds range from 6.9 – 35.0 % dw, while the brown species range from 3.0 – 19.8 % dw and values for green seaweeds range from 7.13 – 26 % dw (Table 2.9). The total protein content of *P. lanosa* obtained in this study lies within values for red seaweeds and is consistent with a value acquired previously for *Polysiphonia* sp (Marsham *et al.,* 2007).

Table 2.9. Global total protein values of red, brown and green seaweeds as determined by the Kjeldahl method.

	Seaweed Species	*Protein (%) dw*
Red	*P. lanosa* [§]	**29.50±0.62**
	P. palmata [a]	8.0 – 35.0
	P. palmata [b]	9.7 – 25.5
	Porphyra sp [d]	24.11
	Porphyra sp [e]	31.4
	Gracilaria cervicornis [f]	19.7
	Ceramium sp [g]	31.2
	Polysiphonia sp [g]	31.8
	Porphyra sp [g]	44.0
	Dumontia contorta [g]	31.7
	Mastocarpus stellatus [g]	25.4
	Osmundea pinnatifida [g]	27.3
	Corallina officinalis [g]	6.9
Brown	*Laminaria digitata* [a]	8 – 15
	Fucus sp [a]	3 – 11
	A. nodosum [a]	3 - 15
	Undaria pinnatifida [e]	19.8
	Laminaria sp [e]	7.5
	Hizikia fusiforme [e]	11.6
	Sargassum vulgare [f]	13.61
	F. serratus [g]	17.4
	Laminaria digitata [g]	15.9
Green	*U. lactuca* [a]	10.0 – 21.0
	U. pertusa [a]	20.0 – 26.0
	U. lactuca [c]	7.13
	U. lactuca [g]	29.0

[§] Present Study; [a] Fleurence, 1999a (and references therein); [b] Galland-Irmouli *et al.*, 1999; [c] Wong & Cheung, 2001; [d] Sanchez-Machado *et al.*, 2004; [e] Dawczynski *et al.*, 2007; [f] Marinho-Soriano *et al.*, 2006; [g] Marsham *et al.*, 2007.

2.6   Conclusions

The aim of this study was to develop optimised methods for the extraction of protein, polyphenols and total protein from *P. lanosa*.

Protein extraction optimisation methods involved the use of different extraction solvents, durations and temperatures. It was demonstrated that all these parameters affected extracted protein yield.   A method, however which involved an initial extraction in deionised water at 4°C, followed by a pellet extraction with 0.1 M NaOH in presence of mercaptoethanol (0.5% v/v) at room temperature was considered to be the optimum. This method yielded a protein concentration of 10.8 mg/g dw. Previous studies for brown, green and red seaweeds demonstrated that the soluble protein content ranged from 1.49 – 43.11, 5.70 – 280.67, and 2.98 – 45.92 mg/g dw respectively (Table 2.7).   Results from this study generally concur with ranges of values from previous findings for red seaweeds and were similar to a value previously established for *P. arctica* (7.51 mg/g dw).   Large variations in extracted protein levels were observed among red, brown and green seaweeds (Table 2.7).   An optimised protein extraction and quantitation method, developed in this study, was subsequently utilised in order to determine and compare baseline levels of extracted protein in various classes of seaweeds, in addition to comparing levels prior to and post heavy metal exposure.

Polyphenol yields of *P. lanosa* also varied greatly with extraction solvent, duration and temperature.   An extraction technique using 70% methanol, carried out overnight at room temperature was chosen as the optimum method.   This method yielded a polyphenol concentration of 12.9 mg/g dw. Previous research studies for brown, green and red seaweeds species demonstrated that polyphenol levels ranged from 60.0 – 245.0, 1.44 – 10.0, and 1.15 – 124.0 mg/g dw respectively (Table 2.8).   Polyphenol levels in this study therefore lie within previous values for red seaweeds.   Previous studies also demonstrated significant variations in polyphenol levels among three main classes of seaweeds (Table 2.8).   An optimised protein extraction and quantitation method, developed in this study, was subsequently utilised in order to determine and compare baseline levels of extracted protein in red, brown and green seaweeds. Furthermore, comparisons were made following heavy metal exposure.

Studies were also conducted in order to determine the effect of oven drying at 60°C on the total protein content of *P. lanosa*. By using a CF value, it was found that a minimal loss of total protein (6.0 mg/g (0.6%)) was experienced. Overall, therefore it was concluded that oven drying of seaweed, prior to total protein determination was an appropriate method. Total protein contents of *P. lanosa* in this study was also confirmed as 295 mg/g dw (29.5% dw). In previous studies, the total protein content of brown, green and red seaweeds ranged from 3.0 – 19.8, 7.13 – 26.0 and 6.9 – 35.0% dw respectively (Table 2.9). Additionally, the total protein content of *Polysiphonia* sp in a previous study yielded 31.8% dw (Marsham *et al*., 2007), a value similar to that established during this research. Overall, significant variations in previous total protein levels of red, brown and green seaweed were observed (Table 2.9). In order to further investigate these variations, the Kjeldahl method of total protein determination was subsequently used to establish and compare baseline levels of total protein in red, brown and green seaweeds, in addition to comparing levels in species exposed to heavy metals.

A unique study was undertaken to develop optimised methods for protein and polyphenol extraction and quantitation of seaweeds. Several extraction methods were examined and subsequent optimised data compared with previous research. Optimised methods were utilised in subsequent research (except where otherwise indicated), involving protein and polyphenol extraction and quantitation of seaweeds. In Chapter 3 optimised methods were used to determine baseline levels of soluble protein, total protein and polyphenol levels of four seaweed species (*P. lanosa, A. nodosum, F. vesiculosus and Ulva* sp.

Chapter 3

Bioaccumulation of Metals by Seaweeds:- Trends and Biochemical Impacts

3. Bioaccumulation of Metals by Seaweeds – Trends and Biochemical Impacts.

3.1 Introduction

Seaweeds contain significant quantities of protein, although nutrient contents vary from species to species, geographical location, temperature (Sánchez-Machado *et al*., 2004), season and environmental growth conditions (Dawczynski *et al*., 2007). Variations in the total protein content of brown, red and green seaweeds have previously been demonstrated (Refer to Chapter 2, Table 2.9).

In addition, seaweeds are a rich source of structurally diverse secondary metabolites, including polyphenols. The functions of these secondary metabolites are defence against herbivores, fouling organisms and pathogens; they also play a role in reproduction and protection from UV radiation. Variation in secondary metabolites is controlled by genetic and environmental factors (Stirk *et al*., 2007). Wide variations in the polyphenol concentrations of red, brown and green seaweeds have also been established (Refer to Chapter 2, Table 2.8).

The metals under investigation in this study include Pb^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Mn^{2+}, Ni^{2+} and As^{3+}. Some heavy metals such as Mn^{2+}, Cu^{2+} and Zn^{2+} are essential micronutrients and commonly are referred to as trace metals. They may limit algal growth if their concentrations are to low however, they can become toxic at higher concentrations (Lobban & Harrison, 1997). Uptake of Zn^{2+} by *Ascophyllum nodosum* and uptake of Co^{2+} by *Laurencia corallopsis* appeared to be by active transport (Sánchez-Rodríguez *et al.,* 2001). In some algal species, a simple ion exchange process involving intracellular polysaccharides is the key uptake mechanism for Zn^{2+} (Lobban & Harrison, 1997). The findings of previous studies indicate that toxic concentrations of Cu^{2+} and Zn^{2+} ions, together with Co^{2+} and Mn^{2+} deficiencies, may be among the factors controlling the growth of some marine macrophytes in deep seawater (Lobban & Harrison, 1997). Concentrations of Fe, Mn, Zn, Cu, Pb, Ni, Cr, Cd, and Ag in brown seaweeds collected from clean sites have previously been reported to contain 0.30 to 479.40 mg/kg dry weight (dw) (Giusti, 2001). Concentrations of As, B, Ca, Cd, Cr, Cu, Co, Fe, Mg, Mn, Mo, Ni, P, Pb, Se, V, and Zn for green species were 0.46 to 51.40

mg/kg dw, while the red seaweeds contained <0.50 to 42.90 mg/kg dw (Pérez *et al.*, 2007).

Co^{2+} is an essential element of vitamin B12 (Mageswaran & Swasubramaniam, 1984). Additionally, the principal roles of Mn^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} are as enzyme cofactors (DeBoer, 1981). Mn^{2+} plays a crucial role in the oxygen-evolving system of photosynthesis and is a cofactor in numerous Krebs-cycle enzymes (Bidwell, 1979). Cu^{2+} is present in plastocyanin, one of the photosynthetic electron-transfer molecules and is a cofactor in various enzyme reactions (Bidwell, 1979). Urease, which catalyses the hydrolysis of urea to ammonium in several microalga contains nickel. Zn^{2+} is an activator of many important dehydrogenases and is involved in protein-synthesis enzymes in higher plants. It is essential in algae, where it most likely plays similar roles. Zn^{2+}, at an optimum concentration of 0.5 nM was shown to be essential by *Porphyra tenera*, without it, chlorophyll and phycobilin productions were hindered and the amount of high-molecular-weight protein decreased (Lobban & Harrison, 1997).

Heavy metals such at Pb^{2+} are not necessary for growth and they can become toxic to algae at extremely low concentrations (e.g., 10-50 μg/L). Cd^{2+} is a serious contaminant for plants and animals, particularly in coastal waters near industrial locations, where concentrations may increase from the normal level of about 0.1 μg/L to several micrograms per litre (Lobban & Harrison, 1997). Although copper is an essential micronutrient, it was shown to be the second most toxic element after mercury. Its toxicity chiefly depends on the presence of free copper ions (Cu^{2+}) in the medium (Kangwe *et al.*, 2001).

Furthermore, a preliminary report assessing the effects of lead on four red algae demonstrated considerable reductions in growth only at unrealistically elevated concentrations (10 mg/L as $PbCl_2$). Even though zinc is generally considered to be actively taken up by seaweeds, it has a comparatively low toxic effect. For example 5–10 g/L Zn^{2+} was required in order to yield a 50% reduction in growth in five intertidal Fucales. In contrast, Cu^{2+} toxicities occurred at 1/100th that concentration while Cd^{2+} and Pb^{2+} toxicities occurred at 2/10th that value (Lobban & Harrison, 1997).

Chromium in the marine environment occurs as Cr^{3+} or Cr^{6+}. The concentration of Cr^{3+} is known to decrease quickly during mixing in estuaries as a result of Cr^{3+} being perhaps converted to particulate Cr^{3+}. The concentration of Cr^{3+} or Cr^{6+} in the Irish Sea has been reported as 0.00 and 0.46 ng/L (Sadiq, 1992).

As^{3+} is an environmental pollutant and can occur from natural sources such as rocks and sediments and also as a result of anthropogenic activities such as coal burning, Cu^{2+} smelting and the processing of mineral ores. Concentrations of arsenic are higher in the aquatic environment than in most areas of land as it is quite water-soluble and may be washed out of arsenic-bearing rocks. Seaweed in particular, is known to contain high levels of As^{3+} when compared to terrestrial plants and this is due to the ability of marine plants to concentrate the arsenic they derive from sea water (Rose *et al*., 2007).

Heavy metal (HM) pollution is an environmental problem of worldwide concern with effluents from various industrial processes representing one of the most important sources of pollution (Murphy *et al.,* 2007). Conventional HM removal processes including ion-exchange, activated carbon adsorption, reverse osmosis, and membrane filtration (Arslan & Pehlivan, 2008) can be expensive or ineffective at low concentrations and may also lead to secondary environmental problems from waste disposal (Murphy *et al.,* 2008). Over the past two decades, attention has been concentrated on identifying materials that can effectively remove heavy metals from aqueous environments. Seaweeds have been shown to be extremely efficient biosorbents with the ability to bind a variety of metals (Volesky & Holan, 1995; Murphy *et al.,* 2007). The outstanding capacity of several seaweeds to accumulate metals is well known, with some (e.g. *Fucus* spp*., Ulva* spp*.*) able to flourish in estuaries and coastal waters impacted by high metal concentrations (Pawlik-Skowrónska *et al.*, 2007). Previous authors have described the many ways in which seaweeds can take up toxic metal ions, these include metal-binding compounds such as, glutathione, phytochelatins (Malea *et al*., 2006) and polyphenols (Ragan *et al*., 1979), (See Introduction, 1.4).

Macroalgae have the ability to concentrate metal ions from seawater and therefore have been widely used as indicators of metal pollution (Malea *et al*., 1995). Additionally, the justification for using organisms as biomonitors, to assess levels of pollution in the

marine environment has been well documented (Stengel *et al.,* 2004). Their local abundance, widespread distribution, sedentary nature and ability to accumulate trace metals to levels well above those found in seawater have resulted in marine macroalgae being widely used as biomonitors of metal pollution (Brown *et al.*, 1999). Furthermore, the analysis of bioindicators, rather than that the direct study of water samples have advantages particularly where economic considerations exclude the use of high-sensitivity methods suitable for water analysis (Stengel *et al.,* 2004).

In recent years, Ireland has seen an increase in its population and industrial activity with the majority of towns along the coastline having undergone rapid expansion. This demographic and urban pressure has led to an amplification of waste discharges into coastal waters (Morrison *et al*., 2008). Over the past few decades, sediment samples have been extensively analysed to measure anthropogenic impacts in the aquatic environment (Giusti, 2001). Sediments act as reservoirs and sinks for trace metals (Mountouris *et al.*, 2002). The concentration of trace metals in sediments does not however provide adequate data on the concentrations of metals available to biota living in the environment. Trace metals in the water column are transferred to sediments with estuaries and coastal areas being the largest recipients of contaminants (Giusti, 2001).

Unpolluted marine and freshwater sediments from many sites around the globe generally contain less than 50 mg/kg Zn^{2+}, up to 20 mg/kg Cu^{2+}, between 2 and 50 mg/kg Pb^{2+}, up to 100 mg/kg Ni^{2+}, less than 60 mg/kg Cr^{3+} and less than 1.0 mg/kg Cd^{2+} (Giusti, 2001).

Measuring the biological responses to trace metals depends upon accurately determining the exposure to the pollutant. This can be difficult since the total metal concentrations in seawater and sediment do not represent the concentrations available to the marine biota (Luoma, 1983). The term bioavailable is used to denote the proportion of a chemical in the environment that might be taken up into an organism. It can also be used to refer exclusively to the quantity of chemical that is actually taken up into an organism. Within the marine environment trace metals can be present in equilibrium among free hydrated metal ions, metal bound in organic (e.g., amines, humic acid) and inorganic (e.g., OH-, CO_3^{2-}) complexes and metal bound to organic and inorganic particulate matter (Brown & Depledge, 1998). The chemical composition of the seawater has a powerful influence on the speciation of metals. In turbid estuarine waters therefore, a large portion of the total metal load is bound in or to organic or

inorganic particulate matter (Salomons & Forstner, 1984). Mixing in estuaries changes metal speciation as the ionic strength increases. Dissolved organic complexes and particulate matter may undergo flocculation and for a number of metals, the result is that a large proportion of the load transported in the river water sinks to the sediments of the estuary. Other metals however, (e.g., Cd) are displaced from particulate matter by chloride ions as a result of the formation of chloride complexes (Elbay-Poulichet *et al.*, 1987). In estuaries, the speciation of the metals that stay in solution is affected by the increasing concentration of anions, particularly chloride and for the majority of metals, free ions constiute a relatively minor proportion of the total dissolved metal concentration (Mantoura *et al.*, 1978).

Some trace metals are available for uptake into organisms from solution only as free ions, whereas others are transported across biological membranes as inorganic complexes (Brown & Depledge, 1998). In experiments where the free Cu species were controlled either by organic chelators or determined by way of ion-selective electrodes, the toxicity and bioavailability correlated with the concentration of free metal ions rather than total dissolved metal concentration (Sanders *et al.*, 1983). These results are consistent with the uptake and toxicity of these metals increasing with decreasing salinity (and thereby free ion concentration) in most estuarine organisms studied (McLusky *et al.*, 1986).

Since the chemistry of sediment is significantly more complicated than that of seawater, the mechanisms by which geochemistry affects metal bioavilability from sediments are not as well understood as those influencing metal speciation in seawater. Despite a number of attempts however, there are generally no applicable, consistent methods to assess bioavilability of metals in such media (Luoma, 1989).

Trace metals that accumulate in seawater and sediments are therefore not necessarily freely available for uptake into biota. A fraction of the metal may be strongly bound in dissolved complexes or on sediment surface or in organic films surrounding particles. This speciation of metals is particularly diffficult to forecast either qualitatively or quantitavely (Brown & Depledge, 1998). Alterations in physicochemical parameters of the environment can strongly influence the relative proportions of the metal species that can be taken up; alterations in pH, redox potential, salinity, temperature, etc., can all considerably influence the bioavailability of metals for uptake into marine organisms (Mantoura *et al.*, 1978). This is an important consideration for biomonitoring studies.

Just because organisms do not contain particularly high levels of trace metal in their tissues, does not rule out the possibility that metals may be present in the environment at elevated levels; they may be strongly bound in highly stable complexes (Brown & Depledge, 1998). As physicochemical conditions change (e.g., after the resuspension of sediments due to turbulence), a rapid conversion of metals to bioavailable ionic forms may result in higher concentrations in biota (Samiullah, 1990). For marine algae, metals are derived almost entirely from the aqueous phase, although it has been proposed by some (e.g., Luoma *et al*., 1982) that metals can be taken up directly from sediments or suspended inorganic particulates (Brown & Depledge, 1998). While these techniques cannot be totally ruled out, it is difficult to confirm since algal surfaces are heavily contaminated by organic matter (Holmes *et al*., 1985) that contains significant quantities of metals (Brown & Depledge, 1998).

3.2   Objectives of Research

In this chapter, optimised extraction methods from Chapter 2 were used to determine and compare the baseline extracted protein, polyphenol, total protein content, in addition to the metal contents (total, intracellular and surface bound) of four seaweed species, *P. lanosa* (Red), *A. nodosum* (Brown), *F. vesiculosus* (Brown), *F. ceranoides* (Brown) and *Ulva* sp (Green).   The purpose of this work was to examine the bioaccumulation of metals by seaweeds and to investigate trends and biochemical impacts. Samples were collected from estuaries along the West coast of Newfoundland and South East coast of Ireland. A diverse range of samples were collected from various sites at each location. Profiling of this type, for the four seaweed species under investigation has never previously been performed. The work covered in this study included:

- Physico-chemical analysis (pH, temperature and conductivity) of seawater, at each site.

- Total protein, extracted protein and polyphenol levels of the four species under investigation were determined in order to establish biochemical profiles for *Polysiphonia lanosa*, *Ascophyllum nodosum*, *Fucus vesiculosus, F. ceranoides* and *Ulva* sp. Profiles of the five species were compared and interspecies and inter-site variations were established. Furthermore, data compiled in this study was compared with reports from the literature.

- Seawater collected from the West coast of Newfoundland and South East coast of Ireland was assayed for its metal content. A wide variety of metals in seawater from Newfoundland and Ireland were examined for the first time and compared with both global and Irish Seawater values.

- Sediment samples collected from the South East coast of Ireland were analysed for their metal contents.

- Total, intracellular and surface bound metal concentrations for the five species were determined and compared. Additionally, comparisons with literature were also undertaken.

- Concentration Factors (CF), the ratio of seaweed metal to seawater metal concentrations were calculated for the four species under investigation in order to determine variation in bioaccumulation power among red, brown and green seaweeds. Comparisons were also made with global CF values.

- Regression analysis was performed in order to establish relationships between protein and polyphenol levels with physico-chemical parameters and seaweed metal concentrations.

This novel study explores the baseline metal, total protein and polyphenol levels of each of the main classes of seaweed available off the South-East coast of Ireland and West Coast of Newfoundland. In addition metal levels (both essential and non-essential) of seawaters collected off the South-East coast of Ireland and West Coast of Newfoundland were also investigated and compared. Metal levels in sediment collected from the Suir Estuary and Fethard-on-Sea were also determined. Overall results from this study therefore hold major significance both nationally and globally in terms of data relating to the biochemical interactions of seaweed and seawater.

3.3 Experimental

3.3.1 Chemicals

- Certified Reference Material Metals on Soil (Trace Metals-Sand 1) Catalogue No., CRM048-050 (RT-Corp, Wiltshire, UK).

- Nitric Acid (69%) (Lennox, Dublin, Ireland).

- Hydrogen Peroxide (35%) (Sigma Aldrich, Dublin, Ireland)

- Multi-Metal Solution (Std 165) (Inorganic Ventures – JVA Analytical Ltd, Dublin, Ireland). (Mn^{2+} 80 ppm, Al^{3+} 80 ppm, As^{3+} 10 ppm, Zn^{2+} 10 ppm, Cr^{3+} 3 ppm, Cu^{2+} 1.5 ppm, Ni^{2+} 1.5 ppm, Pb^{2+} 1.5 ppm, Cd^{2+} 0.5 ppm, Co^{2+} 0.5 ppm.

- BCR Reference Material No. 60 Lagarosiphon Major

- NIST Standards Reference Material Tomato Leaves

3.3.2 Instrumentation

- HACH Drel 2800 System (HACH LANGE Ltd., Dublin 12, Ireland):
  - HACH HQ 30d Fleximeter
  - pHC 101 pH probe
  - CDC 401 conductivity probe
- Microwave Digester: Milestone Microwave Laboratory Systems
- Easy Wave 3 software – version 3.2.1.0
- Mars 5 CEM Microwave Digester (JVA Analytical Ltd, Dublin, Ireland)
- Inductively Coupled Plasma Spectrometer OES (Varian 710-OES) (JVA Analytical Ltd, Dublin, Ireland)
- Seawater Analysis was performed by National Laboratory Service (NLS), Environment Agency, Leeds, England. Analysis was performed with the following instrumentation:
  - ICP-MS
  - Graphite Furnace – AAS (Acid Digested Samples)
  - ICP-OES – (Acid Digested Samples)
  - ICP-MS – (Acid Digested Samples)

3.3.3   Locations

This study compared protein, polyphenol and metal levels in seaweeds from two locations in Newfoundland, Canada (Figure3.1) and two locations in Ireland.

3.3.3.1 Newfoundland

Samples were collected from two locations in Newfoundland, Canada. Newfoundland is an island located on the East Coast of mainland Canada. Samples were collected from five sites on the West coast of the island in Bonne Bay and six sites along the Humber Arm (Figure 3.1).

Bonne Bay is located on the Western side of Newfoundland and separates Great Northern Peninsula from the rest of the island. It is a part of Gros Morne National Park. Bonne Bay has a population of approximately 7000 people. It is separated into two sections: Inner Bonne Bay and Outer Bonne Bay. Inner Bonne Bay consists of two arms and Outer Bonne Bay consists of the entrance to the fjord of Bonne Bay**.**

Located on the Western coast of Newfoundland, Humber Arm is the Southernmost arm of the Bay of Islands. It has the highest concentration of freshwater of the entire bay as a result of inflow from the Humber River.   The Humber is a major river in Newfoundland, making the Bay of Islands an important estuary. Although the river is mainly used for recreational purposes, the bay is still associated with active shipping to and from Corner Brook port.

3.3.3.2 Suir Estuary and Fethard-on-Sea

At 183km, the River Suir is the second longest river in Ireland. It is a wide river ranging from 25-35m in its middle sections. The main channel of the River Suir and its tributaries run primarily through counties Waterford, Tipperary and Kilkenny which form part of the South Eastern River Basin District. The River Suir rises in the Devils Bit Mountains close to Moneygall, and flows in a Southerly course until it meets the Knockmealdown mountain range where the river changes its route Northwards. At Knocklofty, the River Suir turns in an Easterly direction, passing the Comeragh

Mountains and continues on through the city of Waterford until it enters the sea at Waterford Harbour (Waterford County Council, 2010).
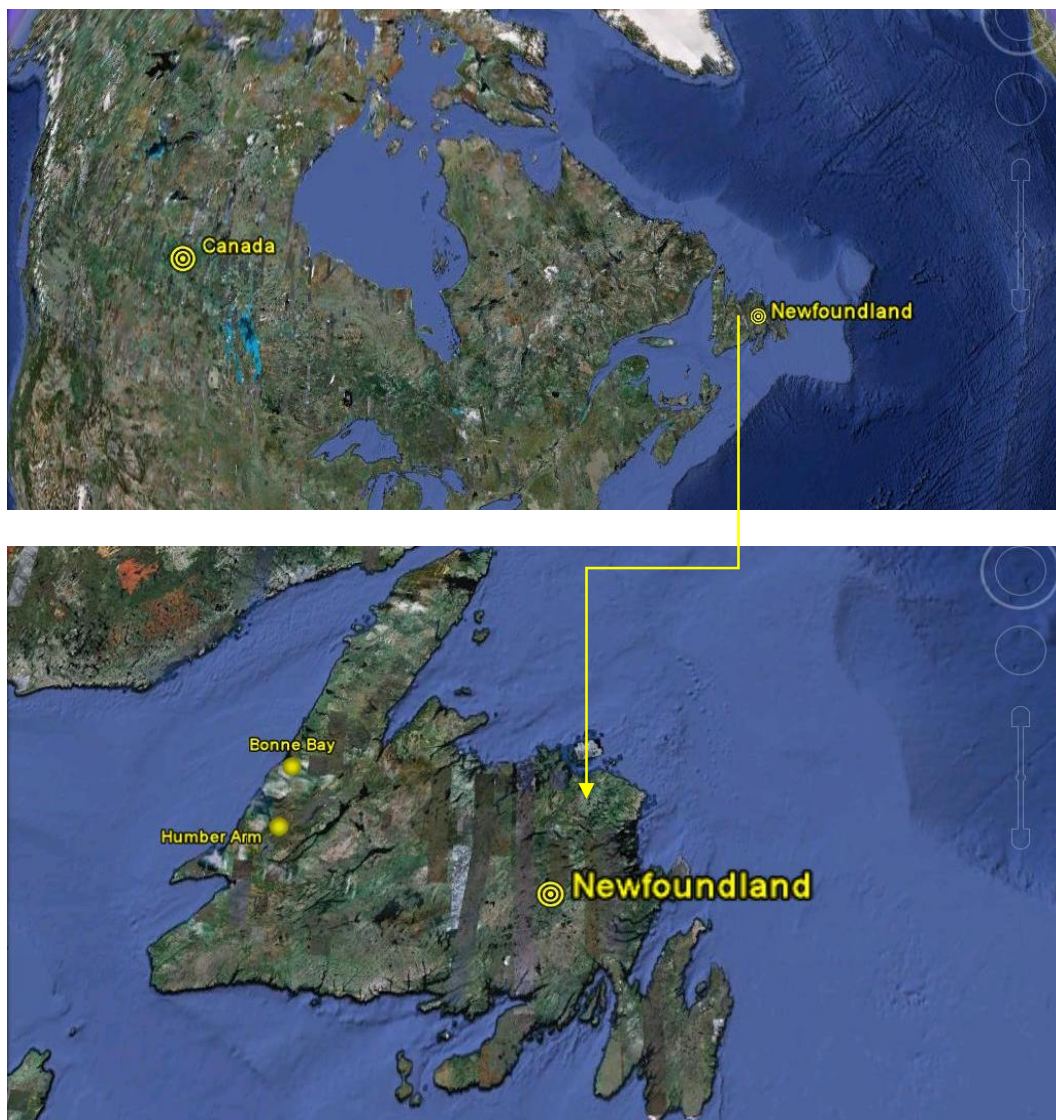
Figure 3.1. Sample collection locations, Bonne Bay and Humber Arm, Newfoundland, Canada (Google Earth, 2010).

Metals under investigation in this study include Cr^{3+}, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Al^{3+}. Chromium has historical significance in the South East of Ireland as it has traditionally been used in the tanning industry. In addition, several zinc mines operate in Ireland and cadmium is a by-product of the zinc mining industry. Copper was also mined intensively in the region throughout the 19th century. Lead too has historical importance due to large scale lead crystal manufacture in Waterford City until 2009. Furthermore several industries in the geographical area use aluminium during the manufacturing

process. Table 3.1 displays a list of all the sites under investigation together with their co-ordinates and seaweeds available.

Table 3.1. Co-ordinates and seaweed species collected from Newfoundland and Ireland sites.

Location	*Site No.*	*Co-ordinates*		*Seaweed species*
Humber Arm	1	48 57.524 N,	57 59.234 W	FV
Humber Arm	3	49 06.229 N,	58 21.703 W	FV, AN
Humber Arm	4	49 03.605 N,	58 11.007 W	FV, AN
Humber Arm	5	49 00.431 N,	58 07.394 W	AN
Humber Arm	6	48 58.457 N,	58 03.704 W	FV, AN
Humber Arm	7	48 58.910 N,	58 00.322 W	FV, AN
Bonne Bay	1	49 33.361 N,	57 50.302 W	FV, AN, FC
Bonne Bay	2	49 29.182 N,	57 55.509 W	FV, AN, FC
Bonne Bay	3	49 35.123 N,	57 56.335 W	FV, AN, PL
Bonne Bay	4	49 30.689 N,	57 52.397 W	FV, AN
Bonne Bay	5	49 28.967 N,	57 44.298 W	FV, AN
Suir Estuary	1	52 15.606 N,	7 06.223 W	FV
Suir Estuary	2	52 15.521 N,	7 03.851 W	FV, AN
Suir Estuary	3	52 16.363 N,	6 59.745 W	FV, AN, PL
Suir Estuary	4	52 14.417 N,	6 58.370 W	FV, AN, PL
Fethard on Sea	5	52 11.890 N,	6 49.577 W	FV, AN, PL, UL

(FV= *F. vesiculosus,* AN = *A. nodosum,* FC = *F. ceranoides,* PL = *P. lanosa,* UL= *Ulva sp*)

3.3.4   Site descriptions

3.3.4.1 Bonne Bay, Newfoundland, Canada

> o   *Site 1: Deer Brook Arm, North of Red Cliff.*

This sampling site is located near a lagoon at Deer Brook Arm. The location plays host to a variety of seaweeds such as *F. vesiculosus, A. nodosum and F. ceranoides.* The brown seaweed *F. ceranoides* was only available at the Newfoundland site. The lagoon itself has a diverse geography. Surface materials consist of drift material, rocks, stone,

including limestone. A wide variety of plants grow at the site. Certain species of amphipods, worms, larvae and flowers which exist at the site cannot be found anywhere else. The lagoon is a pristine location unspoiled by human intervention (Figure 3.2).
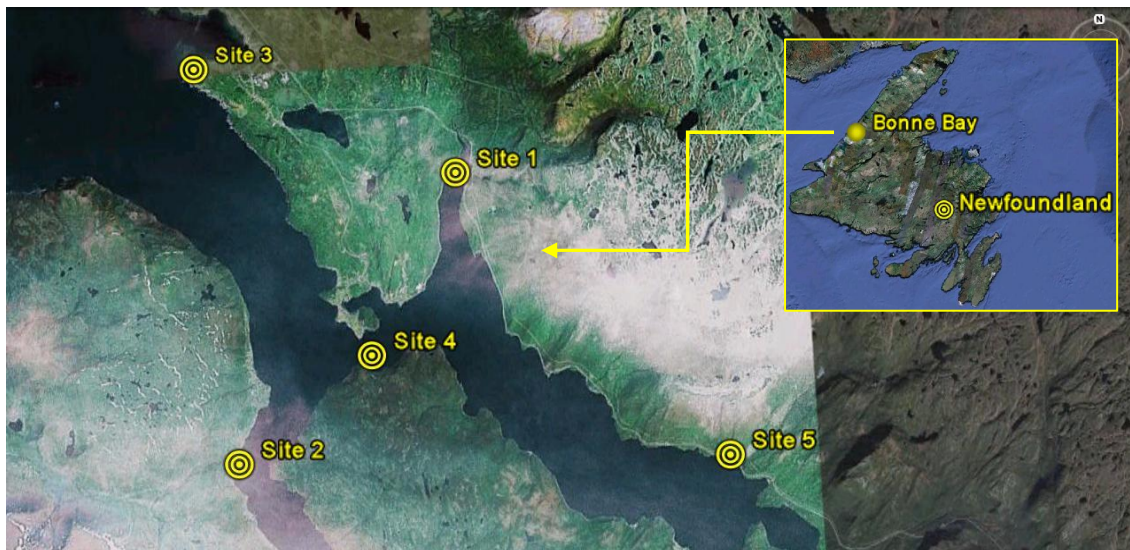
Figure 3.2. Bonne Bay, sites 1 – 5 (Google Earth, Jan 2010).

o *Site 2: Winter House Brook (near Woody Point).*

This location is best known as a fishing wharf. Mackerel and herring are processed on site. Approximately 40% of the process is washed directly into the water, resulting in seawater pollution. Additionally, moderately high volumes of boat traffic on the waters in this area contribute to Cu pollution. Cu is commonly used as an antifouling agent (Baumann *et al*., 2009). *F. vesiculosus, A. nodosum* and *F. ceranoides* were collected at the site (Figure 3.2).

o *Site 3: Salmon Point (near Rocky Harbour).*

Fishing is one of the main activities at Salmon Point. This site which consists of numerous tide pools is located at the outer limit of Bonne Bay. The location which plays host to *P. lanosa, F. vesiculosus and A. nodosum,* is a platform beach that is subjected to severe erosion. Ice rafted boulders however, provide a great deal of shelter from the elements, such as low temperatures and ice. For example, packed ice is capable of removing vegetative covering, resulting in bare rocks. The presence of boulders can prevent these events. High volumes of *A. nodosum* growing on site also act as a protective canopy layer to communities such as Irish Moss, pink coralline algae,

epiphytes etc., against temperature and light extremities. Overall, this is a clean site with a small human population (Figure 3.2).

  o   *Site 4: Gadd's Harbour.*

This is a rugged, rocky site, unspoilt by human activities. Sampling was quite difficult as the site was only accessible by boat. During 2006, this area was severely affected by ice scouring. This had a detrimental effect on the seaweed population. Small quantities of *F. vesiculosus* and *A. nodosum* were available at the time of collection. *P. lanosa*, however was completely eradicated. Gadd's harbour is located opposite Norris Point (approximately 0.5 km), location of Bonne Bay Marine Station. Activities in this area include land reclamation, seabed dredging and aggregate mining (i.e., gravel from the beach is used for concrete). A drainage point in a central location of Bonne Bay releases untreated sewage into the sea. The impact however is much localised as a result of currents (Figure 3.2).

  o   *Site 5: Seal Cove.*

Situated approximately 300m East of Seal Cove, this coastal site was previously subjected to a severe oil spillage 29th August, 1999. An oil tanker, carrying 40,000L lost its load. Much of the oil seeped into the ground and subsequently into coastal waters. Only 4,000 L was recovered. Regrettably however, large volumes of diesel-covered *A. nodosum* were removed from the site during the clean-up stage (Figure 3.2).

3.3.4.2 Humber Arm, Newfoundland, Canada

Figure 3.3 illustrates the six sampling sites at The Humber Arm, Newfoundland.
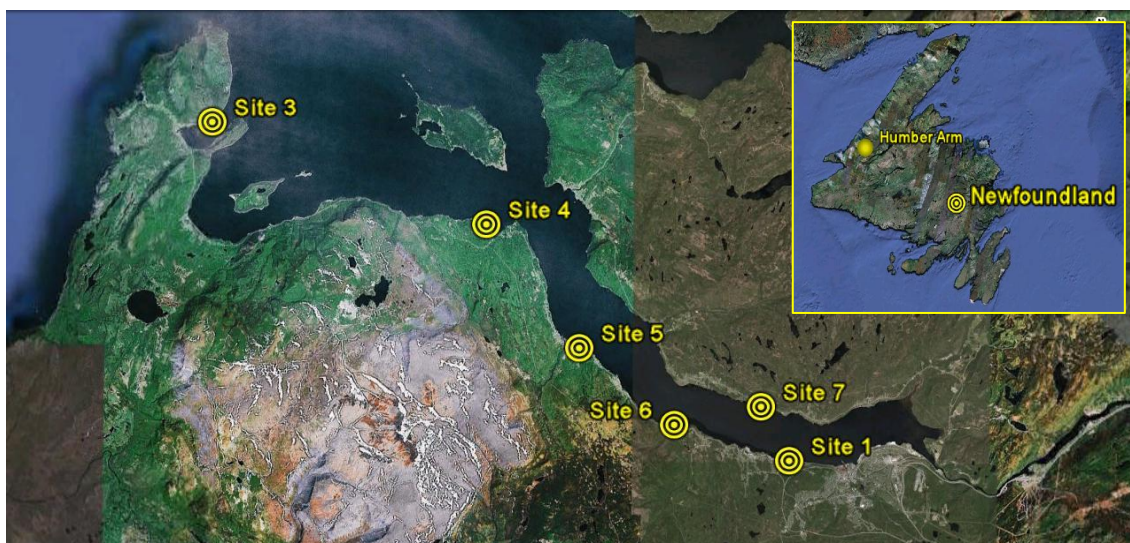
Figure 3.3. Sample collection sites along The Humber Arm.

o *Site 1: Near Corner Brook*

This site is located between the city of Corner Brook and the town of Curling (Figure 3.4). These are the main built-up areas along the Humber Arm. Located on the Bay of Islands, at the mouth of the Humber River, Corner Brook is associated with the pulp and paper mill industry. The city has a population of 20,083 (2006, Canadian Census). Curling, a sub-division of Corner Brook, is located in the Humber Arm. It was originally a fishing community and today is associated with its fish processing industry. Pollution from both industries is ongoing. *F. vesiculosus* was the only seaweed available at this site.
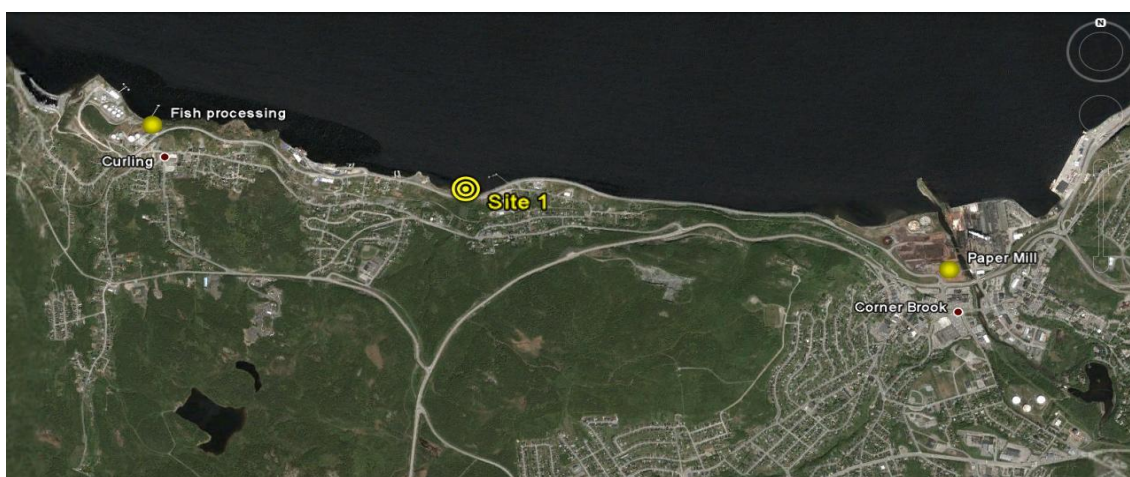
Figure 3.4. Humber Arm Site 1, between Curling and Corner Brook (Google Earth, Jan 2010).

    o   *Site 3: Lark Harbour*

Lark Harbour, a small fishing community, is located at the outer limit of The Humber Arm, on the South side of the Bay of Islands.   The location plays host to *F. vesiculosus* and *A. nodosum.*   The area is unspoilt with a population of <1,100 (2006, Canadian Census).

    o   *Site 4: Frenchman's Cove*

Similar to site 3, Frenchman's Cove is unspoilt and not impacted by industrial pollution. The community had a population of just 166 in 2006 (2006, Canadian Census). *F. vesiculosus* and *A. nodosum* were collected at this site.

    o   *Site 5: Humber Arm South*

As with Sites 3 and 4, this site is unspoilt and the area is not impacted by industrial pollution. *A. nodosum* was the only seaweed available at this site. The town had a population of 1,854 in 2006 (2006, Canadian Census).

    o   *Site 6: Sopers*

Located between the towns of Halfway Point and Mount Moriah, Sopers is a non-industrialised location.   The area is sparsely populated with a population of just 752 (2006, Canadian Census). *F. vesiculosus* and *A. nodosum* were collected at this site.

    o   *Site 7: Irishtown*

This site is approximately 3 km North of Corner Brook. The area is sparsely populated with a population of 1,290 in 2006 (2006, Canadian Census). *F. vesiculosus* and *A. nodosum* were collected at this site.

3.3.4.3   Suir Estuary & Fethard-on-Sea, Ireland

Figure 3.5 illustrates the five sampling sites along the Suir Estuary and Fethard-on-Sea.
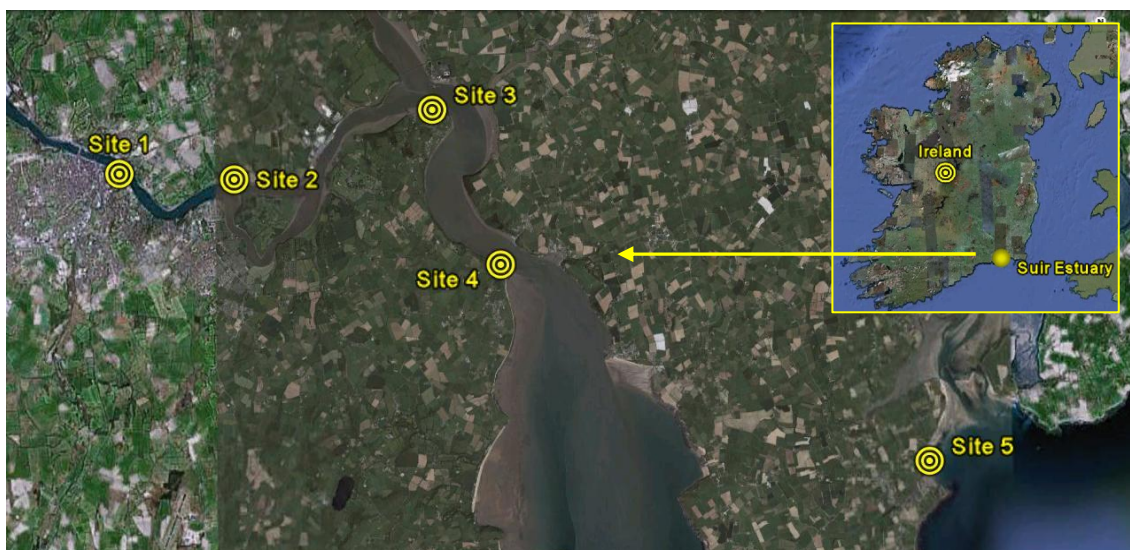
Figure 3.5. Sample collection sites along The Suir Estuary (Sites 1 – 4) and Fethard-on-Sea (Site 5).

- o *Site 1: St. John's River, Waterford City*

St. John's River is a tributary of the River Suir, flowing into the Suir in Waterford City. This sampling site is located within Waterford City, a mid-sized city with a population of 45,748 (Central Statistics Office 2006).   The site is a built-up area and the river environs are subject to heavy traffic. Seaweed samples were collected from the water's edge at low tide. The river itself consisted of dirty, sludge ridden water. *F. vesiculosus* was the only seaweed growing at the site.

- o *Site 2: Ferrybank, Waterford City*

This site is located on the River Suir opposite and downstream from St. John's River. The area itself is quite rural with a low-density population. *F. vesiculosus* and *A. nodosum* were collected at this site.

- o *Site 3: Cheekpoint, Co. Waterford*

Cheekpoint is a small fishing village, located about 9km downstream from Waterford City. During sampling, the water quality was visibly quite poor with reduced clarity. *P. lanosa, F. vesiculosus* and *A. nodosum* were collected at low tide. Upon collection, seaweed samples were coated in a thick viscous sludge. The Great Island Oil Power Station is located on the opposite bank of the river.

     o *Site 4: Passage East, Co. Waterford*

Passage East is a small fishing village located approximately 10km downstream from Waterford City. *P. lanosa, F. vesiculosus* and *A. nodosum* were collected at a site adjacent to Passage East ferry.

     o *Site 5: Fethard-on-Sea, Co. Wexford*

Fethard-on-Sea is a small village located on the Eastern side of the Hook Peninsula in Co. Wexford. There are many clean sandy beaches and outcrops in the vicinity. *F. vesiculosus, A. nodosum, P. lanosa* and *Ulva* sp were collected from Fethard-on-Sea harbour at low tide.

3.3.5 Seaweed Collection

     o *October, 2007 Samples*

Five seaweed species, *P. lanosa* (red)*, A. nodosum* (brown)*, F. vesiculosus* (brown), *F. ceranoides* and *Ulva* sp (green) were collected from various sites at the following locations, in October 2007:

- Fethard-on-Sea, Co. Wexford
- Suir Estuary, Co. Waterford, Ireland
- Bonne Bay, Newfoundland, Canada
- Humber Arm, Corner Brook, Newfoundland, Canada

     o *May, 2008 Samples*

Four seaweed species, *Polysiphonia lanosa* (red), *Ascophyllum nodosum* (brown), *Fucus vesiculosus* (brown) and *Ulva* sp (green) were collected at low tide from Fethard on Sea, Co. Wexford, Ireland (52° 11'53.68" N, 6° 49' 34.64" W), on the 5th of May 2008. The location at Fethard-on-Sea was selected because of the relatively low metal content in both seawater and seaweed (established through preliminary work by the Estuarine Research Group at Waterford Institute of Technology). The relative abundance of the various seaweed species at the site was also a factor.

3.3.6   Seaweed Preparation

The seaweed samples were rinsed thoroughly on site with seawater in order to remove any adhering debris. The range of seaweed collected within each species from various locations on the sampling site was combined to give composite batches. Once harvested, the seaweed was placed in tubs containing seawater and transported to the laboratory within 2 hours. On return to the lab, samples were washed twice in deionised water, blot dried, divided into triplicate samples and oven dried at 60°C for 24h. Samples were ground in a mill and passed through a sieve to give particle sizes ≤500 μm. Samples were stored in airtight polyethylene bottles until required for analysis. Seaweed samples for the determination of intracellular metals were rinsed twice for 10 min with a 5 mM EDTA (pH 8) solution, prior to oven drying, to remove metals adsorbed by the cell wall (García-Ríos *et al*., 2007). The concentration of surface bound metals (mg/kg dw) was calculated by subtracting the concentration of intracellular metals (mg/kg dw) from the concentration of total metals (mg/kg dw).

3.3.7   Seawater Collection and Preparation

Seawater samples were collected in triplicate, in polyethylene bottles decontaminated by rinsing several times with deionised water prior to collection, then rinsing several times on site with seawater.

The seawater samples were combined and mixed well to give composite batches, before being filtered through a 0.45 μm filter (Supor ® Polyethersulfone (PES) membrane). Samples were stored at -20°C in 125ml polypropylene bottles prior to metal analysis the following day.

3.3.8   Seawater analysis – Physico-Chemical Data

Physico-Chemical analysis of seawater was performed on site using a HACH system. Analysis was performed in triplicate.

3.3.9 Seawater – Metal Analysis

Seawater analysis was performed by the National Laboratory Service (UKAS accredited), The Environment Agency, Olympia House, Gelderd Lane, Gelderd Road, Leeds LS12 6DD, England. The Environment Agency carries out analytical work to high standards and within the scope of its UKAS accreditation.

3.3.10 Sediment Sampling (Suir Estuary and Fethard-on-Sea)

Sediments were sampled from the same Suir Estuary and Fethard-on-Sea sites as the seaweed samples. At each site, intertidal sediments (upper 2 cm and upper 20 cm) were collected for total metal analysis.

At each site, twenty samples were taken within an area of approximately 10 m^2. The upper 2 cm samples were removed with a polythene scoop and immediately placed in airtight polythene bags. The upper 20 cm samples were removed with an auger and placed in air tight polythene bags. In the lab, sediments were mixed to form composite samples. Following oven drying at 60 ^{o}C for 24 h, samples were ground using a pestle and mortar and sieved. The < 63 μm sediments were retained for analysis.

3.3.11 Sediment Digestion and Analysis

Representative samples of 0.5 g were digested in 9 ml concentrated nitric acid and 3 ml concentrated HCl using a microwave digestion unit (United States EPA, 1998), (Milestone Microwave Laboratory Systems with Easy Wave 3 software – version 3.2.1.0). The sediment digestion programme is shown in Table 3.2. After cooling, the vessel contents were filtered and diluted to a final volume of 25 ml with ultrapure water. Samples were analysed for total metals using ICP-OES. Certified Reference Materials (Trace Metals-Sand 1) were treated as per sediment samples.

Table 3.2. Microwave digestion programme for sediment samples.

Time (*min*)	Temperature (^{o}C)	Duration (*mins*)
0 – 10	Increased to 130	10
10 – 20	130	10
20 – 30	130 - 200	10
30 – 50	200	20
50 - 70	cooling	20

3.3.12 Total protein determination

Refer to 2.3.8 for Kjeldahl method

3.3.13 Polyphenol Extraction

Refer to 2.3.6.6

3.3.14 Polyphenol Determination

Refer to 2.3.7

3.3.15 Total, Intracellular and Surface Bound Metal Determination

3.3.15.1 Sample Digestion

The following materials were added to appropriately labelled digestion tubes in triplicate: 5ml Nitric Acid, 3ml deionised water, 1ml H_2O_2 and 300mg sample. Stoppers were inserted and lids were securely twisted onto the tubes. The tubes were placed into appropriate segments of a microwave carousel and the samples were subjected to 80^oC, using 600 Watts of power for 2 minutes, then $160\ ^oC$ for 2 minutes and finally $200\ ^oC$ for 4 minutes using 1200 Watts. This method was based on a protocol by Caliceti *et al.* (2002) with modifications.

3.3.15.2 Metal Analysis

When digestion was complete, the carousel was removed and the tubes transferred to a fume cupboard. The contents were carefully transferred to a 25ml volumetric flask and brought to the mark with deionised water. Samples were transferred to appropriately labelled bottles and refrigerated at 4^oC until sample analysis. Analysis was carried out using Varian Inductively Coupled Plasma Spectrometer (Varian 710-OES), using Reference Standards in the range 0 – 1000 ppb. Surface bound metal levels were calculated by subtracting intracellular metal levels from total metal levels. For metals under investigation in this study, the most common oxidation states in the environment are as follows: Cd^{2+}, Cr^{3+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, As^{3+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Al^{3+}. ICP analysis however does not distinguish between oxidative states and therefore analyses for total metals. Metals quoted throughout this work refer to the element speciation as it appears naturally in the environment.

The following Certified Reference Materials (CRM) were used for validation:

- BCR Reference Material No. 60 *Lagarosiphon major*

- NIST Standard Reference Material Tomato Leaves

3.3.16 Calculations to determine Concentration Factors (CF),

(Fehrmann & Pohl, 1993).

$$CF = \frac{\text{Mean metal concentration in seaweed (mg/kg)}}{\text{Mean metal concentration in seawater (mg/L)}} \qquad \textbf{(Equation: 3.1)}$$

3.3.17 Statistical Analysis

3.3.17.1 One-Way Analysis of Variance

Data was analysed statistically using 1-way ANOVA and significant differences were identified using Tukey Post Hoc tests. One-Way ANOVA compares the mean values of a population by analysing variation in the data. In relation to One-Way ANOVA it is assumed that variables are normally distributed and that the samples are independent of each other. One-way analysis of variance (ANOVA) was performed in order to compare the concentrations of metals, protein and polyphenols in different seaweed species. Significant variation is expressed as $p<0.05$.

One-Way ANOVA was performed in order to compare:

- Metal concentrations of *A. nodosum* according to site number for each location.

- Total protein concentrations of *A. nodosum* according to site number for each location.

- Polyphenol concentrations of *A. nodosum* according to site number for each location.

- Metal concentrations of *F. vesiculosus* according to site number for each location.

- Total protein concentrations of *F. vesiculosus* according to site number for each location.

- Polyphenol concentrations of *F. vesiculosus* according to site number for each location.

- Metal concentrations of *P. lanosa* according to site number for Suir Estuary and Fethard-on-Sea.

- Sediment metal levels according to site number for Suir Estuary and Fethard-on-Sea.

- Regression analysis was performed in order to establish relationships between total protein levels in seaweeds with seawater temperature levels.

- Regression analysis was also performed in order to establish relationships between polyphenol levels in seaweeds with seawater conductivity levels.

3.3.17.2 Regression Analysis – Fitted Line Plot

Regression analysis (fitted line plots) of total protein versus total metals, extracted protein versus intracellular metals and polyphenols versus intracellular metals was performed in order to examine relationships between protein and polyphenols concentrations with metals levels in seaweed.

Minitab 15 was used for data analysing. All data were expressed in terms of mean ± 95% Confidence Intervals. Significant variation was expressed as $p<0.05$.

3.4   Results

3.4.1   Seawater Analysis – Physico-Chemical Data

3.4.1.1 pH

Minimal variation in pH values was observed among all sites at each of the four locations. Average pH values for The Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea were 8.11 ± 0.03, 8.14 ± 0.02, 8.13 ± 0.02and 8.17 ± 0.02 respectively. These results were almost identical therefore, differences in the bioaccumulation potential of seaweeds under investigation in this study cannot be explained by pH readings.

3.4.1.2 Temperature

Overall, the highest average seawater temperature was recorded for The Suir Estuary (13.2 ± 0.05 ^{o}C) and the lowest was observed for The Humber Arm (9.54 ± 0.06 ^{o}C), (Figure 3.6).
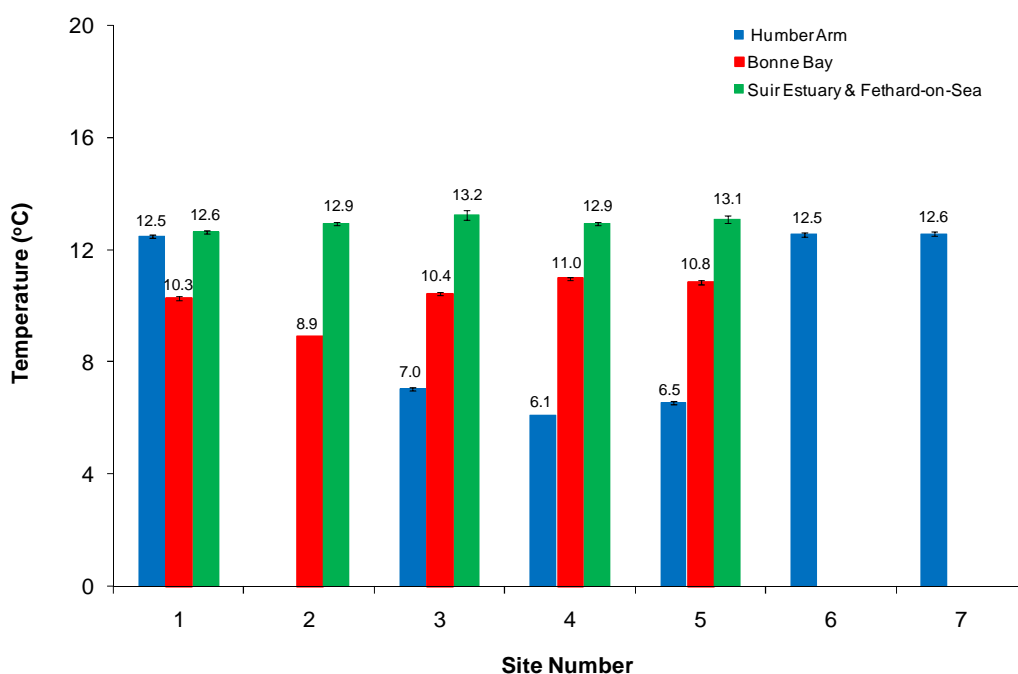
Figure 3.6. Temperature values of seawater collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals. Values for Humber Arm sites 1, 6 and 7 were recorded 3rd October.

Little variation in seawater temperatures were observed along the Suir Estuary. The average temperature for these sites was 12.93 ± 0.08 ^{o}C. The seawater temperature at Fethard-on-Sea was 13.07 ± 0.12 ^{o}C (Figure 3.6). Lower temperatures were recorded for the Bonne Bay sites, with an average temperature of 10.28 ± 0.05 ^{o}C (Figure 3.6). Furthermore, temperature readings of the Bonne Bay sites ranged from 8.9 to 11.0 ^{o}C.

Significant variations were observed for sites along The Humber Arm, where temperatures ranged from 6.1 ± 0.00 ^{o}C at Site 4 to 12.57 ± 0.06 ^{o}C at Site 7. These differences can be explained by sample collection dates. Temperature readings for Sites 1, 6 and 7 were recorded 3rd October, whereas all other readings were collected 16th October. When Sites 1, 6 and 7 were taken into account, the average seawater temperature was 12.52 ± 0.06 ^{o}C. When Sites 3, 4 and 5 however were taken into consideration, little variation in seawater temperature was observed. The average temperature for these sites was 6.56 ± 0.04 ^{o}C (Figure 3.6).

3.4.1.3 Conductivity

Average conductivity readings for the Suir Estuary, Fethard-on-Sea, Bonne Bay and The Humber Arm were 33.46 ± 0.4, 51.11± 0.3, 42.55± 0.06 and 25.06± 0.09 mS/cm respectively. When sites 3, 4 and 5 from The Humber Arm were taken into consideration, a general downstream increase in conductivity was observed. Additionally, the average conductivity reading for these sites was 41.18 ± 0.11 mS/cm (Figure 3.7). See Table 3.3 for a summary of these results.

Similarly, highest conductivity levels at Bonne Bay were recorded for the site furthest downstream (46.10 ± 0.10 mS/cm). Additionally, the highest conductivity levels along the Suir Estuary were recorded for the two sites furthest downstream (42.93 ± 0.21 and 34.70 ± 1.65 mS/cm respectively). The overall highest conductivity value was recorded at the Fethard-on-Sea (51.11 ± 0.03 mS/cm) (Figure 3.7). An overall general downstream increase in conductivity was observed at all locations.
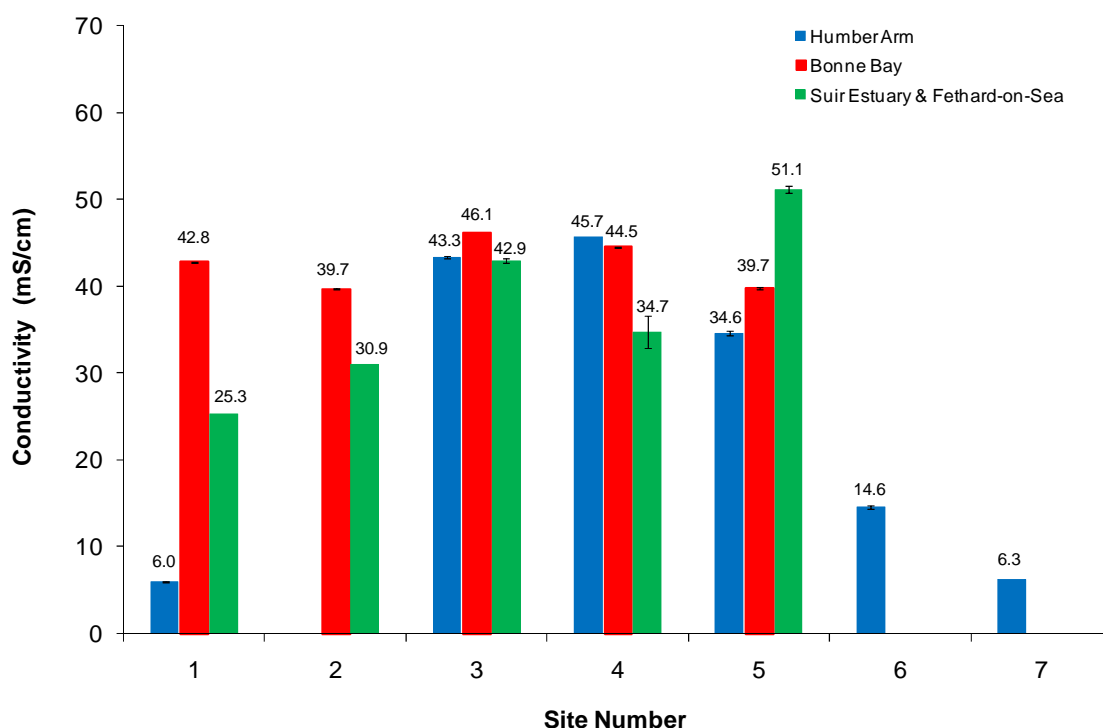
Figure 3.7. Conductivity values of seawater collected. Error bars are calculated based on triplicate runs with 95% Confidence Intervals. Values for Humber Arm sites 1, 6 and 7 were recorded 3rd October. All other results were recorded 16th October.

Table 3.3. Comparison of pH, Conductivity and Temperature for all The Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea.

Physio-Chemical Measurement	*Location*	*Average* (*of all sites*)
pH	Humber Arm	8.11 ± 0.03
	Bonne Bay	8.14 ± 0.02
	Suir Estuary	8.13 ± 0.02
	Fethard-on-Sea	8.17 ± 0.02
Conductivity (mS/cm)	Humber Arm	25.06 ± 0.09
	Bonne Bay	42.55 ± 0.06
	Suir Estuary	33.46 ± 0.46
	Fethard-on-Sea	51.10 ± 0.36
Temperature (^{o}C)	Humber Arm	9.54 ± 0.05
	Bonne Bay	10.28 ± 0.05
	Suir Estuary	12.93 ± 0.08
	Fethard-on-Sea	13.06 ± 0.12

3.4.2 Seawater Metal Analysis

In this study, a wide range of seawater dissolved metals were analysed. Several metals were determined to be below the detection limit, therefore were excluded from images/charts (Table 3.4)

Table 3.4. Limit of detection values for all seawater metals.

Metal	*LOD*	*Unit*	*Metal*	*LOD*	*Unit*
Antimony	10.00	µg/l	Aluminium	110.0	µg/l
Beryllium	10.00	µg/l	Barium	100.0	µg/l
Cobalt	10.00	µg/l	Boron	700.0	µg/l
Molybdenum	30.00	µg/l	Iron	100.0	µg/l
Tin	25.00	µg/l	Manganese	20.00	µg/l
Titanium	20.00	µg/l	Strontium	200.0	µg/l
Vanadium	20.00	µg/l	Chromium	0.500	µg/l
Cadmium	0.040	µg/l	Calcium	10.00	mg/l
Copper	0.200	µg/l	Magnesium	3.000	mg/l
Lead	0.040	µg/l	Potassium	1.000	mg/l
Nickel	0.300	µg/l	Sodium	20.00	mg/l
Zinc	0.400	µg/l	Sulphate, as SO4	5.000	mg/l

There was little variation in concentration for the majority of metals among all sites and locations (Figures 3.8 to 3.16). Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Increases in Na concentration from $393.3 - 10{,}533.3$ mg/L were observed in a downstream direction along the Suir Estuary. This is to be expected as salinity of the seawater increases with increasing distance down the estuary (Lobban & Harrison, 1997). A similar downstream increase was observed for SO_4, K, Mg, Ca, Sr and B. These trends were also observed for the Bonne Bay and Humber Arm locations. Furthermore, downstream increases in seawater salt levels, particularly Na correspond with a general downstream increase in conductivity readings for all locations (3.4.1.3).
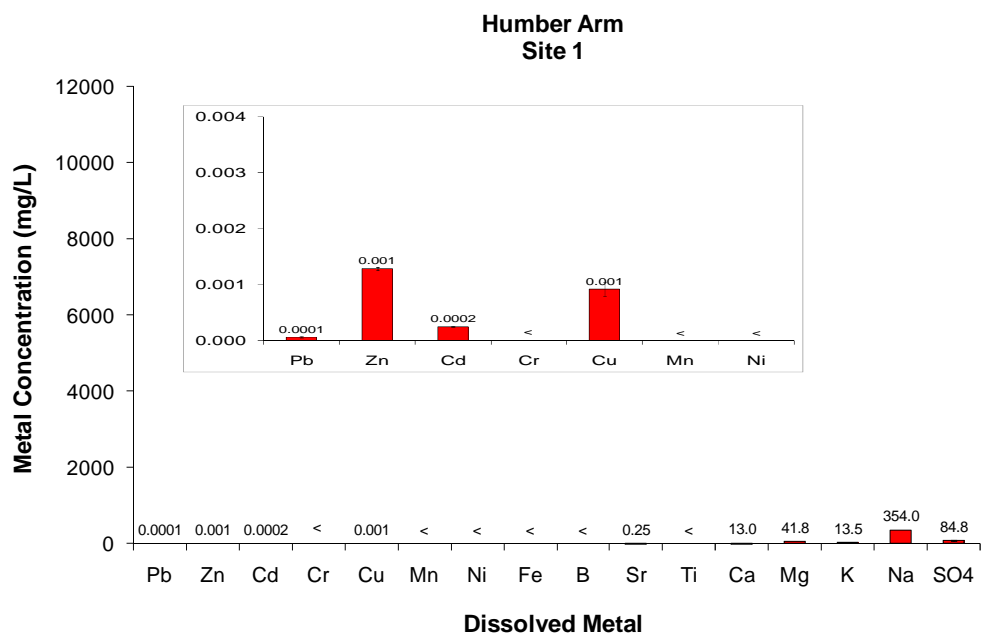
Figure 3.8. Seawater dissolved metal concentrations for The Humber Arm (Site 1). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
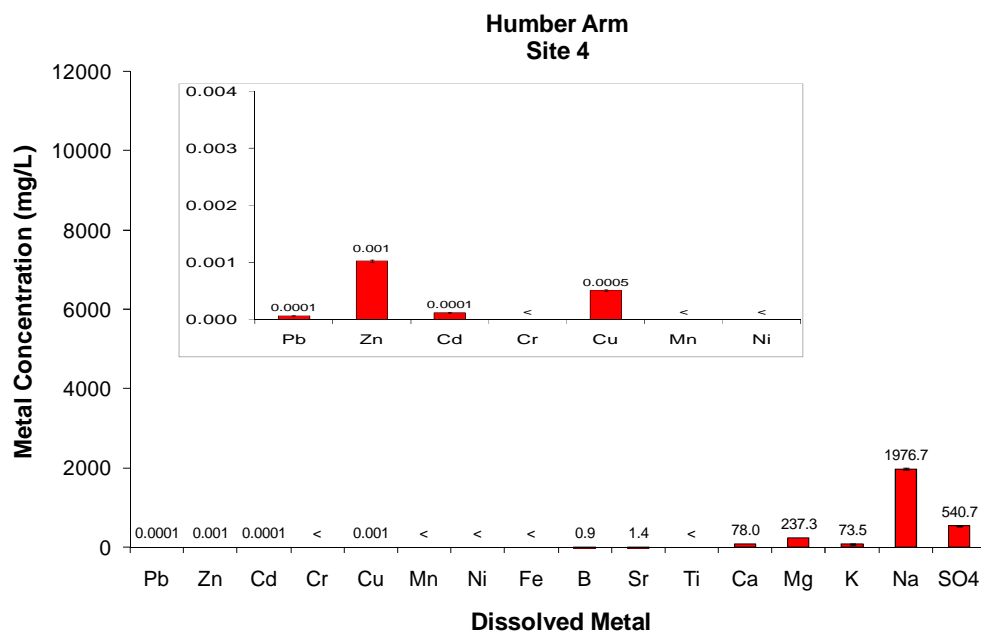
Figure 3.9. Seawater dissolved metal concentrations for The Humber Arm (Site 4). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
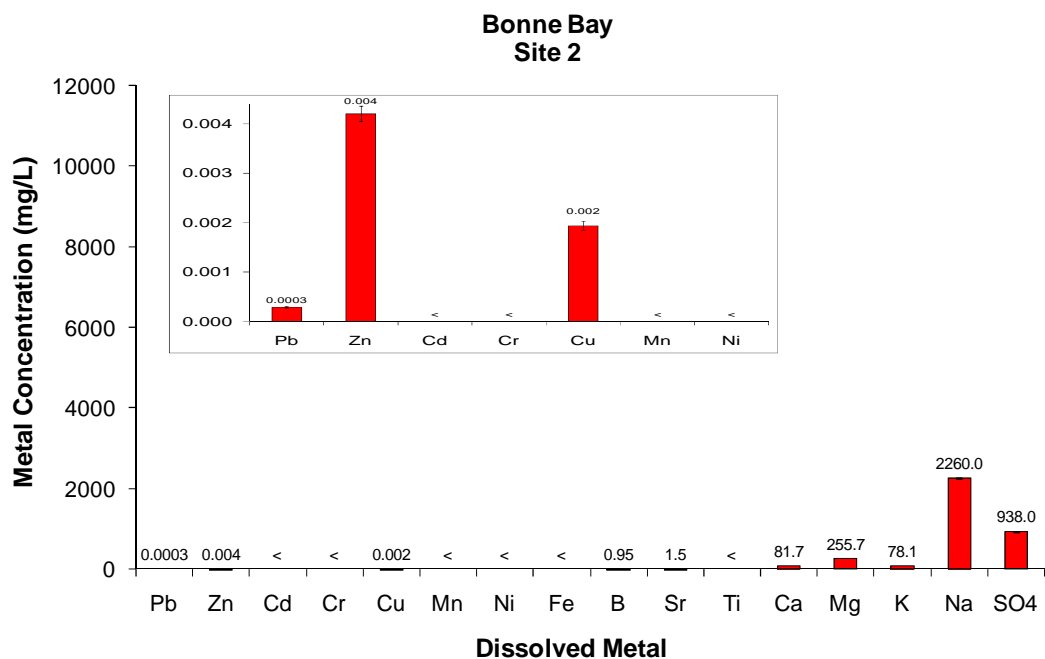
Figure 3.10. Seawater dissolved metal concentrations for Bonne Bay (Site 2). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
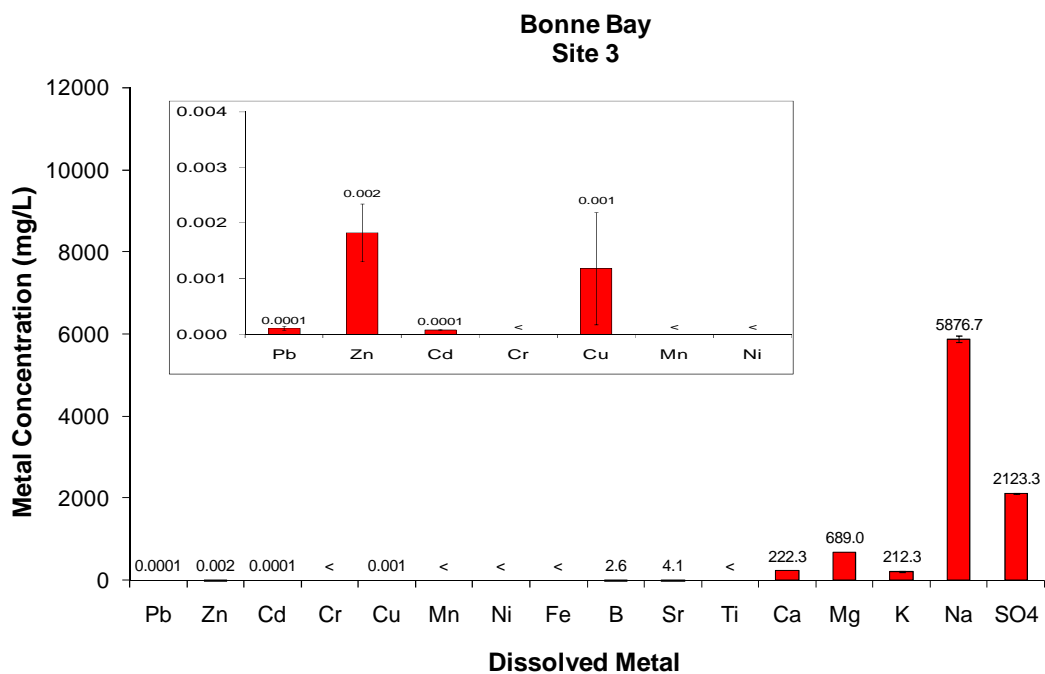
Figure 3.11. Seawater dissolved metal concentrations for Bonne Bay (Site 3). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

154
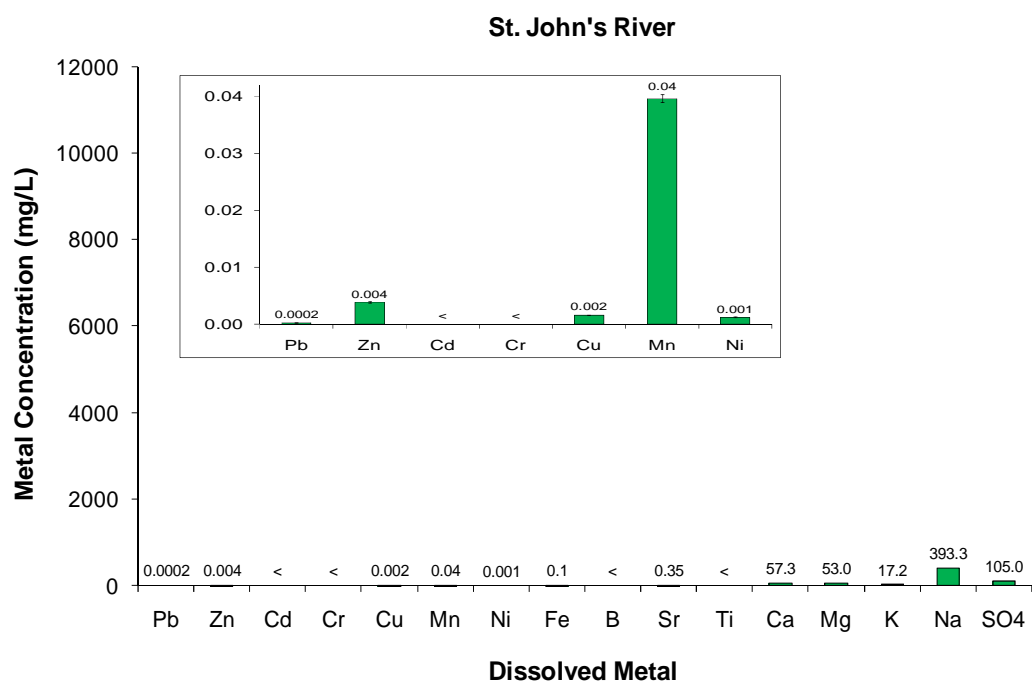
St. John's River

Figure 3.12. Seawater dissolved metal concentrations for Suir Estuary (Site 1). Graph inserts display low ranged metals (< = below the limit of detection). *Insert range for St. John's River is 0.00 – 0.04 mg/L. All other inserts are ranged 0.00 – 0.004 mg/L. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

Ferrybank
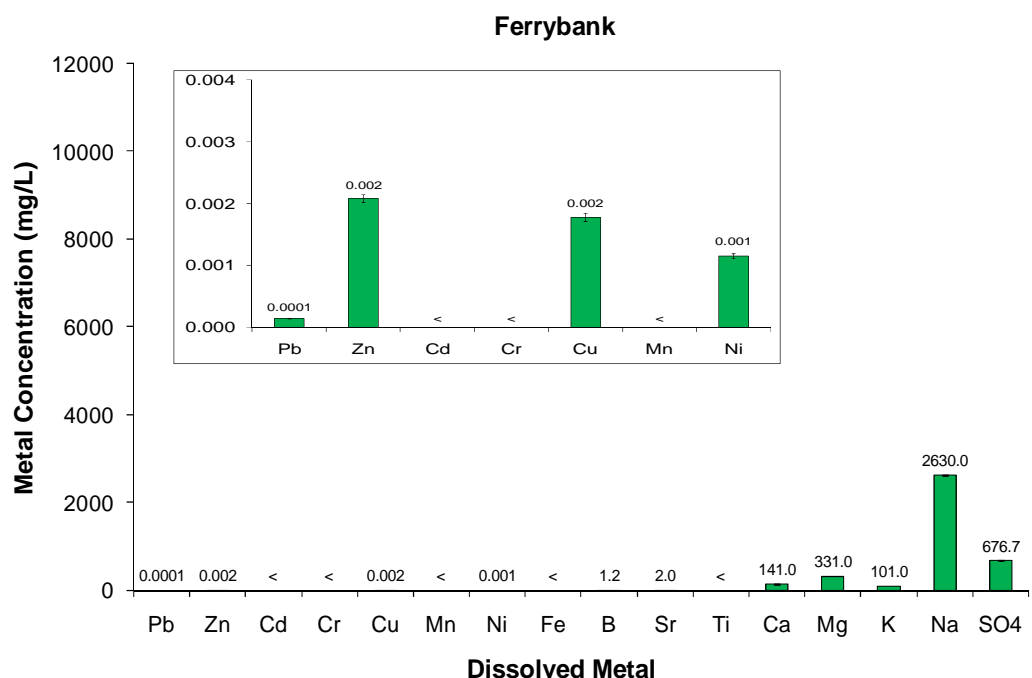
Figure 3.13. Seawater dissolved metal concentrations for Suir Estuary (Site 2). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
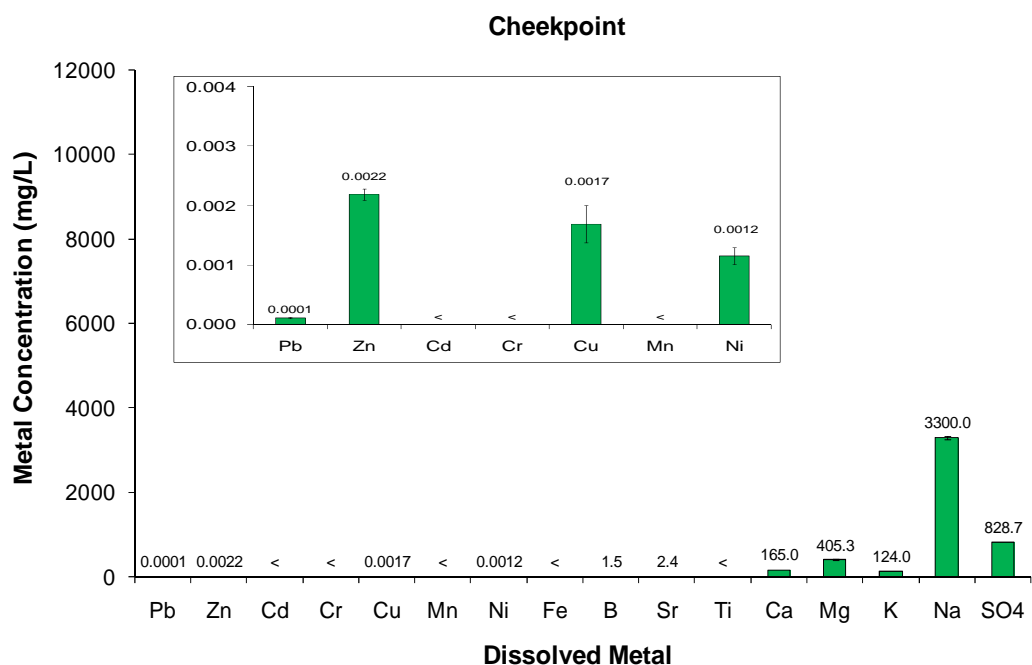
Figure 3.14. Seawater dissolved metal concentrations for Suir Estuary (Site 3). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
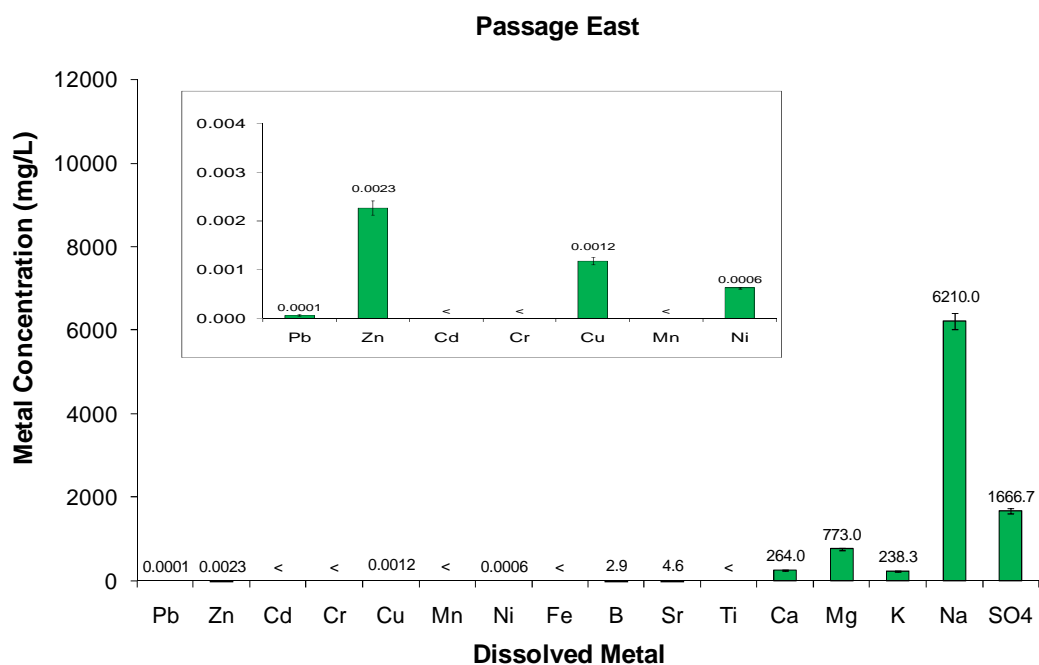
Figure 3.15. Seawater dissolved metal concentrations for Suir Estuary (Site 4). Graph inserts display low ranged metals (< = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
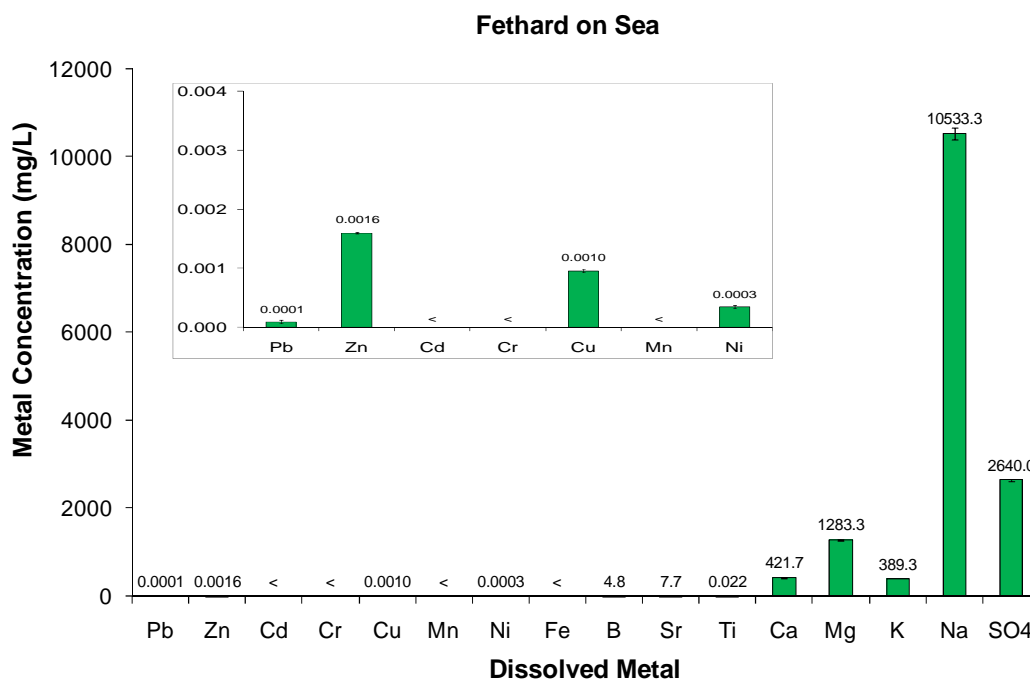
Figure 3.16. Seawater dissolved metal concentrations for Fethard-on-Sea. Graph inserts display low ranged metals ($<$ = below the limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

Mn^{2+} (0.004 mg/L) was only detected at St. John's River, the furthest upstream site on the Suir Estuary (Figure 3.12). Levels of Cr^{3+}, Al^{3+}, Cd^{2+} and Co^{2+} were found to be below the limit of detection, thus indicating clean and uncontaminated seawater with respect to these metals (Figures 3.8 to 3.16). Pb^{2+} levels for the Newfoundland sites ranged from 0.0001 – 0.0003 mg/L (Figures 3.8 to 3.11) and similarly, corresponding values for the Ireland sites ranged from 0.0001 – 0.0002 mg/L (Figures 3.14 to 3.18).

Cu^{2+} for the Newfoundland sites ranged from 0.0005 – 0.002 mg/L, the highest at Bonne Bay (Site 2), (Figures 3.8 – 3.11). Levels of Cu^{2+} for the Irish sites ranged from 0.001 mg/L (Fethard-on-Sea) to 0.002 mg/L (St. John's River), (Figures 3.12 to 3.16).

Levels of Ni^{2+} were below the limit of detection for the Newfoundland sites (Figures 3.8 to 3.11). A downstream decrease in the levels of Ni^{2+} (0.003 – 0.001 mg/L) was observed for the Ireland sites.

Zn^{2+} levels for the Newfoundland sites ranged from 0.001-0.004 mg/L, the highest value observed for Bonne Bay (site 2). Levels for the Irish sites ranged from 0.0016 – 0.004 mg/L, the highest value obtained by St. John's River (Figures 3.8 – 3.16). This higher value may correspond to Zn^{2+} mining in the Suir River catchment area.

Comparisons of seawater metals, collected from Fethard-on-Sea in October, 2007 and May, 2008 demonstrated little variations in their concentrations. Furthermore, results for seawater collected in May, 2008 generally corresponded with global and Irish seawater metal levels from previous studies. Slight variations however, in levels of Pb^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+} were also observed (Table 3.5).

Table 3.5. Comparison of mean seawater metal concentrations from the present study with global seawater metal concentrations reported in previous studies. Calculations were based on triplicate samples with 95% Confidence Intervals.

Dissolved Metal	*Average Concentration (mg/L) Present Study*	*Average Concentration (mg/L) Global Studies*	*Average Concentration (mg/L) Irish Sea* [1]
Lead	0.0001	0.000009[3]	0.0016
Zinc	0.0010	0.0004[2]	0.0066
Cadmium	< 0.00004	0.0000024[3]	0.00041
Cobalt	< 0.01	≤0.00021[4]	——
Chromium	< 0.0005	≤0.00035[5]	——
Copper	0.0010	0.00020[2]	0.0014
Manganese	< 0.02	0.00003[2]	0.002
Nickel	0.0004	0.00018[3]	0.0014
Antimony	< 0.01		
Beryllium	< 0.01		
Molybdenum	< 0.03		
Tin	< 0.025		
Vanadium	< 0.02		
Barium	< 0.1		
Iron	< 0.1		
Titanium	0.0246		
Aluminium	0.1		
Boron	4.9		
Strontium	7.8		
Calcium	411.7		
Magnesium	1260.0		
Potassium	387.3		
Sodium	10333.3		
Sulphate (as SO^4)	2576.7		

[1] Preston *et al*., 1972; [2] Lobban & Harrison 1997; [3] Apte *et al*., 1998; [4] Cannizzaro *et al*., 2000; [5] Cuong *et al*., 2008; < = below the limit of detection.

　　　　 = Metals subsequently analysed throughout this study.

3.4.3   Sediment Metal Analysis

Certified Reference Materials (CRM) were digested and analysed for metal content as per seaweed samples. % recovery of CRMs were as follows: Pb (82±1.0%), Zn (85±0.6%), As (90±0.5%), Cd (87±1.4%), Co (84±1.3%), Cr (84±0.3%), Cu (82±0.3%), Mn (87±0.7%), Ni (84±0.8%) and Al (73±3.5%). In this study, there was a general decrease in all metals downstream along the Suir Estuary (Figures 3.17 to 3.26). With respect to all five sites, Fethard-on-Sea yielded the lowest metal concentrations. St. John's River generally yielded the highest levels. Additionally, there was little variation in metal concentration between surface and 20 cm sediment samples (Figures. 3.17 to 3.26). Generally, levels of Cu^{2+}, Cr^{3+}, Ni^{2+}, Pb^{2+}, As^{3+}, Cd^{2+}, Co^{2+} and Cu^{2+} were low (0.9 – 73.9 mg/kg dw) when compared to Mn and Al (153.9 – 6,937 mg/kg dw). One-way ANOVA demonstrate significant variance ($p<0.05$) for all metals (with the exception of Arsenic) versus site number (Table 3.6). Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals according to site number is demonstrated in Table 3.6. For example no significant variations in the Cd level of sites 2 and 3 were demonstrated however, there were significant variations in the Cd concentration of site 1when compared with all other sites. Furthermore, there were no significant variations in the Ni levels of sites 1, 2 and 3, nor sites 4 and 5.
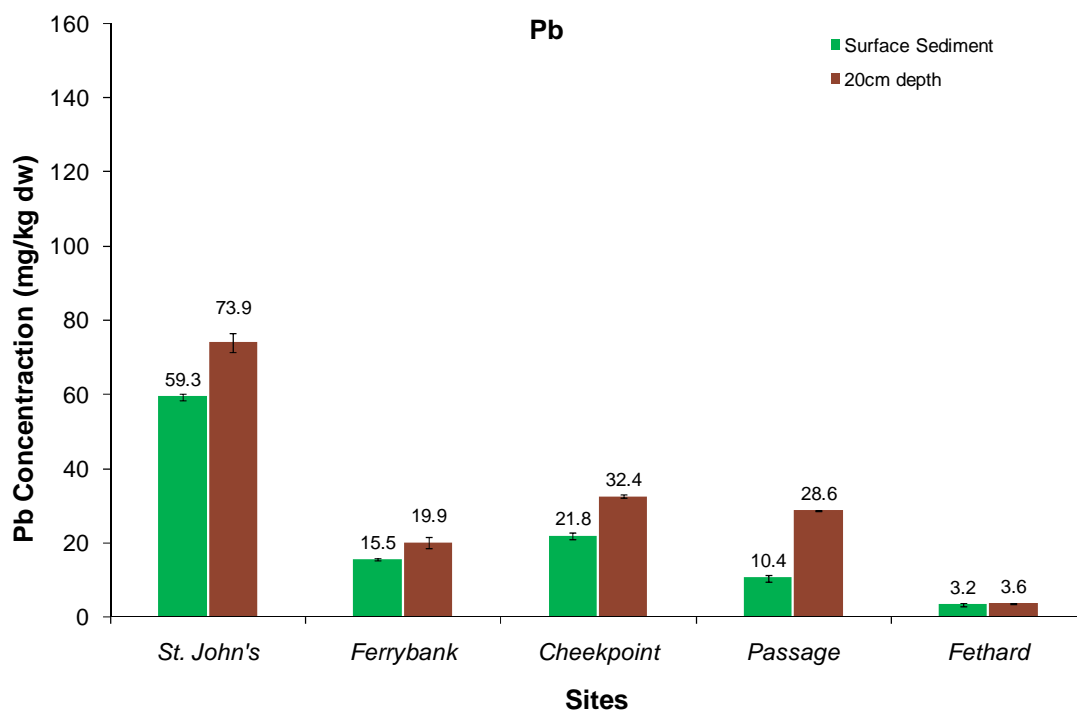
Figure 3.17. Pb levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
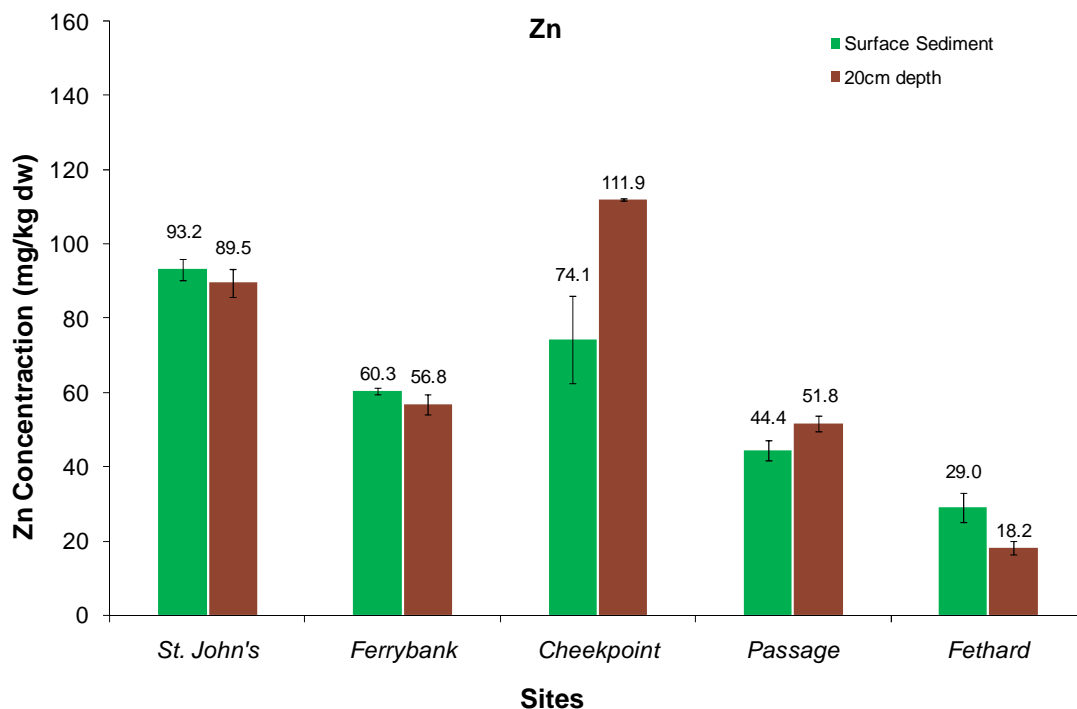
Figure 3.18. Zn levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
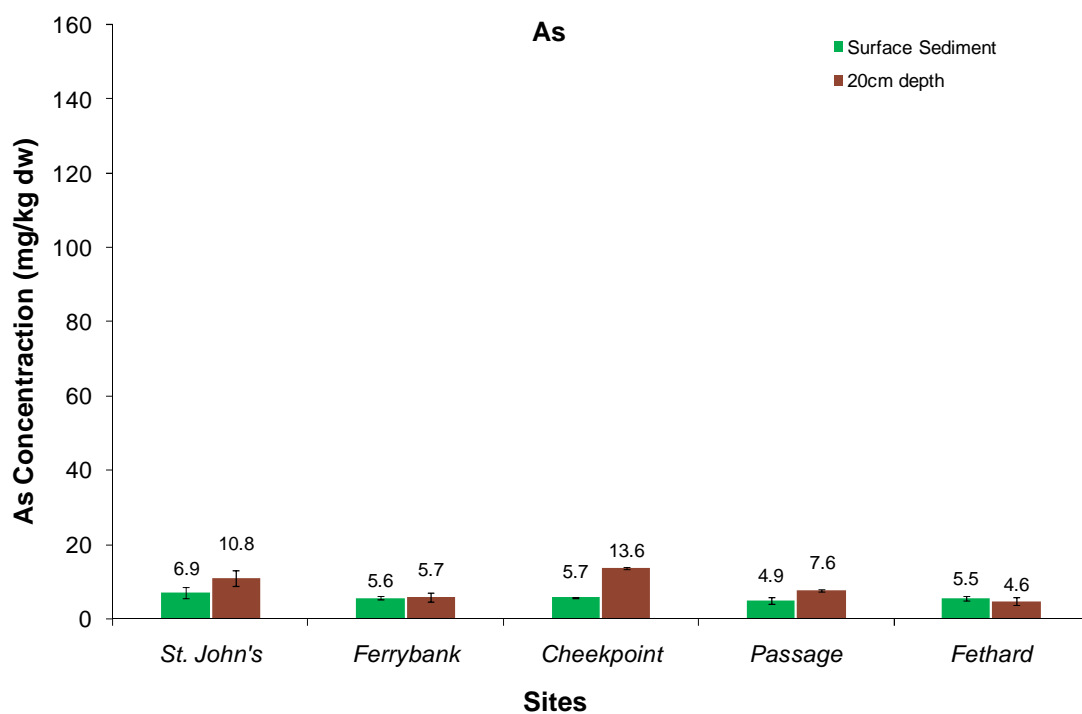
Figure 3.19. As levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
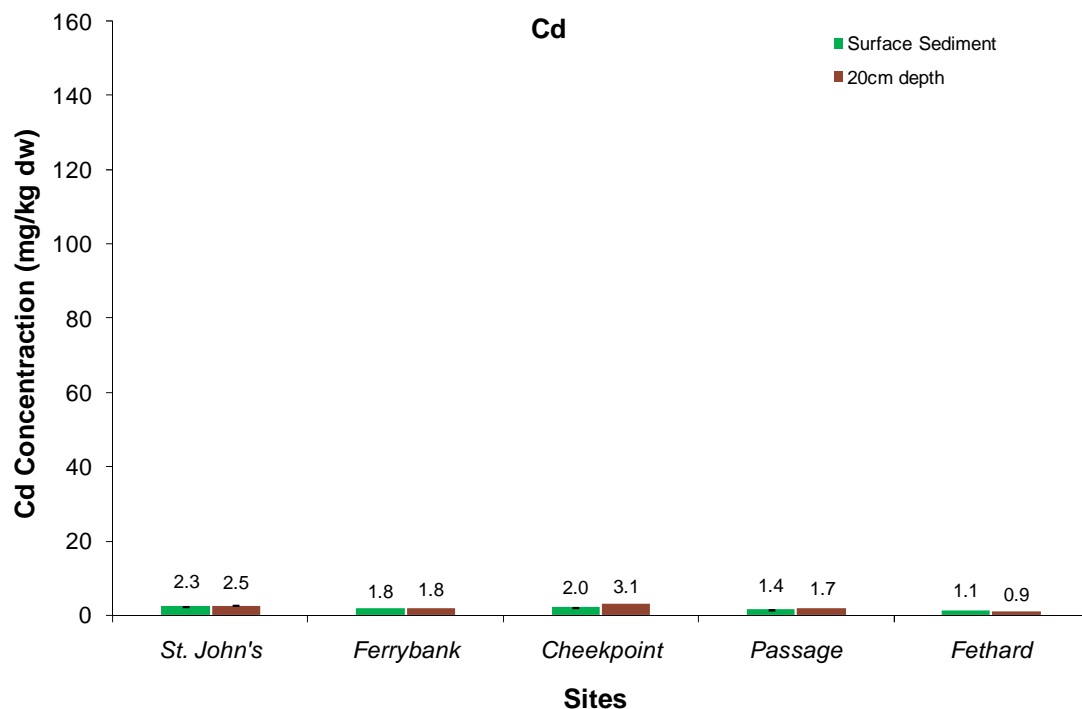
Figure 3.20. Cd levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

161
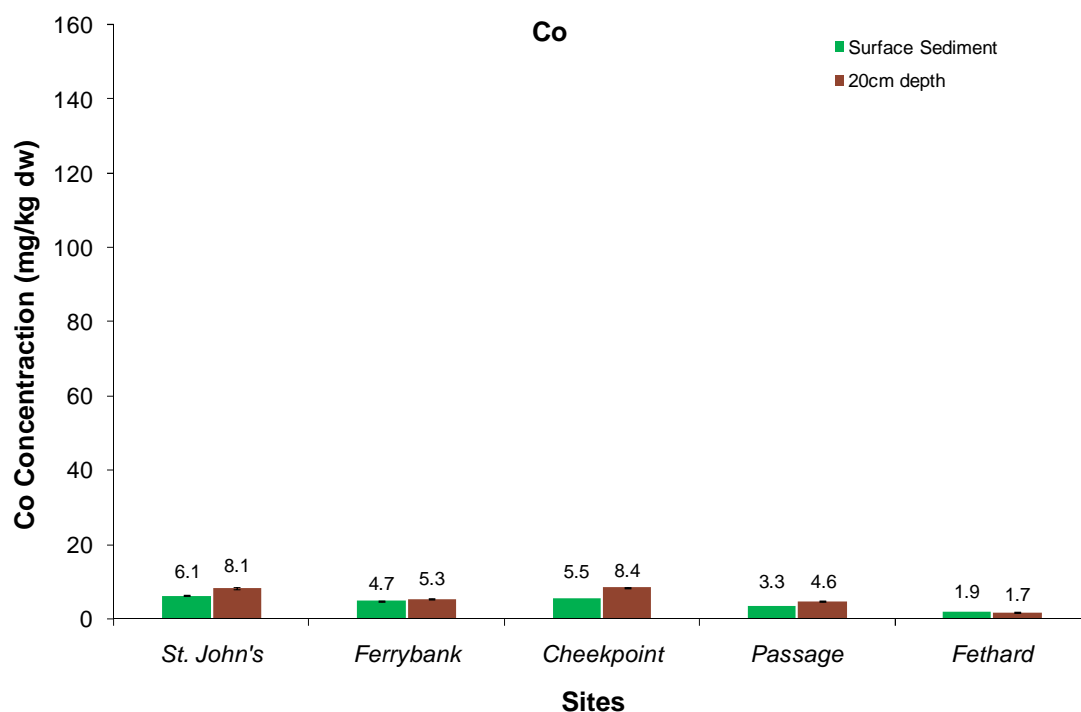
Figure 3.21. Co levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
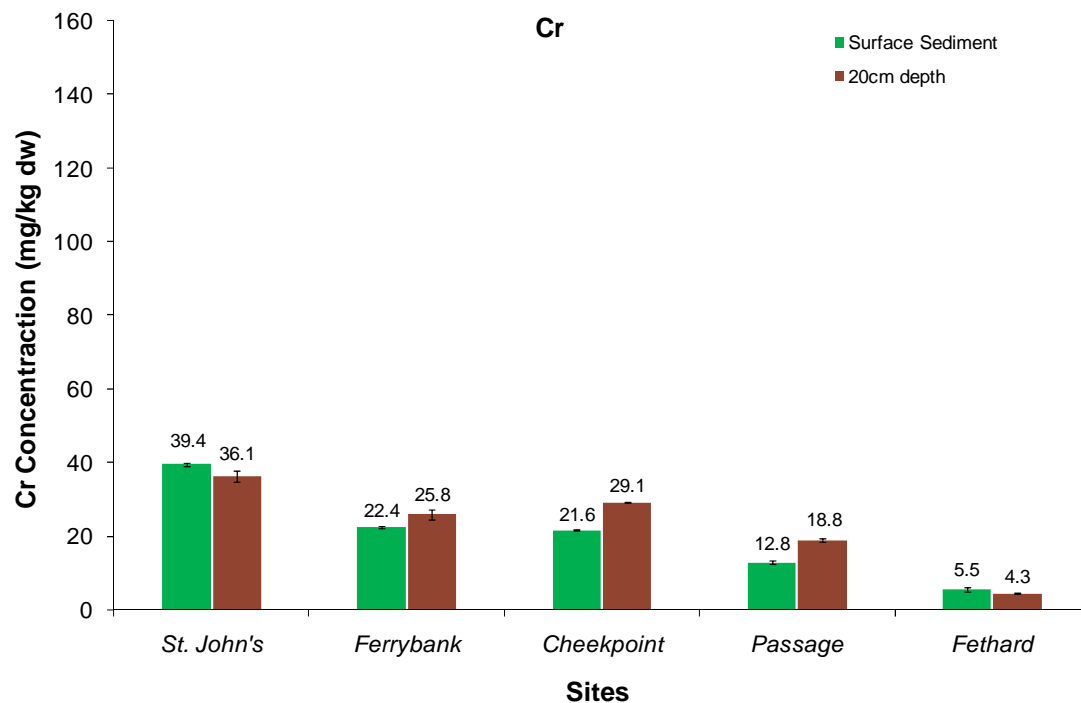
Figure 3.22. Cr levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
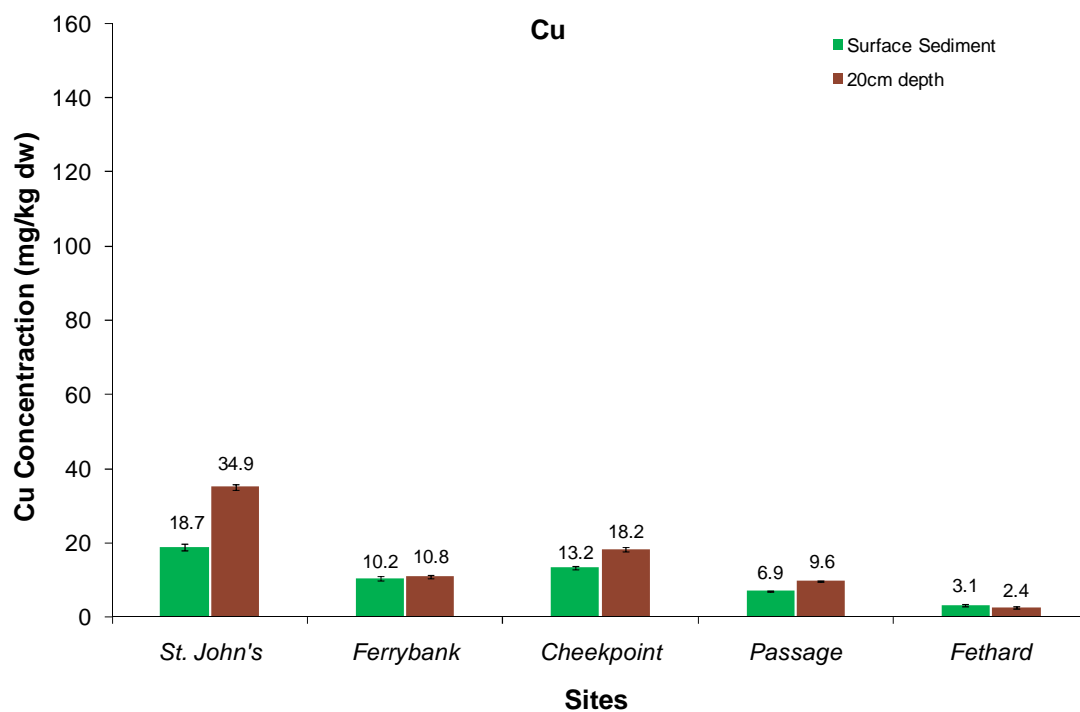
Figure 3.23. Cu levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
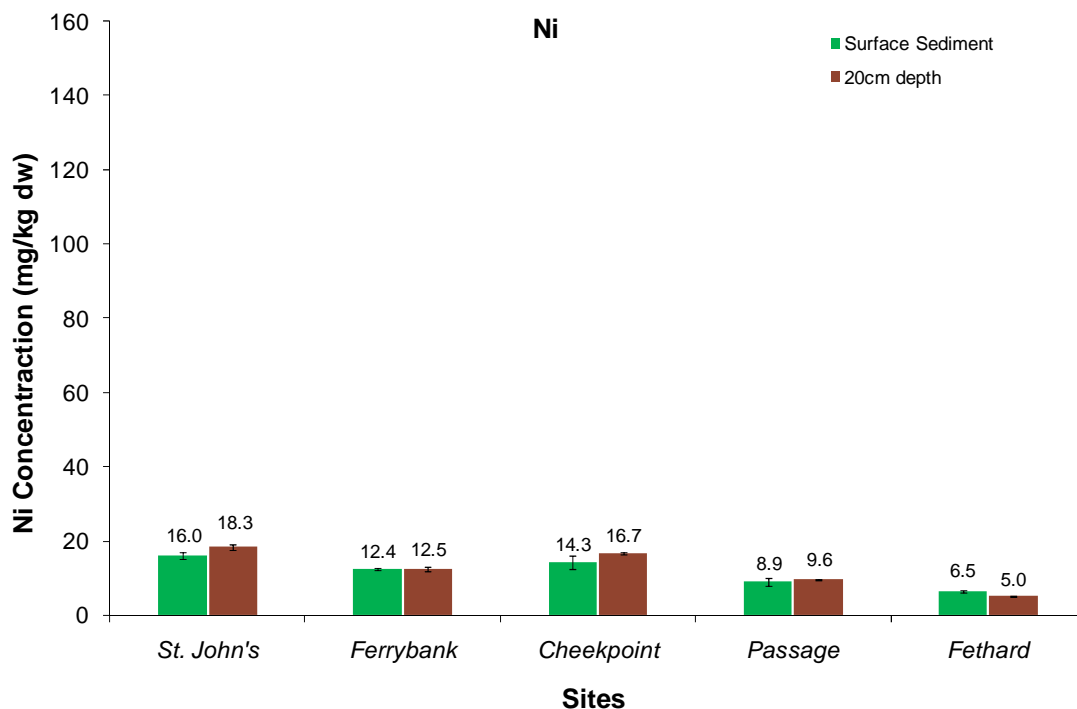
Figure 3.24. Ni levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
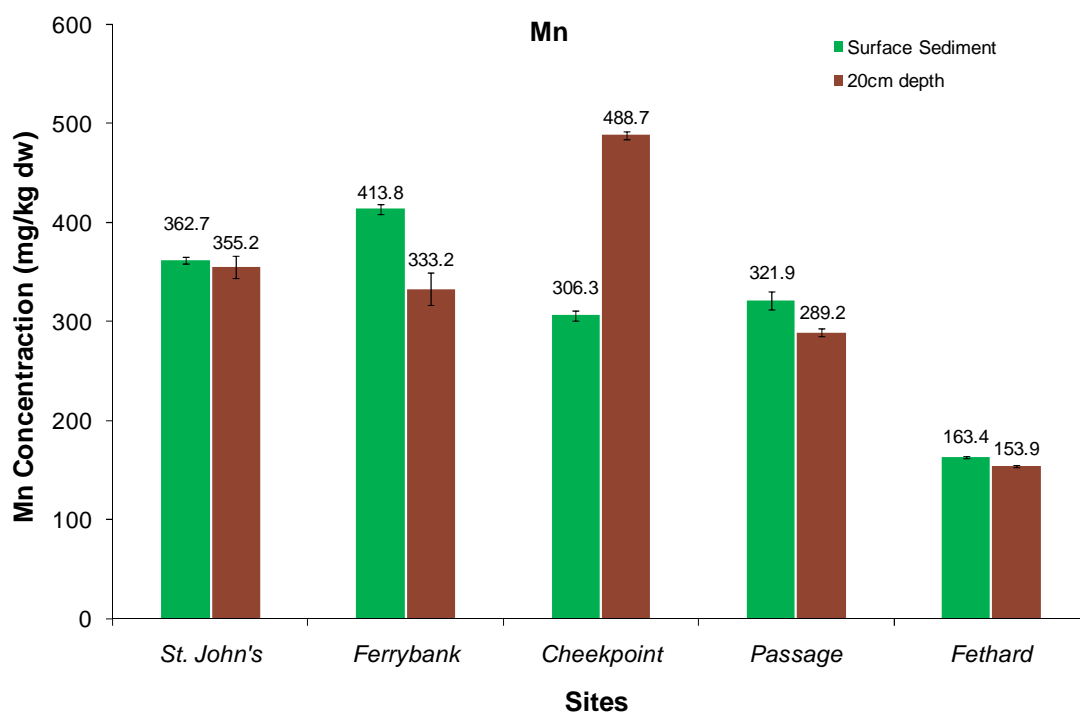
Figure 3.25. Mn levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
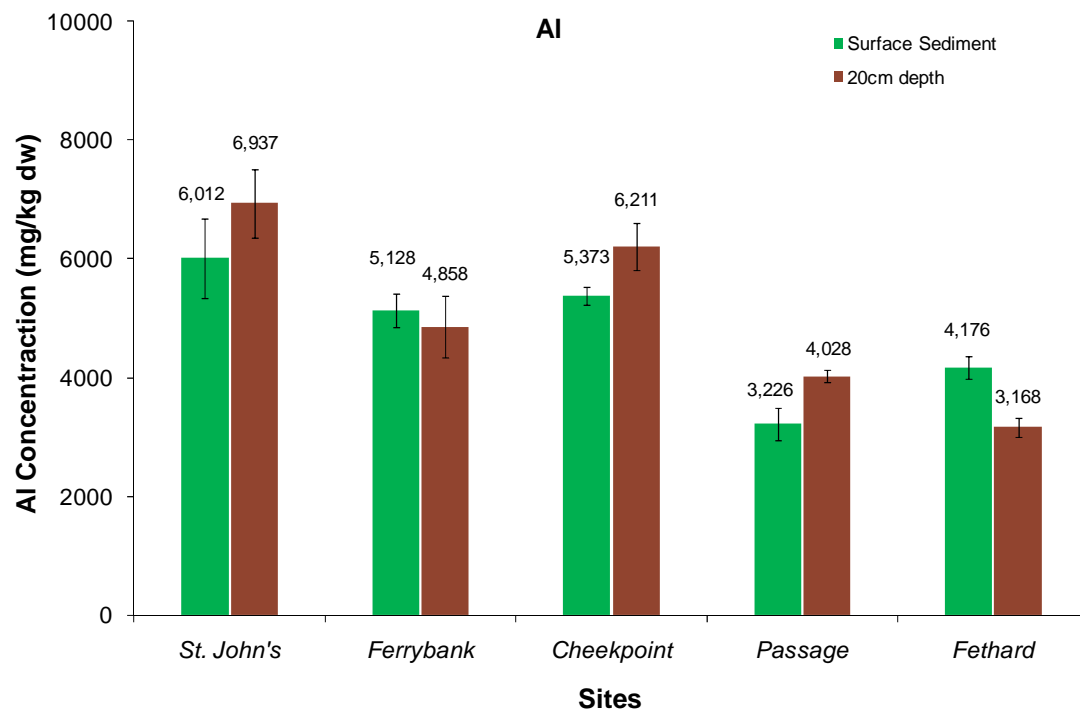
Figure 3.26. Al levels found in sediment samples from various sites along the Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

164

Table 3.6. One-Way ANOVA and post hoc Tukey analysis of surface sediment metal concentration (mg/kg dw) versus site number. Sediment samples were collected from various sites along the Suir Estuary and Fethard-on-Sea. Site numbers are displayed according to their sediment metal concentration.

Range of values for:	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. with highest concs.*	*One-Way ANOVA (p value)*
Cadmium 1.1 – 2.3	[5]	[4]		[2,3]	[1]	0.001
Chromium 5.5 – 39.4	[5]	[4]		[2, 3]	[1]	0.001
Lead 3.2 – 59.3	[5]	[4]	[2]	[3]	[1]	0.001
Copper 3.1 – 18.7	[5]	[4]	[2]	[3]	[1]	0.001
Zinc 29.0 – 93.2	[4,5]	[2]	[3]		[1]	0.001
Arsenic 5.5 – 6.9			[4,5,2,3,1]			0.063
Cobalt 1.9 – 6.1	[5]	[4]	[2]	[3]	[1]	0.001
Manganese 163.4 - 413.8	[5]	[3]	[4]	[1]	[2]	0.001
Nickel 6.5 – 16.0	[4,5]			[1,2,3]		0.001
Aluminium 3226.3 – 6012.1	[4,5]		[2,3]		[1]	0.001

3.4.4 Total Protein Determination

Total protein levels of the brown, red and green seaweeds collected in this study varied according to species. The protein concentration for the brown seaweeds ranged from 3.9 – 12.8 % dw (39-128 mg/g dw), while the red seaweeds ranged from 18.7 – 29.7 % dw (187-297 mg/g dw) and the protein content of *Ulva* sp was 20.6 % dw (206 mg/g dw) (Table 3.7 and Figure 3.27).

Table 3.7. Comparison of total protein content of *F. vesiculosus, A. nodosum, P. lanosa, F. ceranoides* and *Ulva* sp for all four locations. <u>Average</u> total protein values with 95% confidence intervals are presented when all sites at each location are considered.

Seaweed species	*Humber Arm*	*Bonne Bay*	*Suir Estuary*	*Fethard-on-Sea*
F. vesiculosus (*%*)	6.2 ± 0.28	6.6 ± 0.23	12.0 ± 0.17	10.8 ± 0.45
A. nodosum (*%*)	6.5 ± 0.20	5.2 ± 0.25	8.9 ± 0.47	7.4 ± 0.10
P. lanosa (*%*)	——	18.7 ± 0.28	28.9 ± 0.15	25.6 ± 1.06
F. ceranoides (*%*)	——	7.05 ± 0.04	——	——
Ulva sp (*%*)	——	——	——	20.6 ± 0.52

F. vesiculosus and *A. nodosum* collected at sites along the Humber Arm showed a general downstream decrease in total protein. Total protein content of *F. vesiculosus* was more abundant at all sites with the exception of site six when compared with that of *A. nodosum*. Total protein for *F. vesiculosus* and *A. nodosum* at this site was 7.5 and 10.2% dw respectively. *F. vesiculosus* collected at the site furthest downstream (site 3) contained the lowest total protein (4.6% dw). Whereas *F. vesiculosus* collected at the site furthest upstream (site 1) contained the highest total protein (11.4% dw). *A. nodosum* collected at the sites furthest downstream (sites 3 and 4) also contained the lowest protein (4.4 and 3.9% dw respectively). While *A. nodosum* collected from the site furthest upstream (site 6) contained the highest total protein (10.2% dw) (Figure 3.27 and Table 3.7)

One-Way ANOVA ($p<0.05$) demonstrated a significant spatial variation in the total protein content of *F. vesiculosus, A. nodosum* and *P. lanosa* with respect to site number.
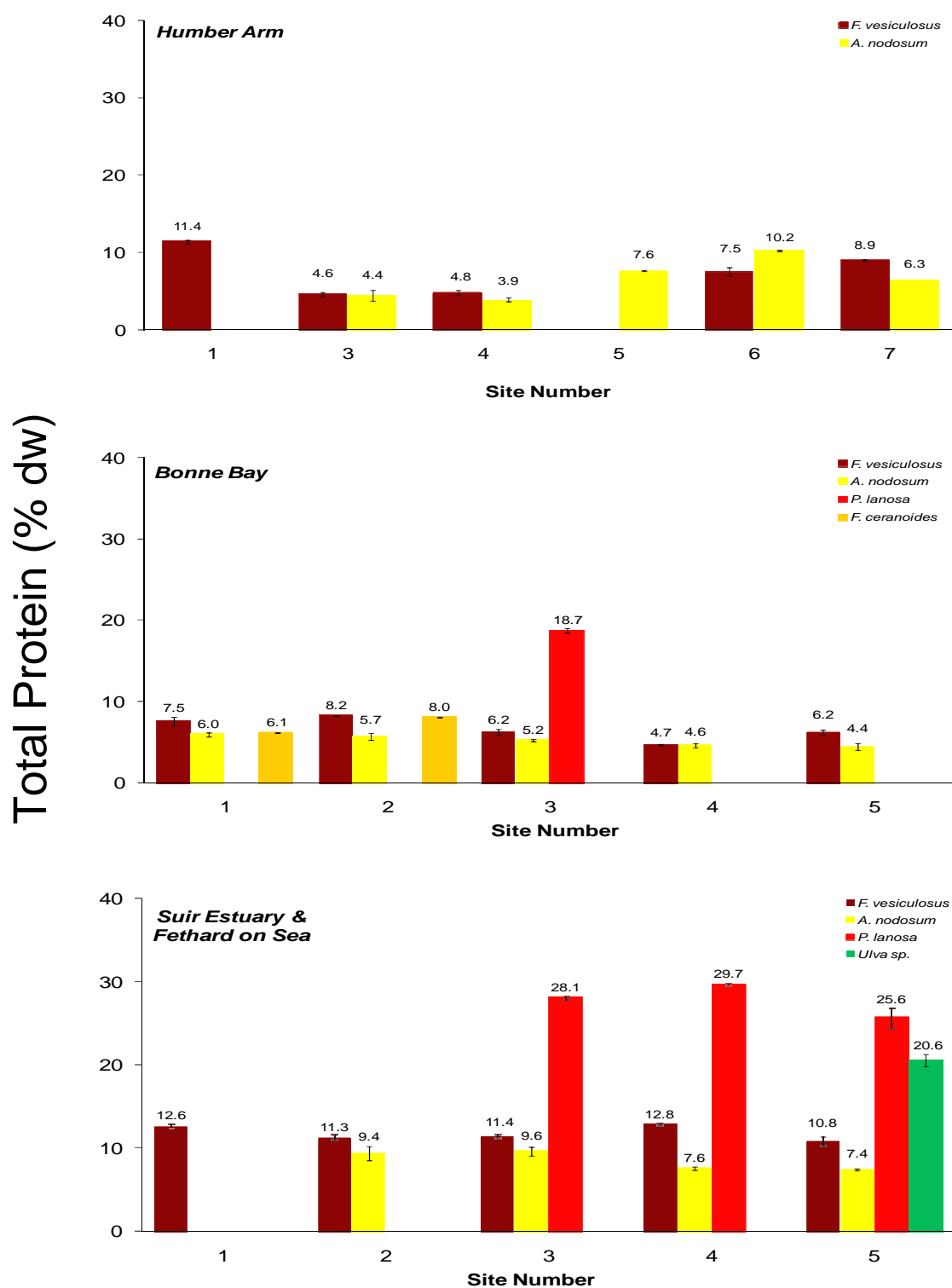
Figure 3.27. Percentage total protein of seaweeds collected from various sites along the Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars are calculated based on triplicate samples with 95% Confidence Intervals.

Seaweeds collected at Bonne Bay showed little variation in total protein content of *F. vesiculosus, A. nodosum and F. ceranoides* among all sampling sites. Protein values for these three species ranged from 4.7-8.2%, 4.4-6.0% and 6.1-8.0% dw respectively. *P. lanosa* contained, by far the greatest total protein (18.7% dw) when compared with that of the brown species (Figure 3.27 and Table 3.7).

There was little variation in total protein content of *F. vesiculosus* collected from all four sites along the Suir Estuary. Values ranged from 11.3 – 12.8% dw. Additionally, there was little variation in the total protein content of *A. nodosum* collected from the four sites. Values ranged from 7.6 – 9.6% dw. *A. nodosum* collected at the site furthest downstream contained the lowest total protein. *P. lanosa* was only available from two sites along the Suir Estuary. *P. lanosa* collected at the site furthest downstream (site 4) contained the highest total protein 29.7% dw, compared with 28.1% from *P. lanosa* collected from site 3 (Figure 3.27, Table 3.7). When all five sites are taken into account however, *P. lanosa* collected at Fethard-on-Sea provided the lowest protein concentration 25.6 % dw. Four seaweed species (*F. vesiculosus, A. nodosum P. lanosa* and *Ulva* sp) were collected from Fethard on Sea. Total protein values for the four species were 10.8, 7.4, 25.6 and 20.6 % dw respectively (Figure 3.27 and Table 3.7).

Brown seaweed collected from the Newfoundland sites contained similar protein levels $\leq 6.6 \pm 0.23$ % dw. Generally, brown seaweed collected from both locations in Ireland contained slightly higher protein when compared to Newfoundland ($\leq 12.0 \pm 0.17$ % dw). The average protein levels in *F. vesiculosus* collected from the Suir Estuary were approximately 41% higher then those collected from Newfoundland. *P. lanosa* collected from Ireland contained ~ 26.9 to ~ 37% more protein than samples collected from Bonne Bay. The green seaweed, *Ulva* sp was only available at one site (Fethard on Sea). When compared with all other seaweeds, this species contained the second highest over all protein concentration (20.6 ± 0.52 % dw) (Table 3.7).

Furthermore, results from this study also demonstrated a negative correlation between the protein content of *F. vesiculosus* and seawater conductivity levels. Generally, higher protein values corresponded to lower conductivity levels and *vice versa* for all three locations (Tables 3.8 to 3.10).

Table 3.8. Fluctuations in protein content of *F. vesiculosus* in relation to conductivity & temperature (Bonne Bay).

	Site No.	Protein (% dw)	Conductivity (mS/cm)	Temperature (^oC)
	1	7.5	42.8	10.3
High Protein →	2	8.2	39.7	8.9
	3	6.2	46.1	10.4
Low Protein →	4	4.7	44.5	11.0
	5	6.2	39.7	10.8

Table 3.9. Fluctuations in protein content of *F. vesiculosus* in relation to conductivity and temperature (Suir Estuary and Fethard-on-Sea).

	Site No.	Protein (% dw)	Conductivity (mS/cm)	Temperature (^oC)
High Protein →	1	12.6	25.3	12.6
	2	11.3	30.9	12.9
	3	11.4	42.9	13.2
High Protein →	4	12.8	34.7	12.9
Low Protein →	5	10.8	51.1	13.1

Table 3.10. Fluctuations in protein content of *F. vesiculosus* in relation to conductivity and temperature (Humber Arm).

	Site No.	Protein (% dw)	Conductivity (mS/cm)	Temperature (^oC)
High Protein →	1	11.4	6	12.5
	3	4.6	43.3	7.0
Low Protein →	4	4.8	34.7	6.1
	6	7.5	14.6	12.5
	7	8.9	6.3	12.6

In relation to temperature, trends for protein concentrations were observed within the three locations. For example, higher temperatures corresponded to lower protein and *vice versa* for both Bonne Bay and Ireland. However, with respect to the Humber Arm, correlations between total protein and temperature were inconclusive (Tables 3.8 to 3.10).

Furthermore, regression analysis-fitted line plot of highest total protein values versus temperature demonstrated a strong positive correlation between protein and temperature (Figure 3.28). This therefore may help explain a general higher protein concentration of seaweeds from the Irish sites.
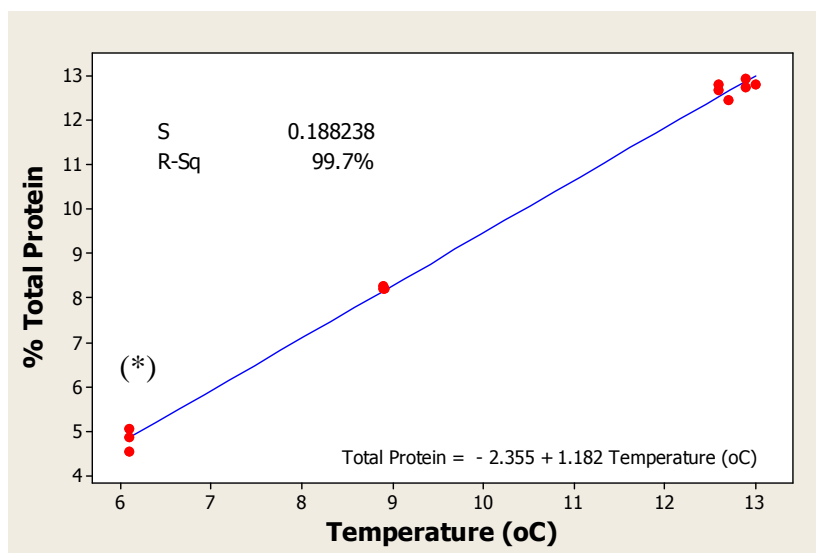
Figure 3.28. Regression Analysis – Fitted Line Plot. Highest protein versus temperature from each location. (*) refers to highest protein content of *F. vesiculosus* collected from the Humber Arm on 16th October 2007. S = Standard deviation of a sample; R-Sq = Regression coefficient measures how well the regression line approximates the real data points.

Furthermore, comparisons of total protein levels of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp, collected from Fethard-on-Sea, in October, 2007 and May 2008 appear to demonstrate seasonal variations in total protein levels.

Results for samples collected from Fethard-on-Sea in May, 2008 demonstrated that the red seaweed, *P. lanosa* contained the highest total protein content (Figure 3.29). The green species, *Ulva* sp contained the second highest total protein levels (19.5% dw), while the brown seaweeds yielded the lowest values (11.1-14.9% dw), (Figure 3.29). Additionally, one-way ANOVA ($p < 0.05$) demonstrated significant variations in the levels of total protein among the four seaweeds under investigation.
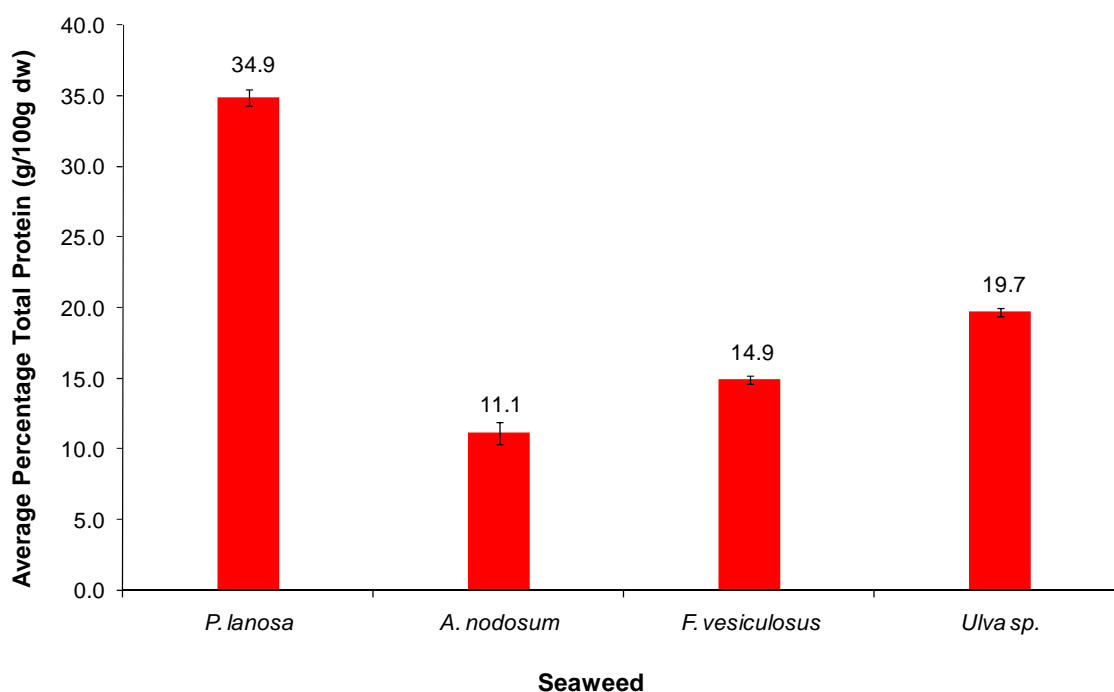
Figure 3.29. Total protein content of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp. Samples were collected from Fethard-on-Sea, Co. Wexford in May, 2008. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

3.4.5   Extracted Protein Determination

Following alkaline extraction (Chapter 2: 2.3.4.6) of seaweeds collected from Fethard-on-Sea, in May, 2008, the brown species, *A. nodosum* and *F. vesiculosus* yielded, by far, the highest extracted protein values (Figure 3.30). Although both *P. lanosa* and *Ulva* sp contained the highest total protein values, 34.9 and 19.7% dw respectively, poor yields were achieved for both species upon protein extraction. Extracted protein values for *A. nodosum, F. vesiculosus, P. lanosa* and *Ulva* sp equate to 9.69, 3.96, 0.57 and 0.47 % dw respectively. Out of the two brown seaweeds, *F. vesiculosus* yielded the higher total protein value (Figure 3.29). The percentage of protein extracted for each species, when the total protein content is taken into consideration, is as follows: *P. lanosa* (1.6%), *A. nodosum*, (87.3%), *F. vesiculosus* (26.6%) and *Ulva* sp (2.4%). One-way ANOVA ($p < 0.05$) demonstrated significant variations in the levels of extracted protein among the four seaweeds under investigation.
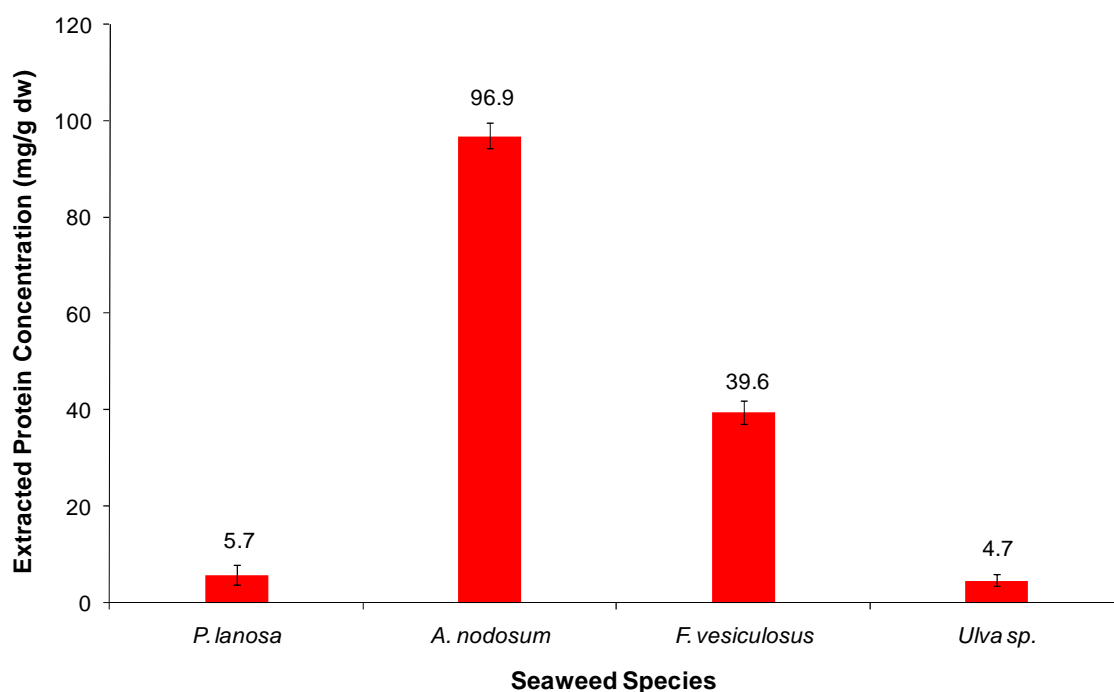
Figure 3.30. Extracted protein concentration of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp. Samples were collected from Fethard-on-Sea, Co. Wexford in May, 2008. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

3.4.6   Polyphenol Determination

Analysis of the October, 2007 samples demonstrated the brown seaweed species contained significantly higher concentrations of polyphenols when compared to those of red and green species.

A general downstream increase in polyphenol concentrations for all seaweed species was observed. Highest polyphenol concentrations were seen for the outermost sampling sites with lowest polyphenol concentrations for those sampling sites furthest upstream (Figure 3.31 and Table 3.11).
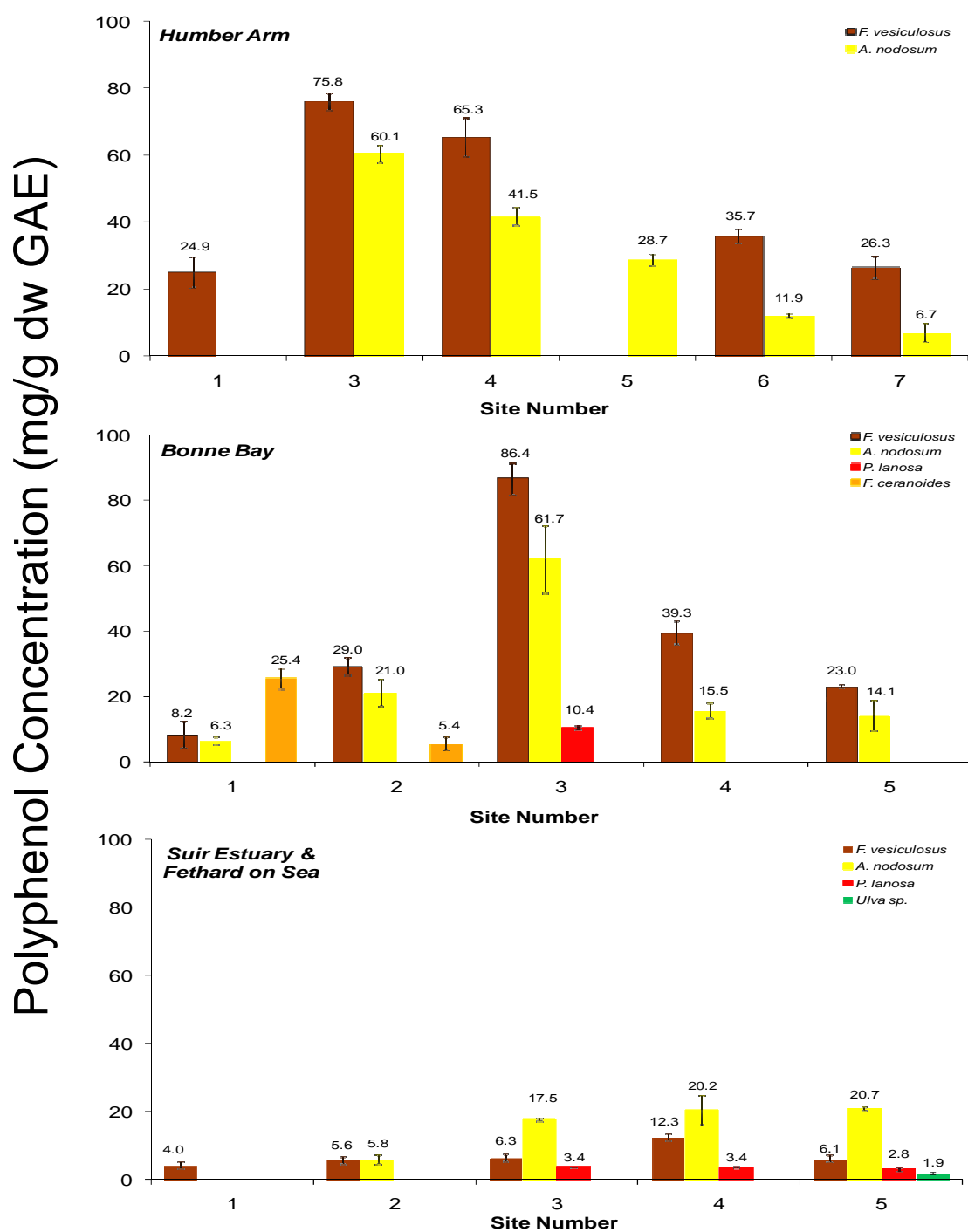
Figure 3.31. Polyphenol concentration of seaweeds collected from various sites along the Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

Table 3.11. Comparison of the highest polyphenol concentrations of *P. lanosa, A. nodosum F.vesiculosus* and *Ulva* sp collected from Humber Arm, Bonne Bay Suir Estuary and Fethard-on-Sea. Errors are calculated based on triplicate runs with 95% Confidence Intervals.

Seaweed species	Humber Arm	Bonne Bay	Suir Estuary	Fethard-on-Sea
F. vesiculosus	75.8 ± 2.20	86.4 ± 8.90	12.3 ± 3.80	6.1 ± 0.47
A. nodosum	60.1 ± 2.10	61.7 ± 4.12	20.2 ± 1.70	20.7 ± 0.43
P. lanosa	———	10.4 ± 0.52	3.4 ± 0.20	2.8 ± 0.42
Ulva sp	———	———	———	1.9 ± 0.24

One-Way ANOVA ($p<0.05$) demonstrated significant spatial variation in the polyphenol levels of *F. vesiculosus* and *A. nodosum* (but not for *P. lanosa*) with respect to site number.

F. vesiculosus from the Newfoundland sites contained approximately 21-75% more polyphenols than *A. nodosum.* A reverse trend however was observed for the Irish sites where *A. nodosum* contained up to 80% more polyphenols than *F. vesiculosus*.

Results from this study also demonstrated a positive correlation between the polyphenol content of *F. vesiculosus* and seawater conductivity. In general, high polyphenol concentrations corresponded to high conductivity values and vice versa for all three locations (Tables 3.12 to 3.14). In relation to temperature, variations in trends were observed for all three locations. Generally, for the Humber Arm, higher temperature values corresponded to lower polyphenol concentrations, whereas the opposite was observed for Bonne Bay (Tables 3.12 to 3.14).

Table 3.12. Fluctuations in polyphenol content of *F. vesiculosus* in relation to conductivity & temperature (Bonne Bay).

	Site No.	Polyphenol (mg/g dw GAE)	Conductivity (mS/cm)	Temperature (°C)
	1	8.2	42.8	10.3
Low Polyphenol →	2	29.2	39.7	8.9
High Polyphenol →	3	86.4	46.1	10.4
	4	39.3	44.5	11.0
	5	23.0	39.7	10.8

174

Table 3.13. Fluctuations in polyphenol content of *F. vesiculosus* in relation to conductivity and temperature (Suir Estuary and Fethard-on-Sea).

	Site No.	Polyphenol (mg/g dw GAE)	Conductivity (mS/cm)	Temperature (^oC)
Low Polyphenol →	1	4.0	25.3	12.6
	2	5.6	30.9	12.9
	3	6.3	42.9	13.2
High Polyphenol →	4	12.3	34.7	12.9
	5	6.1	51.1	13.1

Table 3.14. Fluctuations in polyphenol content of *F. vesiculosus* in relation to conductivity and temperature (Humber Arm).

	Site No.	Polyphenol (mg/g dw GAE)	Conductivity (mS/cm)	Temperature (^oC)
Low Polyphenol →	1	24.9	6.0	12.5
High Polyphenol →	3	75.8	43.3	7.0
	4	65.3	34.7	6.1
	6	35.7	14.6	12.5
	7	26.3	6.3	12.6

Regression analysis demonstrated good correlation between polyphenol concentrations and conductivity, producing a regression value of 96.1% (Figure 3.32). A poor correlation between polyphenol and temperature values was observed ($R^2 = 0.5413$).
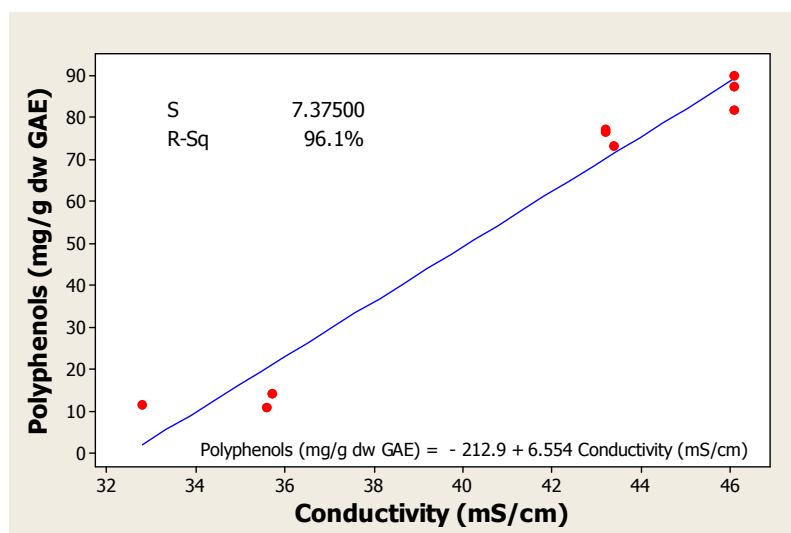
Figure 3.32. Regression Analysis – Fitted Line Plot. Highest polyphenol concentration versus conductivity from each location. S = Standard deviation of a sample; R-Sq = Regression coefficient measures how well the regression line approximates the real data points.

Furthermore, comparisons of polyphenol levels of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva sp,* collected from Fethard-on-Sea, in October, 2007 and May 2008 also appear to demonstrate seasonal variations. For example, the polyphenol concentrations of *P. lanosa, A. nodosum, F. vesiculosus Ulva* sp collected in May, 2008 yielded 12.9, 34.8, 26.9 and 2.2 mg/g dw GAE, respectively (Figure 3.33). Whereas the corresponding values in the October, 2007 samples were 2.8, 20.7, 6.1 and 1.9 mg/g dw GAE, respectively (see Table 3.15). One-way ANOVA ($p < 0.05$) demonstrated significant variations in the levels of polyphenols among the four seaweeds under investigation.
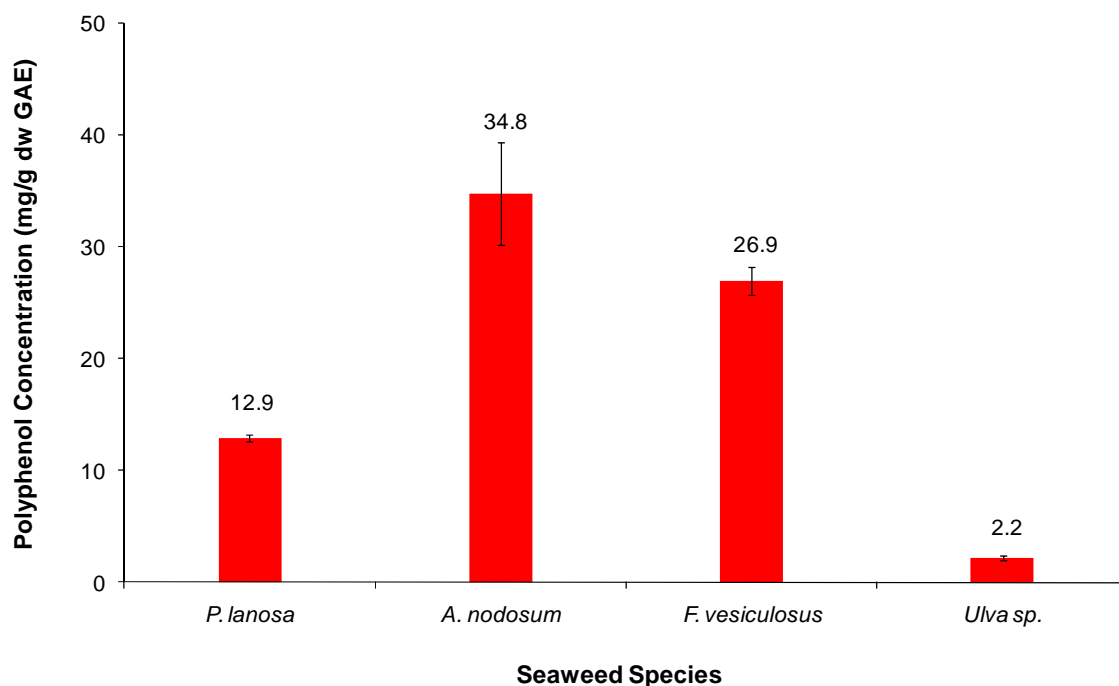
Figure 3.33. Polyphenol concentration of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp. Samples were collected from Fethard-on-Sea, Co. Wexford in May, 2008. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

The average total protein, extracted protein and polyphenol levels of the four seaweeds under investigation are summarised in Table 3.15.

Table 3.15. Summary of average total protein, extracted protein and polyphenol levels of *P. lanosa*, *A. nodosum*, *F. vesiculosus* and *Ulva* sp harvested in May, 2008. Values were calculated based on triplicate samples with 95% Confidence Intervals.

Seaweed Species	Total Protein (% dw)	Extracted Protein (mg/g dw)	Polyphenols (mg/g dw)
P. lanosa	34.9 ± 0.6	5.7 ± 2.0	12.9 ± 0.3
A. nodosum	11.1 ± 0.8	96.9 ± 2.7	34.8 ± 4.6
F. vesiculosus	14.9 ± 0.3	39.6 ± 2.4	26.9 ± 1.2
Ulva sp	19.7 ± 0.3	4.7 ± 1.2	2.2 ± 0.2

3.4.7 Seaweed Metal Analysis

3.4.7.1 Total Metals

Certified Reference Materials (CRM) were digested and analysed for metal content as per seaweed samples. % recovery of CRMs were as follows: Pb (87±2.6%), Zn (96±7.2%), As (81±8.7%), Cd (90±3.4%), Co (84±6.2%), Cr (89±4.7%), Cu (91±0.9%), Mn (100±0.8%), Ni (90±6.1%) and Al (68±1.3%). In general, metal levels varied according to seaweed, location and metal species (Table 3.16 and Figures 3.34 to 3.43). Error bars were calculated based on triplicate samples with 95% Confidence Intervals. With the exception of As^{3+} and Cd^{2+} *P. lanosa* collected along the Suir Estuary had the ability to concentrate the highest proportion of all metals under investigation (Figures 3.34 to 3.43). In relation to As^{3+}, *F. vesiculosus* collected from the Newfoundland sites ranged from 20.2-33.5 mg/kg dw, whereas a slightly larger range was observed for the Suir Estuary/Fethard-on-Sea sites (13.5-46.4 mg/kg dw). The As^{3+} concentration of *A. nodosum* collected from Newfoundland ranged from 16.1-33.2 mg/kg dw. A similar range was obtained for the South-East coast of Ireland. The overall highest As^{3+} concentration was observed for *F. ceranoides* collected in Bonne Bay. *P. lanosa*, contained the overall lowest As^{3+} concentrations (5.8-10.7 mg/kg dw), (Table 3.16 and Figure 3.36).

There was a downstream increase in the As^{3+} content of *F. vesiculosus* and *A. nodosum* collected from The Humber Arm. A similar trend was also observed for *F. vesiculosus*, *A. nodosum* and *P. lanosa* along the Suir Estuary (Figure 3.36). Little interspecies variation in As^{3+} levels were observed for the Bonne Bay samples.

The highest Cd^{2+} uptake was observed for *P. lanosa* collected at site 3 (the furthest downstream) in Bonne Bay (7.7 mg/kg dw). This was followed by *P. lanosa* collected at sites 3 and 4 along the Suir Estuary. Of the brown seaweeds, *F. ceranoides* contained the highest Cd^{2+}, ranging from 1.8-2.1 mg/g dw. No significant trends were observed in relation to a downstream variation of Cd^{2+} levels of the brown seaweeds. A small downstream increase in *P. lanosa* however collected from the Suir Estuary (Sites 3 and 4) was observed, with values ranging from 3.9-5.0 mg/g dw (Figure 3.37).

Table 3.16. Comparison of seaweed metal concentrations (mg/kg dw) for all four locations. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Metal	Seaweed	Humber Arm	Bonne Bay	Suir Estuary	Fethard
Pb	*F. vesiculosus*	0.0-0.2	0-0.2	0.8-1.7	0.9
	A. nodosum	0.0-0.1	0.0-0.1	0.1-0.1	0.0
	P. lanosa	——	0.0	6.5-9.1	5.3
	F. ceranoides	——	0.0-0.0	——	——
Zn	*F. vesiculosus*	8.6-61.6	12.1-54.3	22.2-55.1	37.7
	A. nodosum	16.4-41.3	16.7-71.4	25.0-27.8	59.7
	P. lanosa	——	48.5	238.5-248.4	106.8
	F. ceranoides	——	21.0-65.6	——	——
As	*F. vesiculosus*	16.7-33.5	27.4-32.6	13.5-42.7	46.4
	A. nodosum	16.1-33.2	19.4-28.0	14.7-20.0	32.6
	P. lanosa	——	6.1	5.8-6.8	10.7
	F. ceranoides	——	29.4-36.3	——	——
Cd	*F. vesiculosus*	0.5-0.9	0.7-1.3	0.5-0.8	0.8
	A. nodosum	0.1-0.4	0.2-0.9	0.1-0.2	0.3
	P. lanosa	——	7.7	3.9-5.0	1.4
	F. ceranoides	——	1.8-2.1	——	——
Co	*F. vesiculosus*	1.1-2.7	0.6-1.2	0.9-2.2	1.7
	A. nodosum	0.5-1.0	0.5-1.3	0.6-0.9	0.8
	P. lanosa	——	1.7	2.9-4.8	1.3
	F. ceranoides	——	1.1-1.8	——	——
Cr	*F. vesiculosus*	0.2-1.5	0.1-0.3	0.6-1.3	0.4
	A. nodosum	0.1-0.4	0.1-0.3	0.2-0.3	0.2
	P. lanosa	——	0.9	1.7-4.0	3.6
	F. ceranoides	——	0.1-0.4	——	——
Cu	*F. vesiculosus*	4.1-8.8	3.3-16.8	10.1-37.0	2.4
	A. nodosum	1.9-5.3	1.6-5.5	3.3-4.3	4.5
	P. lanosa	——	29.7	34.4-72.6	36.8
	F. ceranoides	——	7.7-11.3	——	——
Mn	*F. vesiculosus*	112.2-415.2	79.9-146.8	471.5-702.0	231.1
	A. nodosum	11.0-22.5	6.8-34.0	30.0-44.9	47.5
	P. lanosa	——	12.8	689.8-1091.5	185.9
	F. ceranoides	——	16.8-123.0	——	——
Ni	*F. vesiculosus*	4.3-10.8	4.5-9.1	2.9-6.3	3.6
	A. nodosum	1.3-5.3	0.7-2.7	0.5-1.1	1.0
	P. lanosa	——	7.9	8.1-10.0	8.4
	F. ceranoides	——	4.7-6.5	——	——
Al	*F. vesiculosus*	81.3-112.3	24.3-154.8	144.8-257.9	82.7
	A. nodosum	9.3-60.3	6.4-21.5	35.9-41.3	33.5
	P. lanosa	——	257.7	606.2-1485.1	1840.3
	F. ceranoides	——	53.8-142.8	——	——
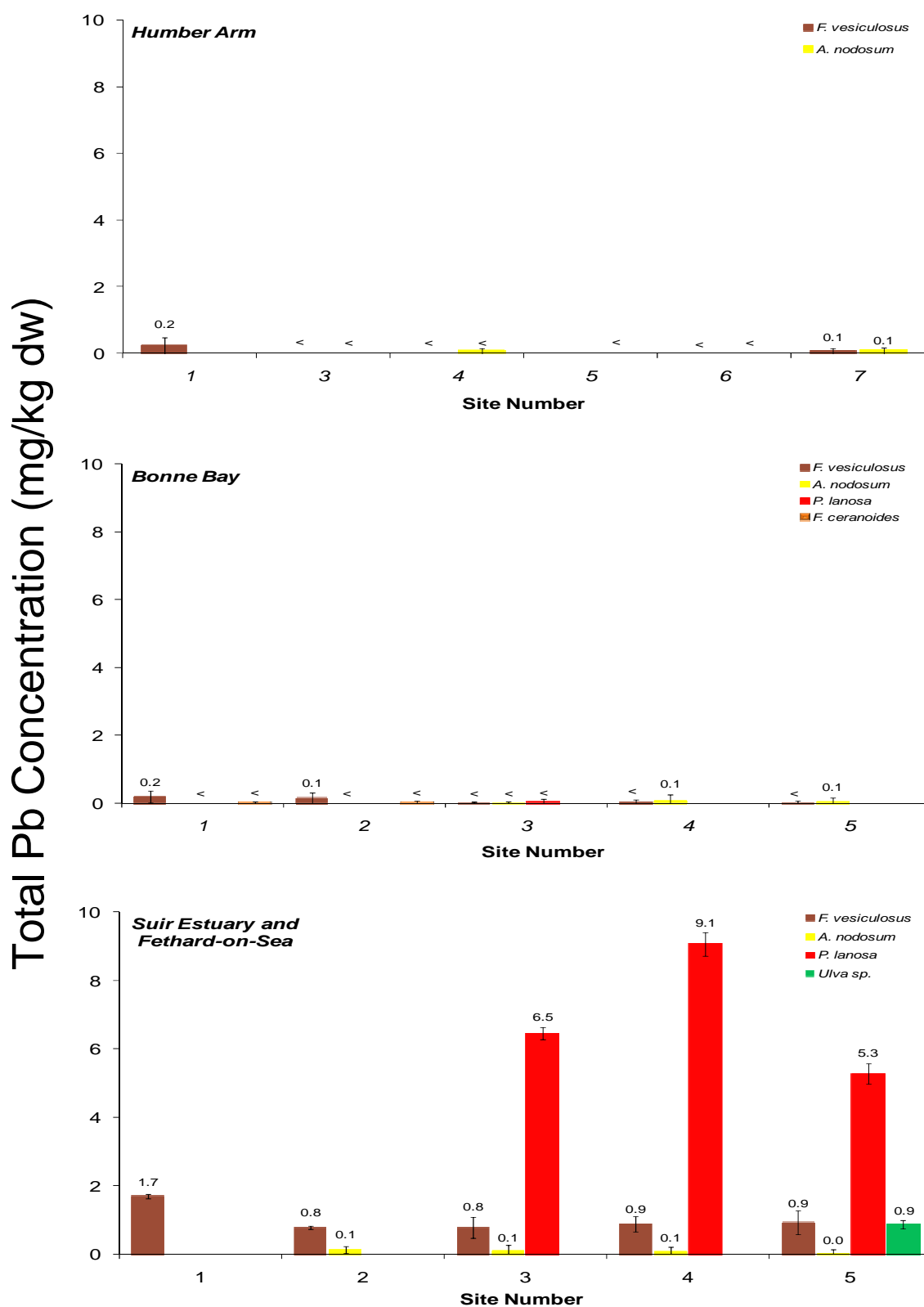
Figure 3.34. Comparison of total Pb concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea (< = below limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
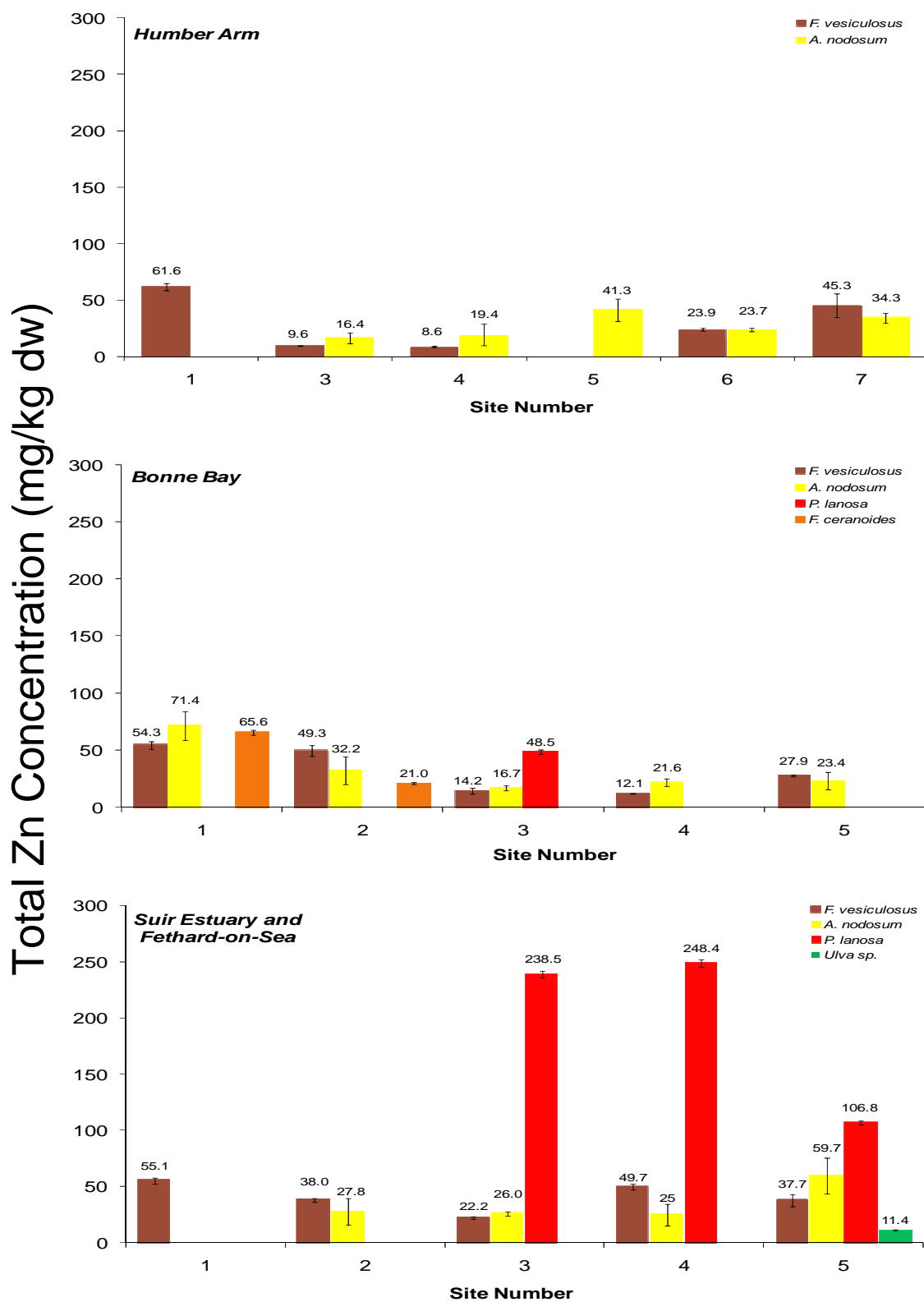
Figure 3.35. Comparison of total Zn concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
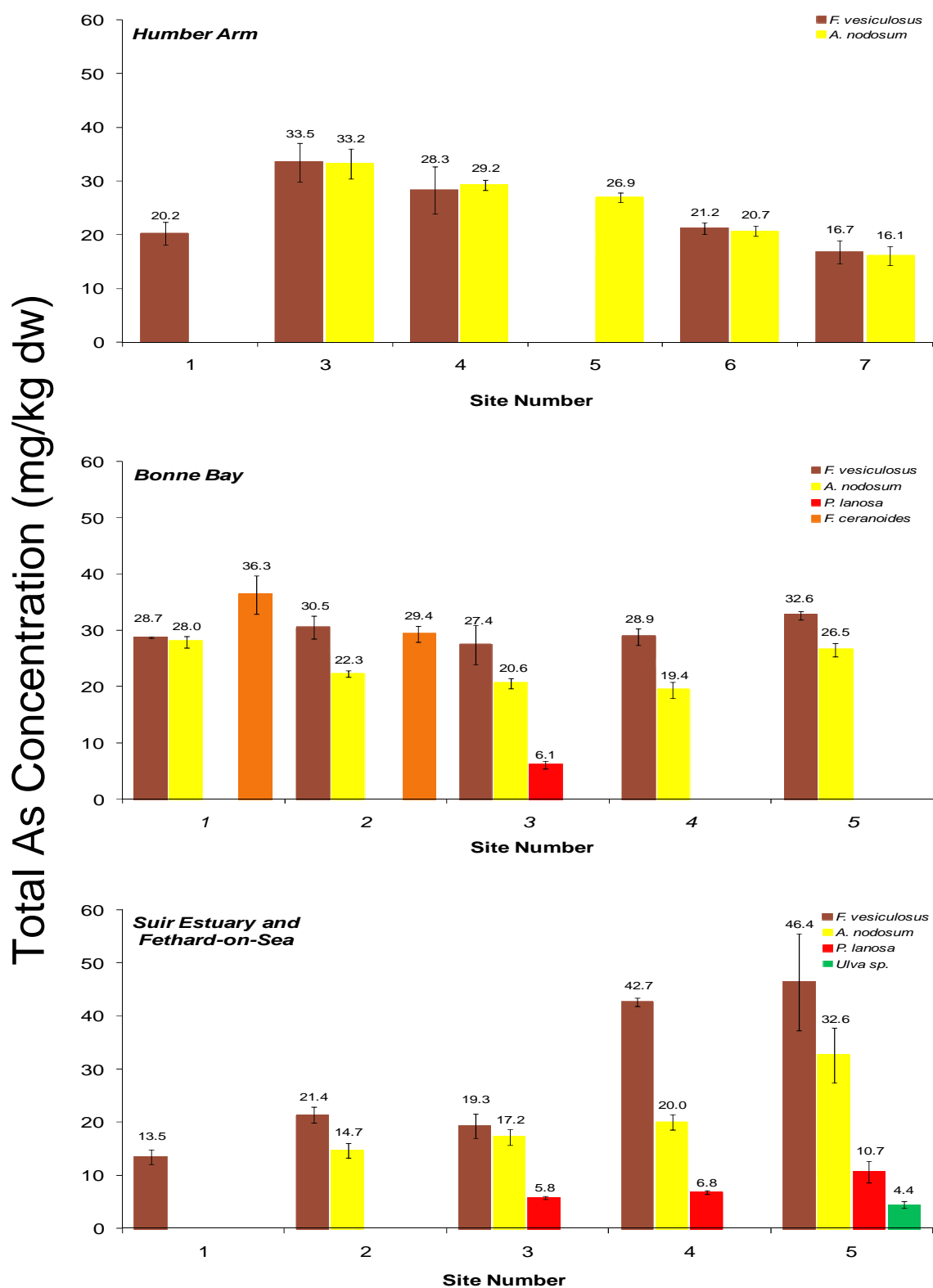
Figure 3.36. Comparison of total As concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
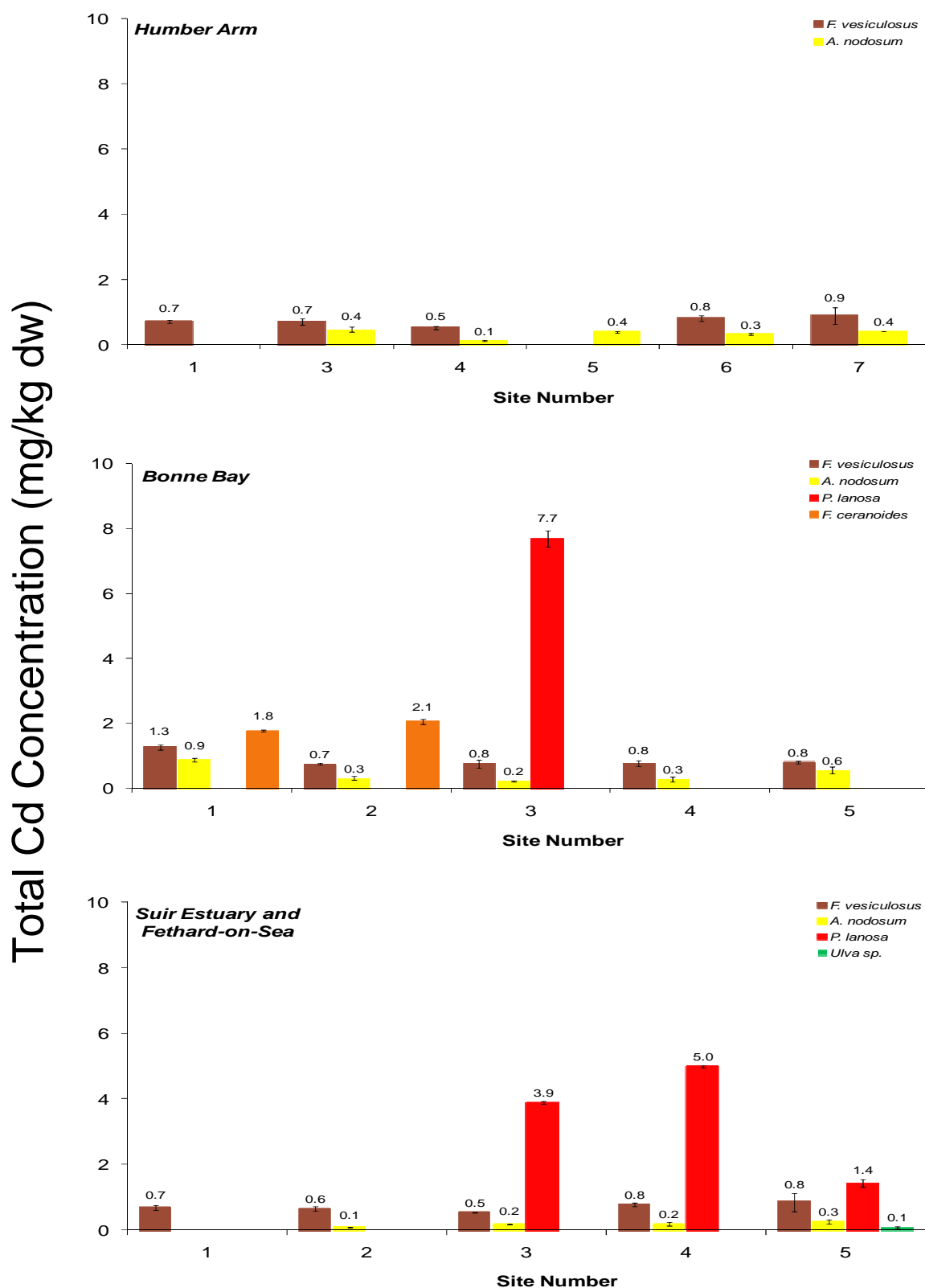
Figure 3.37. Comparison of total Cd concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
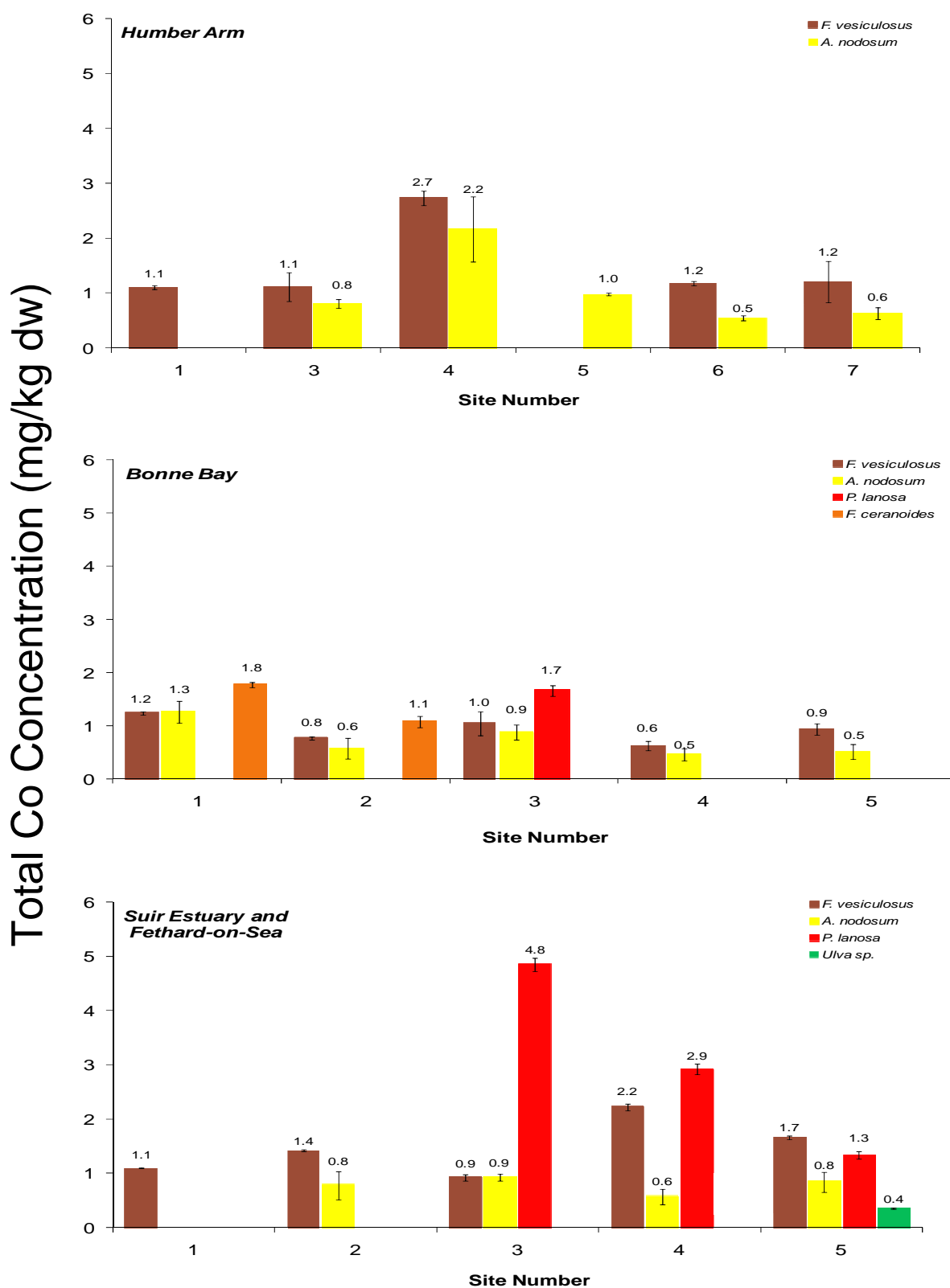
Figure 3.38. Comparison of total Co concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
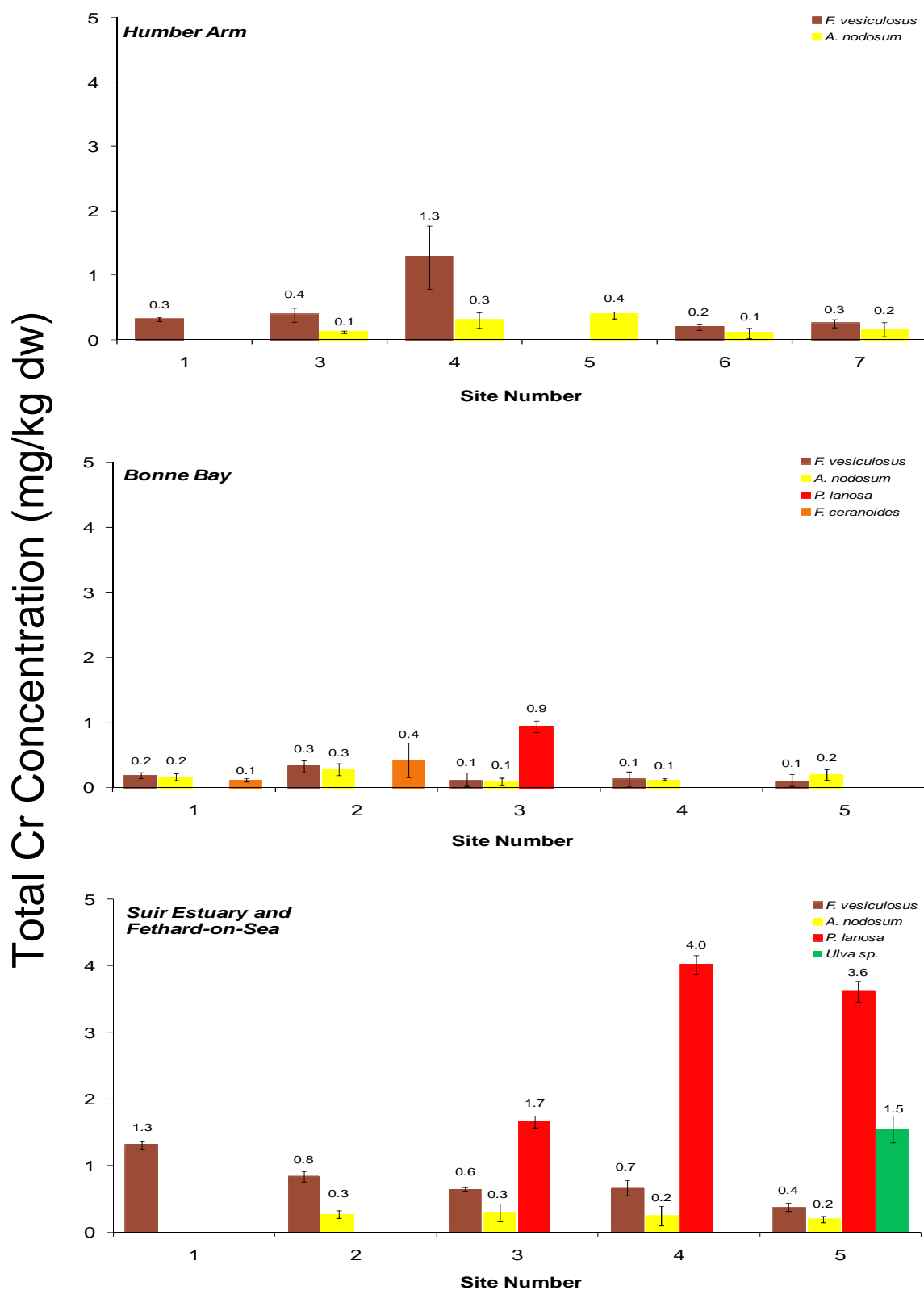
Figure 3.39. Comparison of total Cr concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

184
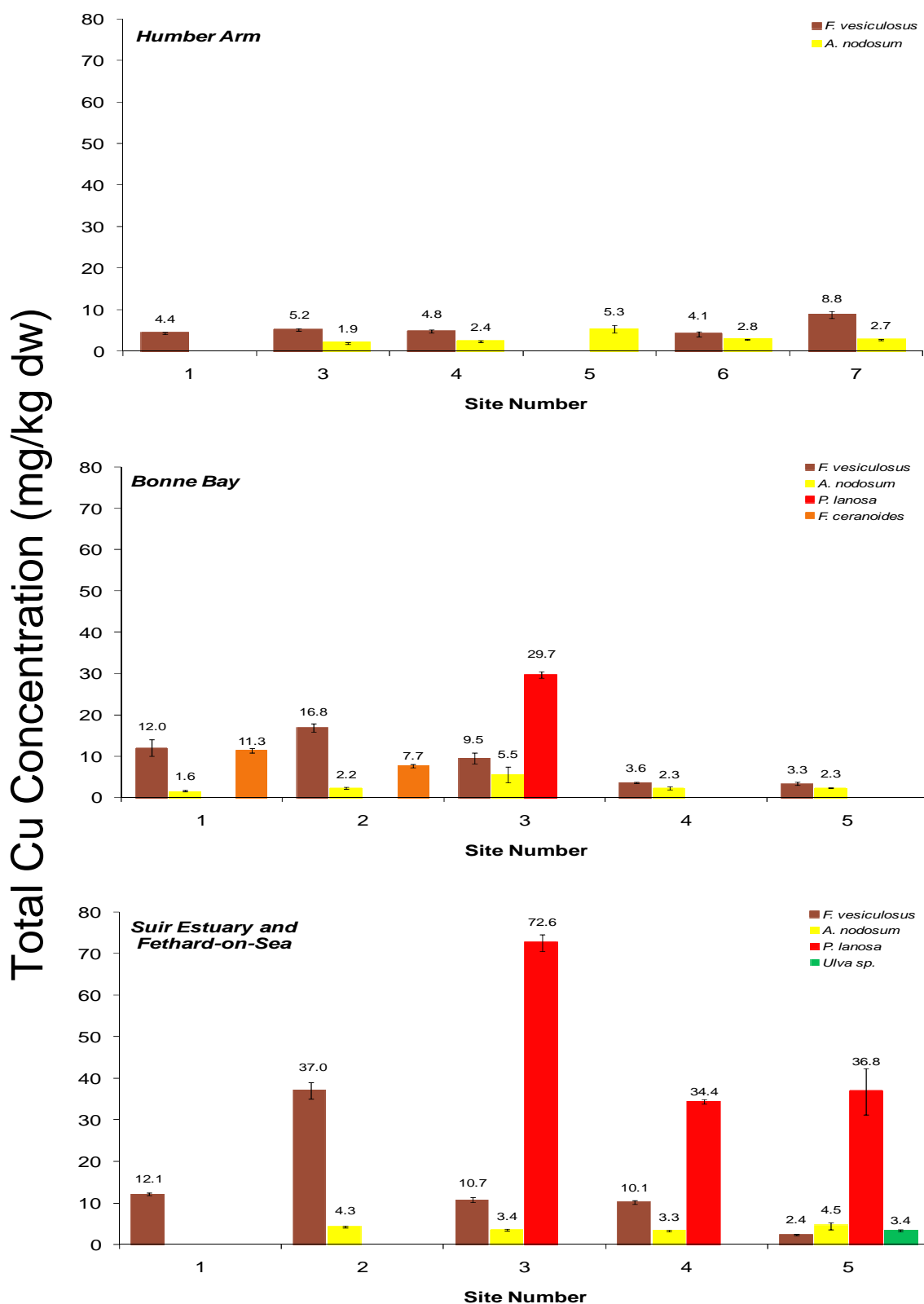
Figure 3.40. Comparison of total Cu concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

185
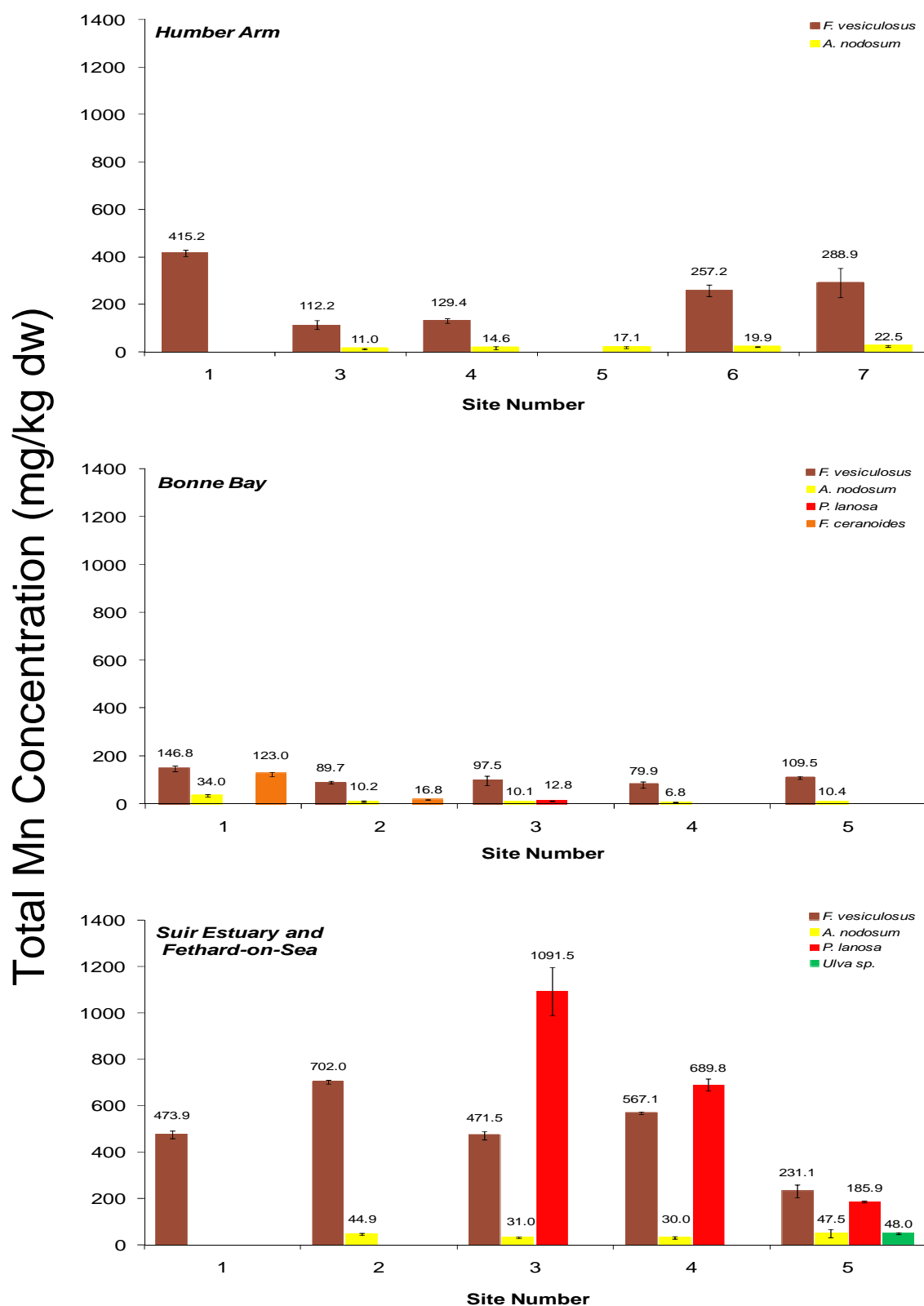
Figure 3.41. Comparison of total Mn concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
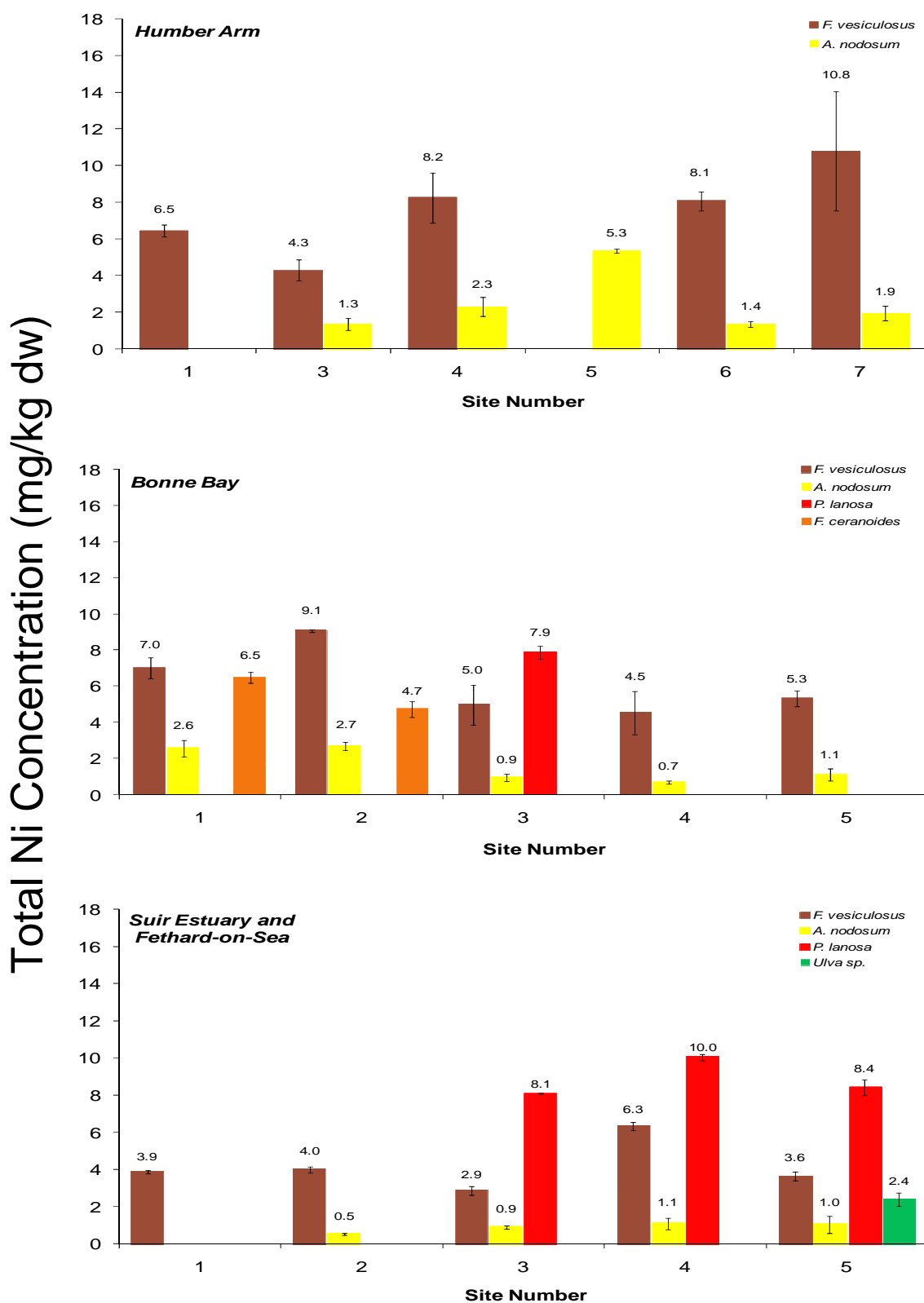
Figure 3.42. Comparison of total Ni concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
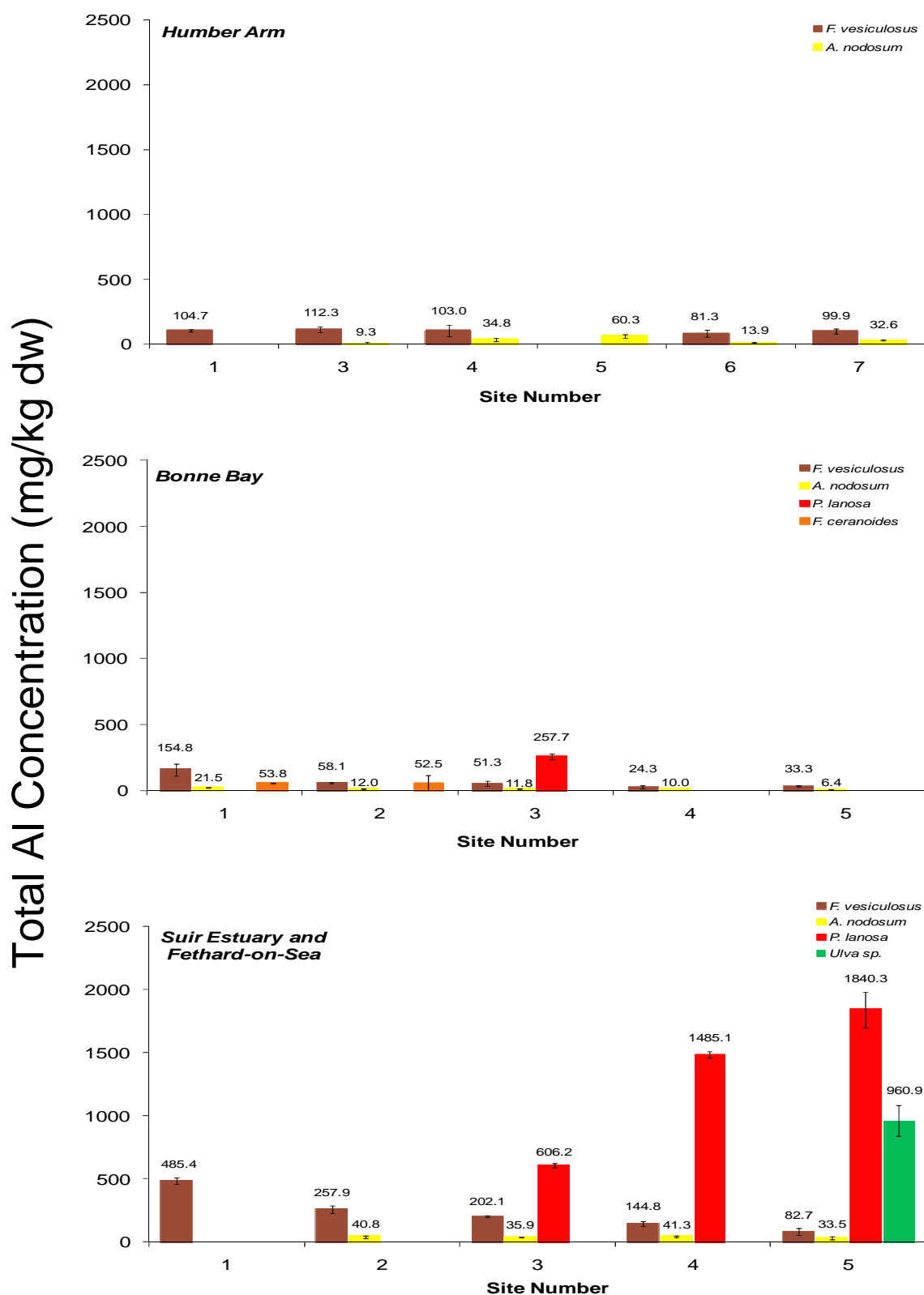
Figure 3.43. Comparison of total Al concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

The second overall highest metal content was observed for Mn^{2+}. *P. lanosa, F. vesiculosus* and *A. nodosum* collected from the Suir Estuary sites yielded the highest levels of this essential nutrient when compared with Corner Brook and Bonne Bay. *P. lanosa* contained the overall highest Mn^{2+} with values ranging from 185.9-1091.5 mg/kg dw. There was however little variation in the Mn^{2+} concentration of *A. nodosum* among all locations (Figure 3.41). A general downstream decrease in the Mn^{2+} concentration of *F. vesiculosus* was observed for the Humber Arm sites (415.2-112.2 mg/kg dw). A similar trend was also observed for the Ireland sites with respect to the same seaweed species. A downstream decrease in the Mn^{2+} levels of *A. nodosum* was also observed for both the Humber Arm and Suir Estuary/Fethard-on-Sea locations. A downstream decrease for *P. lanosa* collected along the South-East coast of Ireland was also reported (1091.5-185.9 mg/kg dw). No apparent trends however were observed for the Bonne Bay sites when all seaweed species were taken into consideration (Figure 3.41).

The lowest overall metal concentration was observed for Pb^{2+} collected along The Humber Arm and Bonne Bay for all seaweed species. The highest value obtained for these sites was 0.2 mg/kg dw and in the majority of cases, Pb^{2+} was below the limit of detection. There was little or no interspecies variation in the Pb^{2+} contents of *F. vesiculosus* and *A. nodosum* collected from sites 2 to 5 along the Suir Estuary/Fethard-on-Sea. The highest Pb^{2+} value along the Suir Estuary however, was obtained at Site 1, St. John's River. Significantly, highest levels of Pb^{2+} in seawater were detected at this site (0.0002 mg/L, compared to 0.0001 mg/L at all other sites.). A downstream increase in Pb^{2+} levels of *P. lanosa* was observed for sites 3 and 4 (6.5-9.1 mg/kg dw). (Figure 3.34).

P. lanosa collected from the Suir Estuary contained the highest overall Cr^{3+} levels (1.7 – 4.0 mg/kg dw). Cr^{3+} levels associated with the brown seaweeds ranged from 0.1-1.3 mg/kg dw. A general downstream decrease in the Cr^{3+} concentration of *F. vesiculosus* was observed for samples collected from the Suir Estuary whereas an opposite trend was observed for *P. lanosa* (Figure 3.39). No significant trends in Cr^{3+} levels of seaweeds were observed for the Newfoundland sites.

P. lanosa, F. vesiculosus and *A. nodosum* collected from the Suir Estuary sites yielded the highest Cu^{2+} levels when compared with The Humber Arm and Bonne Bay. The highest Cu^{2+} concentration was obtained from *P. lanosa* collected from the Waterford,

Site 3 (72.6 mg/kg dw), (Figure 3.40). Of the brown seaweeds, *F. vesiculosus* collected from the Suir Estuary (Site 2) contained the highest levels of Cu^{2+}. Cu^{2+} values for *F. vesiculosus* ranged from 2.4-37.0 mg/g dw at this location ranged, whereas values for the same seaweeds ranged from 3.3-16.6 for Bonne Bay. There was little spatial/interspecies variation with respect to Cu^{2+} levels for the Humber Arm. Significantly, downstream decreases in Cu^{2+} levels for both *F. vesiculosus* and *P. lanosa* were observed for the Ireland sites (Figure 3.40).

The overall highest metal content was observed for Al^{3+}. *P. lanosa, A. nodosum* and *F. vesiculosus* collected from the Suir Estuary/Fethard-on-Sea yielded the highest levels of Al^{3+} when compared with Newfoundland and of those, the red seaweed obtained the overall highest Al^{3+} concentration (1,840.3 mg/kg dw). Values for *F. vesiculosus* and *A. nodosum* belonging to the Irish sites ranged from 82.7-485.4 mg/kg dw and 33.5-40.8 mg/kg dw, respectively. Of the two Newfoundland locations, *A. nodosum* collected from Bonne Bay yielded the overall lowest Al^{3+} (6.4-21.5 mg/g dw). Furthermore, the highest concentration of Al^{3+} was obtained from *F. vesiculosus* (Bonne Bay, Site 1). This is a pristine location, unspoiled by human activity (See 3.3.4). Additionally, with regard to the same locations, the red seaweed, *P. lanosa* yielded the highest Al^{3+} (257.7 mg/g dw), a similar trend to that of Ireland. Overall, a downstream increase with respect to *P. lanosa* and a downstream decrease in *F. vesiculosus* Al^{3+} levels was particularly apparent for the Suir Estuary and Fethard-on-Sea.

The highest Zn^{2+} levels were found in *P. lanosa* from the Suir Estuary (>238.5 g/kg dw). Of the brown seaweeds, *A. nodosum* collected from Site 1, Bonne Bay contained the highest Zn^{2+} (71.4 g/kg dw), followed by *F. ceranoides* (65.6 g/kg dw), at the same location. The lowest Zn^{2+} concentration was yielded by *F. vesiculosus* from Site 4, Humber Arm. A slight downstream decrease in Zn^{2+} levels for *F. vesiculosus* and *A. nodosum* for both Newfoundland locations was observed. A downstream decrease was observed for *F. vesiculosus* and *P. lanosa* for Suir Estuary and Fethard-on-Sea when all five sites are taken into account. With respect to Fethard-on-Sea, the green seaweed, *Ulva* sp, yielded the lowest Zn^{2+} and *P. lanosa* the highest.

P. lanosa collected from the Suir Estuary provided the highest Co^{2+} levels. With respect to Newfoundland, the highest levels of this essential element was found in *F. ceranoides* (1.8 g/kg dw) and *F. vesiculosus* from the Humber Arm (2.7 g/kg dw). The overall lowest Co^{2+} levels were found in *Ulva* sp. A downstream decrease in Co^{2+}

levels of *P. lanosa* from the Suir Estuary was observed corresponding to a general downstream increase for *F. vesiculosus*. Furthermore, a general downstream increase in Co^{2+} levels for *F. vesiculosus* and *A. nodosum* were observed for the Humber Arm. There was no significant variation in Co^{2+} levels for *A. nodosum* when all sites were taken into consideration.

The overall highest levels of Ni^{2+} were found in *P. lanosa* from the Suir Estuary (10.0 g/kg dw), *F. vesiculosus* from Bonne Bay (9.1 g/kg dw) and the Humber Arm (10.8 g/kg dw). Along the Suir Estuary, levels of Ni^{2+} in *P. lanosa*, *A. nodosum* and *F. vesiculosus* ranged from 8.1-10.0, 0.5-1.0 and 2.9-6.3 g/kg dw, respectively. In Bonne Bay samples, Ni^{2+} levels from *A. nodosum*, *F. vesiculosus* and *F. ceranoides* ranged from 0.7-2.7, 4.5-9.1 and 4.7-6.5 g/kg dw respectively. With respect to the Humber Arm, Ni^{2+} concentrations in *A. nodosum* and *F. vesiculosus* ranged from 1.3-5.3 g/kg dw.

3.4.8 Statistical Analysis

In relation to *F. vesiculosus* collected from The Humber Arm, one-way ANOVA ($p<0.05$) of total metals versus site number demonstrated significant variation for all metals with the exception of Pb^{2+} and Al^{3+} (Table 3.17). For example One-Way ANOVA of Zn^{2+} levels in *F. vesiculosus* produced a *p* value of 0.001. Furthermore a downstream increase in As^{3+} and downstream decreases in Ni^{2+}, Zn^{2+} and Mn^{2+} were observed. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *F. vesiculosus* according to site number is demonstrated in Table 3.17. For example post hoc Tukey analysis demonstrated no significant variations in the Cd or Cr levels of sites 1, 3, 6 and 7. However, significant variations in the Mn concentration of site 1 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Ni levels of sites 1, 3, 4 and 6.

Table 3.17. One-Way ANOVA and post hoc Tukey analysis of *F. vesiculosus* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along The Humber Arm. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for:	Site No. with lowest concs.	Site No. with low/mid concs.	Site No. with mid concs.	Site No. with mid/high concs.	Site No. with highest concs.	One-Way ANOVA (*p value*)
Cadmium 0.5-0.9	[4]				[1,3,6,7]	0.039
Chromium 0.2-1.3	[1,3,6,7]				[4]	0.001
Lead 0.0-0.2			[1,3,4,6,7]			0.166
Copper 4.1-8.8	[1,3,4,6]				[7]	0.001
Zinc 8.6-61.6	[3,4]	[6]		[7]	[1]	0.001
Arsenic 16.7-33.5	[1,6,7]				[3,4]	0.001
Cobalt 1.1-2.7	[1,3,6,7]				[4]	0.001
Manganese 112.2-415.2	[3,4]		[6,7]		[1]	0.001
Nickel 4.3-10.8				[1,3,4,6]	[7]	0.030
Aluminium 81.3-112.3			[1,3,4,6,7]			0.596

In relation to *A. nodosum* collected from The Humber Arm, one-way ANOVA ($p<0.05$) of seaweed total metals versus site number demonstrated significant variation for all metals with the exception of Pb^{2+}. (Table 3.18). For example One-Way ANOVA of As^{3+} produced a *p* value of 0.001, whereas a *p* value for Pb^{2+} was 0.333, thereby demonstrating no significant variation for this metal concentration. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *A. nodosum* according to site number is demonstrated in Table 3.18. For example post hoc Tukey analysis demonstrated no significant variations in the Cu^{2+} levels of sites 3, 4, 6 and 7. However, significant variations in the As^{3+} concentration of site 3 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Zn^{2+} levels of sites 3, 4 and 6.

Table 3.18. One-Way ANOVA and post hoc Tukey analysis of *A. nodosum* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along The Humber Arm. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for: (mg/kg dw)	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. highest concs.*	*One-Way ANOVA (p value)*
Cadmium 0.1-0.4	[4]				[3,5,6,7]	0.001
Chromium 0.1-0.4	[3,6,7]				[4,5]	0.004
Lead 0.0-0.1			[3,4,5,6,7]			0.333
Copper 1.9-5.3	[3,4,6,7]				[5]	0.001
Zinc 16.4-41.3	[3,4,6]			[7]	[5]	0.002
Arsenic 16.1-33.2	[6,7]		[4,5]		[3]	0.001
Cobalt 0.5-2.2	[3,5,6,7]				[4]	0.001
Manganese 11.0-22.5			[3,4,5,6,7]			0.031
Nickel 1.3-5.3	[3,4,6,7]				[5]	0.001
Aluminium 9.3-60.3	[3,4,6,7]				[5]	0.001

In relation to *F. vesiculosus* collected from Bonne Bay, one-way ANOVA ($p<0.05$) of seaweed metals versus site number demonstrates significant variation for all metals with the exception of Pb^{2+} (Table 3.19). For example One-Way ANOVA of Cu^{2+} produced a *p* value of 0.001, whereas a *p* value for Pb^{2+} was 0.198, thereby demonstrating no significant variation for this metal concentration. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *F. vesiculosus* according to site number is demonstrated in Table 3.19. For example post hoc Tukey analysis demonstrated no significant variations in the Mn^{2+} levels of sites 2, 3, 4 and 5. However, significant variations in the Cr^{3+} concentration of site 2 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Ni^{2+} levels of sites 3, 4 and 5.

Table 3.19. One-Way ANOVA and post hoc Tukey analysis of *F. vesiculosus* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites in Bonne Bay. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for: (mg/kg dw)	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. with highest concs.*	*One-Way ANOVA (p value)*
Cadmium 0.7-1.3	[2,3,4,5]				[1]	0.001
Chromium 0.1-0.3	[1,3,4,5]				[2]	0.047
Lead 0.0-0.2			[1, 2,3,4,5]			0.198
Copper 3.3-16.8	[4,5]		[3,1]		[2]	0.001
Zinc 12.1-54.3	[3,4]		[5]		[1,2]	0.001
Arsenic 27.4-32.6			[1, 2,3,4,5]			0.031
Cobalt 0.6-1.2	[2,4]		[3,5]		[1]	0.001
Manganese 79.9-146.8	[2,3,4,5]				[1]	0.001
Nickel 4.5-9.1	[3,4,5]	[1]			[2]	0.001
Aluminium 24.3-154.8	[2,3,4,5]				[1]	0.001

In relation to *A. nodosum* collected from Bonne Bay, one-way ANOVA ($p<0.05$) of seaweed metals versus site number demonstrated significant variation for all metals with the exception of Pb^{2+} (Table 3.20). For example One-Way ANOVA of Cd^{2+} produced a *p* value of 0.001, whereas a *p* value for Pb^{2+} was 0.629, thereby demonstrating no significant variation for this metal concentration. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *A. nodosum* according to site number is demonstrated in Table 3.20. For example post hoc Tukey analysis demonstrated no significant variations in the Zn^{2+} levels of sites 2, 3, 4 and 5. However, significant variations in the Cu^{2+} concentration of site 3 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Al^{3+} levels of sites 2, 3, 4 and 5.

Table 3.20. One-Way ANOVA and post hoc Tukey analysis of *A. nodosum* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites in Bonne Bay. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for: (*mg/kg dw*)	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. with highest concs.*	*One-Way ANOVA (p value)*
Cadmium 0.2-0.9	[2,3,4]		[5]		[1]	0.001
Chromium 0.1-0.3	[1,3,4,5]				[2]	0.023
Lead 0.0-0.1			[1,2,3,4,5]			0.629
Copper 1.6-5.5	[1,2,4,5]				[3]	0.001
Zinc 16.7-71.4	[2,3,4,5]				[1]	0.001
Arsenic 19.4-28.0	[2,3,4]				[1,5]	0.001
Cobalt 0.6-0.9	[2,4,5]	[3]			[1]	0.001
Manganese 10.1-34.0	[2,3,4,5]				[1]	0.001
Nickel 0.7-2.7	[3,4,5]				[1,2]	0.001
Aluminium 6.4-21.5	[2,3,4,5]				[1]	0.001

In relation to *F. vesiculosus* collected from Suir Estuary and Fethard-on-Sea, one-way ANOVA ($p<0.05$) of seaweed metals versus site number demonstrates significant variation for all metals with the exception of Cd^{2+} (Table 3.21). For example One-Way ANOVA of Cr^{3+} produced a *p* value of 0.001. Whereas One-Way ANOVA of Cd^{2+} produced a *p* value of 0.074, thereby demonstrating no significant variation for this metal concentration. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *F. vesiculosus* according to site number is demonstrated in Table 3.21. For example post hoc Tukey analysis demonstrated no significant variations in the As^{3+} levels of sites 1, 2 and 3. However, significant variations in the Pb^{2+} concentration of site 1 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Ni^{2+} levels of sites 1, 2 and 5.

Table 3.21. One-Way ANOVA and post hoc Tukey analysis of *F. vesiculosus* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from Suir Estuary and Fethard-on-Sea. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for: (*mg/kg dw*)	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. with highest concs.*	*One-Way ANOVA* (*p value*)
Cadmium 0.5-0.8			[1,2,3,4,5]			0.074
Chromium 0.4-1.3	[5]		[3,4]	[2]	[1]	0.001
Lead 0.8-1.7	[2,3,4,5]				[1]	0.001
Copper 2.4-37.0	[5]		[1,3,4]		[2]	0.001
Zinc 22.2-55.1	[3]		[2,5]		[1,4]	0.001
Arsenic 13.5-46.4	[1,2,3]				[4,5]	0.001
Cobalt 0.9-2.2	[1,2,3]				[4,5]	0.001
Manganese 231.1-702.0	[5]		[1,3]	[4]	[2]	0.001
Nickel 2.9-6.3	[3]		[1,2,5]		[4]	0.001
Aluminium 82.7-485.4	[5]	[4]		[2,3]	[1]	0.001

In relation to *A. nodosum* collected from Suir Estuary and Fethard-on-Sea, one-way ANOVA (p<0.05) of metals versus site number demonstrates significant variation for some metals with the exception of Cr^{3+}, Pb^{2+}, Co^{2+}, Mn^{2+}, Ni^{2+} and Al^{3+} (Table 3.22). For example One-Way ANOVA of As^{3+} produced a *p* value of 0.001, whereas a *p* value for Cr^{3+} was 0.624, thereby demonstrating no significant variation for this metal concentration. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *A. nodosum* according to site number is demonstrated in Table 3.22. For example post hoc Tukey analysis demonstrated no significant variations in the Cd^{2+} levels of sites 3, 4 and 5. However, significant variations in the Zn^{2+} concentration of site 5 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Ni^{2+} levels of sites 3, 4 and 5.

Table 3.22. One-Way ANOVA and post hoc Tukey analysis of *A. nodosum* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from Suir Estuary and Fethard-on-Sea. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for: (mg/kg dw)	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. with highest concs.*	*One-Way ANOVA (p value)*
Cadmium 0.1-0.3	[2]				[3,4,5]	0.004
Chromium 0.2-0.3			[2,3,4,5]			0.624
Lead 0.0-0.1			[2,3,4,5]			0.843
Copper 3.3-4.5	[3,4]				[2,5]	0.016
Zinc 25.0-59.7	[2,3,4]				[5]	0.007
Arsenic 14.7-32.6	[2,3,4]				[5]	0.001
Cobalt 0.6-0.9	[4]				[2,3,5]	0.112
Manganese 30.0-47.5	[3,4]				[2,5]	0.067
Nickel 0.5-1.1	[2]				[3,4,5]	0.101
Aluminium 33.5-41.3			[2,3,4,5]			0.552

In relation to *P. lanosa* collected from Suir Estuary and Fethard-on-Sea, one-way ANOVA ($p<0.05$) of seaweed metals versus site number demonstrates significant variation for all metals (Table 3.23). For example One-Way ANOVA of Cd^{2+} produced *p* values of 0.001. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals in *P. lanosa* according to site number is demonstrated in Table 3.23. For example post hoc Tukey analysis demonstrated no significant variations in the Cu^{2+} levels of sites 4 and 5. However, significant variations in the Ni^{2+} concentration of site 4 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the As^{3+} levels of sites 3 and 4.

Table 3.23. One-Way ANOVA and post hoc Tukey analysis of *P. lanosa* metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from Suir Estuary and Fethard-on-Sea. Site numbers are displayed according to increasing seaweed metal concentration.

Range of values for: (mg/kg dw)	*Site No. with lowest concs.*	*Site No. with low/mid concs.*	*Site No. with mid concs.*	*Site No. with mid/high concs.*	*Site No. with highest concs.*	*One-Way ANOVA (p value)*
Cadmium 1.4-5.0	[5]		[3]		[4]	0.001
Chromium 1.7-4.0	[3]		[5]		[4]	0.001
Lead 5.3-9.1	[5]		[3]		[4]	0.001
Copper 34.4-72.6	[4,5]				[3]	0.001
Zinc 106.8-248.4	[5]			[3]	[4,]	0.001
Arsenic 5.8-10.7	[3,4,]				[5]	0.003
Cobalt 1.3-4.8	[5]		[4]		[3]	0.001
Manganese 185.9-1091.5	[5]		[4]		[3]	0.001
Nickel 8.1-10.0	[5,3]				[4]	0.001
Aluminium 606-1840	[3]		[4]		[5]	0.001

Table 3.24 is a profile of the total metal concentrations of seawater, seaweed and surface sediment samples collected along the Suir Estuary and Fethard-on-Sea. This is the first time that a profile of various metals in seawater, sediment and three seaweed species from five different locations along the Suir Estuary and Fethard-on-Sea has been established.

Table 3.24. Summary of total metal concentrations of seawater, seaweed and surface sediment samples collected along the Suir Estuary and Fethard-on-Sea.

Site	*Total Metal*	*Seawater mg/L*	*Sediment mg/kg*	*Seaweeds mg/kg*		
				F.vesiculosus	*P. lanosa*	*Ulva* sp
St. John's River	Pb	0.0002	59.3	1.7	——	——
Ferrybank		0.0001	15.5	0.8	——	——
Cheekpoint		0.0001	21.8	0.8	6.5	——
Passage East		0.0001	10.4	0.9	9.1	——
Fethard-on-Sea		0.0001	3.2	0.9	5.3	0.9
St. John's River	Zn	0.004	93.2	55.1	——	——
Ferrybank		0.002	60.3	38.0	——	——
Cheekpoint		0.0022	74.1	22.2	238.5	——
Passage East		0.0023	44.4	49.7	248.4	——
Fethard-on-Sea		0.0016	29.0	37.7	106.8	11.4
St. John's River	As	n/a	6.9	13.5	——	——
Ferrybank		n/a	5.6	21.4	——	——
Cheekpoint		n/a	5.7	19.3	5.8	——
Passage East		n/a	4.9	42.7	6.8	——
Fethard-on-Sea		n/a	5.5	46.4	10.7	4.4
St. John's River	Cd	<0.00004	2.3	0.7	——	——
Ferrybank		<0.00004	1.8	0.6	——	——
Cheekpoint		<0.00004	2.0	0.5	3.9	——
Passage East		<0.00004	1.4	0.8	5.0	——
Fethard-on-Sea		<0.00004	1.1	0.8	1.4	0.1
St. John's River	Co	<0.01	6.1	0.2	——	——
Ferrybank		<0.01	4.7	0.3	——	——
Cheekpoint		<0.01	5.5	0.3	0.6	——
Passage East		<0.01	3.3	0.7	0.7	——
Fethard-on-Sea		<0.01	1.9	0.6	0.4	0.3
St. John's River	Cr	<0.0005	39.4	1.3	——	——
Ferrybank		<0.0005	22.4	0.8	——	——
Cheekpoint		<0.0005	21.6	0.6	1.7	——
Passage East		<0.0005	12.8	0.7	4.0	——
Fethard-on-Sea		<0.0005	5.5	0.4	3.6	1.5
St. John's River	Cu	0.0020	18.7	12.1	——	——
Ferrybank		0.0020	10.2	37.0	——	——
Cheekpoint		0.0017	13.2	10.7	72.6	——
Passage East		0.0012	6.9	10.1	34.4	——
Fethard-on-Sea		0.0010	3.1	2.4	36.8	3.4
St. John's River	Ni	0.001	16.0	3.9	——	——
Ferrybank		0.001	12.4	4.0	——	——
Cheekpoint		0.0012	14.3	2.9	8.1	——
Passage East		0.0006	8.9	6.3	10.0	——
Fethard-on-Sea		0.0003	6.5	3.6	8.4	2.4
St. John's River	Mn	0.04	362.7	473.9	——	——
Ferrybank		<0.02	413.8	702.0	——	——
Cheekpoint		<0.02	306.3	471.5	1091.5	——
Passage East		<0.02	321.9	567.1	689.8	——
Fethard-on-Sea		<0.02	163.4	231.1	185.9	48.0
St. John's River	Al	<0.11	6012.0	485.4	——	——
Ferrybank		<0.11	5128.0	257.9	——	——
Cheekpoint		<0.11	5373.0	202.1	606.2	——
Passage East		<0.11	3226.0	144.8	1485.1	——
Fethard-on-Sea		<0.11	4176.0	82.7	1840.3	960.9

In relation to May, 2008 samples, the total metal concentrations of Pb^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+} and Ni^{2+} were generally low among all four seaweed species when compared with Zn^{2+}, Mn^{2+} and As^{3+}. Values for these total metals ranged from $0.3 - 8.0$ mg/kg dw (Table 3.25 and Figures 3.44 – 3.47).

Zn^{2+}, Mn^{2+} and As^{3+} were found to be the dominant total metals for all species under investigation. *P. lanosa,* however, contained the highest total Zn^{2+} (137.6 mg/kg dw). Differences between the brown seaweed total metal were also observed. Concentrations of Zn^{2+}, As^{3+} and Mn^{2+} associated with *F. vesiculosus* were 25, 44 and 149.1 mg/kg dw respectively, whereas, levels of the same metals in *A. nodosum* were 57.5, 38.2 and 29.2 mg/kg dw respectively), (Table 3.25).

Mn^{2+} levels were prominent for both *P. lanosa* and *F. vesiculosus*, while Zn^{2+} levels were also prominent for *P. lanosa* and *A. nodosum* (Figures 3.44 and 3.45).

A significant level of As^{3+} was also associated with the brown seaweeds (Figures 3.45 and 3.46), when compared to As^{3+} levels for *P. lanosa* and *Ulva* sp respectively (Figures 3.44 and 3.47).

Table 3.25. Total metal concentration (mg/kg dw) of four seaweed species under investigation. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Species	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*
P. lanosa	3.1±0.3	137.6±6.1	12.3±1.0	2.4±0.1	1.0±0.1	2.6±0.0	4.8±0.1	150.0±3.8	8.0±0.3
A. nodosum	0.3±0.1	80.9±15.0	42.1±3.3	0.3±0.1	1.2±0.2	0.04±0.1	1.5±0.2	32.7±11.8	1.7±0.5
F.vesiculosus	0.7±0.2	25.0±2.3	44.0±3.0	0.4±0.0	0.9±0.1	0.3±0.0	1.4±0.1	149.1±13.0	3.6±0.5
Ulva sp	1.4±0.1	12.2±0.8	5.7±0.7	0.1±0.0	0.4±0.1	4.2±0.7	3.3±0.2	39.9±2.0	3.9±0.3
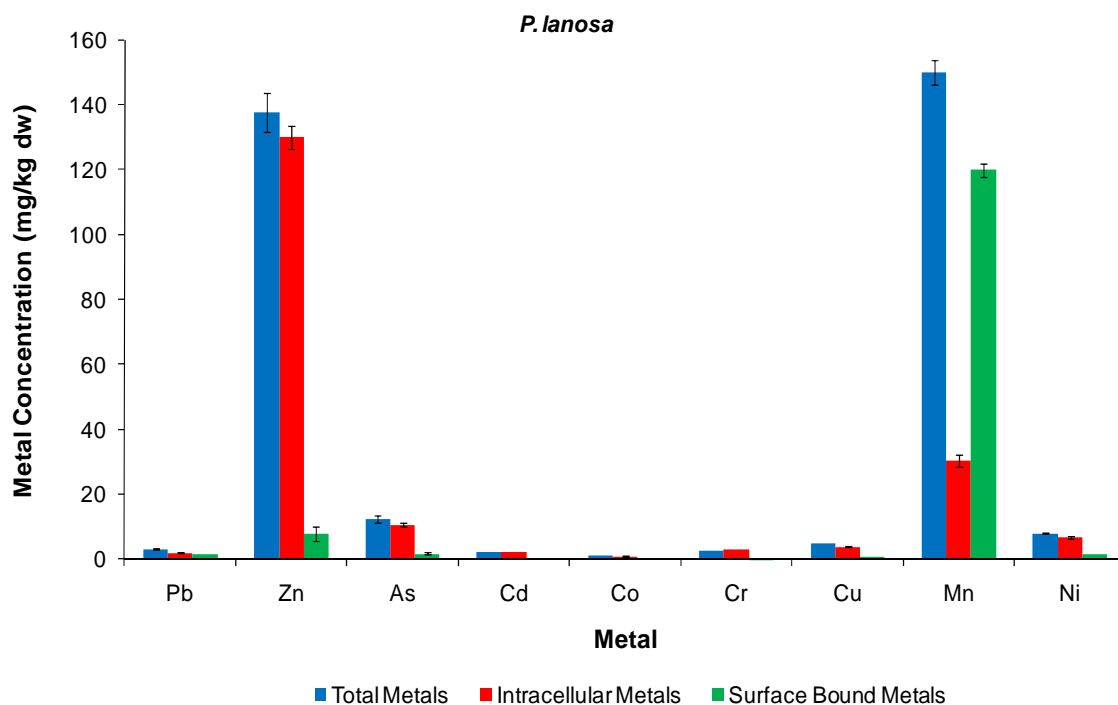
Figure 3.44. Metal content of *P. lanosa.* Error bars were calculated based on triplicate samples with 95% Confidence Intervals.
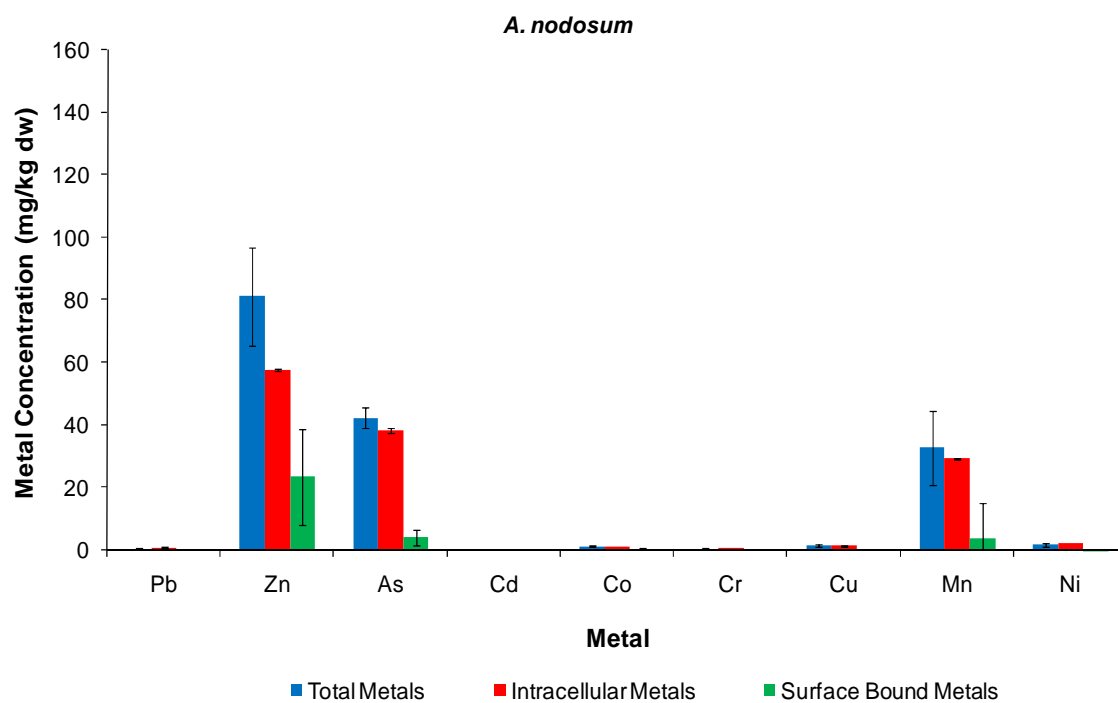
Figure 3.45. Metal content of *A. nodosum.* Error bars were calculated based on triplicate samples with 95% Confidence Intervals.
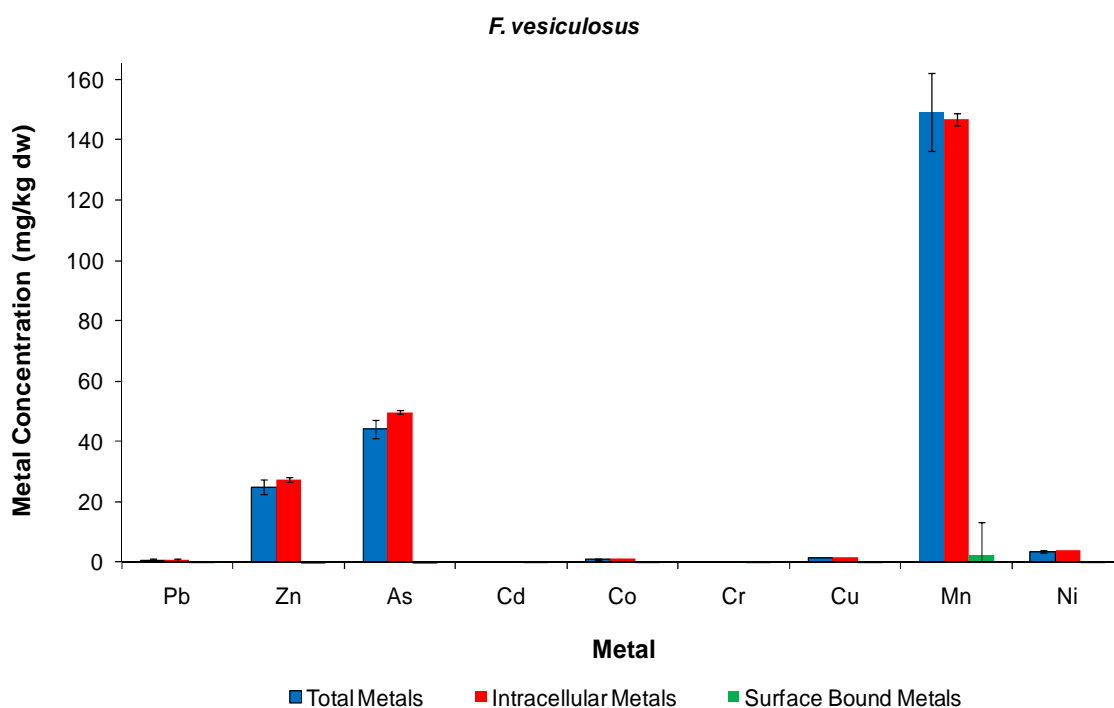
F. vesiculosus

Figure 3.46. Metal content of *F. vesiculosus*. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.
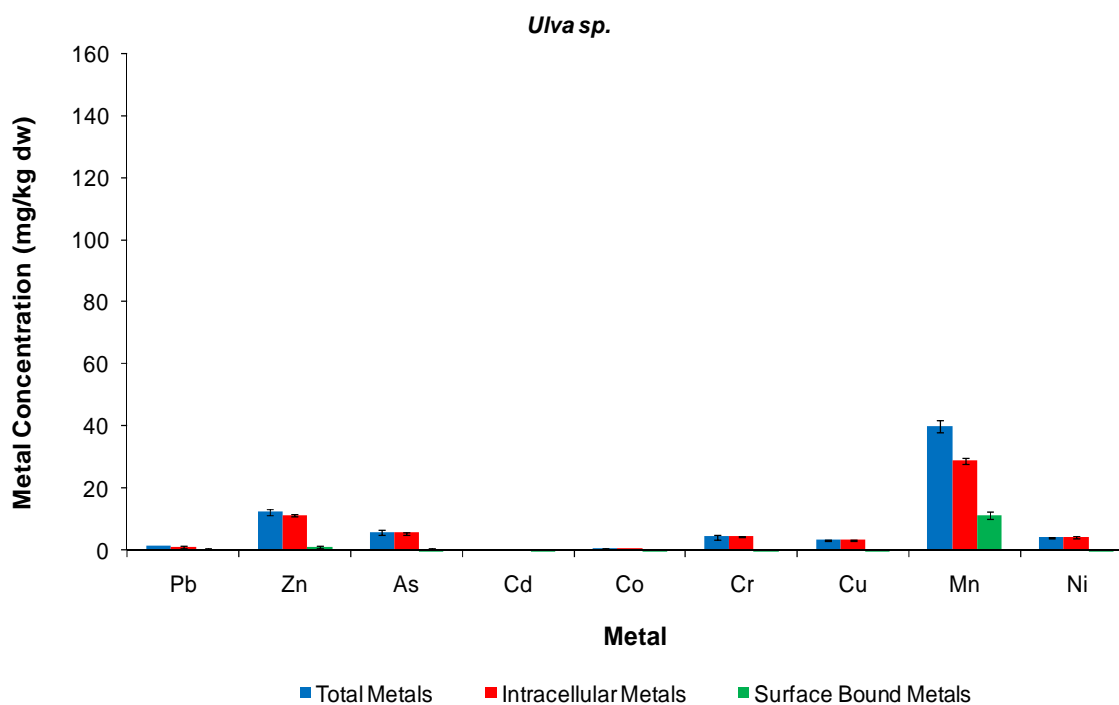
Ulva sp.

Figure 3.47. Metal content of *Ulva* sp. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

One-way ANOVA (p<0.05) demonstrated significant variations in the levels of total metals among the four seaweed species under investigation Table 3.26 displays One-way ANOVA values for all metals versus seaweed species. Seaweeds are grouped and ranked according to their metal concentration. Furthermore, statistical analysis demonstrated the superior metal uptake power of *P. lanosa* in relation to Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Ni^{2+}, whereas *F. vesiculosus* and *A. nodosum* contained the lowest Pb^{2+}, Zn^{2+}, Cd^{2+}, Cr^{3+} and Cu^{2+} and *Ulva* sp yielded the lowest As^{3+} and Co^{2+}. Furthermore, *P. lanosa* and *F. vesiculosus* contained the highest Mn^{2+} levels. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals according to seaweed species is demonstrated in Table 3.26. For example post hoc Tukey analysis demonstrated no significant variations in the Pb^{2+} levels of *F. vesiculosus* and *A. nodosum*. However, significant variations in the Zn^{2+} concentration of *P. lanosa* were demonstrated when compared with all other species. Furthermore, there were no significant variations in the Co^{2+} levels of sites *F. vesiculosus*, *A. nodosum* and *P. lanosa*.

Seasonal variations in the concentration of some metals associated with seaweeds collected from Fethard-on-Sea were also demonstrated. For example, Levels of Cu^{2+} and Mn^{2+} associated with *P. lanosa* were substantially higher in the October samples when compared to those of May, whereas, Zn^{2+} levels in the October *P. lanosa* were lower. Furthermore, seasonal variations in Zn^{2+} and Mn^{2+} associated with *A. nodosum* were observed. For example, Zn^{2+} levels of *A. nodosum* for May and October samples were 80.9 and 59.7 mg/kg dw respectively and Mn^{2+} concentrations for the same months were 32.7 and 47.5 mg/kg dw respectively.

Additionally, variations in Zn^{2+} and Mn^{2+} of *F. vesiculosus* were also observed. For example, Zn^{2+} levels of for May and October samples were 25.0 and 37.7 mg/kg dw respectively while Mn^{2+} concentrations were 149.1 and 231.1 mg/kg dw respectively.

Mn^{2+} associated with *Ulva* sp also varied seasonally. For example Mn^{2+} levels for May and October samples were 39.9 and 48.0 mg/kg dw respectively

Table 3.26. One-Way ANOVA and post hoc Tukey analysis of seaweed metal concentration versus seaweed species. Distribution of seaweed species in order of the lowest to highest total metal concentrations (mg/kg dw) for *F. vesiculosus, A. nodosum, P. lanosa and Ulva* sp collected from Fethard-on-Sea.

Range of values for:	*Species with lowest concs.*	*Species with mid concs.*	*Species with highest concs.*	*One-Way ANOVA p value*
Lead (0.3 - 3.1)	FV, AN	UL	PL	0.001
Zinc (12.2 – 137.6)	FV, UL	AN	PL	0.001
Arsenic (5.7 – 44.6)	UL	PL	AN, FV	0.001
Cadmium (0.1 – 2.4)	FV, AN	UL	PL	0.001
Cobalt (0.5 – 1.2)	UL		FV, PL, AN	0.001
Chromium (0.3 – 4.2)	FV, AN	PL	UL	0.001
Copper (1.4 – 4.8)	FV, AN	UL	PL	0.001
Manganese (32.7 – 150.0)	AN, UL		FV, PL	0.001
Nickel (1.7 – 8.0)	AN	FV, UL	PL	0.001
Sum Total Metals (71.2 – 321.8)	UL	AN, FV	PL	0.001

3.4.8.1 Intracellular Metals

In the majority of cases, the highest proportions of metals under investigation were found to be intracellular (Figures 3.48 to3.57). Error bars are calculated based on triplicate samples with 95% Confidence Intervals.

For all seaweeds under investigation, the highest proportion of Pb^{2+}, As^{3+}, Cr^{3+}, Cu^{2+}, Ni^{2+} and Al^{3+} were found to be intracellular, therefore trends in metal accumulation were similar to those of total metals (Figures 3.48 to 3.57). Pb^{2+}, As^{3+}, Cr^{3+} and Ni^{2+} are non-essential elements therefore, high intracellular concentrations of these metals may indicate the activation of detoxification mechanisms within seaweeds. The highest

proportions of Zn^{2+}, Cd^{2+} and Mn^{2+} were also found to be intracellular for all seaweeds, with the exception of *P. lanosa*. For example, total Mn^{2+} concentration of *P. lanosa* was 1091.5 mg/kg dw, whereas intracellular levels accounted for 248.5 mg/kg dw, therefore surface bound Mn^{2+} accounted for 843.0 mg/kg dw. Additionally, total Cd^{2+} concentration of *P. lanosa* was 3.9 mg/kg dw, and intracellular levels accounted for 0.2 mg/kg dw, therefore surface bound Mn^{2+} accounted for 2.7 mg/kg dw. Furthermore, total Zn^{2+} concentration of *P. lanosa* was 238.5 mg/kg dw, whereas intracellular levels accounted for 6.8 mg/kg dw, therefore surface bound Zn^{2+} accounted for 231.7 mg/kg dw.
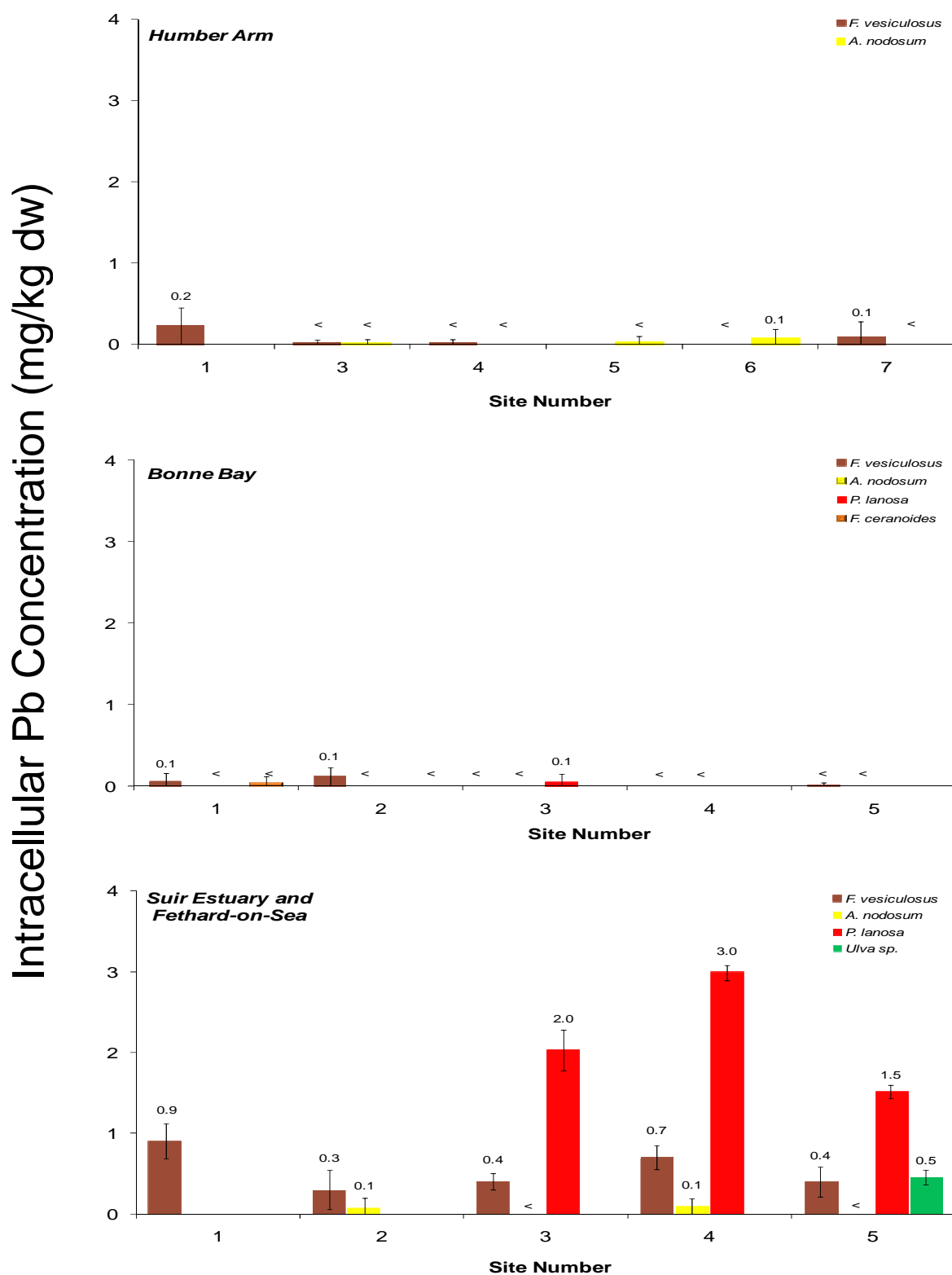
Figure 3.48. Comparison of Intracellular Pb concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. (< = below limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
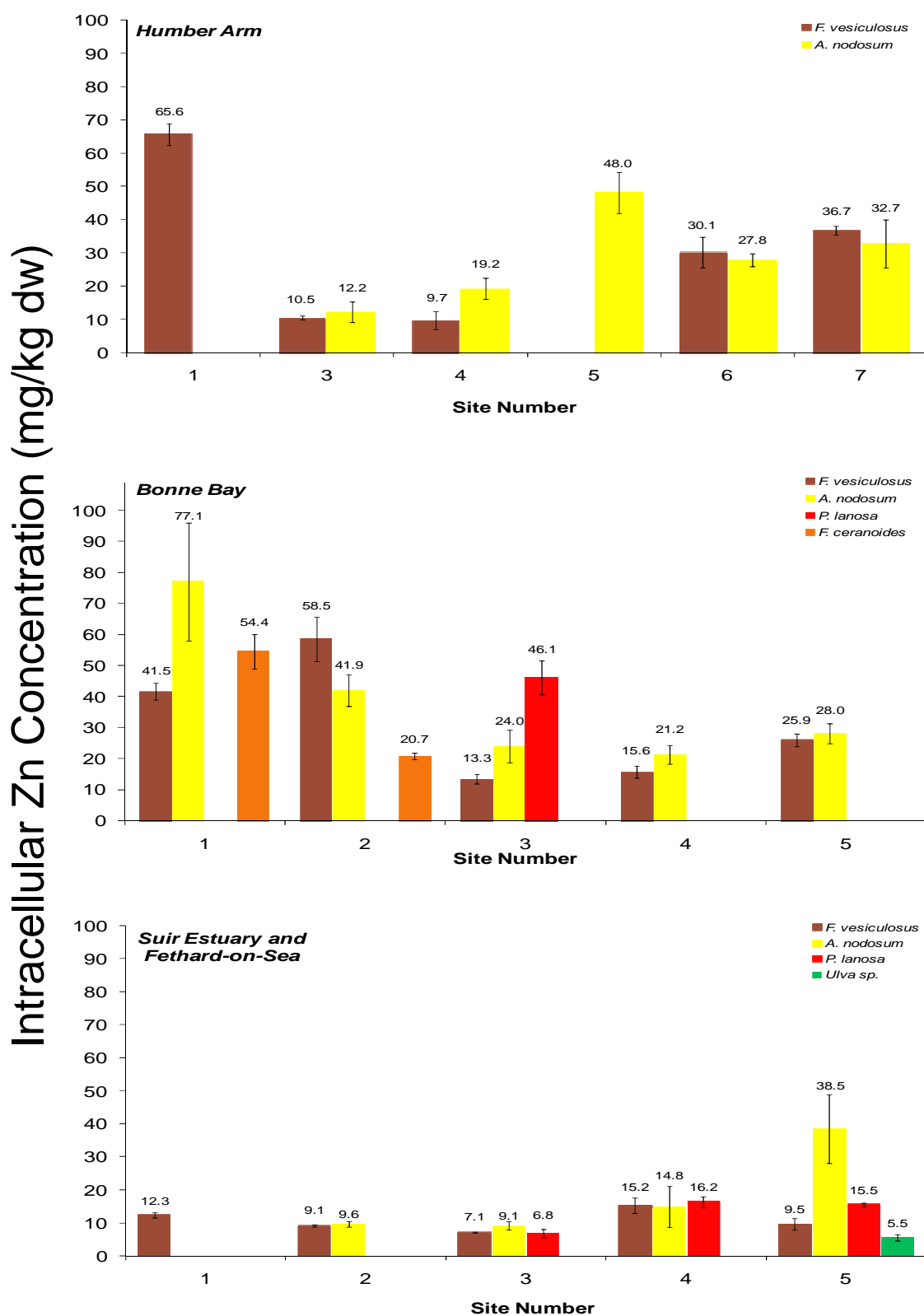
Figure 3.49. Comparison of Intracellular Zn concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
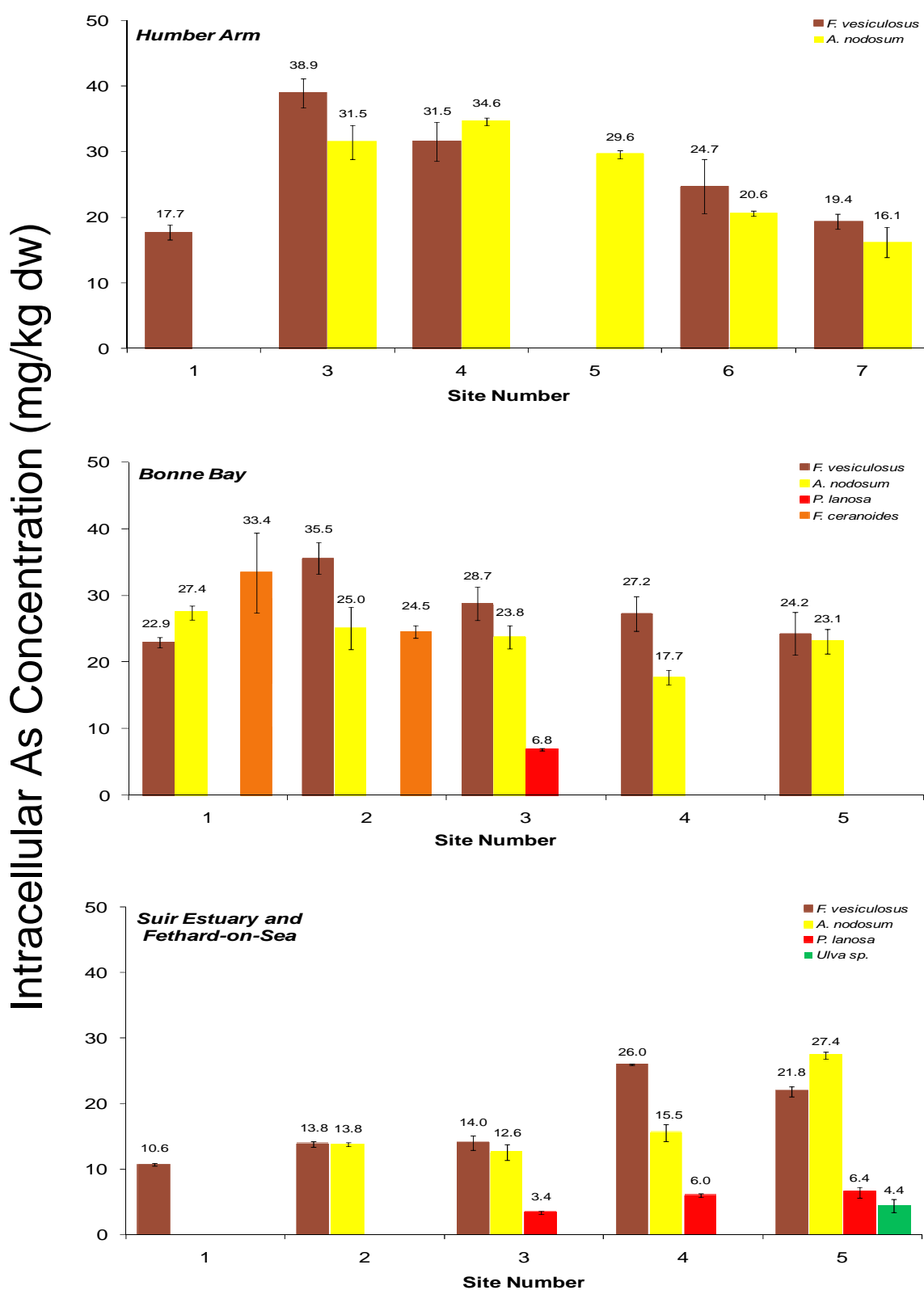
Figure 3.50. Comparison of Intracellular As concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
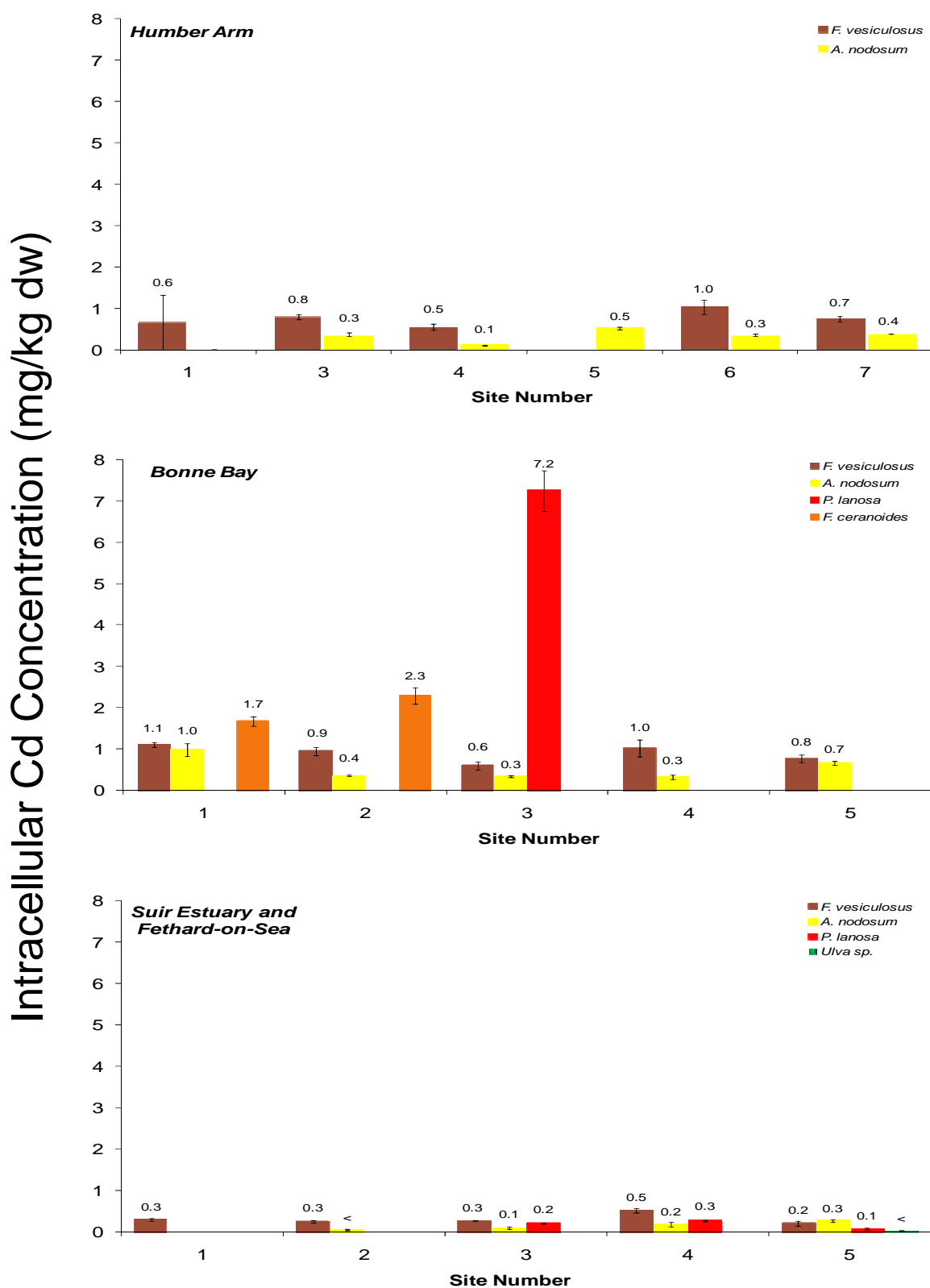
Figure 3.51. Comparison of Intracellular Cd concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. (< = below limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

209
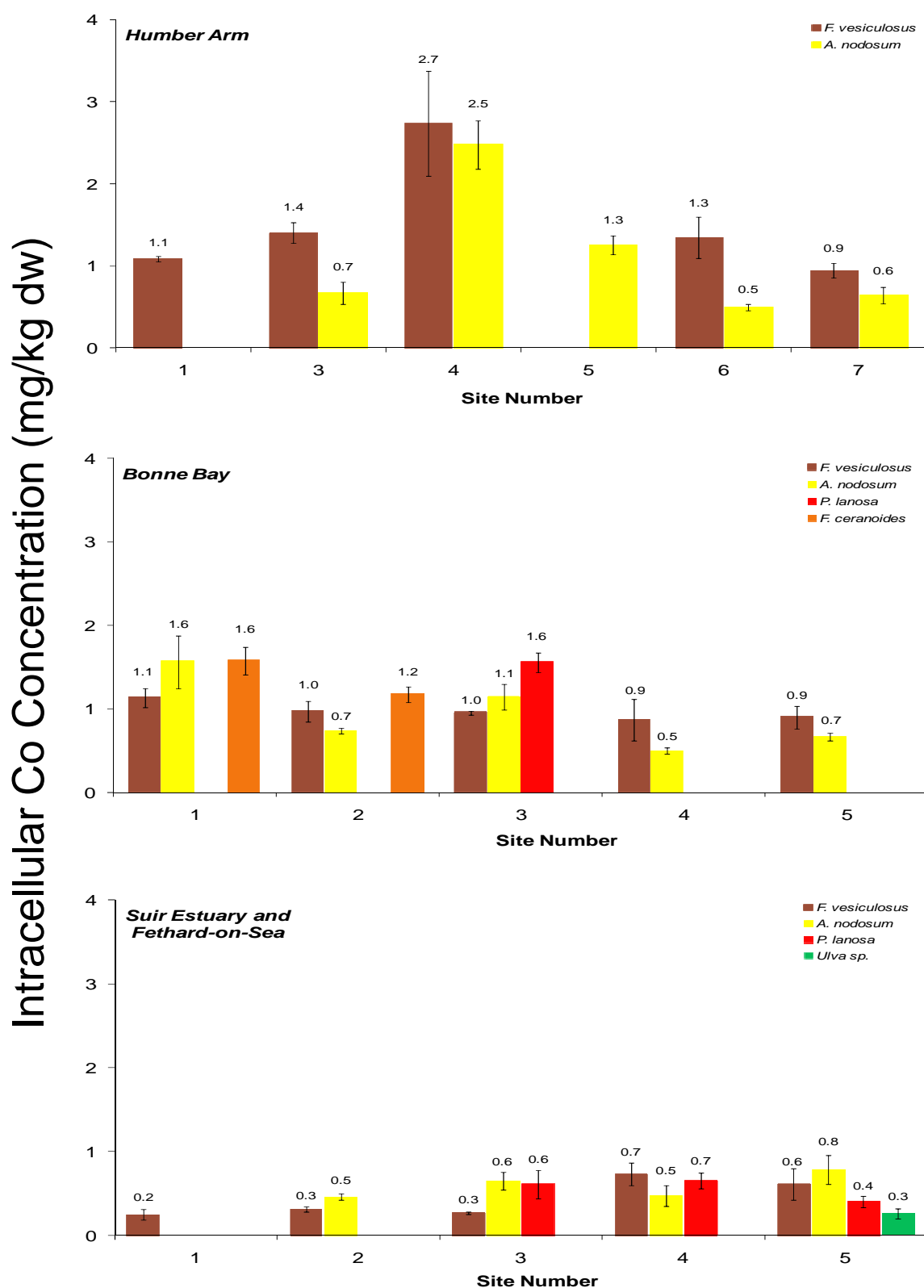
Figure 3.52. Comparison of Intracellular Co concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
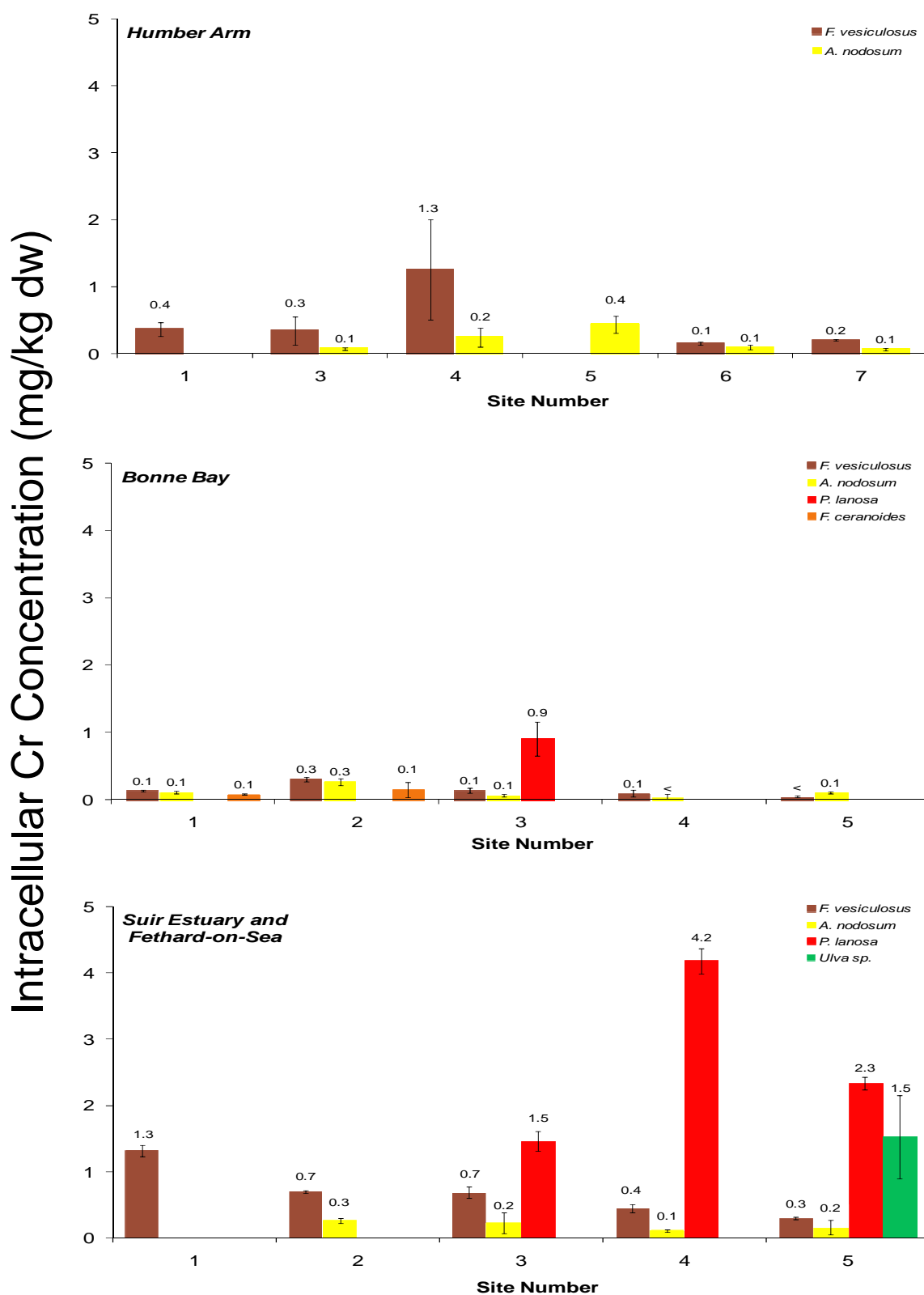
Figure 3.53. Comparison of Intracellular Cr concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. (< = below limit of detection). Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
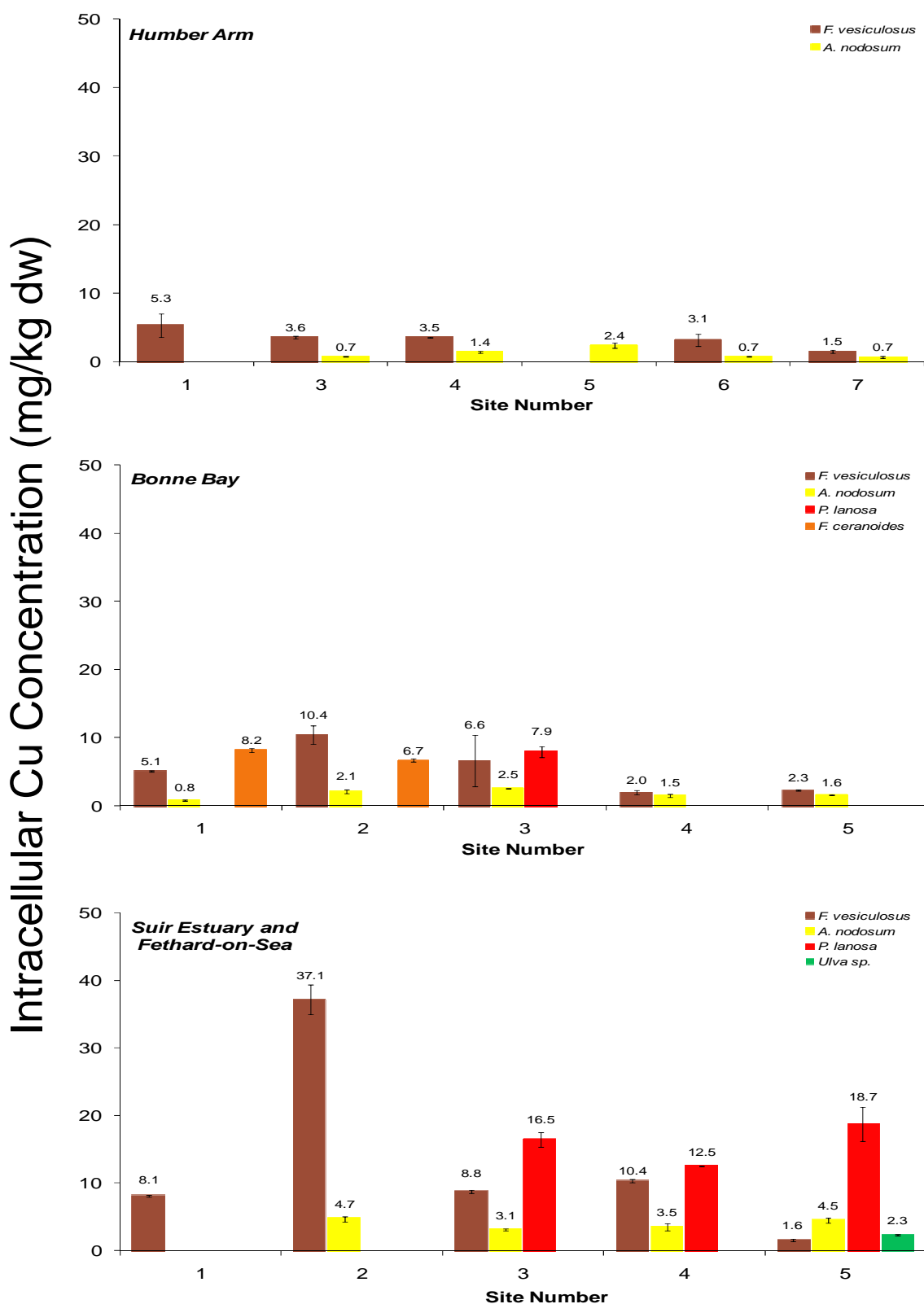
Figure 3.54. Comparison of Intracellular Cu concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
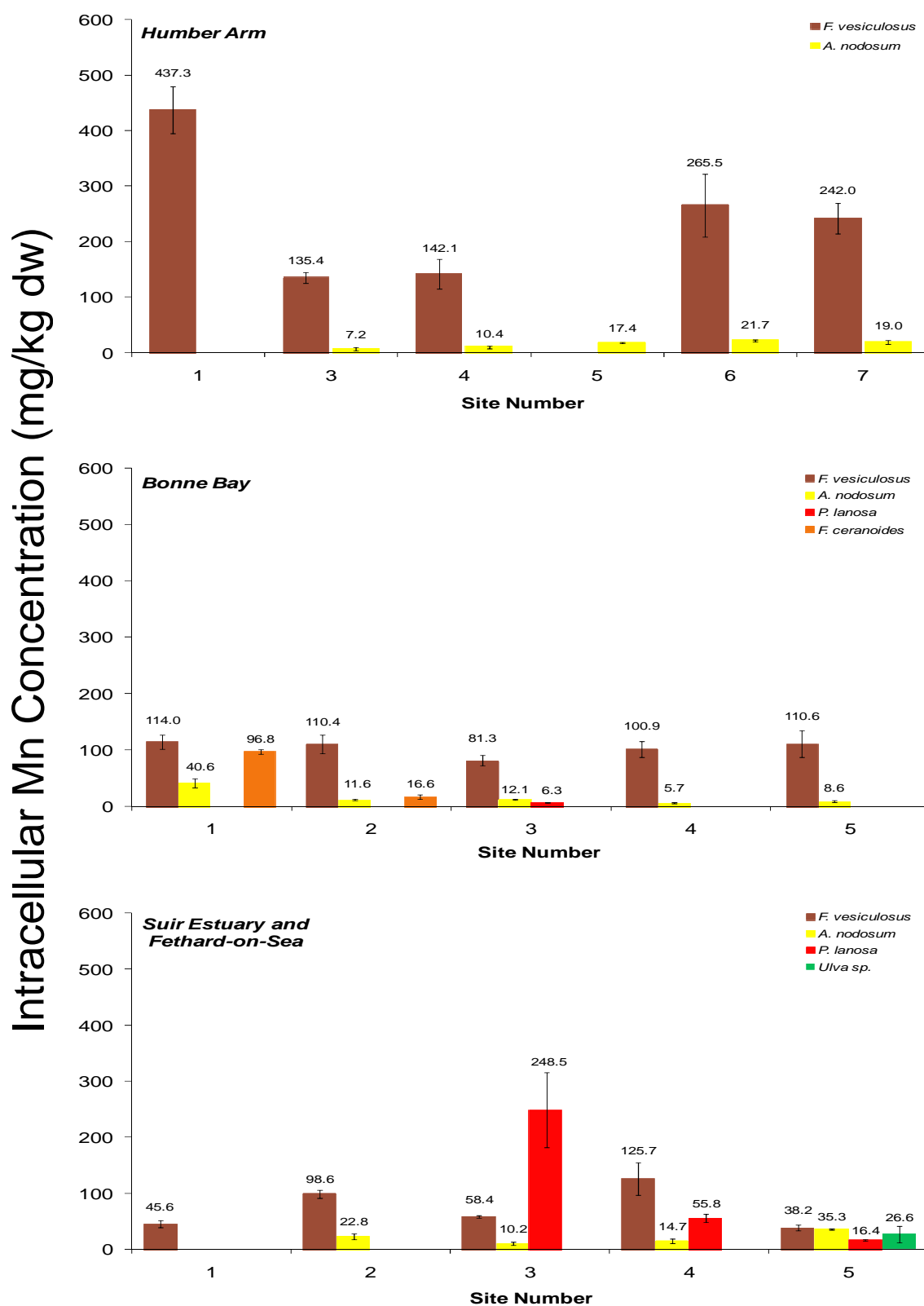
Figure 3.55. Comparison of Intracellular Mn concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.
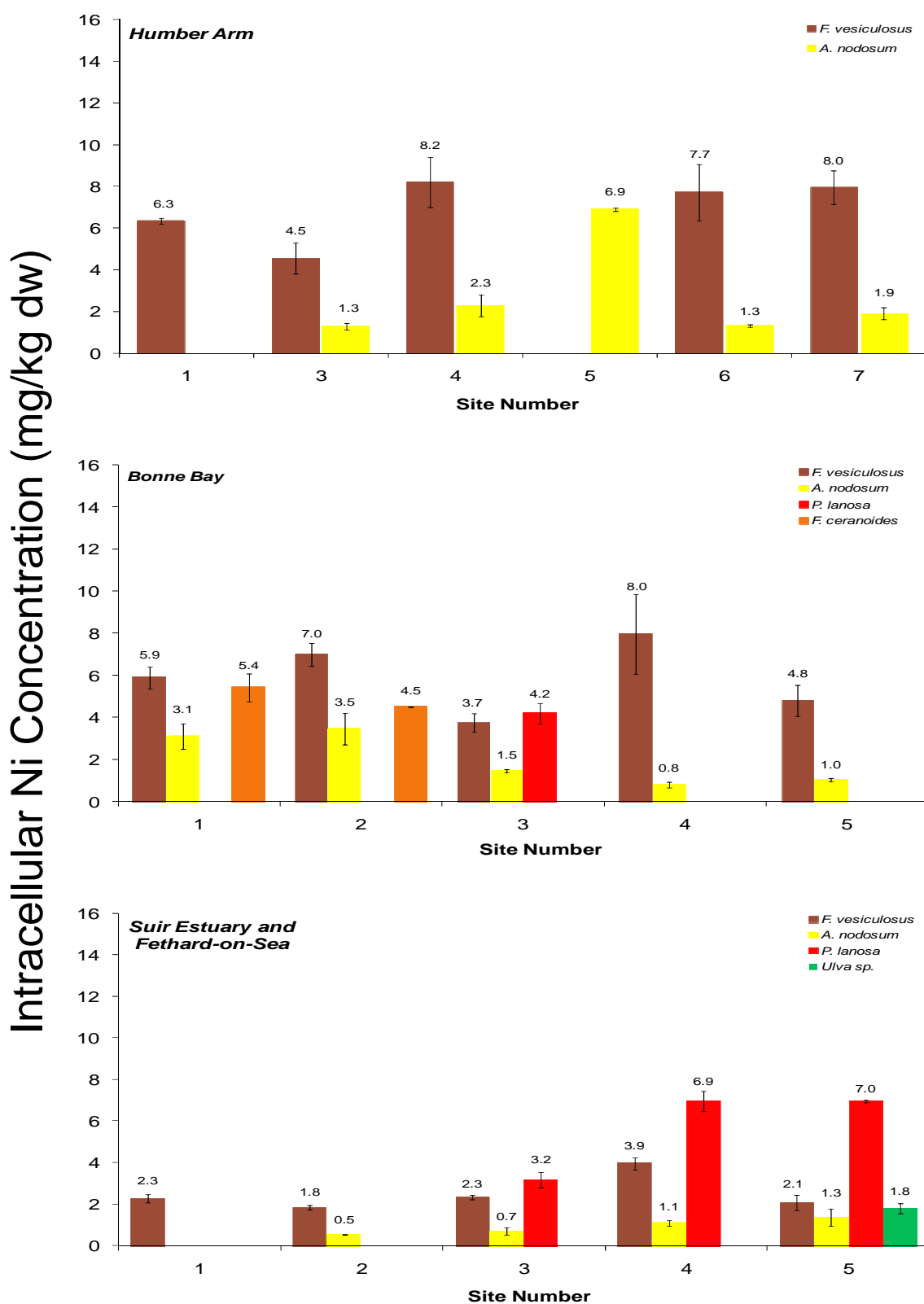
Figure 3.56. Comparison of Intracellular Ni concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

214
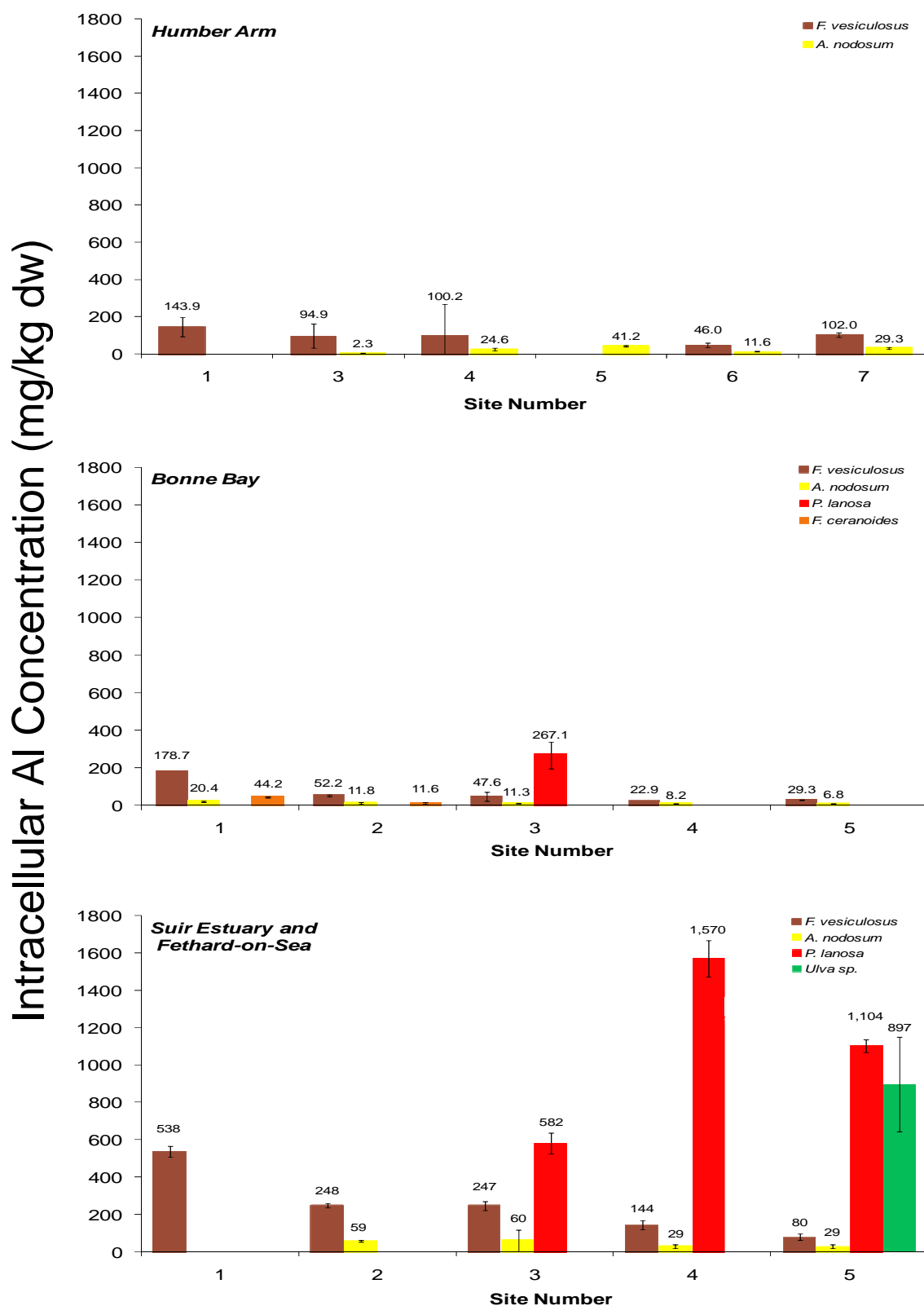
Figure 3.57. Comparison of Intracellular Al concentrations of seaweeds collected from Humber Arm, Bonne Bay, Suir Estuary and Fethard-on-Sea. Error bars were calculated based on triplicate runs with 95% Confidence Intervals.

One-Way ANOVA was performed in order to compare levels of various intracellular metals with site number. Statistical analysis of intracellular metals levels collected from the Humber Arm demonstrated significant variations (p<0.05) in the levels of Zn^{2+}, As^{3+}, Co^{2+}, Cu^{2+}, Mn^{2+} with respect to *F. vesiculosus*. Significant variations however were not observed for Pb^{2+}, Cd^{2+}, Cr^{3+}, Ni^{2+} and Al^{3+} (Table 3.27). Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *F. vesiculosus* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the Zn^{2+} levels of sites 3 and 4. However, significant variations in the As^{3+} concentration of site 3 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Co^{2+} levels of sites 1, 3, 6 and 7.

Table 3.27. One-Way ANOVA (p<0.05) of *F. vesiculosus* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along The Humber Arm.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*0.184*	*0.001*	*0.001*	*0.354*	*0.001*	*0.079*	*0.002*	*0.001*	*0.059*	*0.268*

Furthermore, statistical analysis demonstrated different trends in intracellular metal levels for *A. nodosum* from the same location. With the exception of Pb^{2+}, One-Way ANOVA demonstrated significant variations (p<0.05) for all intracellular metals in relation to site number (Table 3.28). Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *A. nodosum* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the Cd^{2+} levels of sites 3, 6 and 7. However, significant variations in the Cu^{2+} concentration of site 5 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Co^{2+} levels of sites 3, 6 and 7.

Table 3.28. One-Way ANOVA (p<0.05) of *A. nodosum* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along The Humber Arm.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*0.479*	*0.001*	*0.001*	*0.001*	*0.001*	*0.01*	*0.001*	*0.001*	*0.001*	*0.001*

Statistical analysis of intracellular metals levels collected from the Bonne Bay demonstrated significant variations (p<0.05) in the levels of Zn^{2+}, As^{3+}, Cd^{2+}, Cr^{3+}, Cu^{2+}, Ni^{2+} and Al^{3+} with respect to *F. vesiculosus*. Significant variations were not however observed for Pb^{2+}, Co^{2+} and Mn^{2+} (Table 3.29). Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *F. vesiculosus* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the Cd^{2+} levels of sites 1, 3 and 4. However, significant variations in the Zn^{2+} concentration of site 2 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Mn^{2+} levels of sites 1, 2, 4 and 5.

Table 3.29. One-Way ANOVA (p<0.05) of *F. vesiculosus* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites at Bonne Bay.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*0.198*	*0.001*	*0.001*	*0.01*	*0.194*	*0.001*	*0.001*	*0.095*	*0.001*	*0.001*

Significant variations (p<0.05) for all metals with the exception of Pb^{2+} were observed for *A. nodosum* collected from Bonne Bay, a similar trend to that of the same species from the Humber Arm (Table 3.30). Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *A. nodosum* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the Zn^{2+} levels of sites 2, 3, 4 and 5. However, significant variations in the Cr^{3+} concentration of

site 2 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Cd^{2+} levels of sites 2, 3 and 4.

Table 3.30. One-Way ANOVA ($p<0.05$) of *A. nodosum* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites at Bonne Bay.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*1.0*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*

Statistical analysis of intracellular metals levels of *F. vesiculosus* collected from the Suir Estuary and Fethard-on-Sea demonstrated significant variations ($p<0.05$) in the levels of all metals (Table 3.31).. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *F. vesiculosus* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the Cd^{2+} levels of sites 1, 2, 4 and 5. However, significant variations in the Cr^{3+} concentration of site 1 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Cu^{2+} levels of sites 1, 3 and 4.

Table 3.31. One-Way ANOVA ($p<0.05$) of *F. vesiculosus* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along the Suir Estuary & Fethard-on-Sea.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*0.01*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*	*0.001*

Statistical analysis of intracellular metals levels of *A. nodosum* collected from the same locations however demonstrated significant variations ($p<0.05$) for As^{3+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+} and Ni^{2+} . (Table 3.32). Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *A. nodosum* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the As^{3+} levels of sites 2 and 3. However, significant variations in the Cd^{2+} concentration of site 5 were

demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Cu^{2+} levels of sites 3 and 4.

Table 3.32. One-Way ANOVA (p<0.05) of *A. nodosum* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along the Suir Estuary & Fethard-on-Sea.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*0.339*	*0.001*	*0.001*	*0.001*	*0.016*	*0.228*	*0.001*	*0.001*	*0.006*	*0.351*

In relation to *P. lanosa*, statistical analysis demonstrated significant variation (p<0.05) in levels of all metals with the exception of Co^{2+}. Significantly, *P. lanosa* was with only species to demonstrate significant variations of Pb^{2+} levels in relation to site number (Table 3.33). Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various intracellular metals in *P. lanosa* according to site number was demonstrated. For example post hoc Tukey analysis demonstrated no significant variations in the Zn^{2+} levels of sites 4 and 5. However, significant variations in the As^{3+} concentration of site 3 were demonstrated when compared with all other sites. Furthermore, there were no significant variations in the Mn^{2+} levels of sites 4 and 5.

Table 3.33. One-Way ANOVA (p<0.05) of *P. lanosa* intracellular metal concentration (mg/kg dw) versus site number. Seaweed samples were collected from various sites along the Suir Estuary & Fethard-on-Sea.

Statistical Analysis (p<0.05)	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*	*Al*
One-Way ANOVA	*0.001*	*0.001*	*0.001*	*0.001*	*0.054*	*0.001*	*0.005*	*0.001*	*0.001*	*0.001*

Results for the May, 2008 samples demonstrated that 94.4% of Zn^{2+} associated with *P. lanosa* was intracellular, whereas for *A. nodosum*, the corresponding value was 71.1% (Table 3.34). With the exception of Mn^{2+} associated with *P. lanosa*, intracellular metal concentrations exceeded surface bound metal levels (Table 3.34, Figures 3.44 to 3.47).

Table 3.34. Intracellular metal concentration (mg/kg dw) of four seaweed species under investigation. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Seaweed Species	Pb	Zn	As	Cd	Co	Cr	Cu	Mn	Ni
P. lanosa	1.8±0.2	129.9±3.7	10.7±0.7	2.1±0.0	0.8±0.1	2.9±0.0	3.9±0.2	30.2±1.9	6.7±0.3
A. nodosum	0.3±0.2	57.5±0.5	38.2±0.9	0.3±0.1	0.9±0.0	0.5±0.0	1.3±0.1	29.2±0.3	2.3±0.0
F.vesiculosus	0.7±0.4	27.2±0.7	49.6±0.5	0.5±0.0	1.0±0.1	0.3±0.0	1.6±0.0	146.8±2.1	4.0±0.0
Ulva sp	1.1±0.3	11.2±0.3	5.5±0.4	0.2±0.0	0.5±0.0	4.4±0.2	3.2±0.1	28.7±0.9	4.0±0.4

Differences in trends for seaweed intracellular metals were also observed in this study. In the cases of *Ulva* sp, *A. nodosum* and *F. vesiculosus,* high proportions of Mn^{2+} were intracellular (almost 100% for the brown seaweeds and 72% for *Ulva* sp), whereas only 20% Mn^{2+} associated with *P. lanosa* was intracellular (Table 3.34).

3.4.8.2 Statistical Analysis of Intracellular Metals versus Seaweed Species

One-way ANOVA ($p<0.05$) demonstrated significant variations in the levels of all intracellular metals, with the exception of Mn^{2+}, for the four seaweed species under investigation. In the case of Mn^{2+}, One-way ANOVA, demonstrated a significant difference. Statistical analysis also demonstrated that *P. lanosa* was capable of accumulating the highest levels of intracellular, Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Ni^{2+}. Furthermore, *Ulva* sp provided the lowest levels of Zn^{2+}, Cd^{2+} and Co^{2+} intracellularly. The brown seaweeds contained the lowest Cr^{3+} and Cu^{2+}, while *F. vesiculosus* yielded the highest Mn^{2+}. Table 3.35 displays One-way ANOVA values for all metals versus seaweed species. Additionally, seaweeds are grouped and ranked according to their metal concentration. Following 1-way ANOVA, post hoc Tukey analysis of significant variations was performed. The distribution of the various metals according to seaweed species is demonstrated in Table 3.35. For example post hoc Tukey analysis demonstrated no significant variations in the Pb^{2+} levels of *F. vesiculosus. Ulva* sp and *A. nodosum.* However, significant variations in the Mn^{2+} concentration of *F. vesiculosus* were demonstrated when compared with all other species. Furthermore, there were no significant variations in the Cd^{2+} levels of sites *F. vesiculosus* and *A. nodosum.*

Table 3.35. One-Way ANOVA and post hoc Tukey analysis of seaweed intracellular metal concentration versus seaweed species. Distribution of seaweed species in order of the lowest to highest intracellular metal concentrations (mg/kg dw) for *F. vesiculosus, A. nodosum, P. lanosa and Ulva* sp collected from Fethard-on-Sea.

Range of values for:	*Species with lowest concs.*	*Species with mid concs.*	*Species with highest concs.*	*One-Way ANOVA p value*
Lead (0.7 – 1.8)	FV, AN, UL		PL	0.003
Zinc (11.2 – 129.9)	UL	FV, AN	PL	0.001
Arsenic (5.5 – 49.6)	PL, UL	AN	FV	0.001
Cadmium (0.2 – 2.1)	UL	FV, AN	PL	0.001
Cobalt (0.5 – 1.0)	UL	PL	FV, AN	0.001
Chromium (0.3 – 4.4)	FV, AN	PL	UL	0.001
Copper (1.3 – 3.9)	FV, AN	UL	PL	0.001
Manganese (28.9 – 146.8)	PL, AN, UL		FV	0.001
Nickel (2.3 – 6.7)	AN	FV, UL	PL	0.001
Sum Metals (58.8 – 231.7)	UL	AN	FV, PL	0.001

3.4.8.3 Regression Analysis (*Fitted Line Plots)*

Results from this study demonstrate that each of the seaweed species tested had different affinities for different metals and this is reflected in concentrations of total, surface bound and intracellular metal uptake. Furthermore, variations in total protein, extracted protein and polyphenol levels were also observed for the seaweeds under investigation (Table 3.36).

Table 3.36. Comparison of metal, protein and polyphenol levels for four seaweed species.

Seaweed Species	Total Metal (mg/kg dw)	Intracell. Metal (mg/kg dw)	Total Protein (% dw)	Extracted Protein (mg/g dw)	Polyphenol (mg/g dw GAE)
P. lanosa	321.8	188.85	24.9	5.7	12.9
A. nodosum	161.21	131.00	11.1	96.9	34.8
F. vesiculosus	225.2	231.71	14.9	39.6	26.9
Ulva sp	71.21	58.84	19.7	4.7	2.2

Results from regression analysis suggest relationships between protein, polyphenol and seaweed metals. Apparent linear relationships between total protein with Pb^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} were demonstrated (R^2 = 97.1, 95.6, 88.7 and 82.3 respectively). Furthermore, a negative relationship between extracted protein and intracellular Cu^{2+} was observed (R^2 = 74.8). By contrast, apparent negative relationships between seaweed polyphenol levels with intracellular Cr^{3+} and Cu^{2+} were also demonstrated. Additionally, positive relationships between polyphenols and intracellular Co^{2+} and As^{3+} were also demonstrated.

3.4.8.4 Surface Bound Metals

Surface bound metal levels were calculated by subtracting intracellular metal levels from total metal levels.

In this study, the highest proportions of metals under investigation were found to be intracellular (Figures 3.44 to 3.47). Some seaweed species however demonstrated high proportions of surface bound metals and the majority of those were from the Suir Estuary (Tables 3.37 and 3.40).

The high proportions of surface bound Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Pb^{2+} and Cu^{2+} were associated with *P. lanosa* collected from the Suir Estuary and values ranged from 49 – 97% (Table 3.37). Elevated levels of surface bound Zn^{2+} and Mn^{2+} were associated with the brown seaweeds and values ranged from 26-90%. Additionally, a high proportion of surface bound Co^{2+} was related to *F. vesiculosus* (65-82%) (Table 3.37).

Table 3.37. List of seaweeds with high proportions of surface bound metals (%).

Location	*Seaweed Species*	*Surface Bound Metal Range (%)*					
		Zn	Cd	Co	Mn	Pb	Cu
Suir Estuary	*F. vesiculosus*	(66-78)		(65-82)	(78-90)		
Suir Estuary	*A. nodosum*	(36-66)			(26-67)		
Suir Estuary	*P. lanosa*	(86-97)	(93-95)	(69-88)	(77-91)	(67-72)	(49 -77)
Bonne Bay	*P. lanosa*						(73)

In relation to the Newfoundland samples, *P. lanosa* was the only seaweed associated with a high proportion of surface bound metal. In this instance, the only metal with a higher proportion bound to the seaweed surface was Cu^{2+} (73%) (Tables 3.38 and 3.39).

Table 3.38. Seaweed surface bound metals (mg/kg dw) from the Humber Arm. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Metal	*Seaweed*	*Site 1*	*Site 3*	*Site 4*	*Site 5*	*Site 6*	*Site 7*
Pb	*FV*	0.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	——	0.0 ± 0.0	0.0 ± 0.1
	AN	——	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1
Zn	*FV*	0.0 ± 3.2	0.0 ± 0.5	0.0 ± 1.6	——	0.0 ± 2.9	8.6 ± 6.0
	AN	——	4.2 ± 4.0	0.2 ± 6.3	0.0 ± 8.1	0.0 ± 1.7	1.6 ± 5.9
As	*FV*	2.5 ± 1.7	0.0 ± 2.9	0.0 ± 3.7	——	0.0 ± 2.6	0.0 ± 1.6
	AN	——	1.7 ± 2.7	0.0 ± 0.8	0.0 ± 0.8	0.1 ± 0.7	0.0 ± 2.1
Cd	*FV*	0.1 ± 0.4	0.0 ± 0.1	0.0 ± 0.1	——	0.0 ± 0.1	0.1 ± 0.2
	AN	——	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Co	*FV*	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.4	——	0.0 ± 0.1	0.3 ± 0.2
	AN	——	0.1 ± 0.1	0.0 ± 0.4	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.1
Cr	*FV*	0.0 ± 0.1	0.0 ± 0.2	0.0 ± 0.6	——	0.1 ± 0.0	0.1 ± 0.0
	AN	——	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.1 ± 0.1
Cu	*FV*	0.0± 1.0	1.6± 0.3	1.2± 0.2	——	1.0± 0.7	7.3± 0.5
	AN	——	1.2 ± 0.2	1.0 ± 0.2	3.0 ± 0.7	2.1 ± 0.0	2.1 ± 0.2
Mn	*FV*	0.0 ± 7.7	0.0 ±14.7	0.0 ±17.8	——	0.0 ±40.2	47. ±44.1
	AN	——	3.9 ± 2.6	4.3 ± 4.8	0.0 ± 2.6	0.0 ± 1.7	3.5 ± 3.5
Ni	*FV*	0.1 ± 0.2	0.0 ± 0.7	0.0 ± 1.3	——	0.3 ± 0.9	2.8 ± 2.0
	AN	——	0.1 ± 0.2	0.0 ± 0.5	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.3
Al	*FV*	0.0 ±30.5	17.4±43.5	2.8 104.6	——	35.3±19.5	0.0 ±15.2
	AN	——	7.0± 3.4	10.3±10.2	19.1±8.6	2.4± 3.2	3.3± 4.5

Table 3.39. Seaweed surface bound metals (mg/kg dw) from Bonne Bay. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Metal	Seaweed	Site 1	Site 2	Site 3	Site 4	Site 5
Pb	*FV*	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	AN	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.1
	PL	——	——	0.0 ± 0.1	——	——
	FC	0.0 ± 0.1	0.0 ± 0.0	——	——	——
Zn	*FV*	12.7 ± 2.9	0.0 ± 5.6	0.9 ± 2.0	0.0 ± 1.2	2.0 ± 1.5
	AN	0.0 ± 15.8	0.0 ± 8.5	0.0 ± 3.6	0.4 ± 3.2	0.0 ± 5.4
	PL	——	——	2.4 3.8	——	——
	FC	11.2 ± 4.0	0.3 ± 1.0	——	——	——
As	*FV*	5.8 ± 0.4	0.0 ± 2.2	0.0 ± 3.0	1.7 ± 2.0	8.4 ± 1.9
	AN	0.6 ± 1.0	0.0 ± 1.9	0.0 ± 1.3	1.7 ± 1.3	3.4 ± 1.5
	PL	——	——	0.0 0.4	——	——
	FC	2.9 ± 4.7	4.8 ± 1.2	——	——	——
Cd	*FV*	0.2 ± 0.1	0.0 ± 0.1	0.2 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
	AN	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.1
	PL	——	——	0.4 0.4	——	——
	FC	0.1 ± 0.1	0.0 ± 0.1	——	——	——
Co	*FV*	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.2	0.0 ± 0.1
	AN	0.0 ± 0.3	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
	PL	——	——	0.1 ± 0.1	——	——
	FC	0.2 ± 0.1	0.0 ± 0.1	——	——	——
Cr	*FV*	0.1 ± 0.0	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.1
	AN	0.1 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
	PL	——	——	0.0 ± 0.2	——	——
	FC	0.0 ± 0.0	0.3 ± 0.2	——	——	——
Cu	*FV*	7.0 ± 1.1	6.4 ± 1.2	2.9 ± 2.5	1.6 ± 0.2	1.0 ± 0.2
	AN	0.8 ± 0.1	0.2 ± 0.3	3.0 ± 1.0	0.8 ± 0.3	0.7 ± 0.1
	PL	——	——	21.8 ± 0.8	——	——
	FC	3.2 ± 0.4	1.0 ± 0.3	——	——	——
Mn	*FV*	32.7 ± 12.4	0.0 ± 11.4	16.2 ± 14.2	0.0 ± 13.7	0.0 ± 14.5
	AN	0.0 ± 6.6	0.0 ± 2.0	0.0 ± 1.0	1.1 ± 1.2	1.8 ± 1.2
	PL	——	——	6.5 ± 1.1	——	——
	FC	26.2 ± 6.4	0.2 ± 2.7	——	——	——
Ni	*FV*	1.1 ± 0.6	2.1 ± 0.3	1.2 ± 0.8	0.0 ± 1.6	0.5 ± 0.6
	AN	0.0 ± 0.5	0.0 ± 0.5	0.0 ± 0.2	0.0 ± 0.1	0.1 ± 0.2
	PL	——	——	3.7 ± 0.4	——	——
	FC	1.1 ± 0.5	0.2 ± 0.2	——	——	——
Al	*FV*	0.0 ± 22.8	5.9 ± 5.6	3.7 ± 20.8	1.4 ± 6.0	4.0 ± 2.4
	AN	1.1 ± 2.7	0.2 ± 4.7	0.5 ± 1.7	1.7 ± 1.5	0.0 ± 1.7
	PL	——	——	0.0 ± 46.2	——	——
	FC	9.6 ± 3.7		——	——	——

Table 3.40. Seaweed surface bound metals (mg/kg dw) from Suir Estuary & Fethard on Sea. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Metal	Seaweed	Site 1	Site 2	Site 3	Site 4	Site 5
Pb	FV	0.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.2 ± 0.2	0.5 ± 0.3
	AN	——	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.0
	PL	——	——	4.4 ± 0.2	6.1 ± 0.2	3.8 ± 0.2
	UL	——	——	——	——	0.4 ± 0.1
Zn	FV	42.8 ± 1.9	28.9 ± 1.1	15.1 ± 0.5	34.5 ± 2.3	28.2 ± 3.6
	AN	——	18.2 ± 6.3	16.9 ± 1.6	10.2 ± 8.0	21.2 ± 13.2
	PL	——	——	231.8 ±2.1	232.2 ± 2.5	91.3 ± 1.1
	UL	——	——	——	——	5.9 0.8
As	FV	2.8 ± 0.8	7.6 ± 1.0	5.3 ± 1.7	16.7 ± 0.4	24.6 ± 5.0
	AN	——	0.9 ± 0.9	4.6 ± 1.4	4.5 ± 1.4	5.2 ± 2.8
	PL	——	——	2.4 ± 0.3	0.8 ± 0.3	4.3 ± 1.4
	UL	——	——	——	——	0.1 ± 0.9
Cd	FV	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.6 ± 0.2
	AN	——	0.0 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	PL	——	——	3.7 ± 0.0	4.7 ± 0.0	1.4 ± 0.1
	UL	——	——	——	——	0.1 ± 0.0
Co	FV	0.9 ± 0.0	1.1 ± 0.0	0.6 ± 0.0	1.5 ± 0.1	1.1 ± 0.1
	AN	——	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.0 ± 0.2
	PL	——	——	4.2 ± 0.1	2.3 ± 0.1	0.9 ± 0.1
	UL	——	——	——	——	0.1 0.0
Cr	FV	0.0 ± 0.1	0.1 ± 0.0	0.0 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
	AN	——	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.1	0.0 ± 0.1
	PL	——	——	0.2 ± 0.1	0.0 ± 0.2	1.3 ± 0.1
	UL	——	——	——	——	0.0 ± 0.4
Cu	FV	4.0 ± 0.2	0.0 ± 2.1	2.0 ± 0.4	0.0 ± 0.3	0.8 ± 0.2
	AN	——	0.0 ± 0.3	0.3 ± 0.2	0.0 ± 0.4	0.0 ± 0.6
	PL	——	——	56.2 ± 21.9	21.9 ± 0.3	18.1 ± 4.1
	UL	——	——	——	——	1.0 ± 0.1
Mn	FV	428 ± 11.4	603 ± 8.6	413 ± 10.1	441 ± 17.0	193 ± 16.0
	AN	——	22.1 ± 4.9	20.8 ± 3.1	15.3 ± 4.6	12.2 ± 9.3
	PL	——	——	843 ± 84.7	634 ± 16.4	169 ± 2.0
	UL	——	——	——	——	21.3 ± 8.9
Ni	FV	1.6 ± 0.1	202 ± 0.1	0.5 ± 0.2	2.4 ± 0.3	1.6 ± 0.3
	AN	——	0.2 ± 0.0	0.0 ± 0.1	0.0 ± 0.2	0.0 ± 0.4
	PL	——	——	4.9 ± 0.2	3.1 ± 0.3	1.5 ± 0.2
	UL	——	——	——	——	0.6 ± 0.3
Al	FV	0.0 ± 26.7	10.1 ± 21.2	0.0 ± 16.0	0.8 ± 22.7	2.9 ± 22.1
	AN	——	0.0 ± 6.8	0.0 ± 30.9	12.1 ± 9.1	4.7 ± 11.8
	PL	——	——	24.6 ± 36.9	0.0 ± 61.3	736 ± 86.8
	UL	——	——	——	——	64.3 ± 188

Similar trends relating to surface bound metals were also observed in the seaweeds collected from Fethard-on-Sea, in May, 2008. The majority of metals were found to be intracellular. *P. lanosa* contained the highest proportion of both Mn^{2+} and Zn^{2+}.

Surface bound Mn^{2+} levels of *P. lanosa* exceeded intracellular metals by approximately 4:1 (Table 3.41 and Figure 3.58)

Table 3.41. Surface-bound metal concentration (mg/kg dw) of four seaweed species under investigation. Errors were calculated based on triplicate samples with 95% Confidence Intervals.

Seaweed Species	Pb	Zn	As	Cd	Co	Cr	Cu	Mn	Ni
P. lanosa	1.3±0.3	7.7±4.9	1.6±0.9	0.3±0.1	0.2±0.1	0.0±0.0	0.9±0.2	119.8±2.9	1.3±0.3
A. nodosum	0.0±0.2	23.0±7.8	4.0±2.1	0.0±0.1	0.3±0.1	0.0±0.2	0.2±0.2	3.5±6.1	0.0±0.3
F. vesiculosus	0.0±0.3	0.0±1.5	0.0±1.8	0.0±0.0	0.0±0.1	0.0±0.1	0.0±0.1	2.3±7.6	0.0±0.3
Ulva sp	0.3±0.2	1.0±0.6	0.0±0.6	0.0±0.0	0.0±0.1	0.0±0.5	0.1±0.2	11.2±1.5	0.0±0.4
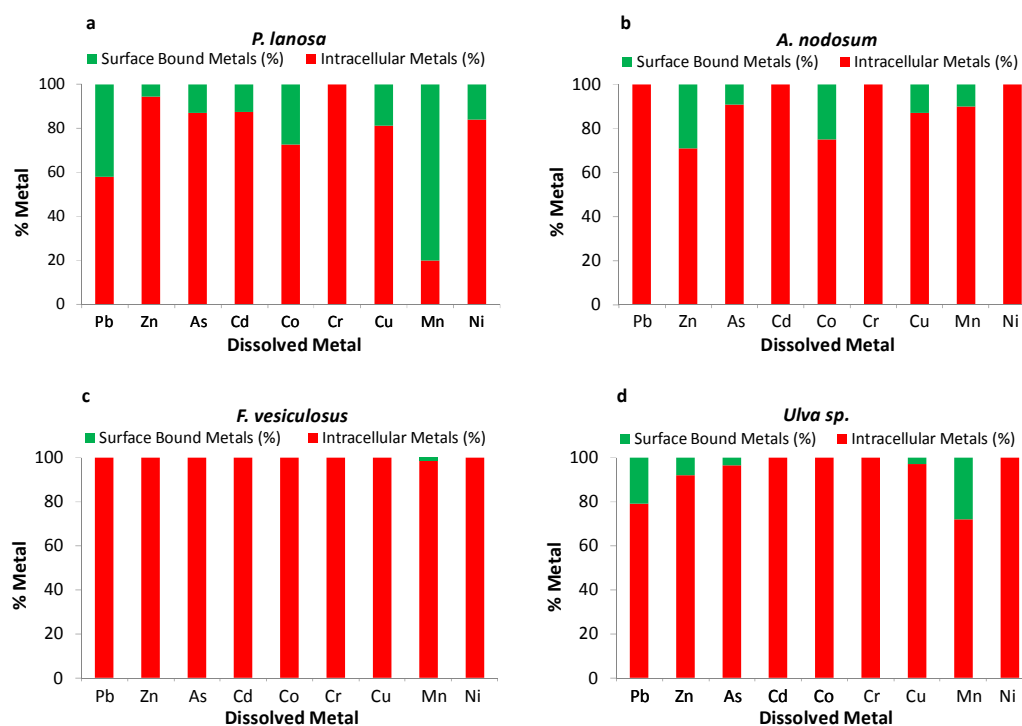
Figure 3.58. % Ratio of intracellular metals to surface bound metals in (a) *P. lanosa,* (b) *A. nodosum,* (c) *F. vesiculosus* and (d) *Ulva* sp collected from Fethard-on-Sea, Co. Wexford in May, 2008.

3.4.8.5 Concentration Factors

Variations in the ability of each species to concentrate the various metals were also confirmed in this study (Figures 3.44 –3.47). Results also exhibit significant inter seaweed variation in CF values. Additionally, CF values varied according to metal

species. For example, the CF values of *P. lanosa* in relation to Pb^{2+} and Zn^{2+} were 31,000 respectively and the CF values of *F. vesiculosus* in relation to Mn^{2+} and Ni^{2+} were 7,455 and 9,000 respectively. In the majority of cases however, *P. lanosa* obtained the highest CF values with respect to the metals under investigation (Table 3.42).

Table 3.42. Comparison of global Concentration Factors (CF) for seaweeds.

	Species	*Pb*	*Zn*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*
Red	*P. lanosa* [§]	31,000	137,600	60,000	100	5,200	4,800	7,500	20,000
	Gracilaria corticata[5]	___	____	22,800	___	____	2,675	23,500	__
	Gelidiella acerosa[5]	___	18,300	____	___	20,000	3,775	21,400	__
	P. umbilicalis[1]	2,000	10,000	660			6,300	7,000	1,100
Brown	*A. nodosum* [§]	3,000	80,900	7,500	120	80	1,500	1,635	4,250
	A. nodosum [2]		13,000				8,600	3,900	4,600
	F. vesiculosus [§]	7,000	25,000	10,000	90	600	1,400	7,455	9,000
	F. vesiculosus [3]	___	≤25,000	≤26,000	___	____	____	____	__
	F. vesiculosus [2]	___	10,000			___	____	6,400	19,000	6,800
	F. vesiculosus [1]	2,400	20,000	2,700	___	____	4,500	23,000	2,800
	Fucus[6]	24,000					7,400		
	S. tenerrimum [5]	___	22,000	____	___	____	1,450	4,700	__
	Padina pavonica [4]	2,450	9,270	3,990	___	11,170	10,360	___	__
Green	*Ulva* sp [§]	14,000	12,200	2,500	40	8,400	3,300	1,995	9,750
	U. lactuca [4]	1,190	8,180	1,430	___	5,280	4,880	___	__
	U. rigida [3]	___	≤26,000	16,000	___	___	___	___	__
	Ulva[6]	49,000					8,600		
	Enteromorpha[6]	45,000					7,700		
	Blidingia[6]	82,000					18,300		

[§] Present Study; [1] Preston *et al*., 1972; [2] Foster 1976; [3] Wang & Dei, 1999; [4] Conti & Cecchetti, 2003; [5] Jadeja & Tewari, 2007, [6] Seeliger & Edwards, 1977.

3.5 Discussion

3.5.1 Seawater Analysis – Physico-Chemical Data

3.5.1.1 pH

Small variations in pH values were observed among all sites at each of the four locations. In a study by Jadeja & Tewari (2007), the bioaccumulation potential of five seaweed species to various metals was investigated in seawater polluted by the waste matter of the soda ash industry. The undiluted soda ash industry effluent is characterised by elevated pH (11.25), ammonia (562.22 μmol/L) and nitrate (187.50 μmol/L). It was concluded that the bioaccumulation of Al^{3+}, Mn^{2+} and Fe^{2+} in four of the seaweeds under examination increased continuously as distance increased from outfall. One species however, *Padina tetrastromatica,* showed a reverse trend. The concentration of Cr^{3+} did not vary for four of the species. There was however a downstream elevation in the concentration of Cr^{3+} for *Gracilaria acerosa.* Additionally, there was a downstream increase in the accumulation of Cu^{2+} in three seaweed species. The reverse trend was observed for *Gracilaria corticata* and *Soleria robusta*. The bioaccumulation of Cd^{2+} in several of species was in a non-detectable range, however with respect to the red seaweeds it was detectable under polluted conditions and non-detectable in control/clean site (Jadeja & Tewari, 2007).

3.5.1.2 Temperature

Little variation in seawater temperatures were observed along the Suir Estuary. Lower temperatures however were recorded for the Bonne Bay sites. Additionally, significant variations in seawater temperature were observed for sites along The Humber Arm. These differences could however be explained by sample collection dates. In a previous study, Marinho-Soriano *et al*. (2006) demonstrated that the protein content of tropical seaweed varied with temperature. Correlations however between temperature and polyphenols were not established.

3.5.1.3 Conductivity

Inter-site variations in seawater conductivity readings were demonstrated. Overall, however a general downstream increase in conductivity levels for each location was observed. The salinity of coastal waters may be significantly reduced following the release of fresh water from rivers. These effects are most prominent near major estuaries (Dring, 1992). The downstream increase (or upstream decrease) in conductivity values observed at The Humber Arm, Bonne Bay and Suir Estuary could therefore be attributed to freshwater input.

For example, surface waters in the Gulf of Alaska undergo a net dilution throughout most of the year as a result of rainfall. The precipitation effect is exaggerated by coastal mountain ranges which enhance the rainfall at or near the coast, resulting in an increase in fresh water at the coast through runoff. Precipitation and runoff alter the salinity levels of coastal waters. Thus precipitation and runoff are vital to the coastal dynamics (Royer, 1979).

In a previous study, the salinity values recorded at various locations along the Irish coast (over six sampling seasons between 1999 and 2000) ranged from 8.3S (contaminated site) to 33.9S (uncontaminated site), (Morrison *et al*., 2008).

Additionally, in a study by Kaimoussi *et al*., (2002), the range of pH and salinity values for several contaminated and uncontaminated seawater along the coast of Morocco were recorded. For sites contaminated by domestic and industrial waste waters, salinity values ranged from 26.41-30.14 ‰ and pH values ranged from 7.89-7.97. Salinity and pH values for sites not impacted by contamination were 33.14 ‰ and 8.15 respectively

Temperature and salinity ranges which were previously observed in coastal waters are even more evident in estuaries. As the mean temperature increases, the mean salinity decreases and the range of each become greater with increasing distance up the estuary. For example, physico-chemical analysis of the Great Bay Estuary in New Hampshire demonstrated an upstream increase in mean temperature from 10.2 to 14.4^{o}C and a downstream increase in mean salinity from 3.4 to 30.3 ‰ (Lobban & Harrison, 1997). Salinity values associated with river mouths depend on the proportions of river water and seawater and these proportions depend on both the tide and the state of the river (Lobban & Harrison, 1997).

In seawater, chloride regularly forms complexes with Zn^{2+}, Cd^{2+}, and, to a lesser extent, Cu^{2+}, but not with Pb^{2+}, and thus the free ion levels of the former metals becomes reduced (Förstner, 1979). In a previous study, it was demonstrated that the uptake of metal (Zn^{2+}) in the seaweed *F. vesiculosus* (L.) decreased with increasing salinity (Munda & Hudnik, 1988). A general decrease therefore in metal uptake with increasing salinity is expected.

3.5.2   Seawater Metal Analysis

Attempting to measure metal levels in seawater is a major problem as metal concentrations are usually very low (ppb) and therefore difficult to measure accurately without sophisticated equipment (Tomlinson *et al*., 1980).

Many indicators have been utilised for facilitating the detection of metal levels in seawater including macroalgae (Stengel *et al*., 2004; Stengel *et al*., 2005; Morrison *et al*., 2008), lichens (Stengel *et al*., 2004), mussels (O'Leary & Breen, 1998; O'Sullivan *et al*., 1991), oysters (O'Sullivan *et al*., 1991), marine mammals (Law *et al*., 1992) and sediment (Fitzgerald *et al*., 2003; Huerta-Diaz *et al*., 2008; Silva *et al*., 2009). The problems with using these indicators however are considerable. For example, variations in species composition and conditions at different locations, season, age of organism, variations in metal levels in different parts of an organism make interpretation of results difficult (Tomlinson *et al*., 1980).

Data regarding the direct analysis of Irish coastal waters is limited. Reported here for the first time is an extensive survey of seawater metal levels from the South-East coast of Ireland. Seawater metal analysis demonstrated that the heavy metals Cd^{2+} and Cr^{3+} were below the limits of detection. Low levels of Cu^{2+} and Pb^{2+} were also detected. Minimal levels of these heavy metals were anticipated as there are no significant pollution sources in the vicinity of the coastal sampling site and the locality is not influenced by industrial activity. Overall, metal analysis of seawater collected from the South-East coast of Ireland confirmed that the sampling site was a clean and uncontaminated location. Global seawater metal levels among sites that are considered clean have previously been reported (Table 3.5).

Results for seawater metal analysis generally corresponded with global seawater metal levels from previous studies. Slight variations however, in levels of Pb^{2+}, Cd^{2+}, Cu^{2+}

and Zn^{2+} were also observed. Furthermore, in a previous study by Preston *et al.* (1972), average dissolved metal concentrations of seawater samples taken along the East coast of Ireland (sampling stations spanned North to South) were determined (Table 3.5). Results from this study demonstrated lower concentrations of Pb^{2+}, Zn^{2+}, Cd^{2+} and Ni^{2+} when compared to those of Preston *et al.* (1972). In this study, seawater samples were collected from a clean site (Fethard-on-Sea), unimpacted by human intervention. Samples analysed by Preston *et al.* (1972) may have originated from slightly polluted areas along the Irish coast.

Small variations in concentration for the majority of metals among all sites and locations were also observed. However, downstream increases in Na^+, SO_4, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and B^{3+} concentration were observed along the Suir Estuary, Bonne Bay and Humber Arm.

Mn^{2+} was detected at St. John's River only. This site was the furthest upstream on the Suir Estuary. Mn^{2+} at this site was 0.04 mg/L, a value higher than previously reported global seawater (0.00003 and 0.002 mg/L), (Lobban & Harrison, 1997; Preston *et al.*, 1972). This may be significant, as St. John's River is the only site subjected to heavy traffic. The phasing out of lead compounds from petrol has led to the search and use of other compounds that have anti-knock qualities. The most promising of such compounds include the organic compounds of manganese, in particular, methyl cyclopentadienyl manganese tricarbonyl (MTT). Manganese compounds, because of their anti-knock characteristics, are now being added to a substantial portion of petrols. This also means, however, that manganese compounds are being emitted from vehicular exhausts, polluting the air at ground level. MMT does not survive the combustion process and inorganic manganese compounds which are formed are emitted. The most prominent of is manganese tetroxide. This compound is thought to be one of the more toxic of the manganese oxides (Joselow *et al.*, 1978).

Cr^{3+}, Al^{3+}, and Co^{2+} were below the limit of detection for all locations, indicating clean and uncontaminated seawater with respect to these metals. Cd^{2+} was found to be below the limit of detection for Suir Estuary and Fethard-on-Sea. Cd^{2+} however was detected at Bonne Bay site 3 and sites 1 and 4 along the Humber Arm. Concentrations of this metal however were low (0.0001 – 0.0002 mg/L). Seawater metal concentrations for all

locations were within previous values obtained for the Irish coast (0.00041 mg/L), (Preston *et al.*, 1972).

Pb^{2+} levels for the Newfoundland and Ireland were similar. All values were relatively consistent with previous global values for Pb^{2+} (0.0016 mg/L), (Preston *et al.*, 1972).

The highest Cu^{2+} value was obtained for Bonne Bay (Site 2). This higher value may reflect relatively higher volumes of boat traffic on the waters of this fishing wharf, which contribute to Cu^{2+} pollution. A slight downstream decrease in Cu^{2+} levels were observed along the Suir Estuary and Fethard-on-Sea. This decrease may be indicative of the salting out process as a result of higher downstream concentrations of salts such as Na^+. Alternatively, slightly elevated levels at the St. John's River site may be as a result of household copper piping in a relatively highly populated area. Overall levels of Cu^{2+} for all locations were generally consistent with previous global values (0.0002 mg/L (Lobban & Harrison, 1997)).

Levels of Ni^{2+} were below the limit of detection for the Newfoundland sites. A downstream decrease in Ni^{2+} levels of was observed for the Ireland sites. This may also be indicative of the salting out process. Overall values however, were relatively consistent with previous seawater Ni^{2+} levels (0.00018 and 0.0014 mg/L (Apte *et al.*, 1998; Preston *et al.*, 1972)).

The highest level of Zn^{2+} obtained by St. John's River. This value may correspond to Zn^{2+} mining in the Suir River catchment area.

Variations in seawater metal levels among sites that are considered clean have previously been reported. For example, in a study by Munksgaard & Parry (2001) average levels of Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+} and Ni^{2+} in seawater collected between April 1998 and November 1999 from the North coast of Australia were 0.013, 0.13, 0.017, 0.77 and 0.43 μg/L respectively, whereas corresponding metal levels of seawater samples collected from the New South Wales coast of Australia were generally lower, 0.009, <0.022, 0.0024, 0.031 and 0.18 μg/L respectively (Apte *et al.*, 1998). Overall higher metal levels were however recorded for seawater samples collected from the coast of Singapore. These samples contained ≤0.62, ≤3.66, ≤0.11, ≤1.16 and ≤0.78 μg/L respectively (Cuong *et al.*, 2008). Variations in metal levels among clean sites may be explained by natural occurrences.

River inputs together with biological and geochemical cycling may perhaps influence the levels of metals in estuaries and coastal seawater (Munksgaard & Parry, 2001). Seawater metal levels reported in this study lie within the range of values from previous studies. The chief sources of heavy-metal pollution are mining, milling and surface finishing industries, discharging a variety of toxic metals such as Cd^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+} and Pb^{2+} into the environment. Since these heavy metals are a valuable resource for different industrial applications, their recovery and recycle holds particular significance. Furthermore, strict environmental regulations require industries to move to cleaner production methods, demanding the development of environmental friendly, low-cost and efficient treatment methods for metal rich effluents (Malik, 2004).

Marine algae have been identified as possessing high metal-binding capacities (Volesky, 2001). The key metal binding mechanisms include ion-exchange and complex formation (Davis *et al.*, 2003). These however may vary according to biomass type, origin and the processing to which it has been subjected. Numerous chemical groups may be responsible for metal biosorption by seaweeds e.g. carboxyl, sulphonate, hydroxyl and amino with their relative significance depending on factors such as the quantity of sites, their accessibility and the affinity between site and metal (Murphy *et al.*, 2008).

3.5.3   Sediment Metal Analysis

A general downstream decrease in all sediment metals was observed along the Suir Estuary. This decrease however is not unexpected as the concentration of metals in coastal waters decrease with distance from the river mouth (Lobban & Harrison, 1997). The downstream decrease in metals corresponds to the downstream increase in seawater NaCl, resulting in the salting out of a number of metals and also the dilution of metals with distance from the river mouth (Lobban & Harrison, 1997). In general, concentrations of Cu^{2+}, Cr^{3+} and Ni^{2+} for all sites along the Suir Estuary lie within global metal levels in sediment from unpolluted sites (Table 3.43). Cd^{2+} levels were $\leq$ 2.3 mg/kg. This value however was still above levels from unpolluted sites. Zn^{2+} levels recorded for the two sites furthest downstream were within values for clean sites. Pb^{2+} levels at St. John's River were above those recorded for clean sites (Table 3.43).

In a study by Fitzgerald *et al* 2003 Cu^{2+} and Pb^{2+} levels of sediment samples collected from salt marshes along the Suir Estuary demonstrated a general downstream decrease. Additionally, Cu^{2+} and Pb^{2+} levels in sediment collected from Cheekpoint were recorded at 9.41 and 28.80 mg/kg respectively. These values were quite consistent with Cu^{2+} and Pb^{2+} levels from the same site in this present study.

Table 3.43 is a comparison of various sediment metals analysed in biomonitoring studies. Comparisons are made between polluted and clean sites, in addition to analysis using varying sediment sizes. Results obtained in this study demonstrate that sediment metal concentrations are generally consistent with global values.

Caution however, must be observed if sediment samples are used as a biomonitoring tool. The natural occurrence of metals in the environment complicates the assessment of potential sediment contamination. Difference in sediment fractions analysed and digestion procedures also limit comparison with data from other studies. The grain size distribution however is possibly the most important factor controlling sediment metal concentrations (Mucha *et al*., 2004). In this study, similar grain sizes ($< 63\mu m$) were obtained from the sites along the Suir Estuary however slightly larger grains ($> 63\mu m$) were collected from Fethard-on-Sea (Table 3.43).

Table 3.43. Global biomonitoring studies using sediment metal analysis (mg/kg dw).

Site	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Ni*	*Mn*	*Al*
St.John's [§]	59.3	93.2	6.9	2.3	6.1	39.4	18.7	16.0	362.7	6012.0
Fethard [§]	3.2	29.0	5.5	1.1	1.9	5.5	3.1	6.5	163.4	4176.0
Polluted [a]	156.0	113.0	___	___	___	35.0	99.0	27.0	1126.0	___
Clean [a]	20.0	11.3	___	___	___	15.0	12.0	23.0	207.0	___
Polluted [b]	68.0	135.0	42.0	___	89.0	___	373.0	77.0	1563.0	___
Clean [b]	31.0	67.0	5.0	___	13.0	___	14.0	37.0	967.0	___
Estuary [d]	45.0	145.0	28.0	0.28	___	___	27.0	22.0	275.0	___
Sediment [*c]	37.0	106.0	___	0.27	___	26.0	19.0	26.0	93.0	19,000
Sediment [†c]	20.0	203.0	___	0.08	___	57.0	74.0	20.0	32.0	4,500

[§] Present Study; [a)] Giusti (2001); [b)] Veinott *et al*., 2003; [c)] Mucha *et al*., 2004 – both sites impacted by domestic sewage and industrial effluents; [d)] Wright & Mason, 1999. [*] and [†] denote sediments of varying grain size.

3.5.4   Total Protein Determination

Seaweed total protein varies according to salinity (Lobban & Harrison, 1997) species and season (Fleurence, 1999a).   Results from this study demonstrated seasonal, interspecies and inter-site variations in total protein.   Protein levels of the brown, red and green seaweeds collected in this study correspond to previously reported values. Generally, the protein content of brown seaweeds is low (3-15% dw (30-150 mg/g dw)) compared with that of the green or red seaweeds (10-47% dw (100-470 mg/g dw)) (Fleurence, 1999a).   The protein content of seaweeds under investigation in this study differed according to species.   *P. lanosa* contained the highest levels of total protein, while the brown seaweeds yielded the lowest.

F. vesiculosus and *A. nodosum* collected from the Humber Arm and *P.lanosa* collected from the Suir Estuary and Fethard-on-Sea demonstrated a general downstream decrease in total protein.   This trend is consistent with higher downstream salinity levels.

Salinity levels are known to influence the protein concentration of seaweed.   Generally, at lower salinity, the protein concentration in seaweed is higher than at higher salinity. For example, at higher salinity (31.03 ‰), the protein content of *F. vesiculosus* was 15.67 % dw, whereas at lower salinity (5.15 ‰), the protein concentration was 17.74 % dw (Lobban & Harrison, 1997).   Results from this study also demonstrated a negative correlation between the protein content of *F. vesiculosus* and seawater conductivity levels.   Generally, higher protein values corresponded to lower conductivity levels and *vice versa* for all three locations

Marinho-Soriano *et al.* (2006) studied the seasonal variations in the chemical composition of tropical seaweeds.   They demonstrated that the protein contents of the various species were significantly different.   Furthermore, results also demonstrated seasonal fluctuations in the protein content of the seaweeds.   These fluctuations were explained by variations in environmental conditions such as temperature, salinity and nutrient availability.   A positive correlation was found between the protein content and nitrogen, whereas a negative correlation was found with water temperature and salinity.

Many previous studies have established the protein content of various seaweeds in order to determine the biochemical composition of seaweed (Fleurence, 1999a; Galland-Irmouli *et al.,* 1999; Wong & Cheung, 2001; Sanchez-Machado *et al*., 2004; Marinho-

Soriano *et al.,* 2006; Dawczynski *et al.*, 2007; Marsham *et al.,* 2007). In this study however, the total protein content of red, brown and green seaweeds collected from several sites along the Suir Estuary/Fethard-on-Sea, Ireland and from Bonne Bay and Humber Arm, Newfoundland were determined. Spatial and interspecies variations were demonstrated in the five seaweeds under investigation. Results in this study demonstrated a possible positive correlation between the total protein content of *F. vesiculosus* and temperature. However, other factors also influence the protein content of macroalgae. Nitrogen availability plays a vital part in the growth and productivity of seaweeds (Topinka & Robbins, 1976). In their study, Topinka & Robbins (1976) demonstrated that the total nitrogen content and growth rate of *F. spiralis* increased with increasing nitrate levels.

Results from this study will therefore contribute to global aquatic and environmental sectors, providing valuable reference material for future research. Furthermore, results demonstrated a temporal variation in the total protein content of seaweeds. This variation is further investigated in Chapter 4.

3.5.5   Extracted Protein Determination

Variations in the levels of extracted protein among the four seaweeds under investigation were also demonstrated. The brown seaweeds yielded the highest soluble protein concentration.

Various studies have previously been performed in order to determine the soluble protein content of red, brown and green seaweeds (Chapter 2, Table 2.7). Extracted protein concentrations for red seaweeds ranged from 4.63 – 310.25 mg/g dw. Dere *et al.*, (2003), reported protein levels in *P. lanosa* as 310.25 ± 168.4 mg/g dw. These samples were collected of the Turkish coast, in May-June, 2001 (a similar sampling period to this study). The red seaweed in this study however yielded 5.7 ± 2.0 mg/g dw. A large degree of error however was associated with the results from Dere *et al.* (2003). Furthermore, temporal variation, position/depth of seaweed in seawater and nutritional levels in the environment are known to affect protein concentration in seaweed (Dere *et al.,* 2003). Additionally, the extraction method utilised also influences the yield of extracted protein (Chapter 2, 2.1). A method of extraction was not reported by Dere *et al.,* 2007. Gordillo *et al.*, (2006), however, studied the biochemical composition of

seaweeds collected from the Norwegian Arctic, in July, 2002. They reported extracted protein levels in *P. arctica* as 7.51 mg/g dw, a value generally more consistent with present findings (Chapter 2, Table 2.7).

Extracted protein levels in *F. vesiculosus* and *A. nodosum* yielded 39.6 ± 2.4 mg/g dw and 96.9 ± 2.7 mg/g dw respectively. Previous studies reported extracted protein levels in brown seaweeds as 4.80 – 43.56 mg/g dw respectively. Gordillo *et al*., (2006), reported protein levels in *F. distichus* as 43.56 mg/g dw, a value which is in good agreement with *F. vesiculosus* in this study. Overall, however previous research in relation to extracted protein levels of *F. vesiculosus* and *A. nodosum* has never previously been performed.

Global extracted protein concentrations of green seaweeds range from 4.56 – 280.67 mg/g dw. Dere *et al.* (2003), reported extracted protein levels in *U. lactuca* as 78.57±75.39 mg/g dw (Chapter 2, Table 2.7), values which were inconsistent with findings for the green seaweed in this study (4.7±1.2 mg/g dw). Again, large degrees of error were associated with results from Dere *et al*. Overall however, published data relating to the extracted protein levels of *Ulva* sp is limited.

The presence of additional cell wall polysaccharides related to both red and green seaweed perhaps further hindered the extraction of proteins from these species. *P. lanosa* and *Ulva* sp yielded the lowest extracted protein concentrations, whereas the brown algae yielded, the highest extracted protein levels. Generally, protein extraction from seaweeds is difficult because of the vast amounts of polysaccharides present in their cell walls. These include alginates of the brown species and carrageenans of some red species. The high concentration of neutral polysaccharides (e.g., xylanes and cellulose) in some red or green seaweeds can further limit protein accessibility (Fleurence, 1999b). A study by Joubert & Fleurence (2008) of *P. palmata* collected during April and July saw the extracted protein values of this red seaweed range from approximately 8.26 – 13.2 mg/g dw) following enzymatic extraction.

For the first time, protein extracted from *F. vesiculosus* and *A. nodosum* was assayed and quantified. Results from the study demonstrated interspecies variations in the extracted protein levels of the four seaweeds under investigation. Additionally, significantly higher levels of extracted protein were obtained for the brown seaweeds when compared to both the red and green species. Furthermore, variations with respect

to global values were also established. Overall results demonstrated the difficulties involved in the extraction of protein from seaweeds which may be attributed to cell wall polysaccharides.

3.5.6 Polyphenol Determination

Results from this study demonstrated temporal, interspecies and inter-site variations in the polyphenol concentrations of seaweed. Generally, the polyphenol levels of red, brown and green seaweeds in this study correspond to previous findings (Chapter 2, Table 2.8).

When compared to previous works, some interspecies differences in polyphenol levels were observed for *A. nodosum, F. vesiculosus* and *Ulva* sp. In this study, *A. nodosum* contained the highest polyphenol levels of 34.8 ± 4.6, whereas, in a study by Connan *et al.,* (2007), methanol extracts of *A. nodosum* collected from the West Coast of Brittany, France in March, 2003, contained ~ 60 mg/g dw. Furthermore, methanol extracts of *A. nodosum* collected from the West Coast of Sweden in October 1992 and 1993 also contained polyphenol levels of ~ 60 – 80 mg/g dw (Pavia *et al.,* 1999). *F. vesiculosus* contained the second highest polyphenol content of 26.9 ± 1.2 mg/g dw GAE. However, in a study by, Wang *et al.,* (2009) polyphenol levels of *F. vesiculosus* collected from the South-West Coast of Iceland, in March 2007 and extracted with 70% acetone were reported as 242 mg/g dw.

In this study, *Ulva* sp yielded the lowest polyphenol level of 2.2 ± 0.2 mg/g dw GAE. The polyphenol levels of *U. lactuca* have previously been determined and values ranged from 8.99 (Wong & Cheung, 2001) to ~30.00 mg/g dw (Wang *et al.,* 2009). In these previous studies, *U. lactuca* samples were collected from North-East Hong Kong in December 1997 and the South-West Coast of Iceland March 2007 respectively and polyphenols were extracted in 80 and 70% acetone respectively.

P. lanosa in this study contained 12.9 ± 0.3 mg/g dw GAE and this value generally lies within values previously reported for red seaweeds (Chapter 2, Table 2.8). Polyphenol levels for this species were however, unavailable in the literature.

Polyphenol extraction by many previous studies has been performed with acetone (Chapter 2, Table 2.8). In this study, optimisation of polyphenol extraction methods demonstrated minimal variation between methanol and acetone extractions of *P. lanosa* (Chapter 2).

A general downstream increase in polyphenol concentrations for all seaweed species was observed. This is to be expected as the concentration of metal binding phenols increase with increasing salinity (Pedersen, 1984). Additionally, results from this study also demonstrated a positive correlation between the polyphenol content of *F. vesiculosus* and seawater conductivity. It should be pointed out however that, other factors besides salinity influence the polyphenol concentrations of seaweeds, they include, nitrogen availability (Ragan & Glombitza, 1986; Arnold *et al.,* 1995), age of seaweed, plant size, tissue part, nutrient levels, herbivory and light intensity (Targett & Arnold, 2001), season (Abdala-Díaz *et al*., 2006).

In studies measuring the phenol content and heavy metal uptake in brown seaweeds, Pedersen (1984) demonstrated an increase in the phenol content of *A. nodosum* and *F. vesiculosus* sampled over increasing distances from a river outlet and along a fjord. It was reported that *F. vesiculosus* contained approximately 40% more phenols than that of *A. nodosum* at different salinities. It was also demonstrated that the phenol content of both species increased with increasing age. Furthermore, the phenol concentration also varied according to tissue type (Pedersen, 1984). These values generally correspond to the polyphenol contents of *F. vesiculosus* and *A. nodosum* from Newfoundland. However, for the Ireland sites *A. nodosum* contained up to 70% more polyphenols than *F. vesiculosus*.

In a previous study, Connan *et al.* (2004) investigated the phenolic content of eight brown seaweeds on a sheltered rocky shore in Brittany, France. They demonstrated no significant variation in the phenolic content of *F. vesiculosus* and *A. nodosum*. Both seaweeds contained ~58mg/g dw. In this study however, *F. vesiculosus* and *A. nodosum* samples collected at the Newfoundland sites contained significantly higher polyphenol concentrations when compared to Irish samples. For example, polyphenol values for *F. vesiculosus* collected from Irish sites ranged from 4.0 – 12.3 mg/g dw GAE whereas polyphenol values for the same species collected from The Humber Arm ranged from 24.9 – 75.8 mg/g dw GAE. The phlorotannin content of brown seaweeds

varies spatially and seasonally due to environmental factors such as nitrogen availability (Ragan & Glombitza, 1986), grazing by herbivores (Targett & Arnold, 1998), solar radiation (Pavia & Brock, 2000), UV- B radiation (Pavia *et al*., 1997).

Patterns of variation in brown algal phlorotannin concentrations are apparent on a worldwide scale. Spatial variation in brown algal phenolic concentrations can be summarised geographical as follows: 1. temperate brown seaweeds are a mixture of species of high and low phenolic levels, 2. phaeophyte phenolic concentrations in temperate Australasia are, on average, much higher than phenolic levels in brown seaweeds from other temperate regions of the world, 3. brown algae in the tropical Pacific and Indo-Pacific have low phenolic levels, and 4. phaeophytes in the western Caribbean (like temperate browns) are a mixture of high- and low-phenolic species (Targett & Arnold, 1998). Additionally, it was suggested in a previous study that the green seaweed, *Enteromorpha compressa* tolerated a copper-enriched environment, and the accompanying oxidative stress, through the accumulation of copper and consumption water-soluble phenolic compounds (Ratkevicius, 2003).

Variations in polyphenol levels may be explained by seasonal and geographical differences. In a study by Plouguerné *et al.* (2006) it was reported that the polyphenol content of the brown seaweed *Sargassum muticum* varied significantly with respect to both location and season. For example, the polyphenol content of *S. muticum* from Point du Diable (Brittany coast) increased from ~4 mg/g dw in February to ~ 30 mg/g dw in May, whereas in Callot Island, these values were ~10 and 60 mg/g dw for the same months (Plouguerné *et al.,* 2006). Furthermore, in a previous study Abdala-Díaz *et al.* (2006) demonstrated both daily and seasonal variations of phenolic compounds in the brown seaweed *Cystoseira tamariscifolia*. Highest levels were observed in Summer (~45 mg/g dw) and lowest in Winter (~7 mg/g dw). The increase of phenolic compounds during Summer suggests that these compounds act as a photoprotective mechanism in ecosystems with high radiation/sunlight.

Previous studies have determined the polyphenol content of various seaweeds (Pavia *et al.,* 1999; Connan *et al*., 2007; Chew *et al*., 2008; Devi *et al*., 2008; Ganesan *et al*., 2008; Matanjun *et al*., 2008; Wang *et al*., 2009). In this study however, the polyphenol content of *F. vesiculosus, A. nodosum, P. lanosa, F. ceranoides* and *Ulva* sp, collected from several sites along the Irish coast and from the West coast of Newfoundland were

determined for the first time. Spatial and interspecies variations were demonstrated in the five seaweeds under investigation. Additionally, correlations between polyphenol levels of *F. vesiculosus* and conductivity were demonstrated. Results from this study will therefore enhance global knowledge in relation to seaweed biochemistry. Temporal variations in the polyphenol levels of seaweed are further investigated in subsequent chapters.

3.5.7   Seaweed Metal Analysis

3.5.7.1 Total Metals

In general, results demonstrated that metal levels varied according to seaweed, location, season and metal species. In relation to samples collected from Fethard-on-Sea, in May 2008, levels of Pb^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+} and Ni^{2+} in *P. lanosa* were generally within levels previously obtained for red seaweeds. Zn^{2+} and Mn^{2+} levels in this seaweed however were significantly higher compared with previous studies (Table 3.44). Additionally, metal concentrations of *F. vesiculosus* and *A. nodosum*, with the exception of As^{3+} and Cr^{3+}, were generally consistent with those previously documented for brown species. Levels of As^{3+} and Cr^{3+} in both *F. vesiculosus* and *A. nodosum* were significantly higher and lower respectively when compared to previous studies.

Minimal information regarding As^{3+} concentrations in seaweeds from clean sites was available in literature to date however data with respect to *F. vesiculosus* from an industrial estuary was documented by Bryan *et al.* (1983). They demonstrated As^{3+} levels of *F. vesiculosus* were lower than those of the present study however, all other metal concentrations (including Cd^{2+}, Cr^{3+}, Pb^{2+}, and Cu^{2+}) in this seaweed were significantly higher (Table 3.44). Furthermore, in the present study, levels of Mn^{2+} in *F. vesiculosus* were higher than those of *A. nodosum.* Giusti (2001) however found significantly higher levels of this metal in *F. vesiculosus* when compared with the present study (Table 3.44).

Binding sites, nutritional requirements and seasonal differences contribute to variations in seaweed metal concentrations. For example, different seaweed species have different affinities for different heavy metals. This may reflect competition between metals for binding or uptake sites in seaweed (Sawidis *et al.,* 2001). In addition, it may sometimes be assumed that all algae have similar requirements, however, due to wide variations in

species it would be imprudent to generalise their nutritional needs (Lobban and Harrison, 1997). Furthermore, concentrations of trace metals in macroalgae vary according to season, especially for Mn^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+} and Al^{3+}(Giusti, 2001). The general trend appears to be an increase in concentration in Winter and early Spring and minimum concentrations in Summer and Autumn (Giusti, 2001). In this present study, seaweed samples were collected during Summer in 2008.

Overall results for seaweed metals demonstrated interspecies variations. Results also demonstrated that *P. lanosa* was able to accumulate the highest levels of Pb^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+} and Ni^{2+}.

Additionally, concentrations of Zn^{2+} and Mn^{2+} in *P. lanosa* far exceeded Zn^{2+} and Mn^{2+} levels in red seaweeds to date (Table 3.44). Sawidis *et al.,* (2001) stated that the concentration of metals in the various seaweed species may reflect their morphology. Seaweeds having a larger surface area were found to have greater heavy metal contents. *P. lanosa* is filamentous seaweed which therefore has a greater surface area than non-filamentous seaweeds. Furthermore, immense quantity of sulphated polysaccharides are associated with the cell wall seaweeds (García-Ríos *et al.*, 2007). Red seaweed polysaccharides may perhaps have a greater ability to bind and accumulate metal ions when compared to brown and green algae (Stengel *et al.*, 2004).

Zn^{2+} and Mn^{2+} were dominant for all four species compared to all other dissolved metals. These species are essential elements and therefore reflect nutritional needs. Variations in these metal concentrations demonstrated that seaweed subjected to the same environmental conditions exhibited a natural variation in elemental uptake. These trends in variability were demonstrated in previous studies. For example, in a study by Żbikowski *et al.* (2006), the abundance of metals in *Enteromorpha* sp decreased in the order Mn>Zn> (Cu, Pb, Ni) > Cd, a trend similar to that of *Ulva* sp in this study. Additionally, Foster (1976) demonstrated high levels of Mn^{2+} and Zn^{2+} in *F. vesiculosus* and *A. nodosum* compared to levels of Cu^{2+} and Ni^{2+} (5.5 – 12 mg/kg dw). Levels of Zn^{2+} and Mn^{2+} in *F. vesiculosus* were 116 and 103 mg/kg dw respectively and levels in *A. nodosum* were 149 and 21 mg/kg dw respectively. Hou & Yan (1998) also demonstrated that Zn^{2+} and Mn^{2+} were the major dissolved metals in the red seaweed *P. urceolate* (45.4 and 103.0 mg/kg dw respectively). Additionally, relatively high levels of As^{2+} in seaweed are not uncommon, for example, concentrations of this dissolved metal in *F. vesiculosus* collected from the North Sea and Baltic Sea demonstrated

relatively high levels (> 20 mg/kg dw) compared to those of Co and Cd (< 2.0 mg/kg dw) (Jayasekera & Ross, 1996).

Table 3.44. Global metal concentrations (mg/kg dw) in seaweeds collected from clean sites.

	Species	*Pb*	*Zn*	*As*	*Cd*	*Co*	*Cr*	*Cu*	*Mn*	*Ni*
Red	*P. lanosa* [§]	**3.10**	**137.6**	**12.30**	**2.40**	**1.10**	**2.60**	**4.80**	**150.0**	**8.00**
	Porphyra columbina [8]	<0.5	42.90	____	3.06	0.51	0.65	5.32	38.50	0.74
	Porphyra columbina [4]	____	4.11	____	8.34	____	0.49	4.28	____	____
	Laurencia johnstonii [6]	____	29.00	____	____	0.99	2.19	____	____	40.0
	Corallina elongata [10]	0.17	12.90	____	0.15	0.05	____	1.23	8.32	0.60
	Gelidium microdon [10]	0.09	17.60	____	0.29	0.11	____	1.34	12.10	4.45
	Chondrus crispus [3]	4.60	48.00	____	0.50	____	1.30	2.60	____	7.90
Brown	*A. nodosum* [§]	**0.30**	**80.90**	**42.10**	**0.30**	**1.20**	**0.40**	**1.50**	**32.70**	**1.70**
	A. nodosum [9]	0.12	____	____	<0.25	bd	1.07	____	____	____
	A. nodosum [3]	7.60	32.00	____	0.60	____	1.80	2.20	____	1.00
	F. vesiculosus [§]	**0.70**	**25.00**	**44.00**	**0.40**	**0.90**	**0.30**	**1.40**	**149.1**	**3.60**
	F. vesiculosus [5]	0.30	17.70	____	0.45	____	1.10	5.60	479.4	0.40
	F. vesiculosus [2]	2.50	514.0	____	4.20	____	____	7.40	____	9.20
	F. vesiculosus [1]	2.40	69.00	18.90	0.73	7.40	2.50	7.30	179.0	10.2
	Sargassum sinicola [6]	____	36.00	7.55	____	3.12	2.82	____	____	____
	Padina pavonica [7]	3.04	50.00	____	0.39	____	3.34	11.90	____	____
	F. sprialis [10]	0.18	15.20	____	1.19	0.25	____	0.95	27.95	6.65
Green	*Ulva* sp [§]	**1.40**	**12.20**	**5.70**	**0.10**	**0.40**	**4.20**	**3.30**	**39.90**	**3.90**
	U. lactuca [4]	1.73	1.98	____	0.55	____	0.33	4.20	____	____
	U. lactuca [7]	1.88	50.00	____	0.17	____	1.62	4.90	____	____
	Ulva sp. [8]	1.72	31.30	2.98	0.46	0.73	1.05	3.81	51.40	4.11
	E. prolifera [4]	4.10	4.92	____	0.70	____	3.05	4.60	____	____
	Codium cuneatum [7]	____	23.00	26.30	____	0.60	2.44	____	____	10.0

[§] present study; [1] Bryan *et al.*, 1983; [2] Pedersen, 1984; [3] Sharp *et al.*, 1988; [4] Muse *et al.*, 1999; [5] Giusti, 2001; [6] Sánchez-Rodríguez *et al.*, 2001; [7] Conti & Cecchetti, 2003; [8] Pérez *et al.*, 2007; [9] Morrison *et al.*, 2008; [10] Wallenstein *et al.*, 2009; bd = below the limit of detection; [1] Samples collected from an industrialised estuary.

Levels of As^{3+} varied according to location and seaweed. The overall highest As^{3+} concentration was observed for *F. ceranoides* collected in Bonne Bay. *P. lanosa*, contained the overall lowest As^{3+} concentrations. There was a downstream increase in the As^{3+} content of *F. vesiculosus* and *A. nodosum* collected from The Humber Arm. A similar trend was also observed for *F. vesiculosus*, *A. nodosum* and *P. lanosa* along the Suir Estuary. Little interspecies variation in As^{3+} levels were observed for the Bonne Bay samples. Values obtained for As^{3+} in this study are generally consistent with those of Caliceti *et al.* (2002) who studied the metal contents of *Fucus* spp of the Venice Lagoon. They reported the range of values for this metal as 8.0-73 mg/kg dw. Additionally, As^{3+} levels in *F. vesiculosus* and *A. nodosum* collected from Newfoundland were similar to those of Phaneuf *et al.* (1999), who studied the metal concentrations of various algae collected along the St. Lawrence River, Canada (Table 3.45). Values of As^{3+} in seaweeds collected off the Irish coast have never previously been recorded. However As^{3+} levels in *F. vesiculosus* collected from the Suir Estuary and Fethard-on-Sea were generally higher than those from the Venice Lagoon (4.4-4.7 mg/kg dw), (Caliceti *et al.*, 2002).

Table 3.45. Average Metal Concentrations (mg/kg dw) for each species of algae collected along the St. Lawrence River (Canada) (Phaneuf *et al.*, 1999).

Seaweed Species	Pb	Zn	As	Cd	Co	Cr	Cu	Ni	Mn	Al
F.vesiculosus	0.44	32.6	31.6	1.4	1.5	1.2	4.1	5.6	158.0	—
A. nodosum	0.16	35.6	23.0	0.46	0.69	0.86	4.2	1.59	19.1	—
U. lactuca	1.64	33.3	6.0	0.22	1.5	4.4	19.2	5.9	409.0	—
P. palmata	0.84	31.2	9.4	0.32	0.49	2.10	8.1	4.4	35.8	—

The highest Cd^{2+} concentration was observed for *P. lanosa*. Of the brown seaweeds, *F. ceranoides* contained the highest Cd^{2+}. Cd^{2+} levels of *F. vesiculosus* and *A. nodosum* collected from the Newfoundland site were similar to those previously recorded for the St. Lawrence River (Table 3.45). Levels of Cd^{2+} in the brown seaweeds collected from the Suir Estuary and Fethard-on-Sea were generally lower than those previously reported for *F. vesiculosus* and *A. nodosum* from the Irish Coast (Table 3.46). For example, Cullinane *et al.* (1982) collected by *F. vesiculosus* and *A. nodosum* from the South Coast. O'Leary & Breen (1997) collected seaweed samples along the Shannon Estuary. Morrison *et al.* (2008) collected *A. nodosum* from six sites along the Irish

Coast, including Cork Harbour, Killimer, Galway Docks, Ballyconneely, Carlingford Lough and Dun Laoghaire. Stengel *et al.* (2004) harvested *P. lanosa, F. spiralis, A. nodosum* and *F. vesiculosus* from Spiddal, Co. Galway.

Table 3.46. Metal Concentrations (mg/kg dw) for each species of algae collected from various sites and locations along the Irish Coast.

Seaweed	Pb	Zn	As	Cd	Co	Cr	Cu	Ni	Mn	Al
F.vesiculosus	——	~48.0[3]	—	——	——	——	0.5-6.4[4]	—	10.0[2]	—
F.vesiculosus	——	34.63-425.26[1]	—	3.0-3.82[1]	——	——	6.91-14.21[1]	—	——	—
A. nodosum	——	40.75-281.74[1]	—	1.63-2.42[1]	——	——	3.49-10.87[1]	—	——	—
A. nodosum	0.118-2.114[5]	~50.0[3]	—	0.105-0.598[5]	0.108-0.917[5]	0.054-1.071[5]	0.55-4.75[4]	—	——	—
U. lactuca	——	~40.0[3]	—	——	——	1.2[2]	7.0[2]	—	23.0[2]	—
P. lanosa	——	~90.0[3]	—	——	——	——	——	—	——	—

[1]Cullinane *et al.,* 1982; [2] O'Leary & Breen, 1997; [3]Stengel *et al.*, 2004; [4] Stengel *et al.*, 2005; [5]Morrison *et al.*, 2008.

The second highest metal content was observed for Mn^{2+} and this was associated with *P. lanosa.* A general downstream decrease in the Mn^{2+} concentration of *F. vesiculosus*, *A. nodosum* and *P. lanosa* was also observed. Downstream decreases in Mn^{2+} levels correspond to a downstream increase in conductivity levels, particularly for the Ireland sites, where the Na levels increased from 393.3-10533.3 mg/L. Increases in the levels of conductivity result in the salting out of dissolved metals. Levels of Mn^{2+} in *F. vesiculosus* from the Humber Arm were significantly higher than those previously obtained by Phaneuf *et al.* (1999) however, levels were consistent with *A. nodosum* (Table 3.45). Concentrations of Mn^{2+} in both *F. vesiculosus* and *A. nodosum* collected from Bonne Bay however were similar to those of Phaneuf *et al.* (1999). Mn^{2+} levels in *F. vesiculosus* from the Suir Estuary were considerably higher than those previously obtained by O'Leary & Breen (1997). However, only slightly elevated levels for *Ulva* sp were found in this study when compared to those of O'Leary & Breen (1997), (Table 3.46).

In the majority of cases, Pb^{2+} was below the limit of detection at Newfoundland locations. The highest Pb^{2+} value along the Suir Estuary however, was obtained at Site 1, St. John's River. Values of Pb^{2+} associated with *F. vesiculosus* and *A. nodosum* for the Newfoundland sites were similar to those for the St. Lawrence River (Table 3.45).

Furthermore, levels of this metal in *A. nodosum* from the South East of Ireland were consistent with those previously obtained for the Irish coast (Table 3.46). Pb^{2+} values associated with *F. vesiculosus* have never previously been obtained from the Irish coast. However, Pb^{2+} levels in *F. vesiculosus* collected from the South East coast of Ireland in this study are generally consistent with values obtained by Caliceti *et al.* (2002) from the Venice Lagoon (1.2-2.0 mg/kg dw).

P. lanosa collected from the Suir Estuary contained the highest overall Cr^{3+} levels. Overall, however Cr^{3+} levels varied with location and seaweed. Previous values of the St. Lawrence River were generally consistent for both *F. vesiculosus* and *A. nodosum* collected from the Humber Arm, however, slightly lower levels were found in the Bonne Bay seaweeds (Table 3.45). Cr^{3+} levels in *A. nodosum* and *Ulva* sp collected from the Suir Estuary and Fethard-on-Sea were similar to those previously recorded for the Irish Coast (Table 3.46).

Seaweeds collected from the Suir Estuary yielded the highest Cu^{2+} levels when compared with those of Newfoundland. The highest Cu^{2+} concentration was obtained from *P. lanosa*. Of the brown seaweeds, *F. vesiculosus* collected from the Suir Estuary contained the highest levels of Cu^{2+}. Levels of Cu^{2+} in *A. nodosum* and *F. vesiculosus* from the Humber Arm were generally consistent with previous values. However slightly more elevated levels in *F. vesiculosus* from Bonne Bay were recorded when compared to Phaneuf *et al.* (1999), (Table 3.45). With the exception of Site 2, concentrations of Cu^{2+} associated with *F. vesiculosus* from the Suir Estuary and Fethard-on-Sea were generally consistent with those previously obtained for the Irish coast. Levels of Cu^{2+} in *Ulva* sp however were half those previously obtained (Table 3.46).

The highest metal concentrations were observed for Al^{3+} with values for seaweeds collected in Ireland were generally higher than those of Newfoundland. Levels of Al^{3+} in seaweeds from the St. Lawrence River and Irish coast have never previously been recorded. This is the first time therefore that levels of Al^{3+} have been established for five seaweed species associated with Newfoundland and Ireland.

The highest Zn^{2+} levels were found in *P. lanosa* from the Suir Estuary. Of the brown seaweeds, *A. nodosum* collected from Bonne Bay contained the highest Zn^{2+}. The lowest Zn^{2+} concentration was found in *F. vesiculosus* from the Humber Arm. Levels

of Zn^{2+} in *F. vesiculosus* and *A. nodosum* from most sites at Bonne Bay and the Humber Arm were consistent with those previously obtained by Phaneuf *et al.* (1999). Zn^{2+} levels of *F. vesiculosus* and *A. nodosum* collected from the Suir Estuary and Fethard-on-Sea were within ranges of values previously seen from the Irish Coast (Table 3.46). Values for *Ulva* sp in this study were lower than previous values, whereas values for *P. lanosa* from the Suir Estuary and Fethard-on-Sea were considerably higher (Table 3.46).

P. lanosa collected from the Suir Estuary provided the highest Co^{2+} levels. With respect to Newfoundland, the highest levels of this essential element was found in *F. ceranoides* (1.8 g/kg dw) and *F. vesiculosus* from the Humber Arm (2.7 g/kg dw). The overall lowest Co^{2+} levels were found in *Ulva* sp. A downstream decrease in Co^{2+} levels of *P. lanosa* from the Suir Estuary was observed corresponding to a general downstream increase for *F. vesiculosus*. Furthermore, a general downstream increase in Co^{2+} levels for *F. vesiculosus* and *A. nodosum* were observed for the Humber Arm. There was no significant variation in Co^{2+} levels for *A. nodosum* when all sites were taken into consideration. Levels of Co^{2+} in *F. vesiculosus* and *A. nodosum* from the Newfoundland sites were generally consistent with those of Phaneuf *et al.* (1999) and values for *A. nodosum* collected from the Suir Estuary and Fethard-on-Sea lie within values previously obtained along the Irish coast (Table 3.46).

The overall highest levels of Ni^{2+} were found in *P. lanosa* from the Suir Estuary. Concentrations of Ni^{2+} in *F. vesiculosus* and *A. nodosum* from both the Newfoundland and Ireland were generally consistent with those of Phaneuf *et al.* (1999). Levels of Ni^{2+} in seaweeds collected along the Irish coast have never previously been recorded. Ni^{2+} levels in *F. vesiculosus* collected along the Irish coast in this study however are consistent with those of the Venice Lagoon (4.4-4.7 mg/kg dw), (Caliceti *et al.*, 2002).

Phaneuf *et al.* (1999) evaluated the metal concentration of various metals along the St. Lawrence River (Canada). It was found that metal concentrations varied according to the seaweed species (Table 3.45). A trend that is supported by results in this study. Information regarding metals from the Irish Coast however is limited (Morrison *et al.*, 2008) and in particular, data relating to Cr^{3+}, Co^{2+}, Pb^{2+} and Cd^{2+} is scarce (Cullinane & Whelan, 1982; Morrison *et al.*, 2008) (Table 3.46).

Generally, data compiled in relation to the Suir Estuary and Fethard-on-Sea lie within previously published results for sites along the Irish coast. One obvious exception however is the Mn^{2+} content of *F. vesiculosus*. Mn^{2+} levels in this seaweed was previously recorded at ~ 10 mg/kg dw. In this study however the corresponding value ranged from 231.1 – 702.0 mg/kg dw. Furthermore, Zn^{2+} levels for all seaweeds collected along the Suir Estuary and Fethard-on-Sea were also within previous ranges of values. However, a survey by Cullinane & Whelan (1982) of Zn^{2+} levels from a number of brown seaweed showed that at one site within Cork Harbour (Ireland) levels of this metal reached 425 mg/kg dw. This value exceed levels from the present study where highest levels of Zn^{2+} for the brown seaweeds was 59.7 mg/kg dw.

It is clear from these results that metal concentrations vary not only from species to species but also with location. Several factors can influence metal concentrations in seaweed. The position on the shore level may increase or decrease metal accumulation in seaweeds, as the time of submersion varies according to location on the shore. Likewise, light exposure, temperature and desiccation also vary according to shore level. All of these aspects affect seaweed rates of metabolism (Lobban & Harrison, 1997) and some will also affect metal bioaccumulation (Munda, 1979). Additionally, photosynthesis may increase the pH on the seaweed surface, thus affecting metal uptake by increasing precipitation. Metal concentrations in different algae from different shore levels could also be attributed to their biochemical composition (e.g., concentrations of polyphenols or the presence of polysaccharides) (Stengel *et al.*, 2004). Salinity also affects the polyphenol concentrations of seaweed. The metal binding phenols increase with increasing salinity (Pedersen, 1984).

In this study, general downstream increases in Cd^{2+}, Pb^{2+}, Cr^{3+} and Al^{3+} concentrations of *P. lanosa* and decreases in Cu^{2+} and Al^{3+} levels of *F. vesiculosus* collected from the Suir Estuary were observed. The chemical speciation of a metal relies on its oxidation state and its interactions with other elements in the system as well as other parameters including, pH, salinity, redox potential, ionic strength, and alkalinity (Lobban & Harrison, 1997). Changes in these variables can result in the transformation of the metal's chemical form and can therefore modify its availability and toxicity. For example, if Cu^{2+} binding takes place through interactions with the carboxyl groups on the seaweed surface, then the chemical changes that reduce the availability of the carboxyl groups for metal complexation should cause a reduction in Cu^{2+} binding

(Fourest & Volesky, 1996). Additionally, Gardea-Torresday *et al.* (2000) studied the effect of esterification on metal uptake. The carboxyl groups of five different biomasses were esterified using acidic methanol. They discovered that all modified biomasses showed significant decreases in Cu^{2+} and Al^{3+} binding however, they also demonstrated that Au^{3+} binding potential slightly increased as the algal carboxyl groups were esterified. In this study, a downstream increase in Na ions was demonstrated for all locations under investigation. Increases in salinity levels may therefore have altered the chemical form of some metals which therefore changed their binding ability. Variations in seaweed metal binding will therefore have implications for both heavy metal detoxification mechanisms and nutritional uptake in seaweeds.

Overall, there was no significant variation for Pb^{2+} levels in relation to *F. vesiculosus* and *A. nodosum*, within each location. Additionally, there was no significant variation for Cr^{3+} levels of the brown seaweeds from Bonne Bay and *A. nodosum* from the Suir Estuary and Fethard-on-Sea. Additionally, in relation to Suir Estuary and Fethard-on-Sea samples, where metal uptake was high for *P. lanosa* within a particular site and low for the brown seaweeds, corresponding polyphenol values were generally low for the red seaweed and high for *F. vesiculosus* and *A. nodosum*. For example, the highest Pb^{2+} uptake was demonstrated by *P. lanosa* and this corresponded to the lowest polyphenol level. The lowest Pb^{2+} uptake was demonstrated by *A. nodosum*, corresponding to the highest polyphenol yields. Similar trends were also observed for Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Al^{3+}, Cr^{3+}, Mn^{2+} and Co^{2+}. Furthermore, seaweeds collected from Newfoundland sites which yielded Pb^{2+} values below the limit of detection demonstrated overall highest polyphenol level. Results may perhaps indicate oxidation/exudation of polyphenols resulting from elevated metal levels in *P. lanosa*. With respect to Irish samples, lowest levels of As^{3+} were observed for *P. lanosa* and this corresponded to the highest total protein when compared to the brown seaweeds, perhaps indicating biochemical changes in protein following heavy metal exposure. However, highest levels of Ni^{2+}, Cu^{2+} and Cd^{2+}, were observed for *P. lanosa* and this corresponded to highest total protein levels, while the lowest levels of these metals in *A. nodosum* corresponded to the lowest total protein. Both Cd^{2+} and Cu^{2+} are heavy metals, therefore elevated levels maybe associated with heavy metal binding protein synthesis in seaweeds. Furthermore Ni^{2+} is an enzyme co-factor (DeBoer, 1981) therefore higher levels in seaweed may reflect nutritional requirements.

Several studies involving the quantitation of metal levels in seaweeds have previously been performed globally (Bryan *et al.*, 1983; Pedersen, 1984; Sharp *et al.,* 1988; Muse *et al.,* 1999; Giusti, 2001; Sánchez-Rodríguez *et al.,* 2001; Conti & Cecchetti, 2003; Pérez *et al.,* 2007; Morrison *et al.,* 2008 and Wallenstein *et al.,* 2009). Limited information however regarding the quantitation of specific metals in seaweeds collected along the Irish coast is available. In this study, the accumulation of nine different metals, in four seaweed species, collected along the South East coast of Ireland is presented for the first time. Furthermore, in this novel investigation, spatial and interspecies variations in metal levels of five different seaweed species were established. Levels of Ni^{2+}, Al^{3+} and As^{3+} in the four seaweed species found off the coast of Ireland were determined for the first time. Furthermore, concentrations of Al^{3+} for all five species under investigation have never previously been determined. Additionally, information regarding metal levels in *F. ceranoides* is rare, therefore for the first time, concentrations of Pb^{2+}, Zn^{2+}, As^{3+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Ni^{2+}, Mn^{2+} and Al^{3+} were determined simultaneously for this seaweed. Seasonal variations in the metal content of seaweeds were further investigated in subsequent chapters.

3.5.7.2 Intracellular and Surface Bound Metals

In the majority of cases, the highest proportions of metals under investigation were found to be intracellular, therefore trends in metal accumulation were similar to those of total metals. García Ríos *et al.*, (2007), demonstrated that following Cd^{2+} exposure studies on two red macroalgae, in one of the seaweeds (*Gracilaria cornea*), 80% of Cd^{2+} was extracellular while in *Chondrophycus poiteaui* almost 100% of the metal was intracellular. No previous studies however have determined the intracellular levels of seaweeds *in situ*. In this study, intracellular metal levels in five seaweed species have been established for the first time. Overall results demonstrated significant variations in the ways in which seaweeds respond to metals in the environment. Interspecies variations in seaweed tolerance to heavy metals and nutritional requirements for essential elements were demonstrated. Furthermore, interspecies variations in intracellular metal were also observed. For example, different trends in intracellular metals were observed for *F. vesiculosus* with respect to Bonne Bay, Humber Arm and Suir Estuary and Fethard-on-Sea. Additionally, spatial variations in metal concentrations for the seaweeds under investigation were also determined.

P. lanosa contained the highest proportion of both Mn^{2+} and Zn^{2+}. Surface bound Mn^{2+} levels of *P. lanosa* exceeded intracellular metals by approximately 4:1. In a study by Garnham *et al.* (1992), it was found that Mn^{2+} uptake in cultures of the estuarine green alga *Chlorella salina* was inhibited by Zn^{2+}. A similar trend was also observed by Sunda & Huntsman (1998a), in the estuarine diatom *T. pseudonana*. It has been established that metal coordination sites of biological ligands are not always specific for a particular metal. Therefore, biological ligands designed to bind an "intended" nutrient metal will also bind a similar competing metal. Competition for binding sites frequently occurs for metals such Mn^{2+} and Zn^{2+}. The inhibition of Mn^{2+} uptake by competing metals such as Zn^{2+} may result both from direct binding of these metals to membrane transport sites and also to intracellular binding of the metal to control sites. Binding to control sites results in a reduction of the cell's capability for feedback regulation of cellular Mn^{2+} (Sunda & Huntsman, 1998a).

This may therefore explain the low intracellular levels of Mn^{2+} in *P. lanosa*. In this study, the low intracellular Mn^{2+} was matched with very high intracellular Zn^{2+}, thus indicating competition for uptake in *P. lanosa*. Inhibition of Mn^{2+} uptake by Zn^{2+} has previously been documented for green algae and estuarine diatoms. Significantly however, this is the first time that such a trend has been observed not only for *P. lanosa* but also for red seaweeds.

In this study, the highest proportions of metals under investigation were found to be intracellular however, some seaweed species demonstrated high proportions of surface bound metals and the majority of those were from the Suir Estuary.

P. lanosa provided the greatest number of metals that were associated with the seaweed surface. This is not surprising, as red algae polysaccharides have a larger capacity to bind and accumulate metal ions when compared to brown and green algae (Stengel *et al.*, 2004). Studies involving the determination of surface bound metals from in situ seaweeds have never previously been performed. In this study, surface-bound metal levels of five seaweed species have been established for the first time. Results demonstrated spatial and interspecies variations in the levels of surface-bound metal. In the first study of its kind, spatial and interspecies variations have been determined for a broad range of metals. Results from this study will therefore enhance significantly global information in relation to the biochemical affects of metal exposure on seaweeds.

3.5.7.3 Regression Analysis (*Fitted Line Plots*)

Metal levels in the various species of seaweed may reflect their morphology, for example, those species containing a larger surface area may possess a greater intracellular concentration. Different seaweed species may have different affinities for different metals and this may reflect competition between metals for binding or uptake sites in seaweed (Sawidis *et al.,* 2001).

In a study by Sawidis *et al.* (2001) it was demonstrated that metal levels in algae depends not only on anthropogenic activities but also on the geology of the specific area. While seaweed can be used effectively to assess the metal levels in the marine environment, not all elevated concentrations necessarily reflect increased levels of contamination (Sawidis *et al.,* 2001). Indeed, the high concentrations of certain metals, e.g., Ni^{2+}, found in seaweed samples of Sawidis *et al.* (2001), reflected the metallic nature of the rock. It is therefore important to take account of a location's geology before attempting to interpret the data.

The geology of the Irish landscape is wide and varied, ranging from basalts and other volcanic rocks to sandstones, limestone, shales and marbles. The rock sequence in South Wexford consist of Precambrian rocks, Lower Palaeozoic rocks, The Cambrian succession, Ordovician successions, Caledonian intrusions, Upper Palaeozoic and Post-Palaeozoic rocks (National Geographical Survey of Ireland, 2010). Rocks connected with the Precambrian era include the Precambrian Shields associated with all continents and contain stretches of folded metavolcanic and metasedimentary rocks surrounded by granite (Greenstone Belts). The Greenstone Belts of the Canadian Precambrian Shield have been studied for many decades because they contain large concentrations of Cu, Ni, Pb and Zn. Furthermore, Greenstone belts of the Superior tectonic province of Canada contain a variety of Mg-rich basalt called komatite. These rocks contain Si, Na, Mg, K, Al and Ca (Faure, 2001). It is clear therefore that the selection of specific species of seaweed must be considered carefully, since different species appear to accumulate metals to differing levels. Information on the physiology and metal-accumulation capacity of a range of seaweed species is necessary before such a selection can be made (Sawidis *et al*., 2001).

Results from this study demonstrate that each of the seaweed species tested had different affinities for different metals and this is reflected in concentrations of total,

surface bound and intracellular metal uptake. Furthermore, variations in total protein, extracted protein and polyphenol levels were also observed for the seaweeds under investigation. Competition for binding sites (Sunda & Huntsman, 1998b), nutritional requirements (Lobban and Harrison, 1997) and seasonal variations (Giusti, 2001) all contribute to variations in metal concentrations of seaweeds.

Relationships between protein, polyphenol and seaweed metals were also investigated in this study. Increases in total protein corresponded to increases of the heavy metals Pb^{2+}, Cu^{2+} and Cd^{2+}. An increase in total protein with a corresponding increase of the heavy metals Cd^{2+}, Cu^{2+} and Pb^{2+} may therefore be indicative of the synthesis of metal binding peptides. For example, some peptides containing the amino acid cysteine are important metal-binding cytosolic ligands (Rijstenbil *et al.,* 1998). These include GSH (Grill *et al.,* 1987) and PC. PC are enzymatically synthesised upon induction by excess heavy metal ions (Gekeler *et al.,* 1988).

Possible negative relationships however, between seaweed polyphenol levels and the intracellular Cr^{3+} and Cu^{2+} were also suggested. Furthermore, possible positive relationships between polyphenols and intracellular Co^{2+} and As^{3+} were also demonstrated. Seaweed polyphenols that are present in the membrane-bound physodes are capable of chelating divalent ions which may be exuded into the surrounding water (Ragan *et al.,* 1979). Marine seaweed polyphenols also possess antioxidant activity (Ganesan, 2008). It has previously been shown that seaweeds exposed to heavy metals contained lower levels of polyphenols than those not impacted (Ratkevicius *et al.,* 2003). Furthermore, Connan & Stengel (2009) saw an increase in the exudation of phenolic compounds from *F. vesiculosus* and *A. nodosum* following exposure to increasing concentrations of Cu^{2+}. Therefore a decrease in polyphenol levels with increasing Cr^{3+} or Cu^{2+} may indicate oxidation or exudation of polyphenols upon exposure to these metals. Conversely, increases in As^{3+} and Co^{2+} concentrations may suggest increases in polyphenol concentrations as a result of induction by these metals in the environment. Possible relationships between seaweed metals and polyphenol concentrations were demonstrated in this study however, other factors that affect the polyphenol levels of seaweeds should also be taken into account, including, plant size, age of seaweed, tissue part, salinity, nutrient levels, herbivory, light intensity (Targett & Arnold, 2001), season (Abdala-Díaz *et al.,* 2006) and nitrogen availability (Ragan & Glombitza, 1986; Arnold *et al.,* 1995).

3.5.7.4 Concentration Factors

The ability of the four seaweeds species under investigation to accumulate metals from the aquatic environment was demonstrated in this study. Variations in the ability of each species to concentrate the various metals however were also confirmed. Results also exhibit significant inter species variation in CF values. Additionally, CF values varied according to metal species. In the majority of cases however, *P. lanosa* obtained the highest CF values with respect to the metals under investigation.

Additionally, when compared with previous studies, significant variations in the CF values were also observed. Most notably, large variations in CF values for Pb^{2+} associated with *Ulva* sp plus Zn^{2+} and Cd^{2+} associated with *F. vesiculosus* were reported (Table 3.42). Seaweed metal concentrations vary according to season (Giusti, 2001), therefore caution must be taken when making comparisons with previous values. For example, in this study, samples used for the determination of CF values were collected in May, 2008. Compared with previous studies, Foster (1976) and Jadeja & Tewari (2007) collected their samples in October/November and December respectively. Furthermore, Seeliger & Edwards (1977) collected their samples in the months of June and October.

Essential metals under investigation in this study were Mn^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}. The significance of these elements, particularly Zn^{2+} and Mn^{2+} was demonstrated for the four seaweeds under investigation in this study (Figures 3.44-3.47). The principle roles of Mn^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+} are as enzyme co-factors (Lobban & Harrison 1997) and Co^{2+} is an essential element of vitamin B12 (Mageswaran & Swasubramaniam, 1984). The presence of an element in seaweed is not however proof that the element is essential. Generally, essential and non essential elements are accumulated in the tissues of algae to concentrations above levels in seawater, giving rise to concentration factors up to 10^3 (Lobban & Harrison 1997). Results from this study further support this finding. Seawater metals (both essential and non-essential) ranged from <0.04 to < 10.00 µg/L (Table 3.39). Corresponding seaweed metal concentrations in brown, green and red seaweeds are referred to in Table 3.46.

For seaweeds to be used as biomonitors, two key criteria must be fulfilled. First of all, they must be sessile so as to reflect environmental conditions at a particular site over a long time. Secondly, the relationship between metal levels in seaweeds and in seawater

must be linear, resulting in a constant concentration factor for a particular metal over a broad range of external concentrations (Jayasekera & Rossbach, 1996). It had been reported that brown seaweeds are best for biomonitoring because of their wide distribution in estuaries (O'Leary & Breen, 1998). Green algae have received the least attention in aquatic contamination applications. This is because their greatest abundance is in fresh water environments (Levine, 1984). Previous studies however have shown that *U. lactuca* can act as a good indicator of Cr^{3+}, Cu^{2+} and Mn^{2+} (O'Leary & Breen, 1998).

Concentration factors for metals in macroalgae vary considerably with respect to seaweed species, metal variety, plant part, time of the year and seaweed origin (Foster, 1976). Furthermore, inter-site comparisons using CF values are affected by seasonal and spatial variations and also by biological variables associated with the organism under investigation (Muse *et al.*, 2006).

It has previously been indicated that brown algae are incapable of regulating the uptake of trace metals. Bioaccumulation therefore, in this species is predominantly passive. This results in exceptionally high tissue concentrations when compared to levels of the surrounding seawater (Jayasekera & Rossbach, 1996). Results from this study generally indicate that brown seaweeds are good accumulators of metals. Out of the two brown seaweeds under investigation, *F. vesiculosus* demonstrated a superior accumulating power with respect to Pb^{2+}, Cd^{2+}, Cr^{3+}, Mn^{2+} and Ni^{2+} when compared with *A. nodosum*. However the best overall bioaccumulator was the red algae, *P. lanosa*. This seaweed generated CF values 2.4 – 24-fold higher than those of brown and green species in relation to all metals under investigation with the exception of two. In those cases, *A. nodosum* and *Ulva* sp demonstrated superior accumulating powers for Co^{2+} and Cr^{3+} respectively.

Information relating to CF values for seaweeds and metals in literature is scarce. The range of metals and variety of seaweeds analysed for their biomonitoring ability is limited. In this study, the CF for four different seaweeds species, encompassing a broad range of metals is demonstrated.

3.6   Conclusions

In this study, seawater physico-chemical data were recorded, protein, polyphenol and metals levels of various seaweeds (*P. lanosa, F. vesiculosus, F. ceranoides, A. nodosum* and *Ulva* sp) were determined, seawater and sediment samples were analysed for their metal contents for the first time.

There was no significant variation with regard to pH readings for all locations under investigation (values ranged from 8.11 – 8.17). Temperature variations were however confirmed among the locations. The lowest average temperatures were for The Humber Arm in Newfoundland and the highest for Fethard-on-Sea (9.54 and 13.06^{o}C respectively). The highest conductivity values were recorded for Fethard-on-Sea (51.10 mS/cm). Overall downstream increases however were observed for all locations.

Little variation in seawater metal levels was observed for the majority of metals among all the sites and locations. Downstream increases in salt concentrations (e.g., Na) however were observed for all locations and the levels were within ranges previously reported.

Sediment samples were collected from the Suir Estuary and Fethard-on-Sea at depths of 2 and 20 cm. A general downstream decrease in all sediment metals, regardless of depth was observed. Additionally, there was little variation in metal concentrations between surface and 20cm sediments. Downstream decreases in sediment metals have previously been reported and levels of Cu^{2+} and Pb^{2+} in this study are consistent with earlier studies from the Suir Estuary (Fitzgerald *et al.,* 2003).

P. lanosa contained the highest total protein and the brown seaweeds the lowest. Brown seaweeds collected from Newfoundland contained similar levels of total protein. However, protein levels in *F. vesiculosus* collected from the Suir Estuary were approximately 48% higher than those from Newfoundland. Additionally, *P. lanosa* collected from the Suir Estuary contained $\leq$ 10.2% more protein than samples collected from Bonne Bay. A positive correlation between total protein and temperature was also demonstrated.   Changes in the protein contents of seaweeds were explained by variations in environmental conditions such as temperature, salinity and nutrients.

Extracted protein levels for *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp were, 5.7 ± 2.0, 96.9 ± 2.7, 39.6 ± 2.4 and 4.7 ± 1.2 mg/g dw respectively. These figures did not correlate directly with corresponding total protein values. The highest levels of total protein were attained by *P. lanosa* and *Ulva* sp, whereas the highest extracted protein was yielded by the brown seaweeds. The disparity in extracted protein with total protein can be attributed to tough cell wall polysaccharides which make extraction of red and green seaweeds difficult. One-way ANOVA ($p<0.05$) demonstrated significant variation among extracted protein levels for all four seaweeds. Extracted protein levels for red, brown and green seaweeds are in general agreement with results from previous studies. Extracted protein values for *F. vesiculosus* and *A. nodosum* are reported for the first time in this study, while only limited information regarding the protein content of *P. lanosa* is available.

Interspecies, spatial and temporal variations in the polyphenol levels of seaweeds were also observed. Polyphenol levels for *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp were, 12.9 ± 0.3, 34.8 ± 4.6, 26.9 ± 1.2 and 2.2 ± 0.2 mg/g dw (GAE) respectively. Additionally, polyphenols are known to vary according to species, phlorotannins with the brown seaweeds, (Ragan & Glombitza, 1986), brominated phenols with the red species (Lundgren *et al*., 1979) and coumarins with the green seaweeds (Pérez-Rodríguez *et al*., 2003). Furthermore, one-way ANOVA ($p<0.05$) demonstrated significant variation among extracted protein levels for all four seaweeds investigated. According to literature, higher polyphenol concentrations are associated with brown seaweeds, while fewer polyphenols are found in red and green varieties (Chapter 1). Polyphenol values in this study generally correlate with this trend. Values obtained in this study for *F. vesiculosus, A. nodosum* and *Ulva* sp were significantly lower that those of previous studies. Furthermore, reporting of polyphenol levels in *P. lanosa* is not available in literature. Polyphenol levels of 12.9 ± 0.3 mg/g dw (GAE) for *P. lanosa* have therefore been reported in this study. A downstream increase in polyphenol levels for all seaweed species was observed. This trend is consistent with previous findings. Pedersen (1984) reported that metal binding phenols are more abundant with increasing salinity.

P. lanosa contained the highest levels of total metals under investigation and the overall highest metal was observed for Al^{3+}. In relation to the Irish sites, Pb^{2+}, Cr^{3+} and Cu^{2+}

levels were highest for sites 1 and 2. This is significant as these sites are in close proximity to Waterford City where a lead crystal industry and a tannery are located in addition to high volume housing. The lowest values were observed for Fethard-on-Sea. There were little variations in Cd^{2+} levels among the sites. Generally, a downstream increase in As^{3+} and Al^{3+} (associated with *P. lanosa*) and a downstream decrease in Cr^{3+}, Zn^{2+}, Co^{2+} and Al^{3+} (associated with *F. vesiculosus*) was observed. Seaweed metal concentrations were generally consistent with previous values for Irish and global values. Furthermore, low levels of As^{3+} were associated with relatively higher total protein levels in *P. lanosa* (and *vice versa*), indicating perhaps, peptide oxidation or exudation following heavy metal exposure. Additionally, high levels of Ni^{2+}, Cu^{2+} and Cd^{2+} were associated with high total protein levels, indicating synthesis of proteins such as PC. The highest proportion of Pb^{2+}, As^{3+}, Cr^{3+}, Cu^{2+}, Ni^{2+} and Al^{3+} were intracellular. Overall, however spatial, interspecies and temporal variations in intracellular metal levels were demonstrated. Some seaweed species however demonstrated high proportions of surface bound metals. For example, in relation to samples collected in October, 2007, high levels of surface bound metals were associated with *P. lanosa*, these included Zn^{2+}, Cd^{2+}, Co^{2+}, Mn^{2+}, Pb^{2+} and Cu^{2+}. Elevated levels of surface bound Zn^{2+}, Co^{2+} and Mn^{2+} were also associated with the brown species (Table 3.37).

The concentration factors for all four seaweeds were calculated and results demonstrate that *P. lanosa* was the best overall bioaccumulator of all metals under investigation with the exception of Co^{2+} and Cr^{3+}. It was also established that *A. nodosum* and *Ulva* sp were best at accumulating Co^{2+} and Cr^{3+} respectively. When compared with literature, significant variations in CF values were observed. For example, large variations in the CF value for Pb^{2+} associated with *Ulva* sp and Zn and Cd associated with *F. vesiculosus* were observed (Table 3.46). Furthermore, in this study, unique data relating to the CF values of four seaweed species and covering a wide range different metals are presented.

Regression analysis established relationships between protein and polyphenol levels with seaweed metal concentrations. Following statistical analysis, associations between total protein and Pb^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} were established (r^2 = 97.1, 95.6, 88.7 and 82.3 %, respectively). Links with extracted protein levels and intracellular Cu^{2+} were

also determined (r^2 = 74.8 %) and furthermore, polyphenol levels were linked with intracellular Cr^{3+} and Cu^{2+} (r^2 = 61.2 and 74.8 %, respectively).

In a unique study, physico-chemical data, seawater metal determination, metal levels in sediment, seaweed protein and polyphenol levels, total, intracellular and surface bound metal levels of five seaweed species, from two different continents have been determined for the first time. Overall results demonstrate spatial, temporal and interspecies variations in protein, polyphenol and metal levels in seaweeds. Additionally, possible links between seaweed protein and polyphenol and metal levels may have been established.

Results from this study will therefore enhance significantly global data regarding the biochemical affects of metal exposure on seaweeds in the environment. Additionally, variations in the ability of several seaweeds to bioaccumulate a wide range of metals will have global implications for the choice of seaweed intended for *in situ* biomonitoring studies.

Chapter 4

Temporal Effects of
Heavy Metal Exposure on Seaweeds

4. Temporal Effects of Heavy Metal Exposure on Seaweeds

4.1 Introduction

This chapter monitors seasonal changes in protein, polyphenol and the relative sensitivity of these seaweeds to heavy metal exposure.

Seaweeds have numerous mechanisms for heavy metal detoxification. For example, seaweeds have cellular peptides (phytochelatins) which are capable of chelating metals and accumulating them in compartments within the cell or exuding them back into the surrounding environment (Merrifield *et al*., 2004). Heavy metal defence mechanisms in algae may also involve polyphenols. These secondary metabolites are capable of binding free metal ions, either in the surrounding medium or intracellularly, resulting in metal compounds that are less toxic to algae (Ragan *et al.,* 1979). A study by Ratkevicius *et al.* (2003), demonstrated a clear inhibitory effect of mining wastes on the concentrations of GSH and soluble phenolic compounds in the green macroalgae *E. compressa*. Furthermore, results of a 30 day study by Toth & Pavia (2000) indicated that the polyphenol levels of seaweeds were not affected by either Cu^{2+} exposure.

Studies have previously been performed in order to determine the effects of heavy metal exposure on macroalgae. For example, in a study by Rijstenbil *et al.* (1998b), the green macroalga, *E. prolifera* synthesised metal binding thiols (PC) following exposure to Cu (Refer to Chapter 1, Section 1.5).

A previous study by García Ríos *et al.* (2007), demonstrated increases in the levels of intracellular Cd^{2+} of the red macroalga, *Gracilaria cornea* following exposure to two different metal levels over 7 days. Following exposure to 0.1 and 1.0 μg/mL Cd^{2+}, intracellular levels of the metal increased from 0.14 ± 0.05 to 1.1 ± 0.7 and 5.0 ± 1.8 respectively. Overall results demonstrated that Cd^{2+} uptake in *G. cornea* was proportional to the $CdCl_2$ concentration in the medium and to the exposure time. Furthermore, García Ríos *et al.* (2007), also demonstrated that in response to Cd^{2+} exposure, small amounts of thiol peptides were synthesised. These peptides produced similar retention times to that of PC (n=2), (García Ríos *et al., 2007).

Several studies have previously been conducted in order to investigate the biochemical effects of heavy metal exposure on microalgae (Gekeler *et al*., 1988; Ahner *et al*., 1994;

Hirata *et al.*, 2001; Ahner *et al.*, 2002; Pawlik Skowrońska, 2000), higher plants (Grill *et al.*, 1987; de Vos, 1992; Schat & Kalff, 1992; de Knecht, 1994; Wu *et al.,* 2001; Hernández-Allica *et al.*, 2006; Mishra, 2006; Aina *et al.*, 2007), aquatic plants (di Toppi *et al.*, 2007) and yeast (Grill *et al.*, 1986). Fewer studies however have been dedicated to the study of brown (Pawlik Skowrońska *et al.,* 2007), green (Rijstenbil *et al.*, 1998b) and red macroalgae (Collén & Davison 1999; García Ríos *et al.*, 2007; Vasconcelos & Leal, 2008). For example, Pawlik Skowrońska *et al.* (2007) reported the presence of PC in native Phaeophyceae (*Fucus* spp.). In exposure studies, Rijstenbil *et al.* (1998b) demonstrated the synthesis metal-binding thiols in the green seaweed *E. prolifera,* only after severe Cu stress. Furthermore, following exposure to Cd^{2+}, small quantities of thiol peptides were synthesised in the red seaweeds *G. cornea* and *C. poiteaui.*

Furthermore, temporal studies investigating seasonal changes in polyphenol levels (Connan *et al.*, 2004; Abdala-Díaz *et al.*, 2006; Peckol *et al.*, 1996; Plouguerné *et al.*, 2006), metal content (O'Leary & Breen, 1998; Brown *et al.*, 1999; Lares *et al.*, 2002; Stengel *et al.*, 2005; Villares *et al.*, 2002) and protein concentration (Martinez & Rico, 2002; Zubia *et al.*, 2003; Fleurence, 1999a) of seaweeds have previously been performed. In this study however, investigations involving the relative sensitivity of seaweeds sampled over different times of the year to heavy metal exposure are reported for the first time.

4.2 Objectives of Research

The aim of this study was to investigate the seasonal effects of heavy metal exposure on seaweeds. During the study, seaweeds collected at different seasons, were exposed to heavy metals at various concentrations and over a range of time periods. Following exposure, changes in total protein, extracted protein, polyphenol and heavy metal concentrations in several seaweeds were monitored.

The overall aims of the study were:

- To determine the effect of heavy metal exposure over 24h on total protein content of three seaweed species.

- To expose *P. lanosa* to Cd^{2+} in order to determine the effect of heavy metal exposure on the extracted protein concentration of this seaweed.

- To expose *P. lanosa* to Cd^{2+} over 6 hours and thus determine the effect of heavy metal exposure on the protein content of this seaweed.

- Due to the apparent influence of heavy metal exposure on glutathione/protein levels and metal uptake in seaweeds, further studies were also carried out to determine the effect of heavy metal exposure on protein content of four seaweed species (*P. lanosa, A. nodosum, F. vesiculosus, Ulva* sp) following exposure to Cd^{2+}, Cr^{2+}, Pb^{2+} and Cu^{2+}.

- A time course study was performed in order to determine the effect of heavy metal exposure on protein concentration, polyphenol concentration and metal accumulation of *P. lanosa*. Samples were exposed to Cd^{2+} for 0 – 6h.

Results from this study will have global implications for understanding the biochemical response of seaweeds collected during different time periods to heavy metals and the subsequent utilisation of these response processes for high value added bioremediation product development.

4.3   Experimental

4.3.1   Chemicals

- 1000 mg/L Cd Standard (Analytical Grade), (Sigma Aldrich Ltd., Dublin, Ireland).
- 1000 mg/L Cr Standard (Analytical Grade), (Sigma Aldrich Ltd., Dublin, Ireland).
- 1000 mg/L Pb Standard (Analytical Grade), (Sigma Aldrich Ltd., Dublin, Ireland).
- 1000 mg/L Cu Standard (Analytical Grade), (Sigma Aldrich Ltd., Dublin, Ireland).

4.3.2   Seaweed Collection and Preparation

Four seaweed species, *Polysiphonia lanosa* (red), *Ascophyllum nodosum* (brown), *Fucus vesiculosus* (brown) and *Ulva* spp (green) were collected at low tide from Fethard on Sea, Co. Wexford, Ireland (52° 11'53.68' N, 6° 49' 34.64' W) (Figure 2.3). Seaweeds were collected at three distinctly different time periods (February/March, May and November). The average temperatures for these seasons were 7.7, 14.3 and 9.5^oC respectively. The seaweed samples were rinsed thoroughly on site with seawater in order to remove any adhering debris. The range of seaweed collected within each species (approximately 20 to 30 plants) from various locations on the sampling site was combined to give composite batches. Once harvested, the seaweed was placed in tubs containing seawater, collected on site and transported to the laboratory within 2 hours.

On returning to the laboratory, seaweeds were immediately placed in holding tanks at a temperature of 12°C (the average temperature at this latitude), in environmentally controlled rooms. Seaweeds were exposed to a light: dark regime of 12:12 hours before and during the experiment. Interpet Airvolution AV4 pumps were used for agitation and aeration. Rectangular tanks containing 5L of seawater (Source: Kilfarrassey, Tramore Co. Waterford (52^o8'4.80" N, 7^o13'54.56"W)) were used as reaction vessels with seaweed present at a concentration of approximately 5mg/ml. Salinity, temperature and pH were kept constant during experiments.

During metal exposure/time course studies, metal/seawater solutions were replaced every 24 h.

4.3.3   24 h Time Course Study (3 Seaweed Species)

Seaweed samples (*P. lanosa, A. nodosum, and, Ulva* sp*)*, collected from Fethard-on-Sea March, 2007 were individually exposed to 1 ppm cadmium over 24 hours. Each species was exposed in vessels designated 0, 6, 12 and 24h. Following exposure, replicate samples (*n=3*) were taken from each vessel at 0, 6, 12 and 24 hours. Samples were washed twice in deionised water (in order to remove any excess metal solution). Samples were then blot dried, placed in airtight freezer bags and then frozen at -20°C until required for % total protein (wet weight (ww)) determination.

4.3.3.1 Total Protein determination

> Refer to 2.3.8 for Kjeldahl method

4.3.4   96 h Time Course Study (*Polysiphonia lanosa*)

P. lanosa, collected from Fethard-on-Sea February 2008 was exposed to 1 ppm Cd for 96 hours.   The seaweed was exposed in individual vessels designated 0, 6, 24, 48, 72 and 96 h. Following exposure, replicate samples (*n=3*) were taken from each tank at 0, 6, 24, 48, 72 and 96 hours and washed twice in deionised water (in order to remove any excess metal solution (Wang & Dei, 1999). Samples were firstly blot dried, then oven dried at 60°C for 24 hours. Finally, samples were ground to a particle size $\leq$ 500 μM and stored in polyethylene bottles until required for analysis.

4.3.4.1 Protein Extraction

> Refer to 2.3.4.6

4.3.4.2 Extracted Protein Determination

> Refer to 2.3.5

4.3.5   Exposure Study (*Polysiphonia lanosa* exposed to 1-5 ppm Cd)

P. lanosa, collected from Fethard-on-Sea February 2008 was exposed to 1 - 5 ppm Cd for 6 hours.   The seaweed was exposed in individual vessels designated 1, 2, 3, 4 and 5 ppm Cd. Post-exposure sample preparation was carried out as per 5.3.4.

4.3.5.1 Protein Extraction

Refer to 2.3.4.6

4.3.5.2 Extracted Protein Determination

Refer to 2.3.5

4.3.6   6 h Exposure Study (4 Seaweed Species)

Seaweed samples (*P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp*)*, collected from Fethard-on-Sea, May 2008, were individually exposed to 1 ppm heavy metals (Cd^{2+}, Cr^{3+}, Pb^{2+} and Cu^{2+}) for 6 hours. Post-exposure sample preparation was carried out as per 4.3.4.

4.3.6.1 Total Protein determination

Refer to 2.3.8 for Kjeldahl method

4.3.6.2 Protein Extraction

Refer to 2.3.4.6

4.3.6.3 Extracted Protein determination

Refer to 2.3.5

4.3.7   6 h Time Course Study (P. lanosa)

P. lanosa, collected from Fethard-on-Sea, November 2008 was exposed to 1 ppm Cd for 1-6 hours. The seaweed was exposed in individual vessels designated 0, 1, 2, 3, 4, 5 and 6 h. Post-exposure sample preparation was carried out as per 4.3.4. Samples for the determination of intracellular metals were rinsed twice for 10 min with a 5mM EDTA (pH 8) solution, prior to oven drying, to remove metals adsorbed by the cell wall (García Ríos *et al.,* 2007).

4.3.7.1 Total Protein Determination

Refer to 2.3.8

4.3.7.2 Protein Extraction

Refer to 2.3.4.6

4.3.7.3 Extracted Protein Determination

Refer to 2.3.5

4.3.7.4 Polyphenol Extraction

Refer to 2.3.6.6

4.3.7.5 Polyphenol Determination

Refer to 2.3.7

4.3.7.6 Metal Analysis – Sample Digestion and Analysis

Refer to 3.3.15

Error bars are calculated based on triplicate samples with 95% Confidence Intervals.
A summary of all seaweed exposure studies is shown in table 4.1.

Table 4.1. Summary of Seaweed exposure studies.

Study No.	*Seaweed Species*	*Metal Exposure*	*Metal Conc. (ppm)*	*Exposure Duration (h)*	*Seaweed Collection Date*	*Analysis*
4.4.1.1	PL, AN, UL	Cd	1	0 – 24	Mar. 2007	Total Protein
4.4.1.2	PL,AN, FV,UL	Cd, Cr,Pb, Cu	1	6	May, 2008	Total Protein
4.4.1.3	PL	Cd	1	0 – 6	Nov, 2008	Total Protein
4.4.2.1	PL	Cd	1-5	6	Feb, 2008	Ex. Protein
4.4.2.2	PL	Cd	1	0 – 96	Feb, 2009	Ex. Protein
4.4.2.3	PL,AN, FV,UL	Cd, Cr,Pb, Cu	1	6	May, 2008	Ex. Protein
4.4.3.1	PL	Cd	1	0 – 6	Nov, 2008	Ex. Protein
4.4.3.2	PL	Cd	1	0 – 6	Nov, 2008	Polyphenols
4.4.3.3	PL	Cd	1-5	6	Nov, 2008	Metals

PL = *P. lanosa*; AN = *A. nodosum*; FV = *F. vesiculosus*; UL = *Ulva* sp.

4.4   Results

4.4.1   Effect of Heavy Metal Exposure on Total Protein Content of Seaweeds

4.4.1.1 24 h Time Course Study (*3 Seaweed Species – March 2007 Samples*)

Following exposure to 1 ppm Cd for 24h, the total protein content of *P. lanosa, A. nodosum* and *Ulva* sp was determined. Initial total protein contents of *P. lanosa, A. nodosum and Ulva* sp were 5.75, 2.98 and 2.46 % wet weight (ww) respectively. Trends in initial total protein values generally agree with findings in Chapter 3 whereby the red seaweeds contained, by far the highest total protein (34.9% dw), followed by *Ulva* sp and *A. nodosum* (19.7 and 11.1 % dw respectively).

Following exposure to 1 ppm Cd an initial increase in total protein content was observed for all three species after 6 hours. This was followed by a decrease in total protein, back to baseline values after 24 hours, a trend observed in all three species (Figure 4.1).

A reduction in total protein (back to original baseline values) was seen subsequently, in all species, up to 24 hours of exposure. This reduction may indicate the complete Cd removal from solution by each of the seaweeds and a return to steady state following heavy metal exposure.

4.4.1.2 6 h Exposure Study on Four Seaweed Species (*May 2008 Samples*)

Results from 4.4.1.1 demonstrated a significant increase in total protein of *P. lanosa, A. nodosum* and *Ulva* sp following exposure to 1 ppm Cd^{2+} for 6h. To further investigate these findings, four different seaweed species were exposed to four different heavy metals. In the study, *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp were exposed to 1 ppm Cd^{2+}, Cr^{3+}, Pb^{2+} and Cu^{2+} over 6h and total protein values were determined.

P. lanosa yielded the highest total protein content of control samples, while *Ulva* sp had the second highest and the brown seaweeds, the lowest (Figure 4.2).
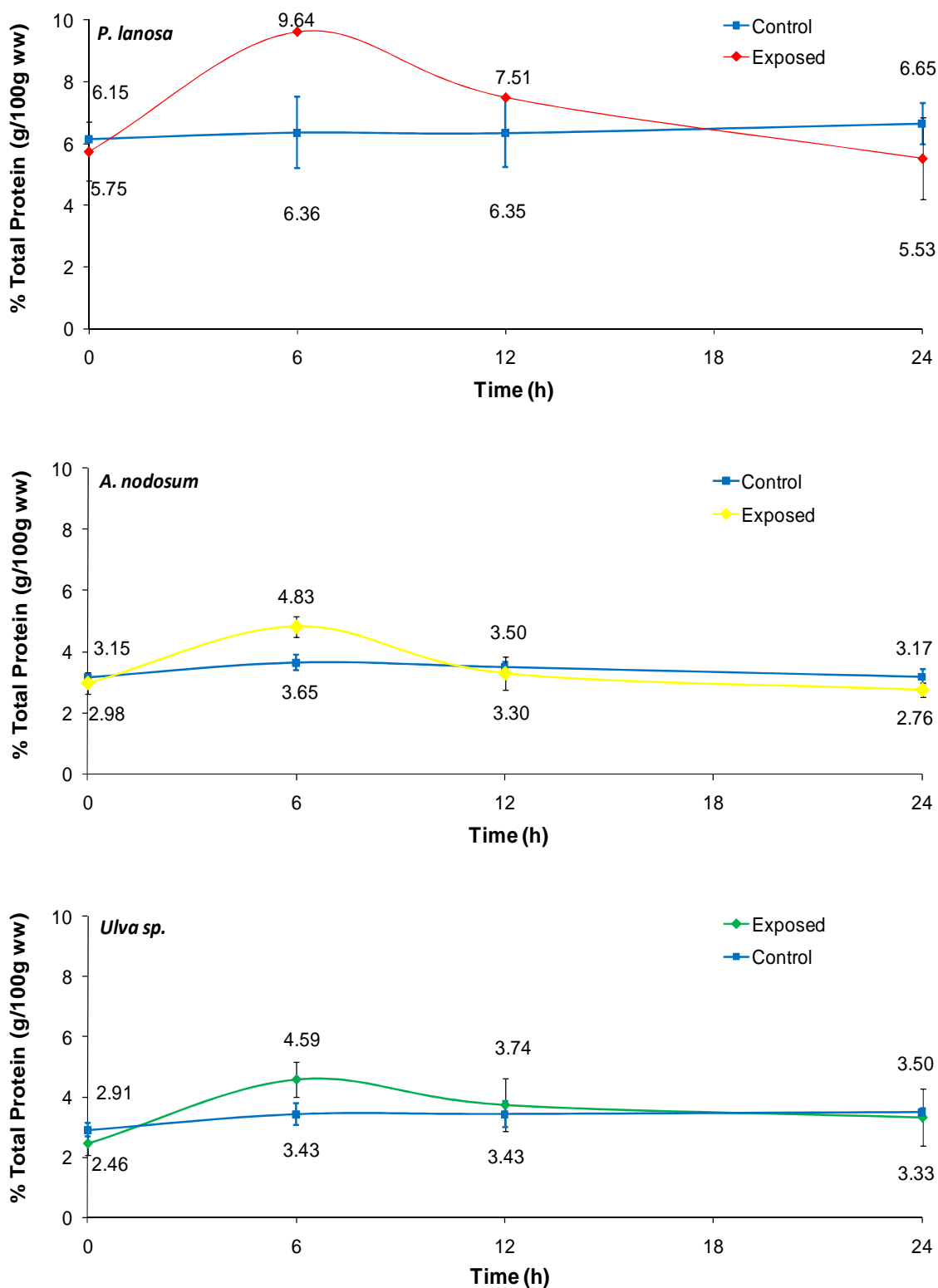
Figure 4.1. % Total protein content of wet *P. lanosa, A. nodosum and Ulva* sp (March 2007), following exposure to 1 ppm Cd over 24 h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

Results however, show no overall change in total protein following heavy metal exposure. Both sets of experiments (4.4.1.1 and 4.4.1.2) were performed in an identical manner, the only difference being the time of seaweed collection. Seaweed samples from 4.4.1.1 were collected in March, 2007 and samples for this study were collected in May, 2008.
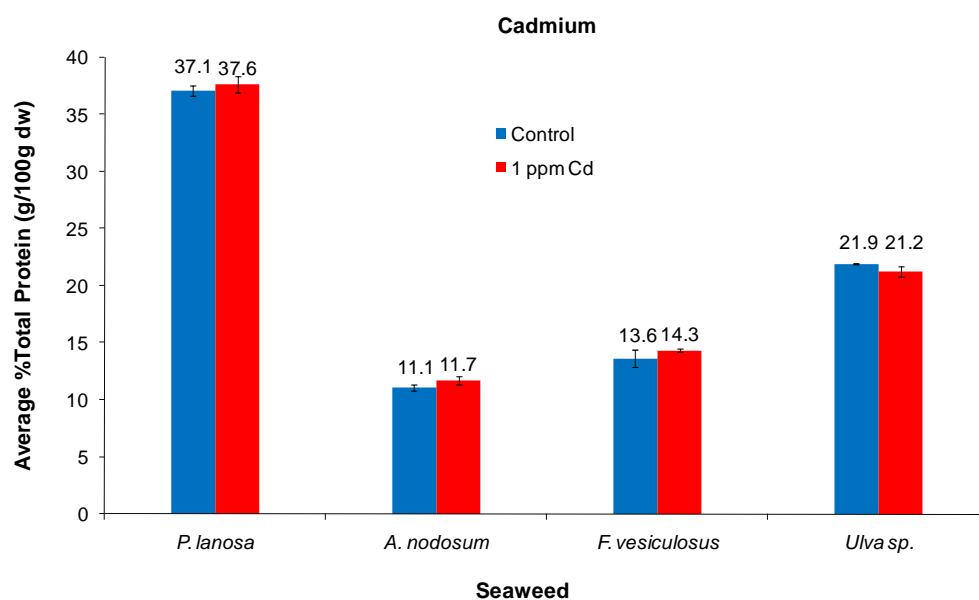
Figure 4.2. Total protein content (% dw) of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm cadmium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.
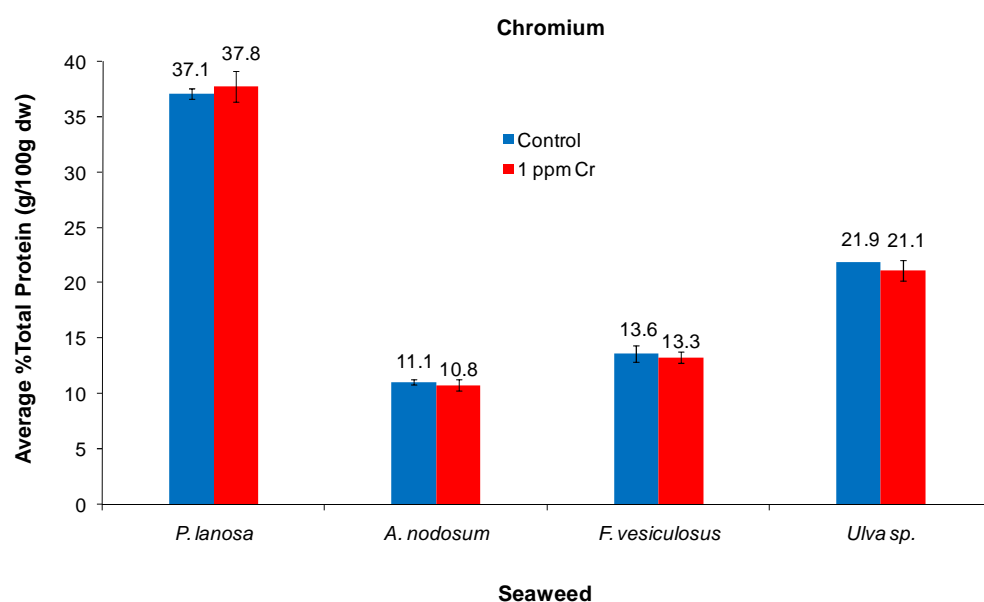
Figure 4.3. Total protein content (% dw) of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm chromium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.
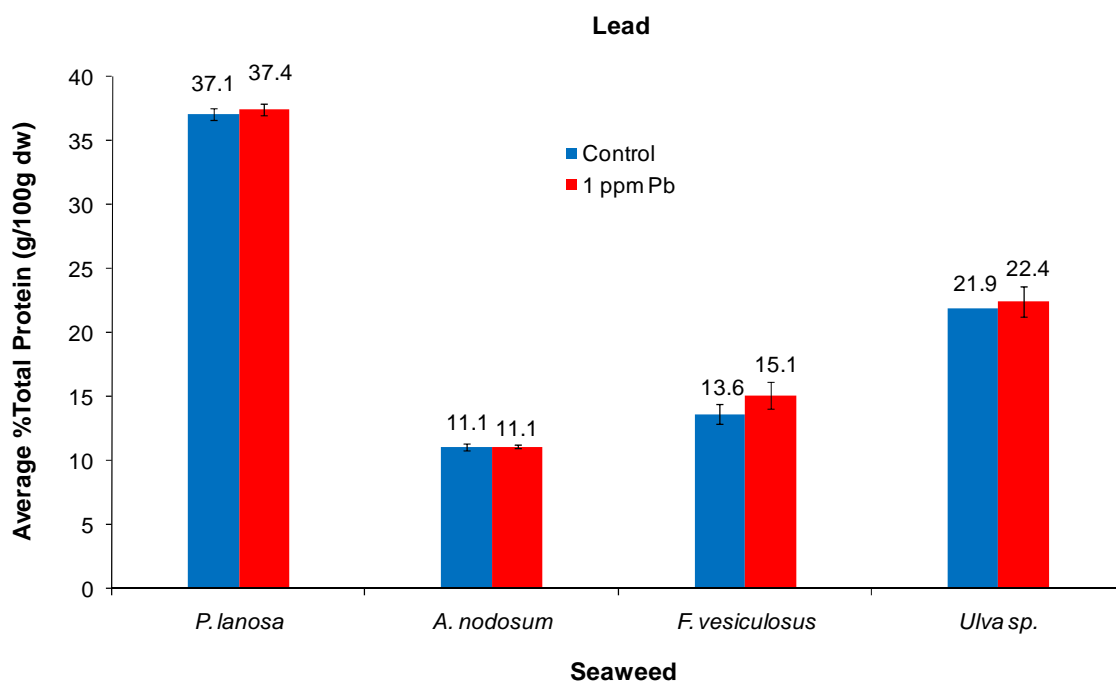
Lead

Figure 4.4. Total protein content (% dw) of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm lead for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.
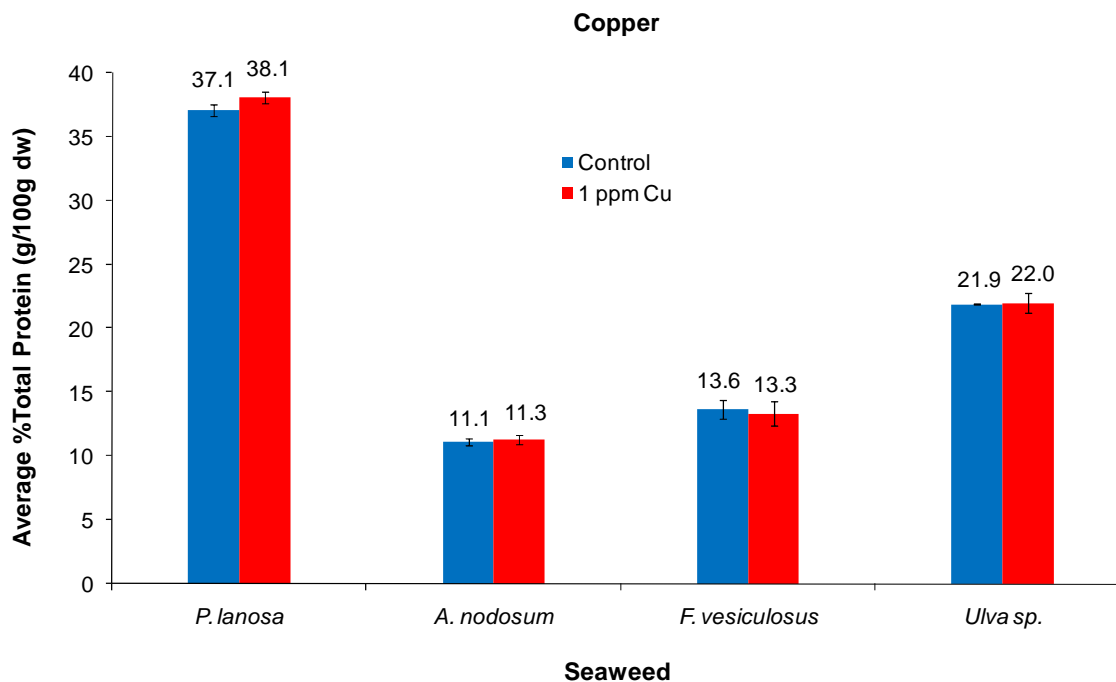
Copper

Figure 4.5. Total protein content (% dw) of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm copper for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

4.4.1.3 Time Course Study (*November 2008 Samples)*

To further investigate a temporal aspect of heavy metal exposure in seaweeds, a short time course study (0 – 6h) was performed with *P. lanosa* (November, 2008) exposed to 1 ppm Cd and the total protein content of each sample set was determined.

Results demonstrate no variation in the total protein content of *P. lanosa* over the 6 hour exposure period, where values ranged from 25.0 ± 0.2 to 25.4 ± 0.1 % dw (Figure 4.6). These results therefore are consistent with those obtained for the May samples and differ with those of the February samples.
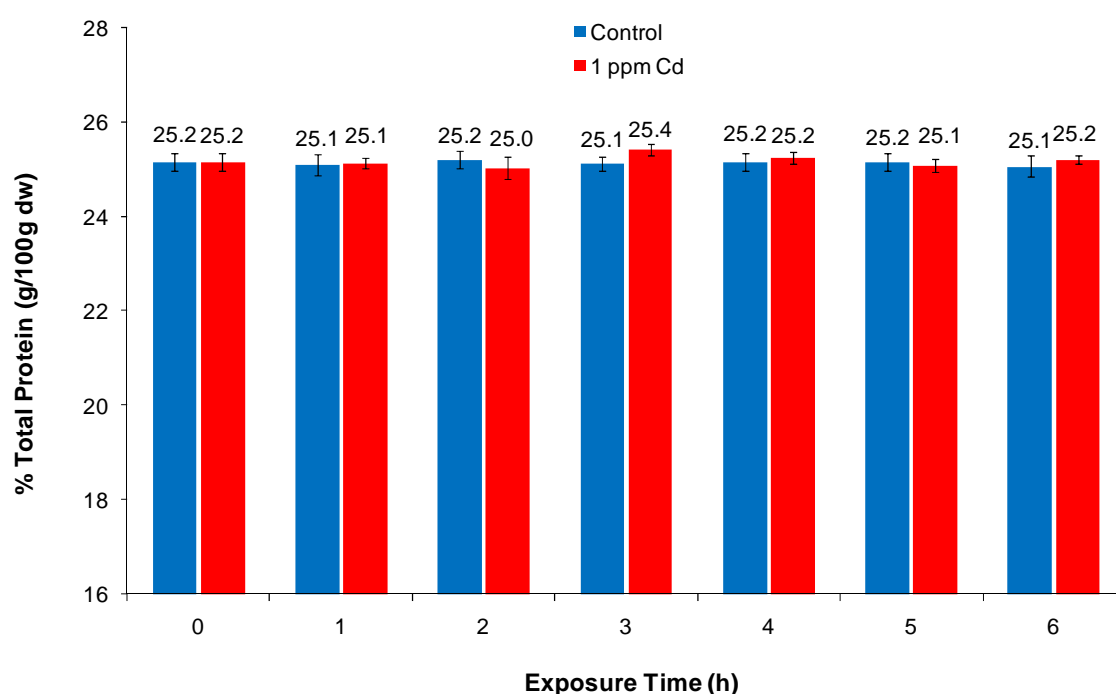
Figure 4.6. Total Protein content of *P. lanosa* (November, 2008) following exposure to 1ppm cadmium for 0 to 6 h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.

4.4.2 Effect of Heavy Metal Exposure on Extracted Protein Content of Seaweeds

4.4.2.1 Effect of Various Cd^{2+} Concentrations on Extracted Protein Content of P.lanosa

Significant temporal variations in total protein were observed for seaweeds exposed to Cd^{2+}. Further investigations involving temporal variations in the extracted protein content of *P. lanosa* following heavy metal exposure were also conducted. *P. lanosa*

(February, 2008), was exposed to 1 – 5 ppm Cd^{2+} for 6 hours and the extracted protein content for each sample was determined.

Significant variations in protein extracts were observed for *P. lanosa* exposed to 1 – 3 ppm Cd^{2+} when compared with control values. Treatment with Cd^{2+} at concentrations up to 3 ppm increased the extracted protein content of *P. lanosa* with the highest protein values detected in samples treated with 1 ppm Cd^{2+}. Treatment with 4 and 5 ppm Cd^{2+} yielded results similar to that of control samples (Figure 4.7).
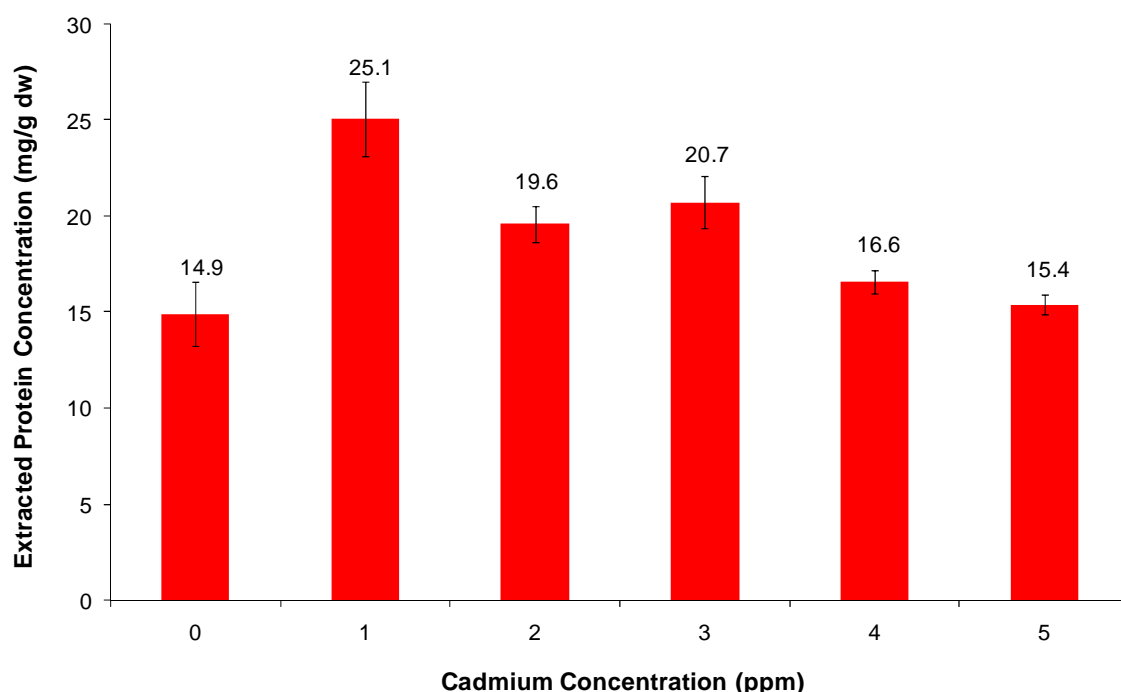
Figure 4.7. Extracted protein concentration of *P. lanosa* (Feb, 2008) following exposure to 1-5 ppm cadmium for 6 h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.

4.4.2.2 96 h Time Course Study (February 2009 Samples)

To further investigate the effect of heavy metal exposure on the extracted protein levels of seaweeds, *P. lanosa* was exposed to 1 ppm Cd for 0 – 96h. Initial extracted protein levels in *P. lanosa* yielded 11.0 mg/g dw. Following exposure however, there was an increase in extracted protein content of *P. lanosa*. This increase was observed for 6, 24, 48 and 72 hour samples. The largest increase was observed at 6 and 24 hours, with a

subsequent decrease for the 48 and 72 hour samples. There was a sharp decrease in extracted protein after 96 hours to below baseline values (Figure 4.8).
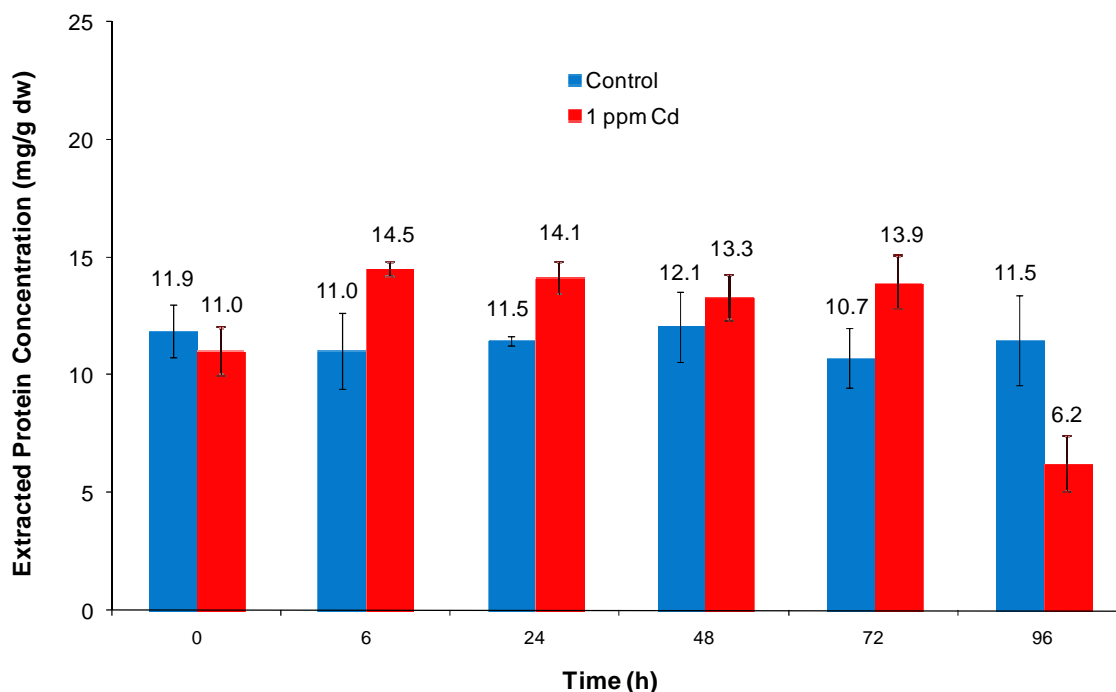
Figure 4.8. *P. lanosa* (Feb, 2009) exposed to 1 ppm cadmium for 0 – 96 h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate

4.4.2.3 6 h Exposure Study on 4 Seaweed Species (*May 2008 Samples)*

Increases in both total and extracted protein levels were consistently observed for February and March *P. lanosa* samples following heavy metal exposure. To further investigate temporal variations in seaweed protein levels following heavy metal exposure, *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp, collected in May, 2008 were exposed to 1 ppm Cd^{2+}, Cr^{3+}, Pb^{2+} and Cu^{2+} for 6h and extracted protein levels examined.

Results demonstrated that initial extracted protein contents varied with species. Extracted protein concentration for *A. nodosum, F. vesiculosus, Ulva* sp *and P. lanosa* were 101.4 mg/g dw, 41.9 mg/g dw, 11.8 mg/g dw and 8.1 mg/g dw respectively (Figures 4.9 to 4.12).
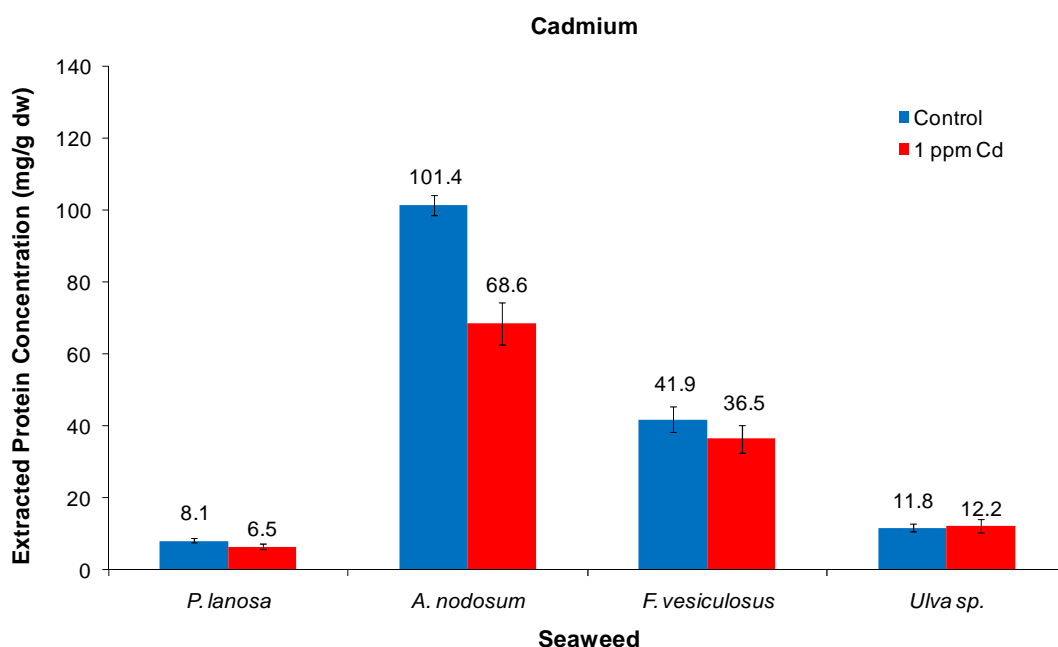
Cadmium

Figure 4.9. Extracted protein content of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm cadmium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.
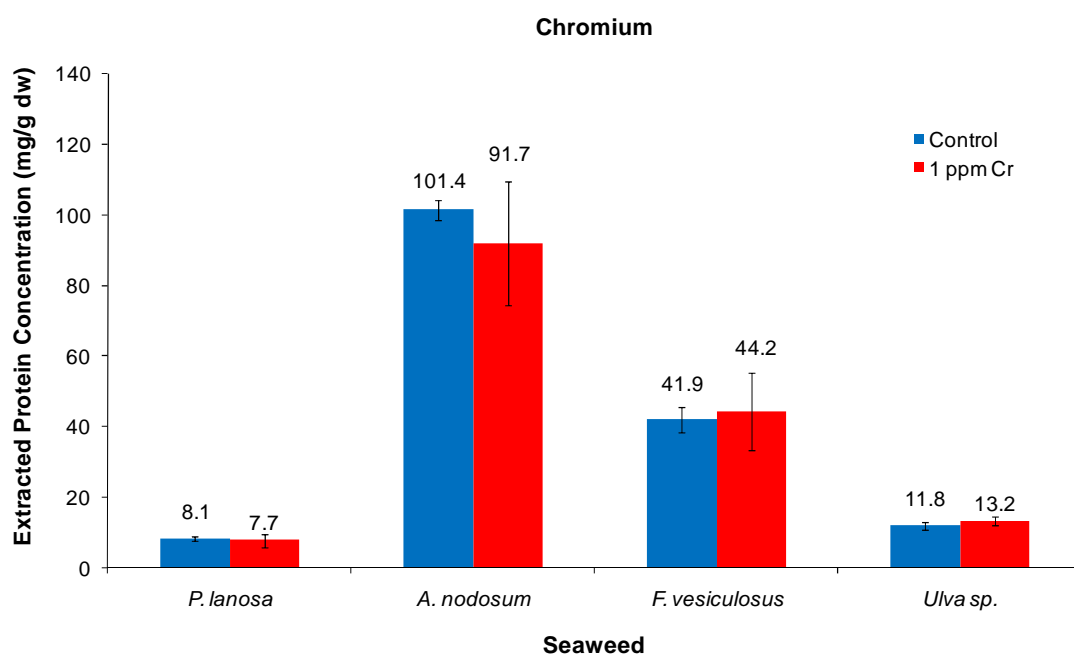
Chromium

Figure 4.10. Extracted protein content of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm chromium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.
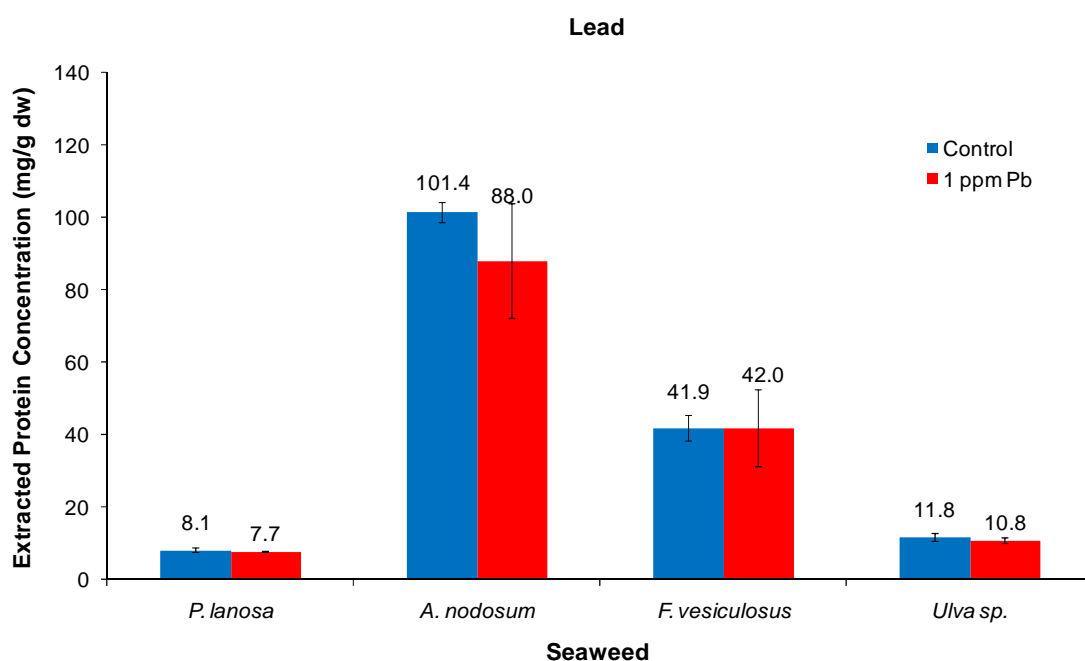
Lead

Figure 4.11. Extracted protein content of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm lead for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.
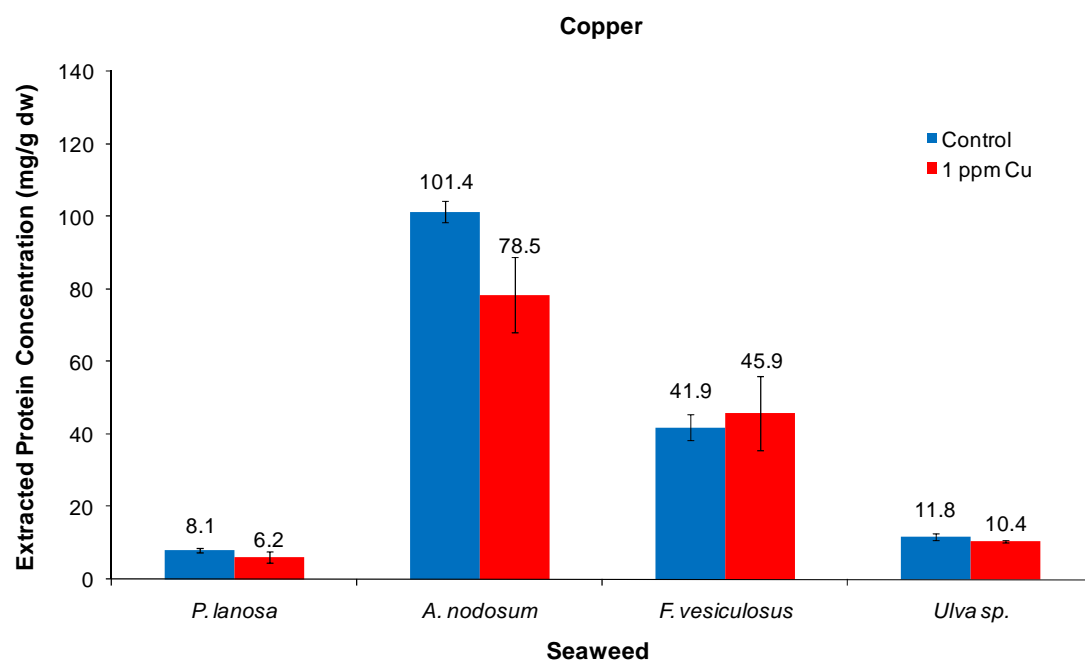
Copper

Figure 4.12. Extracted protein content of *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp (May 2008), following exposure to 1 ppm copper for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.

4.4.3 Effect of Heavy Metal Exposure on Extracted Protein, Polyphenol and Metal Levels in *P. lanosa*

Much of the previous work focused on seaweed samples collected in Feb/Mar and May. In this study, changes in extracted protein, polyphenol and metal levels of *P. lanosa* collected in November, following Cd^{2+} exposure were monitored.

4.4.3.1 Extracted Protein Determination

Results from this study were consistent with the total protein values of 4.4.1.3. There was little variation in the overall extracted protein concentration following 6 hours exposure to 1 ppm Cd^{2+}. The initial and final protein concentrations were 5.1 and 6.7 mg/g dw, respectively. These values however, were within error (Figure 4.13).
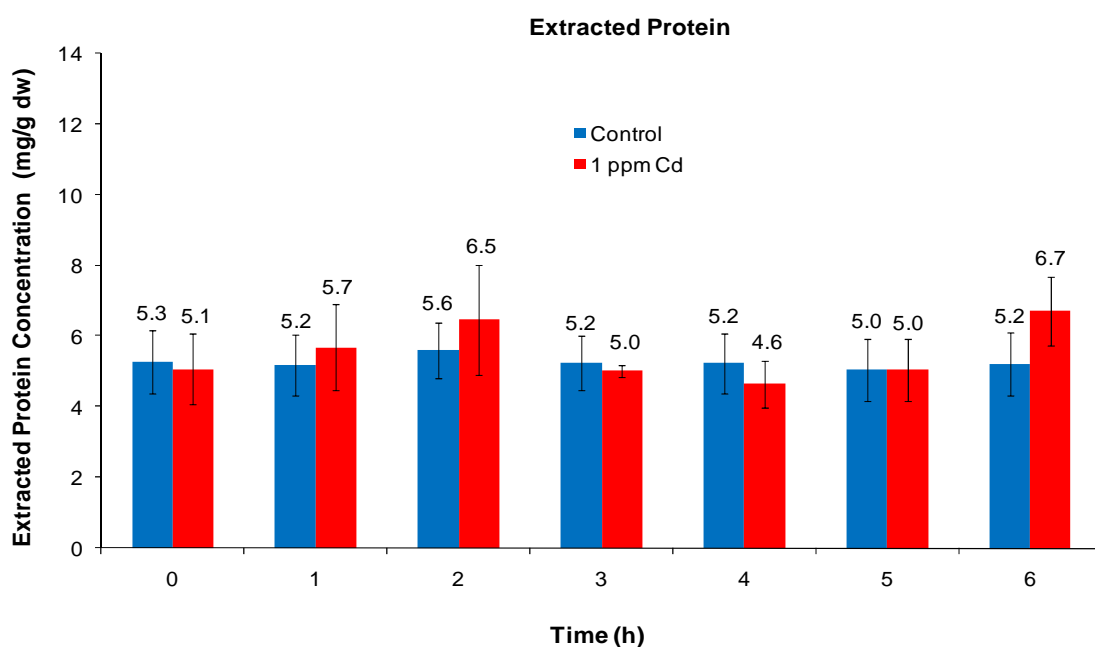
Figure 4.13. Extracted protein concentration of *P. lanosa* following exposure to 1 ppm Cd for 6h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.

4.4.3.2 Polyphenol Determination

Following exposure to 1 ppm Cd^{2+}, small increases in the polyphenol levels of *P. lanosa* were observed after 3h and to a lesser extent, 6h. Furthermore, there was an overall slight increase in polyphenol content from 7.3 (0h) to 9.0 mg/g dw (GAE) over 6 hours

exposure (Figure 4.14). Furthermore One-Way ANOVA ($p<0.05$) demonstrated a significant variation ($p = 0.027$) between 0 h and 6 h polyphenol levels.
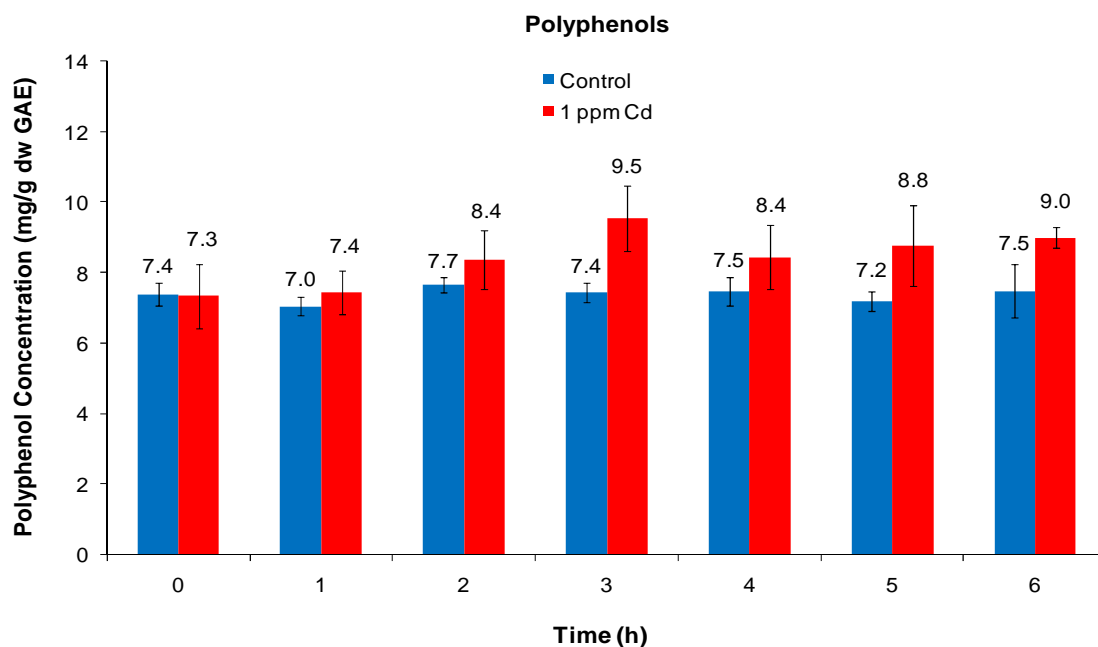
Figure 4.14. Polyphenol content of *P. lanosa* following exposure to 1 ppm Cd for 6h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals. Each replicate sample was assayed in triplicate.

4.4.3.3 Metal Analysis

The overall total Cd^{2+} concentration of *P. lanosa* increased from 2.5 to 43.9 mg/kg dw over 6h. There was a rapid initial increase, resulting in a Cd^{2+} elevation from 2.5 – 28.5 mg/kg dw over the first hour of exposure. Surface bound Cd^{2+} followed a similar trend, with an increase, over 6 h from 0 – 34.3 mg/kg dw. Intracellular Cd increased from 2.5 to 9.7 mg/kg dw (Figure 4.14).
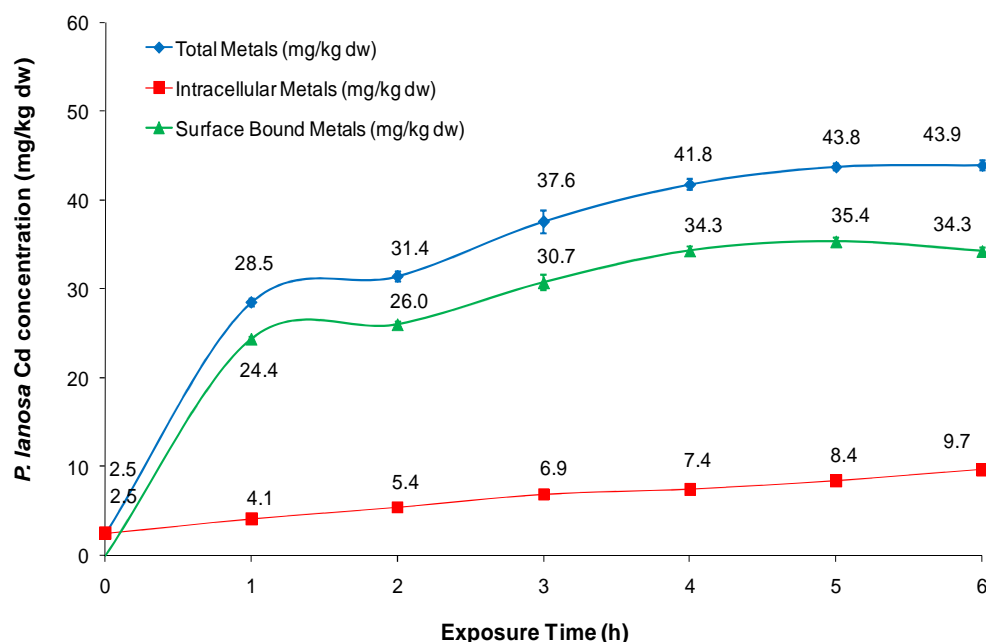
Figure 4.15. Total metal, intracellular metal and surface bound metal concentration of *P. lanosa* following exposure to 1 ppm Cd for 6h. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

Unlike total and surface bound metals, the intracellular increase in Cd^{2+} was of a slower and more linear nature. Both total and surface bound cadmium concentrations levelled off at 5 to 6 hours, whereas intracellular Cd^{2+} levels further increased over 5 to 6 hours from 8.4 mg/kg dw to 9.7 mg/kg dw (Figure 4.14).

Figure 4.16 compares polyphenol concentration with intracellular metal concentration following 6 hours exposure to 1 ppm Cd. Overall, there was a slow but overall, general increase in both intracellular metals and polyphenols concentrations over 6 hours exposure. Intracellular cadmium increased from 2.5 to 9.7 mg/kg dw. Polyphenol levels increased from 7.3 to 9.0 mg/g dw (GAE). Overall, increases in polyphenol levels, as a result of elevated intracellular metal over time, may indicate an initial polyphenolic heavy metal defence system in *P. lanosa*.
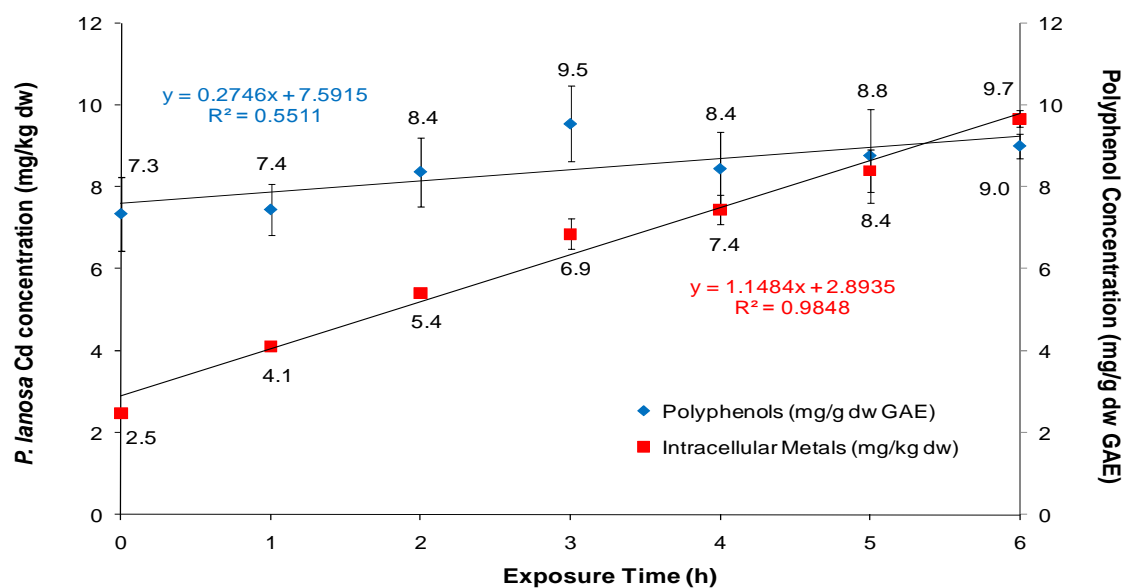
Figure 4.16. Intracellular metal concentration versus Polyphenol concentration of *P. lanosa* following exposure to 1 ppm Cd for 6h.

4.5 Discussion

4.5.1 Effect of Heavy Metal Exposure on Total Protein Content of Seaweeds

4.5.1.1 24 h Time Course Study (*3 Seaweed Species – March 2007 Samples*)

The initial total protein contents of *P. lanosa, A. nodosum and Ulva* sp were 5.75, 2.98 and 2.46 % (ww) respectively. The majority of total protein studies involving seaweeds are performed using dry sample (Chapter 2, Table 2.9). In a study by Azad *et al.* (2007), however the total protein content of brown seaweeds ranged from 2.8 − 6.3 % (ww). This range of values is consistent with the total protein value of *A. nodosum* obtained in this study.

Following Cd exposure, an initial increase in total protein was observed for all three species after 6 hours. This was followed by a decrease in total protein, back to baseline values after 24 hours. In a study by Malea *et al.* (2006), the synthesis of PCs was observed in the green macroalgae *E. prolifera* following exposure to Cd. In this study, a reduction in total protein was subsequently observed after 24 h of exposure. This reduction is perhaps indicative of complete Cd removal from solution by each of the seaweeds. For example, a previous study saw increases in intracellular Cd concentration of a eukaryotic diatom following Cd exposure, with a corresponding increase in PC concentration (Ahner & Morel, 1995). Subsequently, however, there was also a rapid decrease in PC concentration when Cd exposure was alleviated, suggesting that the intracellular PC pool is tightly regulated (Ahner & Morel, 1995). A similar mechanism may also have been observed for the macroalgae, *P. lanosa* in this study.

4.5.1.2 6 h Exposure Study on Four Seaweed Species (*May 2008 Samples*)

To further investigate the findings from section 4.4.1.1, *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp were exposed to 1 ppm Cd^{2+}, Cr^{3+}, Pb^{2+} and Cu^{2+} over 6h and total protein values were determined.

P. lanosa yielded the highest total protein, followed *Ulva* sp, while the brown species were determined to have the lowest. These findings are consisted with results obtained from the baseline study in Chapter 3, where total protein values for *P. lanosa, A.*

nodosum, F. vesiculosus and *Ulva* sp were 34.9, 11.1, 14.9 and 19.7 % dw respectively. Furthermore, results were consistent with previous findings (Chapter 2, Table 2.9.). Generally, the protein concentration of brown seaweeds is low (3-15% of the dry weight) compared with that of the green or red seaweeds (10-47% dw), (Fleurence, 1999a).

Following heavy metal exposure no change in the total protein content of the four seaweeds was observed. The lack of protein induction may therefore indicate different seasonal sensitivities to metal exposure in *P. lanosa*. For example, in a study by Martínez & Rico (2002), it was demonstrated that seawater NH_4^+ concentrations in Summer were generally lower than all other times the year. N is necessary for the synthesis of amino acid (Rijstenbil *et al.,* 1998) such as those required for the induction of GSH, therefore, under such limiting conditions stored matter is diverted to growth, resulting in the dilution of all algal nutrient reserves by the addition of new biomass (Martínez & Rico, 2002). Additonally, Kaczmarska & Dowe (1997) demonstrated that *P. lanosa* from the Bay of Fundy, Canada reproduces from Spring to Autumn and that late October signifies the end of the growing season.

4.5.1.3 Time Course Study (*November 2008 Samples*)

To further investigate a temporal aspect of heavy metal exposure in seaweeds, a short time course study (0 – 6h) was performed with *P. lanosa* (November, 2008) exposed to 1 ppm Cd and the total protein content of each sample set was determined.

Results for the 0 – 6 h exposure study demonstrated no variation in the total protein content of *P. lanosa*. These results therefore are consistent with those obtained for the May samples and differ from those of the February samples. The lack of protein induction in the November samples may also be attributed to seasonal variation to heavy metal exposure in seaweed. As mentioned earlier, the growing season for *P. lanosa* ends in October (Kaczmarska & Dowe, 1997), samples in this study were collected early November. It is possible therefore, that the *P. lanosa* was still in the growing phase and as a result, stored N was still being diverted to growth, thereby preventing the synthesis of compounds such as GSH.

4.5.2   Effect of Heavy Metal Exposure on Extracted Protein Content of Seaweeds

4.5.2.1 Effect of Various Cd^{2+} Concentrations on Extracted Protein Content of P.lanosa

P. lanosa (February, 2008), was exposed to 1 – 5 ppm Cd^{2+} for 6 hours and the extracted protein content for each sample was determined.   The extracted protein concentration corresponds to intracellular protein levels.   Treatment with Cd^{2+} at concentrations up to 3 ppm increased the extracted protein content of *P. lanosa* with the highest protein values detected in samples treated with 1 ppm Cd^{2+}.   Exposure 4 and 5 ppm Cd^{2+} yielded results similar to that of control samples.

A similar trend was also observed by Wu *et al.* (2001).   In their study, the root culture of the plant species *Adenophora lobophylla* was exposed to 0 – 400 μM Cd over three days.   Results demonstrated that there was an increase in protein up to 200 μM Cd^{2+} (32 mg/g dw).   However, further increases in Cd^{2+} concentration (300 – 400 μM) resulted in protein concentrations less then that of control samples (5.4 and 3.7 mg/g dw, respectively).   Results from Wu *et al.* (2001), demonstrated that protein accumulation in a plant species, *A. lobophylla* was sensitive to Cd^{2+} stress and SDS-PAGE analysis of protein extracts established modifications in the protein content components.   Silver staining also revealed complex changes of protein components both in quality and quantity.   However, in a previous study, Malea *et al.* (2006) exposed the macroalga *E. linza* to increasing concentrations of Cd^{2+}.   Following exposure to 0 – 200 μg/L Cd, it was demonstrated that the overall protein content of this seaweed species decreased from 0.15 to 0.11 g/g dw (Malea *et al.,* 2006).

Furthermore, an increase in extracted protein following exposure to 1 ppm Cd over 6h is consistent with previous findings for total protein (4.4.1.1).   Significantly, both sets of seaweed samples for total protein and extracted protein determination were sampled at similar times (March and February respectively).   Results therefore further indicated temporal variations in seaweed sensitivity to heavy metal exposure.   Martinez & Rico (2002) demonstrated that N concentration in seawater is at its lowest in Summer and under such limiting conditions, N reserves in seaweed are utilised for growth during this season.   Highest seawater levels of N however were observed in Winter and this corresponded to highest levels of N compounds in *P. palmata* (Martinez & Rico, 2002). In this study therefore, it is possible that high levels of N both in seawater and *P. lanosa* from the February samples, combined with the lack of seaweed growth in this period,

resulted in a sufficient supply of N for amino acid (and therefore perhaps, GSH) synthesis.

4.5.2.2 96 h Time Course Study (February 2009 Samples)

Following exposure to 1 ppm Cd, there was an increase in the extracted protein content of *P. lanosa* for 6, 24, 48 and 72 h samples. The largest increase was observed at 6 and 24 hours. The increase in extracted protein concentration after 6h exposure is consistent with results from 4.4.2.1 and 4.4.1.1 whereby extracted protein increased from 14.9 to 25.1 mg/g dw and total protein increased from 5.75 to 9.64% ww following 6h exposure to Cd^{2+}. Significantly, all three *P. lanosa* samples were collected during the same time period (February and March). In a previous study, Malea *et al.* (2006) demonstrated the synthesis of PCs in *E. prolifera* following exposure to Cd.

In a study by Martinez & Rico (2002), the yearly variation of nitrogen reserves of *Palmaria palmata*, in relation to seasonal ecological changes was studied. Results demonstrated significant correlations between the seaweed nutrient concentration and nitrate seawater levels. The maximum concentrations of total N compounds were demonstrated during Autumn and Winter because the seaweed stored N rich compounds as a result of high nutrient seawater concentration and additionally, when growth was restricted by low light and temperature. During Spring, lower N concentrations were observed in the growing fronds of *P. palmata*. N seawater concentrations were not detectable during Summer when seaweed nutrient pools were low and growth was reduced and eventually suppressed, thus suggesting nutrient limiting conditions (Martinez & Rico, 2002). Furthermore, Malea *et al.* (2006) demonstrated that the green macroalgae *E. linza* was capable of storing high ammonium reserves in temporarily N-depleted waters. Following Cd exposure however, GSH pools of this seaweed increased (but was susceptible to oxidation). It was proposed that the oxidative effect of Cd in the *Enteromorpha* sps may have been due to N-poor (and therefore, low GSH) conditions. Under N-rich conditions however, GSH pools were adequately high to facilitate the synthesis of PCs (Malea *et al.,* 2006). Furthermore, Rijstenbil *et al.* (1998) investigated the effect of the nitrogen levels on the reserves of metal-binding peptides in a planktonic diatom. It was concluded that in the Summer samples, phytochelatins were only produced in the N-enriched medium when Cu^{2+} concentrations were high whereas, in a non-N-enriched media and at low Cu^{2+} levels PC2 and PC3 were produced in Winter

samples. It therefore evident that PC production in Winter samples of the planktonic diatom are influenced by both Cu^{2+}-dosage and N-enrichment. Similar seasonal trends relating to N enrichment and macroalgae have never previously been published.

The sharp decrease in extracted protein levels after 96 hours exposure maybe indicative of heavy metal toxicity. It is possible that the decrease is a result of changes to intracellular protein following protracted exposure to Cd^{2+} (Figure 4.8). Wu *et al.* (2001), demonstrated an initial increase, followed by a subsequent decrease in the total protein content of a plant species upon exposure to increasing Cd^{2+} concentrations. The decrease in total protein was attributed to Cd^{2+} stress in the plant. Furthermore, Malea *et al.* (2006), demonstrated an overall decrease in the protein content of *E. Linza* following exposure to Cd^{2+} (Malea *et al.,* 2006). The decrease in protein concentration at 96h may also be attributed to exudation of Cd-protein complexes back into the environment. For example, Vasconcelos & Leal (2001b) demonstrated that all tested metals promoted the liberation of exudates (e.g., cysteine- and GSH-like compounds) from both *Porphyra* spp. and *Enteromorpha* spp. Furthermore, both cysteine- and GSH-like ligands were exuded in the presence of Cu, only cysteine-like ligands in the presence of Pb, and only GSH-like ligands in the presence of Cd (Vasconcelos & Leal, 2001b).

4.5.2.3 6 h Exposure Study on 4 Seaweed Species (May 2008 Samples)

P. lanosa, A. nodosum, F. vesiculosus and *Ulva* sp, collected in May, 2008 were exposed to 1 ppm Cd^{2+}, Cr^{3+}, Pb^{2+} and Cu^{2+} for 6h and extracted protein levels examined.

Following heavy metal exposure (with the exception of protein levels of *A. nodosum* following exposure to Cd^{2+} and Cu^{2+}), there was no overall variation in the extracted protein levels of all seaweeds. These results are consistent with results for 5.4.1.2, where no increase in total protein was observed for all four seaweed species following heavy metal exposure. Samples for both sets of analysis were collected in May 2008. Furthermore, results were also consistent with values for 5.4.1.3, where there was no increase in the total protein content of November samples following exposure to $1 - 6$ ppm Cd^{2+}. Results therefore endorse temporal variations in the sensitivity of seaweeds to metal exposure.

Exposure to Cd^{2+} and Cu^{2+} induced a decrease in the extracted protein concentration of *A. nodosum*. Following exposure to Cd^{2+} and Cu^{2+}, extracted protein decreased from 101.4 to 68.6 mg/g dw and 101.4 to 78.5 mg/g dw respectively. It would appear therefore that *A. nodosum* is sensitive to two of the heavy metals under investigation in this study (Figures 4.9 and 4.12). For example, 1 ppm Cd^{2+} induced a decrease in extracted protein of *P. lanosa* over 96h as a result of plant stress initiated by heavy metal toxicity (See 4.4.2.2). A similar result was observed by Wu *et al*., 2001 in relation to a plant species. Malea *et al.* (2006) also demonstrated an overall decrease in the protein content of the macroalgae *E. linza* following exposure to Cd^{2+} (Malea *et al.,* 2006). In a similar way perhaps, exposure to 1 ppm Cd^{2+} and Cu^{2+} over 6h was sufficient to have a similar effect on *A. nodosum*. Alternatively the extracted protein levels may be attributed to exudation of Cd^{2+}-protein and Cu^{2+}-protein complexes or oxidation of GSH. Exudation of metal-GSH exudates were previously observed in the marine macroalgae *Enteromorpha* spp and *Porphyra* spp (Valconcelos & Leal, 2001). Furthermore, exudation of Cd^{2+}-GSH complexes were previously observed in a marine diatom (Tang *et al.,* 2005) and oxidation of GSH was previously observed in *E. prolifera* following exposure to Cu (Rijstenbil *et al.,* 1998b).

4.5.3 Effect of Heavy Metal Exposure on Extracted Protein, Polyphenol and Metal Levels in *P. lanosa*

4.5.3.1 Extracted Protein Determination

Results for samples collected in November demonstrated little variation in the overall extracted protein concentration following 6 hours exposure to 1 ppm Cd^{2+}. Results from this study further support temporal variations in the protein concentrations of seaweeds following heavy metal exposure.

4.5.3.2 Polyphenol Determination

Following heavy metal exposure, small increases in the polyphenol levels of *P. lanosa* were observed after 3h and to a lesser extent, 6h (Section 1.4.3.2). In a study by Wallenstein *et al.* (2009), the response of the brown seaweed *Cystoseira abies-marina* to Cd^{2+} exposure over four weeks was investigated. They observed a gradual increase of Cd^{2+} levels of the seaweed which they attributed to the possible synthesis of new

polyphenols. The changes in polyphenol levels of *P. lanosa* following Cd^{2+} exposure may therefore be indicative of an intracellular defence against heavy metal exposure.

4.5.3.3 Metal Analysis

The total Cd^{2+} concentration of *P. lanosa* increased substantially from 2.5 to 43.9 mg/kg dw over 6h. An initial rapid increase was observed for both total and surface bound Cd^{2+}. In contrast the intracellular increase in Cd^{2+} was of a slower and more linear nature. This trend is in agreement with literature relating to growing cells/microalgae where an initial rapid phase of biosorption is followed by a slower, metabolism-dependent active uptake of metals (Malik, 2004; Garnham *et al*., 1992).

Furthermore, in a previous study by Wang & Dei (1999), the red alga *Gracilaria blodgeti* was exposed to 10 µg/L Cd^{2+} over 47h. They observed an initial rapid adsorption of Cd^{2+} within the first 2h of exposure, this was followed by a linear pattern of metal uptake. This latter observation was supported by Lee & Wang (2001) who also observed a linear pattern of accumulation between 2 and 8h following the exposure of the green seaweed *U. fisciata* to 2 µg/L Cd^{2+} over 8h. In both cases, it was assumed that metals were internalised during the accumulation period. This assumption was substantiated by García Ríos *et al.* (2007), who exposed the red seaweed *Chondrophycus poiteaui* to 1 µg/ml Cd^{2+} for 3 days. Following exposure, the total Cd^{2+} concentration of the seaweed increased from 0.19 − 78.6 mg/kg dw, the greater proportion of which was intracellular. Intracellular levels of Cd^{2+} increased from 0.24 to 72 mg/kg dw.

There is very little published data relating to intracellular accumulation of heavy metals in seaweed. This however, is the first study to determine both total and intracellular trends in *P. lanosa* following exposure to Cd^{2+}.

Overall results from this study demonstrated temporal variations in seaweed protein levels following exposure to heavy metals. Heavy metals therefore may have the ability to induce increases in total and extracted protein during February and March however, no such effect was observed for May and November samples. Other factors however are also known to affect the protein levels in seaweed, including light intensity (Cronin & Hay, 1996) and nitrogen availability (Rijstenbil *et al.,* 1998). Amino acid synthesis (and thus protein synthesis) required nitrogen (Rijstenbil *et al.,* 1998). It has been

demonstrated that increases in nitrate uptake with increasing nitrate levels have previously been established for several seaweed species and it is generally accepted that uptake is via active transport (Lobban & Harrison, 1997). Furthermore, Cheng *et al.,* 2010 demonstrated that nitrogen uptake in *U. lactuca* increased with light intensity. Additionally, Cronin & Hay (1996) demonstrated that the protein content of the brown seaweed, *Dictyota ciliolate* also increased with light intensity.

4.6   Conclusions

The aim of this study was to monitor the effects of temporal variation in the protein, polyphenol and metal levels in seaweeds and the relative sensitivity of the seaweeds to heavy metals. A series of heavy metal exposure studies were performed including, time course studies and heavy metal exposure studies at various heavy metal concentrations. Studies were performed on several seaweed species collected in Feb/Mar, May and November (2007 to 2008) from Fethard-on-Sea, Co. Wexford.

Following an initial 24 hour time course Cd^{2+} exposure study (March samples), an increase in total protein was observed in *P. lanosa, A. nodosum* and *Ulva* sp after 6 hours. A drop in total protein (back to original values) however for all three species was subsequently observed up to 24h. The initial increase may perhaps indicate induction of peptides, such as phytochelatins, in all three species. The decrease however, back to baseline levels may indicate complete Cd^{2+} removal from solution by each of the seaweeds. A previous study saw increases in intracellular Cd^{2+} concentration of eukaryotic algae following Cd^{2+} exposure, with a corresponding increase in PC concentration (Ahner *et al*., 1995). A rapid decrease in PC concentration of the marine microalgae *Thalassiosira weissflogii* however was observed when Cd^{2+} exposure was alleviated (Ahner & Morel, 1995). Similar data relating to seaweed have not previously been published.

The exposure of *P. lanosa* (February samples), to increasing concentrations of Cd^{2+} was also investigated. Following exposure to Cd^{2+}, increases in extracted protein were observed at 1 to 3 ppm Cd^{2+}. 1 ppm Cd^{2+} induced the highest increase, from 14.9 ± 1.7 to 25.1 ± 2.0 mg/g dw. There was however no obvious increase in extracted protein at 4 and 5 ppm Cd^{2+}. In a previous study, Wu *et al.* (2001) demonstrated an increase in the protein content of the plant, *A. lobophylla*, up to 50 μM. No apparent increases in protein however were observed at 150 – 200 μM Cd.

In order to investigate the seasonal sensitivity of seaweed to heavy metals, further time course studies were undertaken whereby *P. lanosa* (February samples) was exposed to Cd^{2+} over 96 hours and extracted protein levels were assayed. Results from this study indicated increases in extracted protein for the 6 to 72 hour exposure periods. This may indicate phytochelatin synthesis over these time periods. There was however, a drastic

drop in extracted protein from 11.0 ± 1.0 to 6.2 ± 1.2 mg/g dw after 96h exposure. The reduction seen at 96 h may also be explained by a heavy metal toxic effect.

Four seaweed species, collected in May, 2008, were subsequently exposed to Cd^{2+}, Cr^{3+}, Pb^{2+} and Cu^{2+} for 6 hours. Results from these exposure studies demonstrated that there were no changes in total protein content for any of the species. Additionally, no variation in the extracted protein levels was observed apart from two exceptions. Cd^{2+} and Cr^{3+} exposure resulted in a decrease in the extracted protein content of *A. nodosum*. Results indicated a temporal change in the ability of the seaweeds to induce protein synthesis following heavy metal exposure.

Further investigations were undertaken, whereby short-term exposure studies were performed on *P. lanosa*, collected in November, 2008. Following exposure to Cd^{2+} for 6 hours, no increases in total protein and extracted protein were observed. Over this time period, an increase in polyphenol concentrations from 7.3 ± 0.9 to 9.0 ± 0.3 mg/g dw (GAE) was observed. This increase corresponded to a slow elevation in intracellular Cd^{2+} from 2.5 ± 0.02 to 9.7 ± 0.2 mg/kg dw, following six hours exposure. In addition, a substantial increase in both total and surface bound Cd^{2+} (2.5 ± 0.1 to 43.9 ± 0.5 and 0.0 ± 0.02 to 34.3 ± 0.4 mg/kg dw respectively) was observed. An initial rapid increase over the first hour of exposure was followed by a levelling off over the subsequent 5 hours. This levelling off may therefore indicate the saturation of the cell wall with heavy metals over time. This trend generally agrees with literature whereby an initial rapid phase of biosorption is followed by slower uptake of metals (Malik, 2004; Garnham *et al*., 1992).

Results from this study demonstrated temporal variation with respect to seaweed protein concentration following exposure to heavy metals. It was shown that heavy metals have the ability to induce increases in total and extracted protein during February and March however, no such effect was observed for May and November samples.

When seaweeds are subjected to a mixture of metals, glutathione (GSH) may be used in a number of ways (i) to combat oxidative stress, (ii) for the production of phytochelatins (PCs) and/or (iii) to complex certain metals (Pawlik-Skowrońska *et al*., 2007). Consumption of GSH by PC synthesis induces an increase in the rate of GSH synthesis to restore basal levels (García-Ríos *et al.,* 2007). The production of cysteine however,

for PC synthesis does not only require sulphate but also nitrogen (Rijstenbil *et al.*, 1998). The ability to produce these peptides may therefore be related to seaweed nitrogen reserves.

Evidence of protein induction in macroalgae is demonstrated for the first time in *P. lanosa* collected in Feb/March following exposure to heavy metals. No such increase however was observed for samples collected in May and November. Furthermore, a linear increase in intracellular Cd^{2+} corresponded to an overall increase in the polyphenol levels of *P. lanosa*, sampled in November, following heavy metal exposure. Overall results from this study demonstrate seasonal sensitivities of *P. lanosa* to heavy metal exposure. These seasonal trends warrant further investigation.

Chapter 5

Investigation into the Temporal Effects of Heavy Metal Exposure on the Total Protein, Extracted Protein, Polyphenol Levels and Metal Uptake of *Polysiphonia lanosa*

5. Investigation into the Temporal Effects of Heavy Metal Exposure on the Total Protein, Extracted Protein, Polyphenol Levels and Metal Uptake of *Polysiphonia lanosa*

5.1 Introduction

Research carried out in Chapter 4 related to the biochemical effects of heavy metal exposure on seaweeds. Results from the study demonstrated that protein, polyphenol and metal levels in seaweeds were not only influenced by heavy metal exposure but also by seasonal variations. In this chapter, a temporal study was performed in order to investigate the seasonal effects of heavy metal exposure on total protein, extracted protein and polyphenol levels, together with heavy metal uptake capacity of red seaweed. Furthermore, potentiometric titrations and FTIR analysis was performed in order to monitor changes and compare the metal binding capacity of *P. lanosa* at different times of the year. Results from Chapter 4 demonstrated an increase in protein and polyphenol levels of *P. lanosa* collected in February/March, following exposure to 1 ppm Cd^{2+} over 6h. In this chapter therefore, *P. lanosa* exposure studies were conducted under these same exposure conditions.

Seaweeds are extremely efficient biosorbents with the capacity to bind a range of metals. The seaweed cell wall plays an important role in binding. This is due to the occurrence of various functional groups such as carboxyl, amino, sulphate and hydroxyl groups, which are capable of acting as binding sites for metals (Murphy *et al.*, 2007). The main mechanisms of binding include ionic interactions and complex formation between metal cations and ligands on the surface of the seaweeds. Biosorption may be based on one or more of the following mechanisms: ion-exchange, physical adsorption, complexation and precipitation (Murphy *et al.*, 2007).

Many techniques have previously been performed to study metal binding to algal biomass. These include potentiometric titrations, infrared spectroscopy (Fourest & Volesky, 1996), X-ray photoelectron spectroscopy (Sheng *et al.*, 2004) and Atomic Force Microscopy (Gunning *et al.*, 1998).

Several authors have used potentiometric titrations in order to establish the number of acidic functional groups on the biomass surface, including *Sargassum fluitans* (Fourest & Volesky, 1996), *Sargassum* sp and *S. filipendula* (Davis *et al*., 2000) and *F. vesiculosus, P. palmata* and *Ulva* spp (Murphy *et al*., 2007) and consequently to predict heavy metal binding to the surface. The analysis of potentiometric titration curves demonstrates the influence of pH on the deprotonation of the functional groups associated wit the biomass surface, which are capable of binding metals (Esposito *et al*., 2002; Pagnanelli *et al*., 2003).

Fourest and Volesky (1996) demonstrated that potentiometric titrations provided information concerning the amounts of strong and weak acidic functional groups in the biomass as well as allowing the pKa characteristic of the algae to be determined. Strong acidic groups generally refer to sulphonate functional groups, while weak acidities are carboxyl groups.

For pH values larger than the pKa, binding sites are primarily in the dissociated form and therefore can exchange H^+ with metal ions in solution. To quantify the potential metal cation uptake by seaweeds, the number of functional groups and their associated pKa values must firstly be determined. In general, the protonated biomass may be titrated with a base (usually sodium hydroxide). The analysis of results usually shows that the cell wall seems to have two or more main functional groups responsible for metal sorption (Davis *et al*., 2000).

If the only active sites are those titrated, the metal sorption abilities of the selected biosorbents are strongly influenced by pH as reported by the following equilibrium equation:

$$Me^{2+} + 2B\text{-}H \longrightarrow B_2Me + 2H^+ \quad \textbf{(Equation: 6.1)}$$

Where Me^{2+} is the heavy metal in solution (divalent form), B-H corresponds to the protonated biomass and B_2Me represents the complex biomass-metal (Esposito *et al*., 2002).

Knowledge relating to the chemical composition of the algal cell wall may help to elucidate the metal binding mechanisms of seaweeds. In this study potentiometric

titrations were performed for the first time in order to determine seasonal changes in the number of acidic functional groups on the biomass surface of *P. lanosa*. The quantity of functional groups available corresponds to the metal binding capacity of the seaweed. Therefore, direct relationships between surface bound Cd^{2+} of *P. lanosa* and its functional groups were determined. Furthermore, comparisons in levels of total protein, extracted protein and polyphenol, in addition to heavy metal uptake were made for *P. lanosa* collected at three different times of the year. Furthermore, this work uses complementary techniques (potentiometric titrations and FTIR analysis) to provide a clear understanding of metal binding in algal cell walls.

5.2 Objectives of Research

The aims of this research were to carry out a temporal study and thus determine the seasonal changes in total protein, extracted protein, polyphenol and metal concentration of *Polysiphonia lanosa,* prior to and after exposure to 1 ppm Cd.   The overall aims of this study were as follows:

- To determine temporal variations of baseline total protein, extracted protein and polyphenol levels of *P. lanosa* and compare these values with those in literature. Following exposure to Cd^{2+}, seasonal variation in total protein, extracted protein and polyphenol levels were determined and compared with baseline values.

- Temporal changes in the total, intracellular and surface bound Cd^{2+} concentrations of *P. lanosa* collected at different times of the year were investigated and these values were compared with those in literature. Following exposure, the seasonal variation in Cd^{2+} uptake levels were determined and the impacts of the metal uptake ability of *P. lanosa* were investigated.

- Temporal correlations between concentrations of protein and polyphenols with seaweed metal levels were investigated.

- Potentiometric titrations and FTIR analysis were conducted in order to determine and compare the quantity of metal binding sites of *P. lanosa* collected in February, May and November. Additionally, temporal changes in the ability of *P. lanosa* to bind metals were monitored.

- Correlations between the numbers of binding sites for each sampling month and the concentrations of surface bound metals of *P. lanosa* were determined.

Previous research has demonstrated seasonal variations in total protein (Marinho-Soriano, 2006), extracted protein (Joubert & Fleurence, 2008) and polyphenol (Kamiya *et al*., 2010) levels of seaweed. In the first study of its kind, all three parameters were studied simultaneously for the red seaweed *P. lanosa*. Furthermore, the effects of heavy metal exposure on the protein and polyphenol levels of *P. lanosa* were also assessed for the first time. Additionally, in an original study, potentiometric titrations and FTIR analysis were employed to determine seasonal changes in the cell wall functionalities of seaweed.

5.3   Experimental

5.3.1   Chemicals

- Sodium Chloride (Solid) Ridel de Haën, Germany

- Hydrochloric acid (37%) LabScan ltd., Dublin, Ireland

- Buffers pH 4 (± 0.02) and 7 (± 0.02) Ridel de Haën, Germany

5.3.2   Instrumentation

- Glove box – Plas labs Inc., Model No. 818GB

- Digilab Scimitar Series InfraRed spectrometer (JVA Analytical Ltd, Dublin, Irl)

- FTIR analysis was performed using: MIRacle[TM] Single Reflection HATR accessory, Bio-Rad Win-IR Pro 3.1 software (JVA Analytical Ltd, Dublin, Ireland) and DTGS detector (JVA Analytical Ltd, Dublin, Ireland).

5.3.3   Seaweed Collection and Preparation

P. lanosa was collected from Fethard-on-Sea Co. Wexford, Ireland (52.39°N, 6.94°W), in February, May and November 2008. The samples were rinsed thoroughly with seawater in order to remove any adhering debris. The plants collected from various locations on the site were combined to give composite batches. On returning to the laboratory, seaweeds were immediately placed in holding tanks, containing seawater, at a temperature of 12°C. Seaweeds were exposed to a light: dark regime of 12:12 hours before and during the experiment. Interpet Airvolution AV4 pumps were used for agitation and aeration. Rectangular tanks containing 5 L of seawater (Source: Kilfarrassey, Tramore Co. Waterford (52°8'4.80"N, 7°13'54.56"W) were used as reaction vessels. Salinity, temperature and pH were kept constant during the experiments.

5.3.4   Seaweed Exposure

P. lanosa, collected from Fethard-on-Sea in February, May and November, 2008 was exposed to 1 ppm Cd^{2+} for 6h. Following exposure, replicate samples (n=3) were taken and washed in deionised water, in order to remove any excess metal solution (Wang & Dei, 1999). Samples were blot dried, then oven dried at $60^{o}C$ for 24h. Samples were ground to a particle size of ≤500μm and stored in polyethylene bottles until required for analysis.

Following exposure, a portion of seaweed was rinsed twice, for 10 minutes in a 5.0 mM EDTA solution pH 8.0 in order to remove any surface bound metals. These samples were subsequently rinsed twice in deionised water, blot dried, then finally, oven-dried for 24h at 60°C. Samples were ground to a particle size of ≤500μm. These samples were used to determine intracellular metal concentrations.

5.3.5   Total Protein Determination

Refer to 2.3.8

5.3.6   Protein Extraction

Refer to 2.3.4.6

5.3.7   Extracted Protein Determination

Refer to 2.3.5

5.3.8   Polyphenol Extraction

Refer to 2.3.6.6

5.3.9   Polyphenol Determination

Refer to 2.3.7

5.3.10 Metal Analysis

Refer to 3.3.15

5.3.11 Protonation Titrations

5.3.11.1 Protonation of the Biomass

5 g of biomass particles were protonated by washing with 250mL of 0.1M HCl. This treatment ensured that any remaining salts e.g. Ca^{2+}, Mg^{2+}, Na^{2+}, K^+ were removed from the seaweed surface. The suspension was allowed to stir for 6h to ensure that equilibrium had been reached. The biomass was filtered under vacuum and washed with distilled water until a constant conductance was obtained for the filtrate. The protonated biomass was oven-dried at 60°C for 24h and stored in polyethylene bottles until required (Murphy *et al*., 2007).

5.3.11.2 Titration of biomass

200 mg of protonated biomass was dispersed in 100mL of a 1mM NaCl solution. Titrations were carried out by stepwise addition of 0.25mL of 0.1M NaOH (prepared with boiled distilled water) to the flask while the suspension was stirred under a nitrogen atmosphere (Glove Box- Model 818-GB/220, Plas Labs, Inc.). After each addition, the system was allowed to equilibrate until a stable pH reading was obtained. Conductivity was measured using a WTW LF 538 Conductivity meter with WTW TetraCon® 325 probe. pH measurements were recorded using a Mettler Toledo MP 220 pH meter with a Mettler Toledo Inlab® 413 pH electrode. The pH electrode was calibrated with buffers pH 4 (± 0.02) and pH 7 (± 0.02) prior to use and the conductivity probe was checked by measuring the conductivity of a 0.01M KCl solution. Potentiometric titrations were carried out in triplicate with errors bars calculated to 95% confidence intervals (Murphy *et al*., 2007).

5.3.12 Fourier Transforms Infra-Red (FTIR) analysis of protonated *P. lanosa* samples.

Protonated February, May and November sample spectra were obtained using a Digilab Scimitar Series InfraRed spectrometer, employing a MIRacle^{TM} single reflection HATR accessory with up to 815 lbs of pressure to keep the sample in optical contact with the diamond. Data processing was performed using Bio-Rad Win-IR Pro 3.1 software. Spectra were recorded using a DTGS detector in the wave number range 4,000-700 cm^{-1} at a resolution of 2. An average number of scans of 40 was used to generate spectra.

5.4   Results

5.4.1   Total Protein Determination

Seasonal differences in total protein values for control February, May and November samples were observed (29.8, 37.1 and 25.2 % dw, 298, 371, 25.2 mg/g dw) respectively) (Figure 5.1). Highest total protein levels were observed between February and May, whereas lowest values were recorded for *P. lanosa* collected in November.
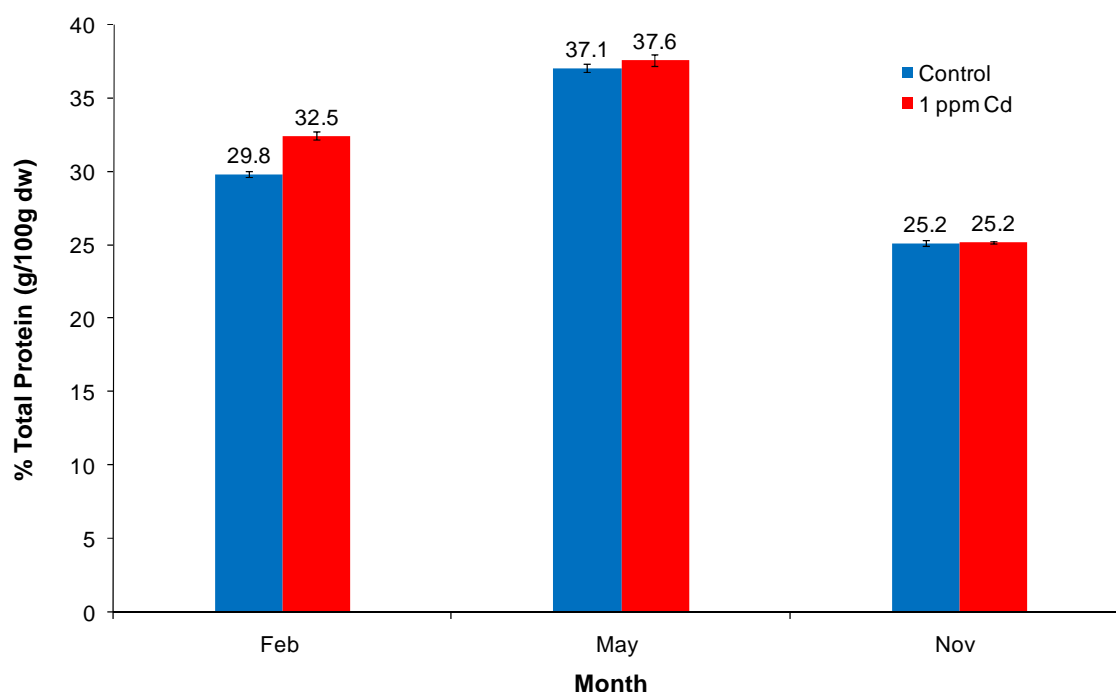
Figure 5.1. Total protein content of February, May and November *P. lanosa,* following exposure to 1 ppm cadmium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

Table 5.1 compares the seasonal variation of total protein for red seaweeds. In this study, a similar trend to that of *P. palmata* was attained in that May yielded the highest total protein and November the lowest. In all cases however, May samples yielded the highest total protein. This is the first time that, temporal changes in the total protein content of *P. lanosa* have been documented.

Table 5.1. Seasonal variation in total protein content of red seaweeds

Seaweed Species	Feb % Total Protein (dw)	May % Total Protein (dw)	November % Total Protein (dw)
P. lanosa [§]	29.8 ± 0.2	37.1 ± 0.3	25.2 ± 0.2
P. palmata [1]	11.0	17.5	9.0
G. cervicornis [2]	14.5	21.5	19.0

[§] Present study; [1] Martinez & Rico, 2002; [2] Marinho-Soriano *et al*., 2006.

Following exposure to Cd^{2+} for 6 hours, results demonstrated an increase in the February total protein from 29.8 to 32.5 % dw (298 to 325 mg/g dw). In contrast, there was no increase in May or November total protein following exposure. This is consistent with results from Chapter 5.

5.4.2 Extracted Protein Determination

Initial protein concentration for February, May and November samples were recorded as 14.9 ± 1.4, 8.1 ± 0.6 and 5.1 ± 1.0 mg/g dw respectively. As with total protein values, seasonal variations in the extracted protein levels for control samples were observed. In contrast however, February had the highest extracted protein (14.9 mg/g dw) and November the lowest (5.1 mg/g dw) (Figure 5.2).

Following exposure to 1 ppm Cd^{2+}, results for extracted protein demonstrated a similar trend to that of total protein. Both February samples produced obvious increases in total and extracted protein (29.8 – 32.5 % dw and 14.9 – 25.1 mg/g dw respectively). In addition, however, there was little or no change in May or November extracted protein (Figure 5.2). This trend was also observed for extracted protein values in Chapter 4, whereby Cd^{2+} exposure induced increased in extracted protein for February *P. lanosa* only (Figure 4.7 and 4.8).
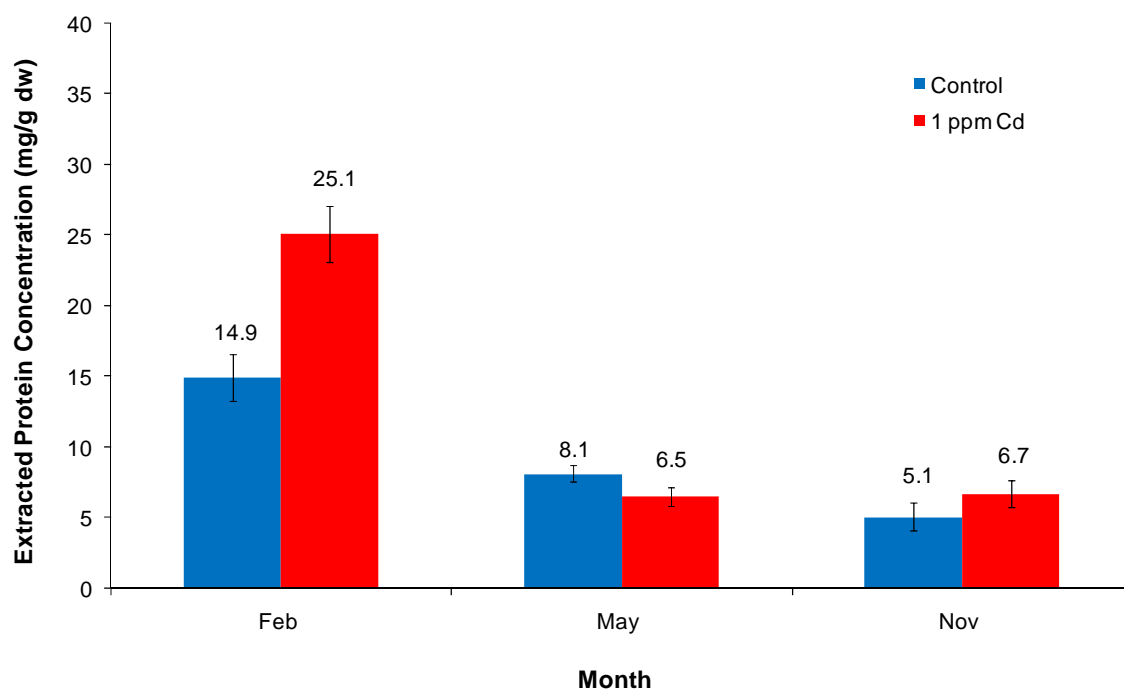
Figure 5.2. Extracted protein content of February, May and November *P. lanosa,* following exposure to 1 ppm cadmium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

5.4.3   Polyphenol Determination

Results for control samples reveal no significant differences in polyphenol concentrations for February and May *P. lanosa*. The November samples yielded a slightly lower polyphenol concentration when compared with the other two sampling periods (Figure 5.3).
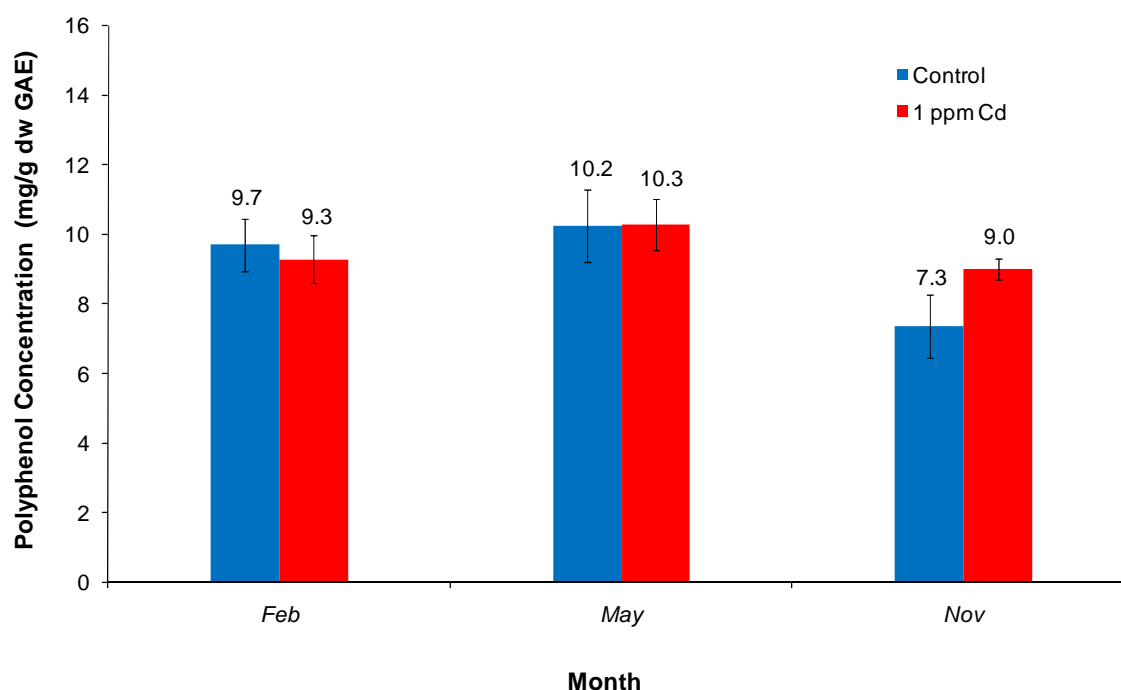
Figure 5.3. Polyphenolic concentration of February, May and November *P. lanosa,* following exposure to 1 ppm cadmium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

Table 5.2 demonstrates the seasonal variations in polyphenol levels of various seaweeds. In relation to the brown species, all seaweeds, with the exception of *S. confusum* yielded highest polyphenol levels in May.

Table 5.2. Comparison of seasonal variations of seaweed polyphenols

	Seaweed Species	Feb Polyphenols (mg/g dw)	May Polyphenols (mg/g dw)	November Polyphenols (mg/g dw)
Red	**P. lanosa [§]**	**9.7 ± 0.7**	**10.2 ± 1.0**	**7.3 ± 0.9**
Brown	*F. vesiculosus* [1]	12.5	45.0	20.0
	C. tamariscifolia [2]	10.0	52.0	40.0
	S. muticum [3]	9.0	60.0	8.0
	S. confusum [4]	75.0	60.0	127.0
	S. hemiphyllum [4]	50.0	75.0	70.0

[§] Present Study; [1] Peckol *et al*., 1996; [2] Abdala-Díaz *et al*., 2006; [3] Plouguerné *et al*., 2006; [4] Kamiya *et al*., 2010.

There was no change in the concentration of February or May polyphenols following heavy metal exposure. The polyphenolic content of November samples however increased slightly from 7.3 to 9 mg/g dw following exposure to Cd^{2+} (Figure 5.3).

5.4.4 Seaweed Metal Analysis

Results from this study demonstrated that the initial total cadmium content for all three control samples, ranged from 2.5 – 2.8 mg/kg dw. Intracellular metals for May and November were 2.4 and 2.5 mg/kg dw respectively. February yielded the lowest intracellular Cd^{2+} content at 0.8 mg/kg dw. February, May and November surface bound metals were 2.0, 0.3 and 0 mg/kg dw respectively (Figure 5.4).

Table 5.3 illustrates seasonal variation of Cd in various seaweed species.

Table 5.3. Seasonal variation of Cd (mg/kg dw) in various seaweed species.

Seaweed Species	Feb/Spring	May/Summer	November/Winter
P. lanosa [§]	**2.8 ± 0.05**	**2.7 ± 0.1**	**2.5 ± 0.05**
U. rigida [1]	1.0	0.95	0.2
P. columbina [2]	6.0	3.75	9.8
Ulva sp [2]	2.5	0.80	2.0

[§] Present Study; [1] Haritonidis & Malea, 1999; [2] Perez *et al.*, 2007.

Statistical analysis (*p<0.05*) however demonstrated significant variations in the total Cd^{2+} concentration of *P. lanosa* between February/May and November samples. The *p* value for One-Way ANOVA was 0.002. Statistical analysis of intracellular Cd^{2+} also demonstrated significant variations with respect to season (*p* value was 0.0).
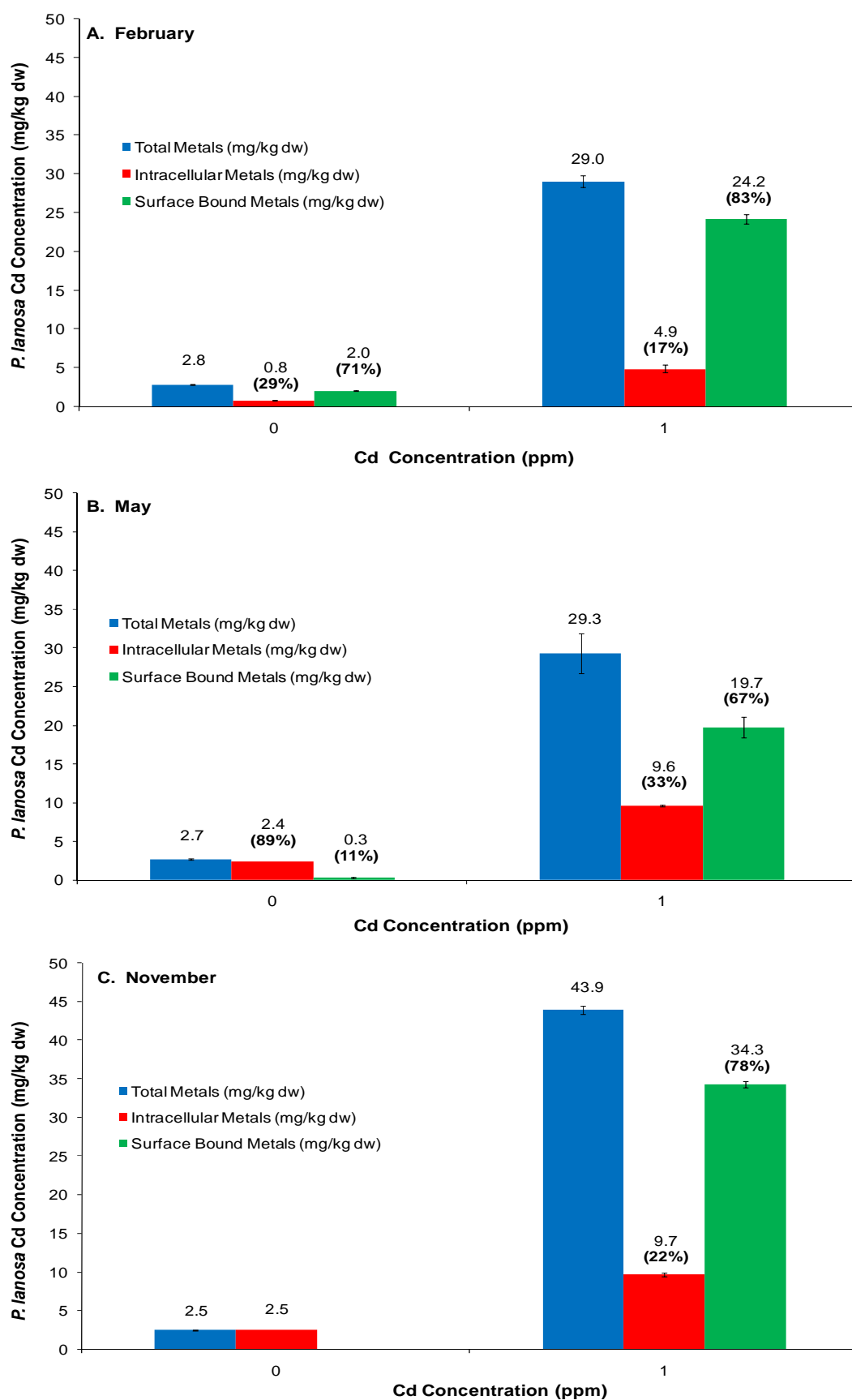
Figure 5.4. Metal concentration in *P. lanosa* sampled in February, May and November, following exposure to 1 ppm cadmium for 6 hours. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

Following exposure, total, intracellular and surface bound metal concentrations of *P. lanosa* increased significantly for all three months (Figure 5.4). Intracellular February, May and November Cd^{2+} accounted for 17, 33 and 22% dw of total Cd^{2+} respectively (Figure 5.4). February, May and November surface bound Cd^{2+} accounted for 83, 67 and 78% dw of total Cd^{2+} respectively (Figure 5.4). Lowest intracellular uptake of Cd^{2+} (17%) was associated with the *P. lanosa* sampled in February. Additionally, February was the only month to see an increase in total protein and extracted protein following heavy metal exposure. The lowest intracellular levels of Cd^{2+} were therefore capable of inducing an increase in protein during this period.

One-Way ANOVA demonstrated significant variations in intracellular levels of Cd^{2+} of the exposed *P. lanosa,* with respect to month sampled ($p = 0.001$). Furthermore, significant variations in total Cd^{2+} between Feb/May versus November samples were also demonstrated ($p = 0.001$).

A summary of control values are demonstrated in Table 5.4. Furthermore One-Way ANOVA ($p <0.05$) demonstrated significant variations in levels of total and intracellular Cd^{2+} ($p = 0.001$ and $p = 0.002$. respectively), in addition to concentrations of total protein ($p = 0.001$), extracted protein ($p = 0.001$) and polyphenols ($p = 0.01$) for control samples, when times of the year are taken into account.

Table 5.4. Summary of control values for protein, polyphenols and metals.

Control Values	Feb	May	Nov	One Way ANOVA (p<0.05)
Total Metals (mg/kg dw)	2.8	2.7	2.5	0.002
Intracellular Metals (%)	29.0	89.0	100.0	0.001
Surface Bound Metals (%)	71.0	11.0	0.0	——
Total Protein (% dw)	29.8	37.1	25.2	0.001
Extracted Protein (mg/g dw)	14.9	8.1	5.1	0.001
Polyphenols (mg/g dw GAE)	9.7	10.2	7.3	0.01

Regression analysis (fitted line plot) demonstrated a negative correlation for control levels of intracellular Cd^{2+} and extracted protein when the time of year is taken into account. No correlations between total protein versus total Cd^{2+} ($R^2 = 12.6\%$) and polyphenols versus intracellular Cd^{2+} ($R^2 = 0.0\%$) were however demonstrated.

A summary of exposed values are demonstrated in Table 5.5. Additionally, One-Way ANOVA (*p*<0.05) demonstrated significant variations in levels of total and intracellular Cd^{2+} (*p* = 0.001 and *p* = 0.001, respectively), as well as concentrations of total protein (p = 0.001), extracted protein (*p* = 0.001) and polyphenols (*p* = 0.024) for exposed samples, when times of the year are taken into account.

Table 5.5. Summary of exposed values for protein, polyphenols and metals.

Exposed Values	*Feb*	*May*	*Nov*	*One Way ANOVA (p<0.05)*
Total Metals (mg/kg dw)	29.0	29.3	43.9	0.001
Intracellular Metals (%)	17.0	33.0	22.0	0.001
Surface Bound Metals (%)	83.0	67.0	78.0	———
Total Protein (% dw)	32.5	37.6	25.2	0.001
Extracted Protein (mg/g dw)	25.1	6.5	6.7	0.001
Polyphenols (mg/g dw GAE)	9.3	10.3	9.0	0.024

Regression analysis (fitted line plot) also demonstrated a strong negative correlation for exposed levels of intracellular Cd^{2+} and extracted protein in relation to time of year. Furthermore, a strong negative correlation for exposed total Cd^{2+} and total protein was also demonstrated. No correlations between polyphenols versus intracellular Cd^{2+} (R^2 = 0.0%) were however demonstrated.

5.4.5 Potentiometric Titrations

Potentiometric titrations have been used by previous researchers to determine the quantity and nature of acidic binding sites on the seaweed surface (Fourest & Volesky, 1996; Davis *et al.,* 2000; Murphy *et al*., 2007; Herrero *et al*., 2008). Once the quantity of available functional groups has been established, their relationship with the maximum metal binding capacity of the seaweed can be determined.

5.4.5.1 Potentiometric Titration Curves

The various amounts of acidic groups in the biomass and their corresponding pKa values were evaluated by identifying the inflection points of the titration curves (Figure 5.5). However, this can be quite difficult and a better indication of the position of these inflections was obtained from first derivative plots of average pH titration data (Figure 5.6). The location of each peak on the x-axis gives the number of acidic groups on the

biomass surface (Murphy *et al*., 2007). Figure 5.5 illustrates the first derivative plots obtained for *P. lanosa* collected in February May and November. Temporal variations in the first derivative plots of seaweed are presented for the first time.
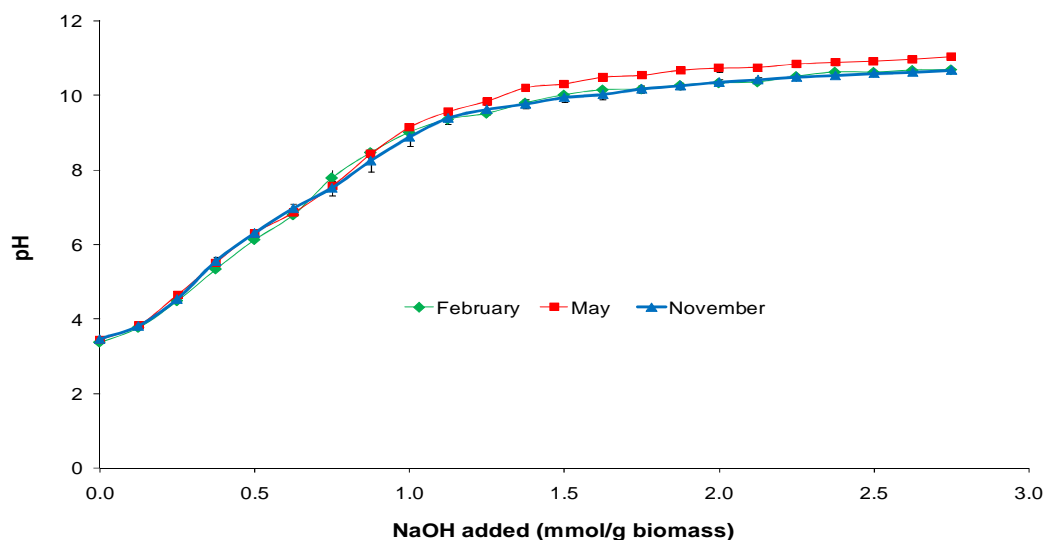
Figure 5.5. Potentiometric Titration Curves for February, May and November *P. lanosa*. Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

5.4.5.2 First Derivative Plots

The number of strong acidic groups was determined from the first peak in each of the first derivative plots while the total number of acidic groups for a given weight of biomass was determined from the final peak. The number of weak acidic groups was then calculated by difference (Figure 5.6). Once these values were established, the corresponding pKa values were then identified from the original titration curve (Figure 5.5).
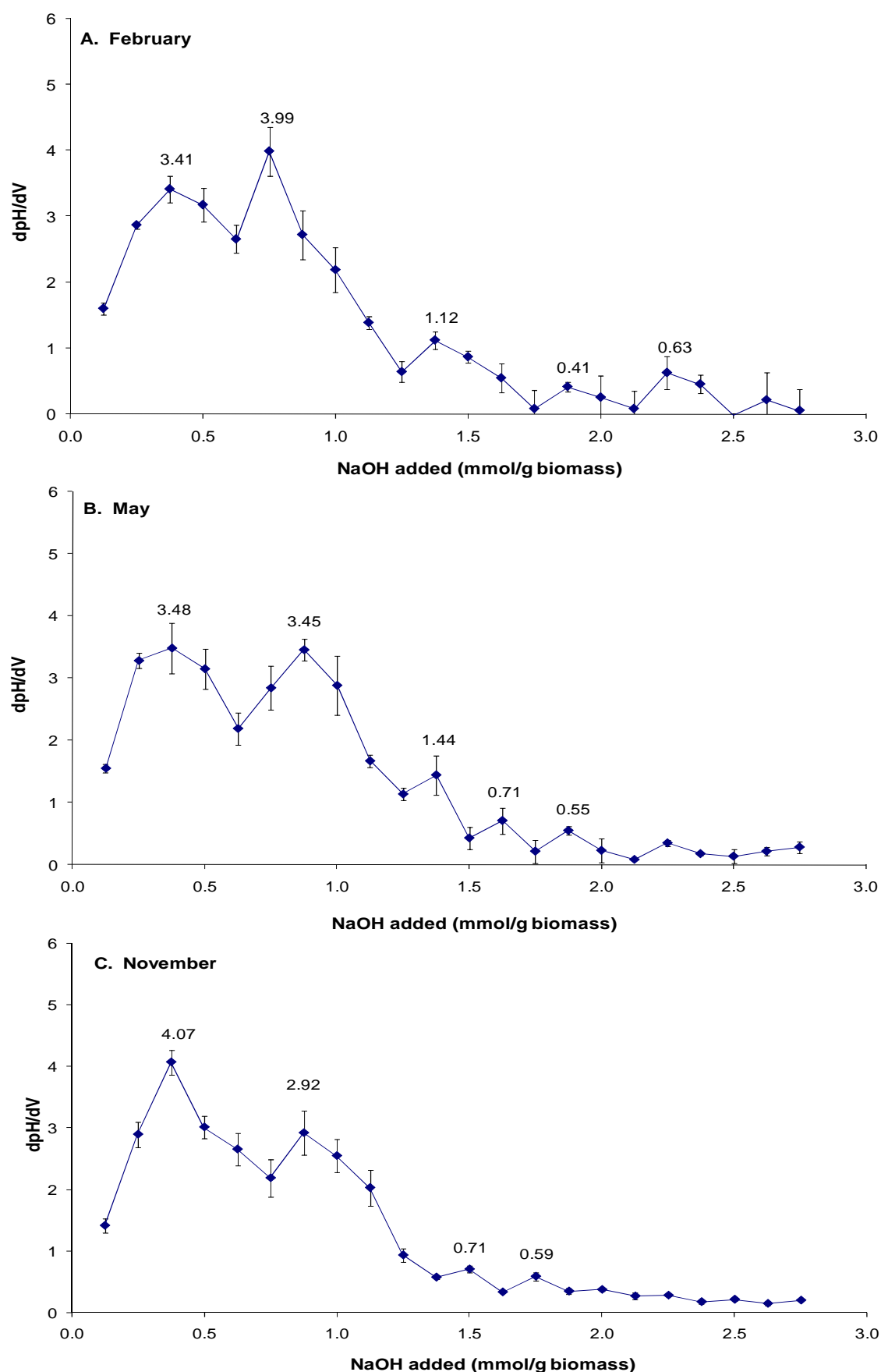
Figure 5.6. First Derivative plots of average pH titration data for November *P. lanosa*.

Error bars were calculated based on triplicate samples with 95% Confidence Intervals.

5.4.5.3 pKa Values

The pKa values and the number of acidic groups on the biomass surface are summarised in Table 5.6. The results of potentiometric titrations demonstrated the presence of hydroxyl, amino and carboxyl functions groups for all three months (Table 5.6).

The pKa values obtained experimentally in this study (pKa 5.34, 5.52 and 5.55 for February, May and November samples respectively), (Table 5.6) generally correspond to carboxyl groups (Murphy *et al.,* 2007).

Additionally, while February and May samples produces five pKa peaks, the November *P. lanosa* produced only four. February and May *P. lanosa* had 2 to 3 pKa values ~ 10 (hydroxyl groups), the November sample only had one in this region (Table 5.6).

Furthermore, pKa values ~ 7-9 corresponding to amine groups were observed for all three sampling periods (Table 5.6)

Table 5.6. pKa values and number of acidic groups of February and May *P. lanosa* seaweed samples as determined by titration. Errors are calculated based on triplicate samples with 95% Confidence Intervals.

Month	pKa Values	*Acidic Groups* (*mmol/ g biomass*)		
		Total	**Strong**	**Weak**
February	5.34 ± 0.1	2.25 ± 0.25	0.38 ± 0.20	1.87 ± 0.23
	7.79 ± 0.2			
	9.80 ± 0.1			
	10.28 ± 0.1			
	10.52 ± 0.1			
May	5.52 ± 0.1	1.88 ± 0.05	0.38 ± 0.10	1.5 ± 0.08
	8.42 ± 0.1			
	10.2 ± 0.1			
	10.49 ± 0.1			
	10.68 ± 0.1			
November	5.55 ± 0.1	1.75 ± 0.07	0.38 ± 0.18	1.37 ± 0.13
	8.25 ± 0.3			
	9.94 ± 0.1			
	10.17 ± 0.1			

It is generally thought that carboxyl groups (weak) are primarily responsible for metal sorption, (especially in brown seaweeds) (Murphy *et al*., 2007). Figure 5.9 indicates the presence of additional inflection points in the carboxyl region. It can be suggested therefore that these inflection points may in fact represent the different orientations of constituent carboxyl groups thus leading to more than one observed pKa for these groups. These may also be linked to different carboxyl groups available for metal binding. The repeatability of these inflections over triplicate runs indicates that these are not just artifacts of the experiment but true inflection points (Murphy *et al*., 2007).

5.4.5.4 Acidic Groups

All three *P. lanosa* samples contained the same number of strong acidic groups (0.38 mmol/g biomass). Significant differences however were demonstrated among the numbers of total acidic groups for *P. lanosa* collected in February, May and November (2.25, 1.88 and 1.75 mmol/g biomass, respectively), (Table 5.6). Weak acidic groups, demonstrated significant variation for February, May and November samples (1.87 ± 0.23, 1.5 ± 0.08 and 1.37 ± 0.13 mmol/g, respectively), (Table 5.6).

5.4.6   FTIR analysis of protonated *P. lanosa* samples.

In this study, Mid-Infrared spectroscopy was used to investigate the temporal changes in the surface functionality of seaweed for the first time. Table 5.7 demonstrates the commonly observed stretching frequencies in seaweed FTIR spectra.

FTIR analysis demonstrated that the main groups associated with metal binding in *P. lanosa* were carboxyl, amino and sulphonate groups. (Figure 5.7). Arrows indicate symmetric $-SO_3$ stretching. Furthermore, FTIR analysis demonstrated seasonal variations in the (Amide) 1600-1500 cm^{-1} region. For example, bands in the 1516 cm^{-1} region appear in the protonated February and May samples. However, for the November samples, this band appears in the 1530 cm^{-1} region. Variations in the distributions of the carboxyl groups (~1600 and ~ 1034 cm^{-1}) associated with the cell wall surface were observed for the November samples when compared to those of the February and May samples (Figure 5.7).

Table 5.7. Commonly observed stretching frequencies in seaweed FTIR spectra (Clothup *et al.,* 1990).

Wavenumber (cm^{-1})	Assignment
3200-3500	Bonded –OH, -NH stretching
2920	Asymmetric stretch of aliphatic chains (-CH)
2854	Symmetric stretch of aliphatic chains (-CH)
1740	Free C=O stretch of COOH
1630	Asymmetric C=O (COOH)
1530	-NH stretch
1450	Symmetric C=O (COOH)
1371	Asymmetric –SO_3 stretching
1237	C-O stretch of COOH
1160	Symmetric –SO_3 stretching
1117	C-O (ether)
1033	C-O (alcohol)
817	S=O stretch
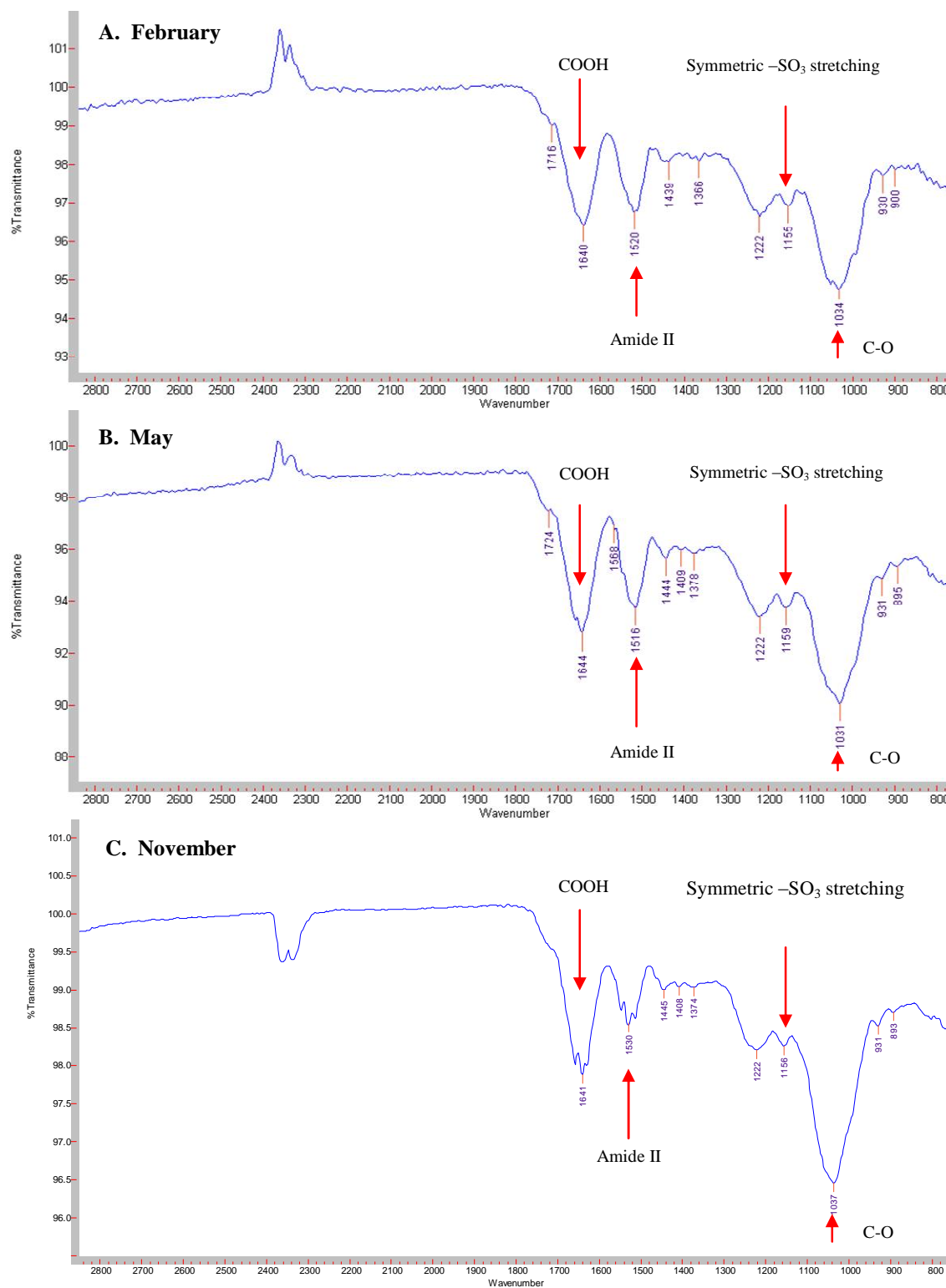
Figure 5.7. FTIR spectrum of (A) February, (B) May and (C) November Protonated *P. lanosa* Biomass. Sample spectra from triplicate samples are shown.

5.5   Discussion

5.5.1   Total Protein Determination

Results from this study demonstrated seasonal variations in the total protein content of *P. lanosa*. The total protein content of seaweed is known to vary from season to season (Fleurence, 1999). An annual monitoring of protein level from *Palmaria palmata* collected on the French Atlantic coast between February 1996 and January 1997 demonstrated that the protein content of these algae can vary between 9 and 25% dw. Seasonal variations in the total protein content of seaweeds are attributed to temperature, salinity and nutrient availability (Marinho-Soriano *et al*., 2006). Furthermore, studies carried out in Chapter 3 of this study demonstrated a strong positive correlation between the total protein content of *F. vesiculosus* and temperature (Chapter 3, Figure 3.30).

The seasonal variation of the algal protein content was also reported for various seaweed species such as *Gracilaria cervicornis* (red) and *Sargassum vulgare* (brown), collected from the North-West coast of Brazil between July 2000 and June 2001 (Marinho-Soriano *et al*., 2006). The lowest protein for the red seaweed was found between November and February and the highest protein between April and August. Furthermore, the lowest protein for the brown seaweed was between October and June, while the highest between July and September (Marinho-Soriano *et al*., 2006). In a study by Martinez & Rico (2002), *P. palmata* was collected from the North coast of Spain between June 1998 and July 1999 and total protein levels monitored. It was demonstrated that the lowest total protein was found during August and November and the highest during March and May. These values are relatively consistent with findings from this study, whereby the highest total protein concentrations were yielded during Summer (37.1 % dw (371 mg/g dw)) and the lowest during Winter (25.2% dw (252 mg/g dw)).

Furthermore, seasonal changes in total protein correspond to seaweed growing seasons. Generally, the highest growth rates are during Spring and Summer. For example, the red seaweed *Polysiphonia elongata* from the coast of Wales reached its optimum growth rate in early Spring (Jones & Williams, 1966). Furthermore the highest growth rate of the brown algae *Laminaria japonica* was between December and October

(Honya *et al*., 1994). Additionally, in their study, Stengel & Dring (1997) showed that the growth of *A. nodosum* is extremely seasonal, with low (but not zero) growth rates during November and December and highest growth rates in late Spring and early Summer (Stengel & Dring, 1997). At the start of the growing season, seaweed proteins consist of reserve proteins and protoplasmic proteins. As a result of rapid growth however, the reserve proteins are utilised to produce protoplasm; and synthesis of proteins and consequently growth will continue as long as inorganic N is present in the seaweed. By August, the reserve proteins may be converted to protoplasm and the absence of inorganic N in the seaweed (and seawater) will reduce the growth rate (Black & Dewar, 1949).

Following exposure to Cd^{2+} for an increase in the total protein content of the February samples from 29.8 to 32.5 % dw (298 to 325 mg/g dw) was observed. This increase is perhaps indicative of the synthesis of proteins on exposure to Cd^{2+}. The increase also corresponds to a previous 24 h exposure study (Chapter 5, Figure 5.1), which saw an increase in *P. lanosa* (March) total protein following 6 h exposure to 1 ppm Cd^2

No increases in the May or November total protein levels were observed following exposure. This too is consistent with results from Chapter 5. The increase in protein for only the February samples following heavy metal exposure maybe indicative of the synthesis of metal-binding peptides. For example, nitrogen is required for PC synthesis and the highest N in *P. palmata* was observed in Autumn and Winter (Martínez & Rico, 2002). Furthermore, it was also demonstrated that Cd^{2+}-stimulated PC synthesis of *E. Prolifera* was associated with N-rich algal pools (Malea *et al*., 2006).

5.5.2   Extracted Protein Determination

Seasonal variations in the extracted protein levels for control samples were observed. February had the highest extracted protein (14.9 mg/g dw) and November the lowest (5.1 mg/g dw). Variations may reflect seasonal changes in algal cell wall polysaccharides (Cossan *et al*., 1990). Cell wall polysaccharides makes seaweed protein extraction difficult (Fleurence *et al.,* 1998a). Therefore, seasonal changes in the cell wall polysaccharide composition of *P. lanosa* may have contributed lower soluble protein yields in May and November. For example, carrageenans, the sulphated

polysaccharides found in the cell wall of red seaweed were found to vary with season (Cossan *et al.*, 1990). In addition, at lower temperatures, the plasma membrane lipids of submerged plants become less permeable (Fritioff *et al.*, 2005). Information regarding the temporal changes in extracted protein levels of seaweeds is limited however. Joubert & Fleurence (2008), studied changes in the concentrations of extracted protein from *P. palmata* collected along the French coast during the months of April and July. They found that the April samples contained more than 2.5 times more protein than that of the July samples (4.62 and 1.65 mg/g dw* respectively). This variation was attributed to the N content of red seaweeds and of seawater. Even though *P. lanosa* contained higher extracted protein levels when compared with *P. palmata*, a similar trend was observed in that Spring seaweeds contained higher protein levels than those collected in Summer. * Based on fresh weight (fw) to dry weight (dw) conversion factor for *P. palmata* (Gordillo *et al*., 2006).

Following exposure, increases in the extracted protein levels of *P. lanosa* were observed for the February samples, with no change in May or November values. Induction of protein in the February samples and the lack of protein increases in May and November samples may be indicative of seasonal variations in seawater N (Martínez & Rico, 2002; Rijstenbil *et al.*, 1998a). Additionally, growth in *A. nodosum* was shown to be strongly seasonal (Stengel & Dring, 1997), this therefore may influence algal nutrient utilisation, rendering amino acids unavailable for protein synthesis during the growing season.

Rijstenbil *et al.* (1998a) observed an increase in the PC concentration of *T. pseudonana* (diatom) following exposure to Cu^{2+} in Winter samples only (Rijstenbil *et al.,* 1998a). Furthermore, Cd stimulated the synthesis of PC in *E. prolifera*, suggesting that in N-rich algae, glutathione pools were sufficiently high for PC synthesis (Malea *et al.,* 2006). Induction of metal binding peptides in the February samples may therefore be reflected in an increase in the overall protein content, both total and extracted, following exposure to Cd^{2+}. Phytochelatins are induced by, and bind to, heavy metals. The strength of induction depends on the metal species and Cd^{2+}has been reported as a good inducer of PCs in *Salix viminalis* (willow) (Landberg & Greger, 2004). It is perhaps not surprising therefore to see an increase in protein concentration of the February samples of *P. lanosa* following exposure to this heavy metal.

A strong dependence of *P. palmata* growth on nutrient enrichment in Summer was demonstrated by Martínez & Rico (2002). It was subsequently suggested that nutrient demands for growth were greater than external N supply. These limiting conditions resulted in the reserved material being diverted to growth. This therefore contributed to the decrease of all algal nutrient pools by the addition of new biomass (Martínez & Rico, 2002). The total protein and extracted protein content of May and November *P. lanosa* did not change following Cd^{2+} exposure (Figures 5.1 and 5.2). Rijstenbil *et al.* (1998) however demonstrated a strong decrease in cysteine and glutamate pools of a diatom as a result of nitrogen depletion. Perhaps therefore, a depletion of stored N caused by algal growth resulted in a deficiency in the levels of N available for GSH and subsequently, PC synthesis.

The seasonal variation in total N of the brown algae *Macrocystis intergrifolia* and *Nereocystis luetkeana* were previously demonstrated by Rossell & Srivastava (1985). The highest N content for *M. integrifolia* was found in March samples and lowest concentrations from May – July (1981) samples. Nitrogen contents were determined in duplicate on a Carlo-Erba model 1106 elemental analyser (Rossell & Srivastava, 1985). This N trend is relatively consistent with seasonal protein values in this study. High N in March corresponded to high levels of protein in February and May *P. lanosa*. Utilisation of N as a result of algal growth saw a depletion of N pools from May – July resulting in lower protein levels in November samples.

5.5.3 Polyphenol Determination

Results demonstrated seasonal variations in the polyphenol content of *P. lanosa*. Previous studies have shown that the polyphenolic content of brown seaweeds varies with season due to environmental factors such as nitrogen availability (Ragan & Glombitza 1986; Arnold *et al.*, 1995) and UV radiation (Pavia *et al.*, 1997; Pavia & Brock 2000). Much of the work regarding seasonal variations of algal polyphenols focuses on brown seaweeds (Ragan & Jensen, 1978; Peckol *et al.*, 1996; Abdala-Díaz *et al.*, 2006; Plouguerné *et al.*, 2006; Kamiya *et al.*, 2010). Reported here for the first time are seasonal variations in the polyphenol levels of the red seaweed *P. lanosa*. Seasonal variations in the polyphenol levels of seaweed have previously been observed. In a study by Abdala-Díaz *et al.* (2006) it was demonstrated that higher phenolic

concentrations in the apical tissue of the brown seaweed *C. tamariscifolia* were observed in months with the greatest irradiance. There was an exponential increase in phenolic compounds in the first half of the year as daily irradiance increased from February to June. By contrast, there was an exponential decrease in phenolic compounds in the second half of the year as daily irradiance decreased from June to November (Abdala-Díaz *et al*., 2006). Seasonal variations in phenolic compounds are species specific, but the highest values are generally observed during the Summer and lowest values during Autumn and Winter (Connan *et al*., 2004).

It is thought polyphenol compounds could act as a photoprotection mechanism against solar radiation by either the adsorption of incident rays or as a result of antioxidant activity. For example, in Summer, high solar irradiance can induce synthesis of radical oxidant species, thus the increase in polyphenol concentrations could act as an antioxidant defence (Abdala-Díaz *et al*., 2006).

Additionally, Arnold *et al.* (1995) investigated the variation in polyphenolic content of the tropical brown alga *Lobophora variegate* as a function of nitrogen availability. Results from their study demonstrated that the phenolic content of the seaweed was inversely proportional to algal tissue nitrogen. They concluded that that nitrogen availability plays a crucial role in determining phlorotannin levels in brown algae. These support previous findings whereby N levels of seawater/seaweed vary with season with highest levels in Winter (Rijstenbil *et al.,* 1998). This corresponded to low levels of polyphenols at higher N levels.

There was no change in the concentration of February or May polyphenols following heavy metal exposure. The polyphenolic content of November samples however increased slightly from 7.3 to 9 mg/g dw following exposure to Cd^{2+} (Figure 5.3). As with high solar irradiance (Abdala-Díaz *et al*., 2006), metals can induce oxidative stress in plants through the creation of reactive oxygen species (ROS), (Ratkevicius *et al*., 2003). The increase in polyphenol levels of the November samples therefore corresponded to heavy metal exposure. *P. lanosa* in this study did not induce polyphenol synthesis in the February and May samples following Cd^{2+} exposure. However, February samples saw an increase in both total and extracted protein following Cd^{2+} exposure, indicating a different mode of defence to combat heavy metal exposure during this period. Seasonal variations in the polyphenol levels of seaweed

following heavy metal exposure have never previously been published. This study therefore provides novel information regarding seasonal variations in the biochemical response of *P. lanosa* to heavy metal exposure.

It is highly likely that heavy metal resistance in algae involves polyphenols that are capable of binding free metal ions, either in the surrounding medium or intracellularly, resulting in compounds that are less toxic to the algae (Toth & Pavia, 2000). It has been reported that brown algal phlorotannins are able to bind divalent metal ions (Ragan *et al.*, 1979). In a study by Toth & Pavia (2000), *A. nodosum* was exposed to Cu^{2+}. Results from their study suggested that phlorotannins chelated copper ions. It is not known however if this binding occurred intracellularly or in the extraction solution (Toth & Pavia, 2000). It has previously been suggested that seaweeds can exude phlorotannins-Cu complexes into the surrounding environment as a detoxifying mechanism (Toth & Pavia, 2000). It has also been suggested that exudation of polyphenolic compounds into the environment plays a significant ecological role (Toth & Pavia, 2000; Abdala-Díaz *et al.*, 2006). Furthermore, high levels of Cd^{2+} and Cu^{2+} have been associated with the physodes of *Fucus vesiculosus* this therefore may support the thought that phlorotannins can act as an internal detoxification mechanism in this seaweed (Toth & Pavia, 2000).

Overall results demonstrated possible temporal variations in seaweed sensitivity to heavy metal exposure whereby the February samples were associated with protein increases and November samples with increases in polyphenol levels. As mentioned in Chapter 4 however, the protein levels in seaweeds are also influenced by factors such as nitrogen availability (Rijstenbil *et al.,* 1998) and light intensity (Cronin & Hay, 1996). Furthermore, polyphenol levels are also affected by nitrogen availability (Arnold *et al.,* 1995) and light intensity (Abdala-Diaz *et al.,* 2006). In a study by Arnold *et al.* (1995), it was concluded that the polyphenol concentration of the brown alga *L. variegate* was inversely related to seaweed nitrogen levels. Furthermore, Abdala-Diaz *et al.* (2006) demonstrated that the phenolic concentration of the brown alga *Cystoseira tamariscifolia* increased with increasing irradiance.

5.5.4   Seaweed Metal Analysis

Results demonstrated little seasonal variation in the Cd^{2+} concentration of *P. lanosa* and furthermore levels of this metal were relatively low for all three sampling periods. Low levels of Cd^{2+} were not unexpected as all samples were taken from a clean and unpolluted site in Fethard-on-Sea, Co. Wexford. Seasonal variations in seaweed metal levels are considered mainly as a result of seasonal growth trends (Riget *et al*., 1997). For example, in a study by Haritidonitis & Malea (1999), they demonstrated that the seasonal variation of metals in the green seaweed, *U. rigida* was associated with growth patterns. Concentrations were found to be higher in Autumn, when biomass was at its minimum and lower in Spring when biomass was at its maximum. Increases in biomass corresponded to the decrease of seaweed metal levels as a result of dilution. Additionally, seasonal fluctuations in seawater metal levels are known to affect the metal concentration of macroalgae (Haritidonitis & Malea, 1999).

Several studies regarding seasonal changes in the total metal concentrations of seaweeds have previously been published (Brown *et al*., 1999; Haritonidis & Malea, 1999; Perez *et al*., 2007; Morrison *et al*., 2008). Very little information concerning intracellular and surface bound metal levels in seaweeds is available in the literature (Pawlik-Skowrońska, 2000; García Ríos *et al*., 2007) and no information regarding the seasonal fluctuations of intracellular and surface bound metals following heavy metal exposure have been reported.

The impact of seaweed sampling time on the ability of a seaweed to uptake metals is presented for the first time. Heavy metals are known induce defence mechanisms in algae. Therefore knowledge regarding the mechanism of heavy metal biosorption and accumulation in seaweed and its dependence on season is important in establishing the impact of metal emissions to the environment in different seasons.

Following exposure, Cd^{2+} concentrations of *P. lanosa* increased significantly for all months. It has previously been demonstrated that seaweeds have the ability to concentrate metals from the surrounding environment (Chapter 3).

Total and intracellular metal uptake is dependent on both metal concentration and duration of exposure (Pawlik-Skowrońska *et al.,* 2007). In a study by García-Ríos *et al.* (2007) involving two red macroalgae species, *Gracilaria cornea* and *Chondrophycus poiteaui*, it was also demonstrated that the total Cd^{2+} uptake of the two species was proportional to the Cd^{2+} concentration and exposure time. The greater proportion of metal in *G. cornea* (80%) was found to be extracellular while *C. poiteaui* accumulated most of the Cd^{2+} (~100%) intracellularly. In a study by Herrero *et al.* (2008) the metal uptake kinetics of the red alga *Mastocarpus stellatus* demonstrated an initial rapid Cd^{2+} uptake signifying that the biosorption process occurred chiefly on the surface of the alga, as a result of active binding sites on the cell wall surface. The initial rapid uptake was followed by a slower stage, corresponding to intracellular accumulation (Herrero *et al*., 2008).

Temporal variations in intracellular Cd^{2+} concentrations of *P. lanosa* may perhaps be attributed to seasonal variations of seaweed plasma membrane and cell wall polysaccharides. For example, Fritioff *et al.* (2005) carried out a study which examined the affect of temperature on heavy metal uptake by submersed plants. They demonstrated that changes in temperature altered the composition of the plasma membrane lipids. These changes subsequently altered the plant membrane fluidity, resulting in a reduction of the membrane permeability at lower temperatures and thus lower metal uptake (Fritioff *et al*., 2005). This would explain therefore why May (Summer) samples had the highest % intracellular Cd^{2+} uptake (33%), while February and November samples had a significantly lower intracellular uptake, accounting for 17 and 22% respectively (Figure 5.5).

Seasonal variations in surface bound Cd^{2+} levels of *P.lanosa* were also observed and may be attributed to seasonal alterations of cell wall polysaccharides which are responsible for metal sorption. For example, carrageenans are sulphated polysaccharides present in the cell wall of red seaweeds (Romera *et al*., 2007). Cosson *et al.* (1990) demonstrated the seasonal variation of carrageenans from four Rhodophyceae. Maximum concentrations of carrageenans were established for the end of Spring and minimal levels in Autumn. Maximum levels of carrageenans in Spring correspond to highest concentrations of surface bound Cd^{2+} in the February *P. lanosa*.

The lowest intracellular Cd^{2+} (17%) was associated with the *P. lanosa* sampled in February. Additionally, February was the only month to see an increase in total protein and extracted protein following heavy metal exposure. The lowest intracellular levels of Cd^{2+} were therefore capable of inducing an increase in protein during this period. The ability to induce protein synthesis in this month may be attributed to enhanced seawater and seaweed N levels. Furthermore, following exposure, the highest levels of intracellular Cd^{2+} (9.7 mg/kg dw) were associated with *P. lanosa* sampled in November, corresponding to an increase in polyphenol levels for this month. November was the only month to see an increase in polyphenol levels. No increases in total or extracted protein levels were observed for this month. Results therefore demonstrate two possible heavy metal defence mechanisms in seaweeds at different times of the year.

Significant variations in the ability of *P. lanosa* to bind Cd^{2+} were observed. In order to establish whether these variations were linked to surface binding groups, both the quantity and type of these groups required further investigation.

5.5.5 Potentiometric Titrations

5.5.5.1 pKa Values

The pKa values of *P. lanosa* sampled in February, May and November corresponded to carboxyl, amino and hydroxyl groups. Metal sorption in brown seaweeds is primarily associated with the carboxyl groups (Murphy *et al.,* 2007). Their presence therefore in this study demonstrates their significance in the metal sorption of the red seaweed.

Seasonal variations in the number of pKa peaks were also observed. These results are significant and therefore demonstrate seasonal variations to the cell wall functionalities and thus changes to the cell wall composition of *P. lanosa*. These changes are reflected in seasonal variations in the uptake of Cd^{2+} in *P. lanosa*.

Research into biosorption has predominantly focused on the study of brown algae and to a lesser extent with green and red algae. Brown algae cell walls contain cellulose, alginic acid and sulphated polysaccharides. As a result, carboxyl and sulphate are the major active groups in this variety of algae. Red algae also contain cellulose, but the

major compounds associated with these species are sulphated polysaccharides (agar and carragenates). Green algae are chiefly cellulose and a high proportion of the cell wall is glycoprotein. These compounds contain several functional groups (e.g., amino, carboxyl, sulphate, hydroxyl, etc.,) which may play an important role in the biosorption mechanism (Romera *et al*., 2007).

It is generally thought that carboxyl groups (weak) are primarily responsible for metal sorption (Murphy *et al*., 2007). The presence of additional inflection points in the carboxyl region were also observed in this study. These inflection points may in therefore signify the different orientations of constituent carboxyl groups thereby leading to more than one observed pKa for these groups. These may also be linked to different carboxyl groups available for metal binding.

Sulphonate groups usually only contribute to metal binding at low pH and their typical pKa values are in the range 1.0-2.5 (Sheng *et al*., 2004; Murphy *et al*., 2007). Apparent pKa values in this range were therefore not detected by titration in this study. FTIR analysis of samples however was performed in order to confirm the presence of such groups (5.4.6).

Hydroxyl groups in seaweed cell wall polysaccharides are significantly weaker than carboxyl groups and therefore may only interact with cations at a higher pH (~ pH >10). Therefore surface hydroxyl groups only play a significant role in binding at very high pH values (Davis *et al*., 2003).

Algal proteins have also been known to interact with metal ions, particularly between pH 6-9 and protonated amino groups have a pKa value of around 8 (Murphy *et al*., 2007). In the present study, seaweeds displayed pKa values in this region. February samples displayed pKa values of 7.79 and 8.84 while May samples had a value of 8.42 and November produced a pKa of 8.25.

5.5.5.2 Acidic Groups

P. lanosa samples collected in February, May and November contained the same number of strong acidic groups. Variations in the numbers of total acidic groups were however observed. Potentiometric titrations have been carried out predominantly for

brown seaweeds (Fourest & Volesky, 1996; Murphy *et al*., 2007). Conversely, there is very little information regarding potentiometric titration analyses for red seaweeds. Murphy *et al.* (2007) however used potentiometric titrations to evaluate the number of total acidic groups in *P. lanosa*. The seaweed was found to contain 1.81 mmol/g acidic groups. This result correlates quite favourably with results for May *P. lanosa* samples in this study (1.88 ± 0.05 mmol/g). Overall however, February *P. lanosa* contained quite a significantly higher number of total acidic groups when compared with May and November samples. Seasonal variations in the number of weak acidic groups were also observed (February samples yielding the highest values). Furthermore, values established by Murphy *et al.* (2007) corresponded to those of May *P. lanosa* in this study. The seasonal variation in seaweed acidic groups is presented for the first time.

Carrageenans are sulphated polysaccharides present in the cell wall of red seaweeds (Romera *et al*., 2007). A study by Cosson *et al.* (1990) demonstrated a seasonal variation of carrageenans from four Rhodophyceae. The carrageenan content was found to be maximal at the end of Spring and minimal in Autumn. In addition, the carrageenan concentration positively correlated with the growth of seaweed under investigation. Furthermore, Dawes (1974) demonstrated an increase in the carbohydrate content of red seaweeds during early Spring with a sharp drop during mid-Spring and then a gradual increase through Summer and Autumn. Variations in the carrageenan content of seaweeds influence the biosorption potential.

February samples were found to contain the highest number of acidic groups and November the lowest. These findings correspond to the concentration of surface bound metals on *P. lanosa* control samples whereby the February samples yielded the highest proportion of surface bound Cd^{2+} and November the lowest.

Results demonstrated significant variations in the number of total and weak acidic groups among the three months examined and this significantly influenced the concentration of surface bound metals of February, May and November in *P. lanosa*. It is apparent that the weak carboxyl groups are significantly more abundant than the strong sulphonate groups (Table 5.6). It is also evident that any correlation between metal binding capacity and the amount of acidic groups is related to the amount of weak acidic groups.

Potentiometric titrations demonstrated seasonal variations in the functional groups of *P. lanosa*. These variations may be attributed to seasonal changes to cell wall carrageenans. Following exposure, February *P. lanosa* contained the highest concentration of surface bound metals (83%) and this corresponded to the highest total and weak acidic groups. In order to further investigate the seasonal variations of the *P. lanosa* surface, FTIR analysis was performed in an attempt to observe changes in bands associated with seaweed metal binding. Variations in intracellular metal levels can also be explained by seasonal variation of the seaweed plasma membrane. Fritioff *et al.* (2005) examined the influence of temperature on heavy metal uptake by submersed plants demonstrated that changes in temperature altered the composition of the plasma membrane lipids. This in turn altered the plant membrane fluidity, resulting in lower membrane permeability at low temperatures and lower metal uptake. This is reflected in the levels of intracellular Cd^{2+} of *P. lanosa*. Following exposure, *P. lanosa* sampled in May, recorded the highest proportion of intracellular Cd^{2+}.

5.5.6 FTIR analysis of protonated *P. lanosa* samples.

FTIR results demonstrated that the main groups associated with Cd^{2+} binding in *P. lanosa* were carboxyl, amino and sulphonate groups. Sulphonate groups only usually influence metal binding at low pH therefore, their typical pKa values are in the range 1.0 – 2.5 (Sheng *et al.*, 2004). Apparent pKa values within this range were not detected by titration in this study. Using FTIR analysis, -SO_3 bands for protonated *P. lanosa* were previously observed at 1156 cm^{-1} by Murphy *et al.*, 2007. Subsequent FTIR analysis of the protonated samples in this study also confirmed the presence of sulphonate groups in all three study samples (Figure 5.7). Arrows indicate symmetric – SO_3 stretching. Furthermore, FTIR analysis demonstrated seasonal variations in the 1600-1500 cm^{-1} region. For example, bands in the ~ 1516 cm^{-1} region appear in the protonated February and May samples. However, for the November samples, this band appears in the 1530 cm^{-1} region. Bands in this region correspond to the amide II groups, a frequently observed stretching frequency in seaweed FTIR spectra (Murphy *et al.*, 2007). Different distributions of the carboxyl groups (~1600 and ~ 1034 cm^{-1}) available at the cell wall surface were observed for the November samples when compared to those of the February and May samples (Figure 5.7). Variations in band regions will therefore contribute to overall changes in the metal binding capacity of the seaweed

surface. In this unique study, potentiometric titrations and FTIR analysis demonstrated seasonal variations in the quantity and type of functionality available at the surface of *P. lanosa*. These variations play a key role in the ability of seaweeds to bind metals and therefore are significant in the bioaccumulation of metals by seaweed.

5.6 Conclusions

The aim of this temporal study was to establish whether there are seasonal changes in total protein content, extracted protein concentration, polyphenol concentration and metal in *P. lanosa.* The seasonal dependency of *P. lanosa* to accumulate Cd^{2+} was also investigated in order to gain an insight into the parameters which influence metal bioaccumulation and the sensitivity of seaweeds to metals throughout the year.

Seasonal variations in total protein were observed, with May *P. lanosa* containing the highest levels. This is in good agreement with published data for other seaweed types. Higher protein levels were observed during the end of Winter and Spring whereas lower quantities were recorded during Summer (Fleurence, 1999a).

Variation in the extracted protein levels for *P. lanosa* were also observed with February samples yielding the highest extracted protein and November samples, the lowest. This is in contrast to total protein values where May samples yielded the highest total protein. Seasonal variation in levels of extracted protein may reflect seasonal changes in seaweed cell wall or plasma membrane. It was shown that carrageenans (Cossan *et al.*, 1990) and plasma membranes (Fritioff *et al.*, 2005) vary with season, making protein extraction during May and November more difficult.

Increases in February total protein and extracted protein were observed following exposure to Cd^{2+}. This perhaps indicates synthesis of metal-binding peptides following heavy metal exposure. In response to Cd^{2+} exposure, low quantities of thiol peptides and PC were synthesised in two red macroalgae species, *G. cornea* and *C. poiteaui* and this corresponded to increases in intracellular levels of this metal (Garcia-Rios *et al.*, 2007).

Results for control samples demonstrated seasonal variation in polyphenol levels for *P. lanosa* for the first time, with November samples containing the lowest concentration. Seasonal variations in polyphenol levels are in general agreement with published data for other seaweed species. Polyphenol levels in seaweed vary with season due to environmental factors including nitrogen availability (Ragan & Glombitza, 1986) and UV radiation (Pavia & Brock, 2000).

Cd^{2+} exposure had little or no effect on polyphenol concentrations in February and May samples. However, there was a slight increase in November polyphenols (7.3 to 9.0 mg/g dw GAE). This increase perhaps also indicates polyphenol induction following heavy metal exposure. There are conflicting reports as to the role of polyphenols in defence against heavy metal exposure. These compounds can bind free metal ions intracellularly or in the surrounding medium (Ragan *et al.*, 1979). Additionally, metals can induce oxidative stress in plants due to the production of ROS (Ratkevicius *et al*., 2003). *P. lanosa* in this study did not induce polyphenol synthesis in the February and May samples following heavy metal exposure. February samples however exhibited an increase in both total and extracted protein following Cd^{2+} exposure, indicating a different mode of defence to combat heavy metal exposure during this period.

Seasonal variations of total, intracellular and surface bound Cd^{2+} levels of seaweeds were demonstrated in this study for the first time. Seasonal variations in seaweed metal levels have been attributed to seasonal growth trends (Riget *et al*., 1995).

Highest intracellular metal levels were recorded for May *P. lanosa* (33%) and the lowest for the February samples (17%). The highest levels of surface bound metals was obtained for February (83%) and the lowest for May *P. lanosa* (67%). Seasonal changes in the seaweed plasma membrane and cell wall polysaccharides (Cossan *et al*., 1990; Fritioff *et al*., 2005) may contribute to variations in levels of intracellular and surface bound metals, prior to and following heavy metal exposure.

Potentiometric titrations of *P .lanosa* samples demonstrated seasonal variations in total and weak acid groups of the seaweed surface. Higher total and weak acidic groups therefore indicate a greater heavy metal binding ability for the February samples. This was reflected in the concentration of surface bound Cd^{2+} in the February sample. Seasonal variations are attributed to seasonal changes in carrageenan levels, as demonstrated by Cossan *et al.* (1990). Changes in the level of carrageenans influence the binding ability of seaweeds. February contained the highest surface bound Cd^{2+} and November the lowest. These values correspond to levels of total and weak acidic groups, whereby February contained the highest number of acidic groups and November the lowest. FTIR analysis demonstrated that the main groups associated with metal binding in *P. lanosa* were carboxyl, hydroxyl, amino and sulphonate groups. There was also evidence of seasonal changes in the types of –NH and C=O functionalities

available on the surface of *P. lanosa*. This seasonal variation of functionality could play a key role in seaweed bioaccumulation of metals and sensitivity of seaweeds to metal exposure.

Previous studies have shown seasonal changes in total protein (Marinho-Soriano, 2006), extracted protein (Joubert & Fleurence, 2008) and polyphenol (Kamiya *et al*., 2010) levels of seaweed (predominantly brown species). In this novel study however, all three parameters were studied simultaneously for the red seaweed *P. lanosa*. Furthermore, the effect of heavy metal exposure on the total protein, extracted protein and polyphenol levels of seaweed samples were also demonstrated for the first time. Additionally, in an original study, potentiometric titrations and FTIR spectroscopy were used to determine seasonal changes in types and quantity of cell wall functional groups of *P. lanosa.*

Chapter 6

Development of

Separation and Visualisation Methods

For *P. lanosa* Protein Extracts

6. Development of Separation and Visualisation Methods for *P. lanosa* Protein Extracts

6.1 Introduction

Research carried out in Chapter 5 of this thesis demonstrated that the impact of heavy metal exposure on extracted protein levels of *P. lanosa* varied according to season. *P. lanosa*, sampled in February, 2009, which was exposed to Cd^{2+}, saw an initial increase in extracted protein after 6 h exposure. This initial increase was followed by subsequent variations in extracted protein levels up to 72 h. The main aims of this study were to further investigate changes in these protein extracts. In this chapter, gel filtration chromatography, HPLC analysis and SDS Polyacrylamide Gel Electrophoresis (PAGE) methods were developed to gain a further insight into changes in *P. lanosa* protein extracts following exposure to heavy metals.

Gel filtration chromatography is based on the separation of proteins, peptides and oligonucleotides with respect to size. Protein molecules migrate through a layer of porous beads, dispersing into the beads to greater or lesser degrees, depending on size. Smaller molecules disperse further into the pores and therefore migrate through the layer more slowly, whereas larger molecules may not enter the beads and therefore move through the layer of beads more quickly. Retention varies according to both molecular weight and three-dimensional shape of the protein (Porath & Flodin, 1959). Previous studies have employed the technique in the separation of proteins derived from heavy metal exposed higher plants (Grill *et al.*, 1985; Wu *et al.*, 2001) and microalgae (Wikfors *et al.*, 1991). Fewer studies however have been dedicated to the separation of seaweed proteins. For example, Hu & Wu (1998) examined variations in the elution profile in the red marine macroalga *Kappaphycus alvarezii*. Figure 6.1 demonstrates the changes in gel filtration fraction profiles from algal fronds treated with 100 μM $CdCl_2$ for 9 days (Hu & Wu, 1998). The Cd treated sample contained two more peaks, which contained more than 80% of the Cd detected in the soluble extract.
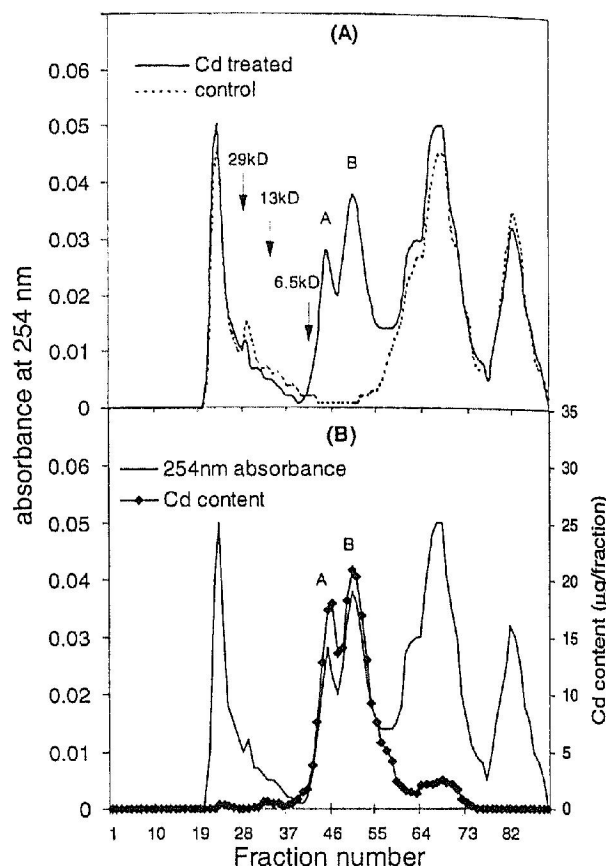
Figure 6.1. Gel filtration chromatography of soluble cell extract on a column of Sephadex G-50. (A) the elution profile of soluble extracts prepared from control algal fronds (dotted line) and from algal fronds treated with 100 μM CdCl$_2$ for 9 days (solid line). Peak positions of molecular weight standards are marked (arrows). (B) The Cd content and the profile of the soluble extract prepared from Cd-treated algal fronds (Hu & Wu, 1998).

HPLC techniques have also been employed in the analysis of seaweed protein extracts. For example, García-Ríos *et al.* (2007) studied the thiol peptide production of two red macroalgae, *Gracilaria cornea* and *Chondrophycus poiteaui* which were exposed to low concentrations of Cd^{2+} (0.89 and 8.9 μM CdCl$_2$), over 7 days. Results demonstrated no significant synthesis of PCs in both species. García-Ríos *et al.* (2007) also demonstrated that the thiol group content/intracellular Cd^{2+} ratio in *C. poiteaui* was 2:1, which was sufficient to bind all intracellular Cd^{2+} without PC synthesis. This response suggested that red macroalgae have detoxification systems that include active metal uptake by the plasma membrane and efficient intracellular metal binding by complexes, possibly smaller thiol-compounds. Low PC synthesis has also been observed in the

green macroalga *Enteromorpha* spp. (Malea *et al*., 2006). Alternative intracellular mechanisms therefore, other than PC production may also be responsible for metal detoxification in some marine macroalgae.

Non-protein reduced sulphur like cysteine and glutathione might be the major thiol compounds involved in the detoxification of all intracellular Cd^{2+} in this macroalga whereas PCs may play only a minor role (García Ríos *et al*., 2007).

SDS PAGE has been employed in previous studies to determine baseline protein characteristics of various seaweeds (Price, 1987; Fleurence *et al*., 1995a; Galland-Irmouli *et al*., 1999; Rouxel *et al*., 2001). In this unique study however, the protein profile of *P. lanosa*, following heavy metal exposure were assessed for the first time using SDS PAGE.

In this work, methods were developed in order to fractionate protein extracts (derived from seaweed, exposed to Cd for 0 – 72 h). Subsequently HPLC analysis was used to monitor changes in both crude and peak protein fractions. Finally, SDS-PAGE of crude extracts was performed in order to separate and identify changes in pattern and the molecular weights of proteins from *P. lanosa* following heavy metal exposure.

6.2   Objectives of Research

Chapter 5 of this study showed that *P. lanosa* (sampled in February, 2009), which was subjected to heavy metal exposure, demonstrated variations in the levels of extracted protein over 72 h. In this chapter, gel filtration chromatography, HPLC analysis and SDS PAGE methods were developed to further monitor changes in *P. lanosa* protein extracts following exposure to heavy metals.

The main aims of this study were to develop the following methods:

- Gel filtration chromatography for the fractionation of protein extracts derived from seaweed, exposed to Cd for 0 – 72 h.

- HPLC analysis to monitor changes in both crude and peak protein fractions following gel filtration chromatography.

- SDS-PAGE of crude extracts in order to separate and monitor metal-induced changes in the molecular weights of proteins.

6.3   Experimental

6.3.1   Chemicals

- Blue Dextran Dye – D-5751 (Sigma-Aldrich, Dublin, Ireland)

- Folic Acid Standards ~ 98% - F-7876 (Sigma-Aldrich, Dublin, Ireland)

- Lysozyme – Cat # 1.05281.0010 (Merck)

- Myoglobin $\geq$ 90% SDS PAGE (Sigma-Aldrich, Dublin, Ireland)

- Trypsinogen – Cat # T9011 from Bovine Pancreas (Sigma-Aldrich, Dublin, Ireland)

- Sephadex G50 – DNA Grade; G-50 medium (S6022, 9-11 ml/g), (Sigma-Aldrich, Dublin, Ireland)

- Tris buffer – Cat # M10622483 (Molekula/Lennox, Dublin, Ireland)

- Tris-HCl – Cat # M10637269, Min. Purity 99% (Molekula/Lennox, Dublin, Irl)

- Stock acrylamide – 30 % acrylamide/Bis 29:1 (Sigma-Aldrich, Dublin, Ireland)

- Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, Dublin, Ireland)

- Ammonium persulphate solution (Sigma-Aldrich, Dublin, Ireland)

- Tetramethylethylenediamine (TEMED)(Sigma-Aldrich, Dublin, Irl, Cat.# T8133)

- Sucrose (Sigma-Aldrich, Dublin, Ireland)

- 0.5 % bromophenol blue (Sigma-Aldrich, Dublin, Ireland)

- Low Molecular Weight Marker Standards, Cat # 17044601 (Amersham, GE Healthcare),

- Protsil1ProteoSilver Silver Stain Kit (Sigma-Aldrich, Dublin, Ireland)

- L-Glutathione – Reduced (Min. 99%) (Sigma-Aldrich, Dublin, Ireland)

- TFA - Superpurity (Sigma-Aldrich, Dublin, Ireland)

- Acetonitrile – HPLC Grade (Sigma-Aldrich, Dublin, Ireland)

6.3.2   Instrumentation

- Gel Chromatography Column and Kits

- Syngene Genius Bioimaging System

- Syngene – Genesnap Image Acquisition Software

- Varian Cary 50 UV spectrophotometer (JVA Analytical Ltd., Dublin, Ireland)

- Biorad Fraction Collector (Model 2110)

- Hewlett Packard 1050 HPLC Spectrophotometer

- ATTO AE 6450 protein gel system (Bio-Rad)

- C-18 (X Terra MS) HPLC column (150 x 4.6 mm) with 5 μm particle size

6.3.3   Seaweed Collection and Preparation

Seaweeds were collected at low tide from Fethard-on-Sea Co. Wexford, Ireland (52.39°N, 6.94°W), February, 2009. Samples were prepared as per Chapter 4, Section 4.3.2

6.3.4   72 h Time Course Study (*Polysiphonia lanosa*)

The 72h time course study was performed as per Chapter 4, Section 4.3.4.

6.3.4.1 Protein Extraction

Refer to Section 2.3.4.6

6.3.5   Sephadex G50 Chromatography

(Porath & Flodin, 1959)

6.3.5.1 Preparation of the Resin: Sephadex G50

The column volume ($\Pi r^2 h$) was calculated in order to determine the volume of Sephadex G50 required. The longer the column, the better the separation. The wider the column, the greater the capacity. The column used was 1.75 x 79 cm. The column volume was 159 ml. 1.5 times the required volume was measured in a graduated cylinder.

The suspension at this stage was too thick to be poured directly into the column. The gel therefore was first diluted with deaerated eluent buffer to the required consistency. To do this the resin was diluted to twice the required volume. The eluent buffer for this application was 10mM Tris buffer (pH 7). The suspension was stirred with a glass rod to make the mixture homogeneous.

6.3.5.2 Packing and Preparation of the Column

All components of the column were cleaned and rinse with 20% ethanol. The packing reservoir was attached tightly to the top of the column. The column was then mounted vertically on a stand. Eluent buffer was poured into the column glass tube to about 1/3 full. The column was gently tapped to remove any air bubbles. With the base of the column still sealed, the resin was added using the extra capacity in the reservoir and a glass rod to let it flow easily down the sides. The lid of the reservoir was attached, with tubing connecting it to the pump. The base of the column was opened and the pump turned on to pack the column at a constant pressure. The column was packed at $1cm^3$ per min until the gel bed had reached a constant height (approx. 2 hours). The column was then washed with buffer from the reservoir at a flow rate of approximately 1 cm^3/min for approx. 1 hour. The pump was stopped and the column outlet was closed. The packing reservoir was removed.

Tubing from a column cap was connected to a peristaltic pump and thereafter to a 3-way valve and thence to a reservoir of buffer. The pump was turned on to fill all the tubing with buffer. This assembly was attached to the top of the column, via the column cap. To start the column washing with reservoir buffer, the valve was opened and the pump turned on. A maximum flow rate of 1 ml/min was maintained at all times while washing. The assembly was washed with two column volumes of buffer.

6.3.5.3 Determination of the Void Volume of the Column

The determination of the Void Volume of the column was carried out using Blue Dextran (4mg/0.5ml of buffer) (mol wt, 2,000,000). Void volume of a column = volume of liquid in the column (around the resin beads) which must elute off the column before any of the components of the sample can reach the base of the column. Blue Dextran is used to measure void volume as it has a molecular weight of 2 million and so cannot pass into any

of the available beads and passes through the column directly behind the void volume material.

Before use, the column was thoroughly washed with eluate buffer and the flow rate was checked. Using the 3-way valve, the Blue Dextran sample was pumped onto the column. The eluate was immediately collected into a graduated cylinder. The 3-way valve was changed to follow the flow of Blue Dextran sample with buffer from the reservoir. The washing procedure with buffer continued until the Blue Dextran eluted. The volume at this time was recorded, (the Void Volume of the column). The column was washed until all the Blue Dextran had left the column and the tubing.

6.3.5.4 Standard and Sample Application to the Column

The following standards were prepared in the eluent buffer (2 mg/ml) as illustrated in Table 6.1. 1 ml of each standard was applied to the column.

Table 6.1. Sephadex G50 Standards prepared for column chromatography calibration.

Standard	*Mol. Wt.* *(kDa)*
Trypsinogen	24.0
Myoglobin	17.8
Lysozyme	14.3
Folic Acid	0.44

6.3.5.5 Fractionation, Elution and Detection

After first collecting the void volume, a fraction collector was used to collect the eluate in individual fractions of 3 ml (approx 90 drops). The total column volume (Vt), was determined, by calculating the volume of the resin-packed portion of the column and adding approx. 10 ml. for the tubing involved. This Vt was the maximum eluate collected.

Monitoring of individual fractions was carried out using the Varian Cary 50 UV spectrophotometer with proteins detected at 280 nm. The molecular weights of unknown samples were determined using a standard curve (Grill *et al.*, 1987).

Standard curve data: The elution volumes of the standards were divided by the elution volume of the blue dextran (Ve/Vo) and plotted against the log of the standard molecular weights.

6.3.6 HPLC Analysis (HP 1050 HPLC)

6.3.6.1 Chromatographic conditions

(Based on García Ríos *et al*., 2007 and Clemens *et al*., 2001)

- The separation was carried out using a 150 x 4.6 mm C-18 (X Terra MS) HPLC column with 5 μm particle size.
- Solvent A: 100% acetonitrile.
- Solvent B: 0.1% trifluoroacetic acid (TFA) in ultrapure water.
- The flow rate of the solvent pump was 1.0 mL/min.
- Sample Size: 25 μl
- Isocratic elution (50/50 Solvent A/Solvent B)

6.3.6.2 GSH Calibration

Glutathione standards were prepared in triplicate, by serial dilution, ranging from 0.39 to 5 mg/ml. A preliminary scan of GSH was performed and the λ max was determined to be 220 nm.

6.3.6.3 HPLC Analysis of Crude Sample and Fractions

HPLC analysis was performed on seaweed protein extracts (following seaweed exposure to 1 ppm Cd for 0 – 72h). Studies included the analysis of (i) crude seaweed protein extracts at 220 and 280 nm and (ii) analysis of gel filtration peak fractions at 220 nm.

6.3.7 SDS-PAGE and Silver Staining

SDS-PAGE of protein extracts from *P. lanosa* exposed to 1 ppm Cd for 0 – 72 hours was performed using the Laemmli method (Laemmli, 1970). Silver staining is a procedure used for detecting proteins separated by gel electrophoresis. It is a sensitive technique for the visualisation of proteins with a detection level of 0.3-10 ng. Protein

visualisation relies on the binding of silver ions to amino acid side chains, principally the sulfhydryl and carboxyl groups. This is followed by reduction to free metallic silver. The protein bands are visualised as dots where the reduction takes place. The protein distribution within the gel is linked to the difference in oxidation-reduction potential between the space on the gel taken up by proteins and the free neighbouring sites (Gromova & Celis, 2004).

6.3.7.1 Electrophoresis

- *Separating Gel Preparation*

16 mls of 12 % separating gel was prepared per two gels as illustrated in Table 6.2.

Table 6.2. Separating gel preparation for SDS-PAGE.

Reagent	*Volume (mls)*
1.875 M Tris-HCl, pH 8.8	3.2
Ultrapure water	6.16
Stock acrylamide – (30 % acrylamide/Bis 29:1)	6.4
10 % SDS solution	0.160
10% ammonium persulphate solution	0.080
TEMED (Tetramethylethylenediamine)	0.005

- *Stacking Gel Preparation*

10 mls of 3% stacking gel was prepared per two gels as illustrated in Table 6.3.

Table 6.3. Stacking gel preparation for SDS-PAGE.

Reagent	*Volume (mls)*
0.6 M Tris-HCl, pH 6.8	1.0
Stock acrylamide – (30 % acrylamide/Bis 29:1)	1.36
Ultrapure water	7.5
10 % SDS	0.100
10 % ammonium persulphate solution	0.050
TEMED (Tetramethylethylenediamine)	0.005

- *Electrode Buffer Preparation*

400 mls of electrode buffer was prepared (per gel tank) as follows: 6g tris base, 26.8g glycine and 1g SDS were placed in a volumetric flask and made up to 1L using ultrapure water.

- *Sample Buffer Preparation*

Sample buffer was prepared as per Table 6.4.

Table 6.4. Sample buffer preparation for SDS-PAGE.

Reagent	*Volume (mls)*
0.6 M Tris-HCl pH 6.8	2.0
10 % SDS	0.2
Sucrose	2.0g
β-Mercaptoethanol	0.100
0.5 % bromophenol blue	2.0

- *Low Molecular Weight Marker Standard Sizes* (*Table 6.5*).

Table 6.5. Low molecular weight markers prepared for SDS-PAGE.

Protein	*Molecular Weight (kDa)*
Phosphorylase b	97.0
Albumin	66.0
Ovalbumin	45.0
Carbonic anhydrase	30.0
Trypsin inhibitor	20.1
α-Lactalbumin	14.4

- *Sample Preparation*

0.5 ml protein extract samples (0.03 mg/ml) were mixed with 0.5ml sample buffer. The sample solutions were then heated for five minutes at 95 $^{\circ}$C, in order to denature the proteins, before applying to gels.

- *Electrophoretic Conditions*

The ATTO AE6450 protein gel system was used for SDS-PAGE protein gel procedures. The system was set-up and electrophoresis was carried out as per manufacturer's instructions. Each sample migration was performed in duplicate. 5 μl of standard and 10 μl of samples (concentration ~ 0.03 mg/ml) were applied to wells. Electrophoresis was performed at 200 Volts and 130 mA, until the blue band of the sample buffer reached the end of the gel.

6.3.7.2 Staining and destaining of protein gels

- *Preparation*

Ultrapure water was used throughout the procedure as it is crucial for low background and high sensitivity staining.

- *Fixing solution*:

50 ml of ethanol, 10 ml of acetic acid and 40 ml of ultrapure water were combined and mixed well.

- *30% Ethanol solution*:

30 ml of ethanol and 70 ml of ultrapure water were combined and mixed well.

- *Sensitiser solution*:

1 ml of ProteoSilver Sensitizer and 99 ml of ultrapure water were combined and mixed well. The solution was used within 2 hours of preparation.

- *Silver solution*:

1 ml of ProteoSilver SilverSolution and 99 ml of ultrapure water were combined and mixed well. The solution was used within 2 hours of preparation.

- *Developer solution*:

5 ml ProteoSilver Developer 1, 0.1 ml ProteoSilver Developer 2 and 95 ml of ultrapure water were combined and mixed well. The solution was used within 20 minutes of preparation.

- *Staining*

Following electrophoresis the gel was placed overnight in a clean plastic tray containing 100 ml of the fixing solution. After the fixing solution was decanted, the gel was washed for 10 minutes using 100 ml of the 30% ethanol solution. When the ethanol solution was decanted, the gel was washed for 10 minutes in 200 ml of ultrapure water. Once the water was decanted, the gel was incubated for 10 minutes in 100 ml of the sensitiser solution. When the sensitiser solution was decanted, the gel was washed twice, for 10 minutes using 200 ml of ultrapure water. After the water was decanted the gel was allowed to equilibrate in 100 ml of the silver solution for 10 minutes. When the silver solution was decanted, the gel was washed in 200 ml of ultrapure water for 1 minute. Once the water was decanted, the gel was let to develop for 5 minutes using 100 ml of the developer solution. After 5 minutes, 5 ml of the stop solution was added to the developer solution in order to stop the developing reaction. The developer/stop solution was decanted after 5 minutes. Finally, the gel was washed with 200 ml of ultrapure water for 15 minutes and this was followed by UV detection of gel bands.

All errors are calculated based on triplicate samples with 95% Confidence Intervals (Except where otherwise stated).

6.4   Results

6.4.1   Sephadex G50 Gel Filtration Chromatography

6.4.1.1 Standards and Standard Curve

Results from standard curve data demonstrated sequential elution of standards in the order: Trypsinogen, Myoglobin, Lysozyme and Folic Acid.   The elution sequence corresponded to decreasing molecular weight of standards (Figure 6.2).
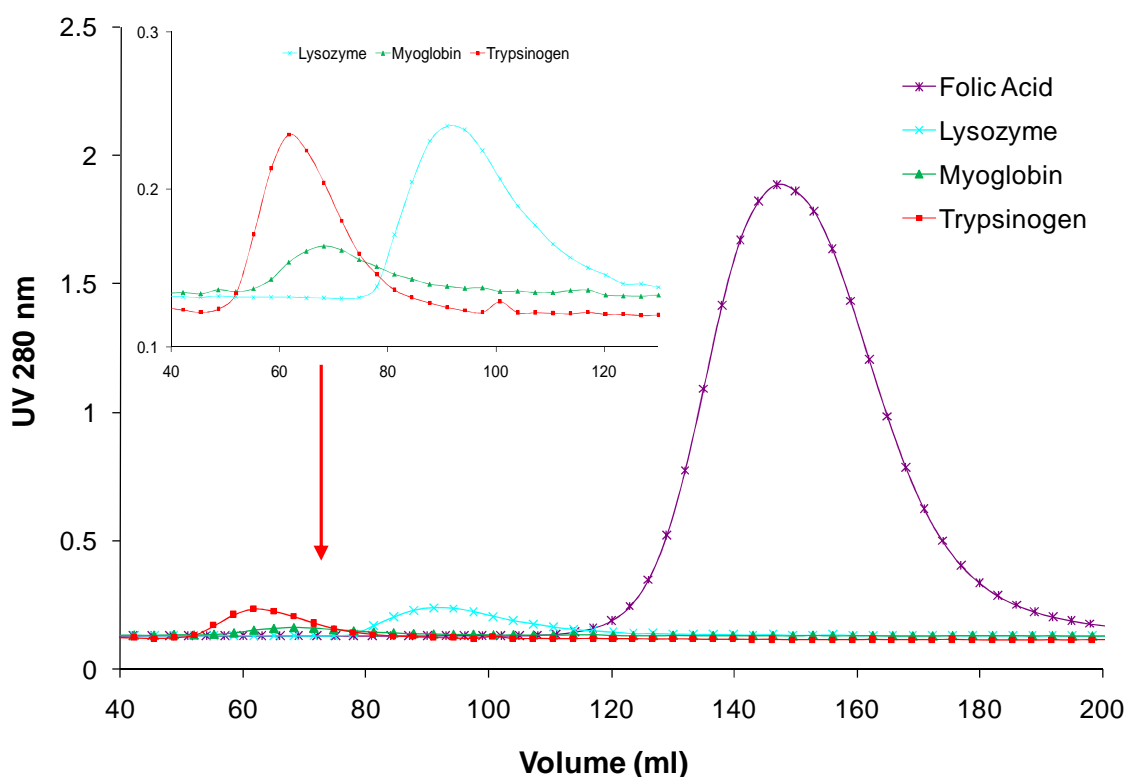
Figure 6.2. UV analysis of gel filtration standards following elution with absorbance measured at 280 nm.   Analysis was carried out in duplicate with 95% confidence intervals.

The standard curve created from the standard curve data is shown in Figure 6.3. Duplicate standards were run and results demonstrated no variations among replicates, therefore error bars are absent from the standard curve.
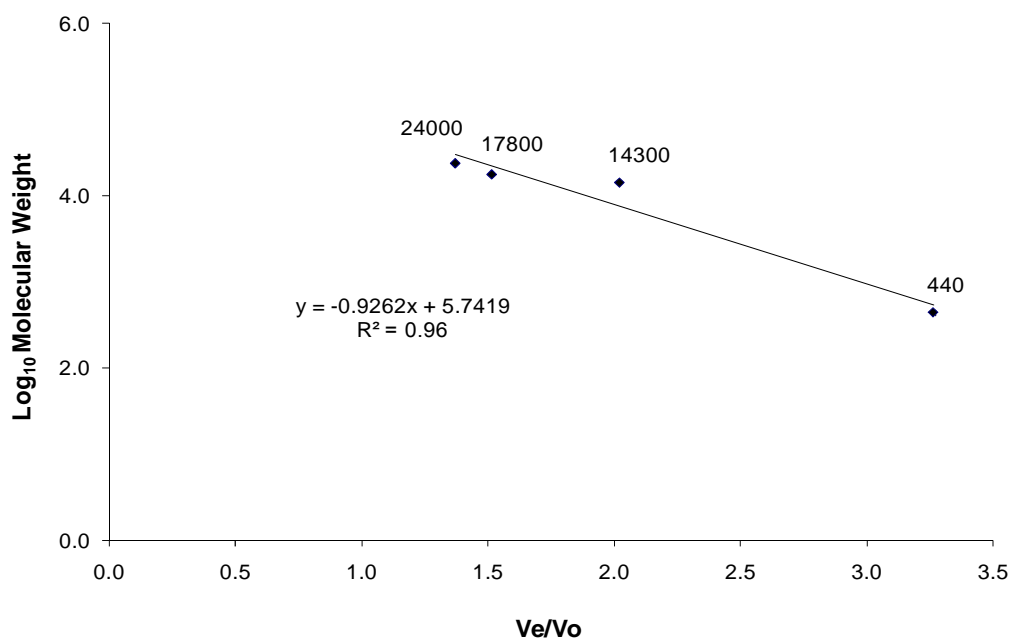
Figure 6.3. Sephadex G50 Standard Curve. Trypsinogen = 24 kDa; Myoglobin = 17 kDa; Lysozyme = 14 kDa and Folic Acid = 0.440 KDa.

6.4.1.2 Protein Extract Analysis

P. lanosa was exposed to 1 ppm Cd for 0 − 72h. Gel filtration chromatographic analysis of protein extracts was as follows:

- Column Volume: 158.84 ml
- Void Volume: 45 ml
- Fraction size: 3 ml

Initial gel chromatography analysis was performed using a smaller 22ml column (Figure 6.4 A). However in an attempt to increase resolution, a longer 158 ml column was used (Figure 6.4 B). Results from both sets of gel chromatography were almost identical. Results following gel chromatographic analysis of protein extracts showed similar trends for all 0 − 72 h exposure samples, whereby each sample produced two main peaks. There was an initial increase in absorbance following the void volume for all five samples. The molecular weights of these peak 1 fractions were in the range 48 − 52 kDa (Figure 6.4 and Table 6.6).
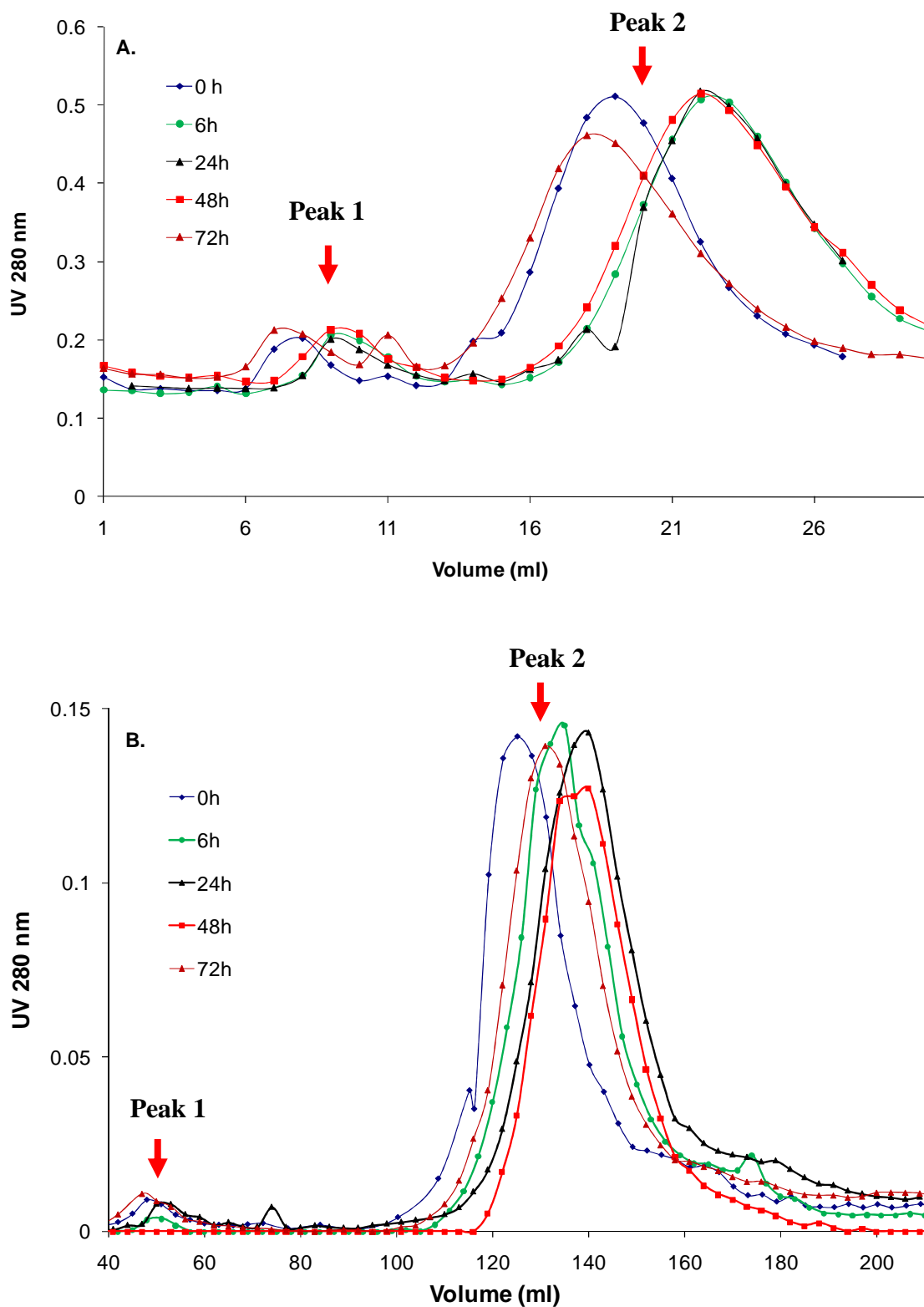
Figure 6.4. UV analysis (λ max = 280 nm) of *P. lanosa* protein extracts exposed to 1 ppm Cd for 0 - 72h. *P. lanosa* samples were collected from Fethard-on-Sea, Co. Wexford, in February, 2009. A. Gel filtration chromatography using a 22 ml column and B. using a 159 ml column.

Table 6.6. Calculations to determine the molecular weight of peak 1 fractions.

Exposure Time (h)	Vo (ml)	Ve (ml)	Ve/Vo	log mw	Mol. wt. of Peak Fractions (kDa)
0	45	51	1.133	4.697	49.77
6	45	51	1.133	4.697	49.77
24	45	50	1.111	4.717	52.12
48	45	50	1.111	4.717	52.12
72	45	47	1.044	4.778	59.98

Table 6.7 illustrates the calculations performed in order to determine the molecular weight of protein peak 2 fractions. The molecular weights of this peak were in the range 0.750 – 1.507 kDa. This range of values generally corresponded to the molecular weights of PC (n=2-5), (Table 6.8). Furthermore, the molecular weight range of samples at half the peak height was 0.565 – 2.301 kDa.

Table 6.7. Calculations to determine the molecular weight of peak 2 fractions.

Exposure Time (h)	Vo (ml)	Ve (ml)	Ve/Vo	log mw	Mol. wt. of Peak Fractions (kDa)	PCn Estimated Equivalents
0	45	125.2	2.782	3.18	1.507	PC(n=5)
6	45	135	3.000	2.98	0.948	PC(n=3)
24	45	140	3.111	2.86	0.750	PC(n=2)
48	45	140	3.111	2.86	0.750	PC(n=2)
72	45	131	2.911	3.06	1.146	PC(n=4)

Table 6.8 demonstrates the amino acids involved in the synthesis of phytochelatins and their corresponding molecular weights.

Table 6.8. Molecular weights of phytochelatins.

Molecule	Molecular Weight (g/mole)
Cysteine	121.15
Glutamic Acid	147.13
Glycine	75.07
GSH ((cys-glu)$_n$ – gly)) (n=2-11)	343.35
PC(n=2)	611.65
PC(n=3)	879.94
PC(n=4)	1148.23
PC(n=5)	1416.50

6.4.2   HPLC Analysis

6.4.2.1 Glutathione (*GSH*) Calibration Curve

GSH standards were prepared from a 5mg/ml solution. Subsequent standards were prepared following serial dilution (Table 6.9).

Table 6.9. GSH Standard Curve Data at 220 nm.

Concentration (*mg/ml*)	Average A (*mAU*)
5	4847 ± 14.1
2.5	2386 ± 8.5
1.25	1179 ± 5.7
0.625	534 ± 7.1
0.313	238 ± 12.7
0.156	103 ± 5.7
0.078	28 ± 2.8
0.039	10 ± 0.7

The GSH peak was detected at an average retention time of 1.58 min. for all standards. A typical GSH peak can be seen in Figure 6.5.   The standard curve produced a regression value of 0.9999 (Figure 6.6).
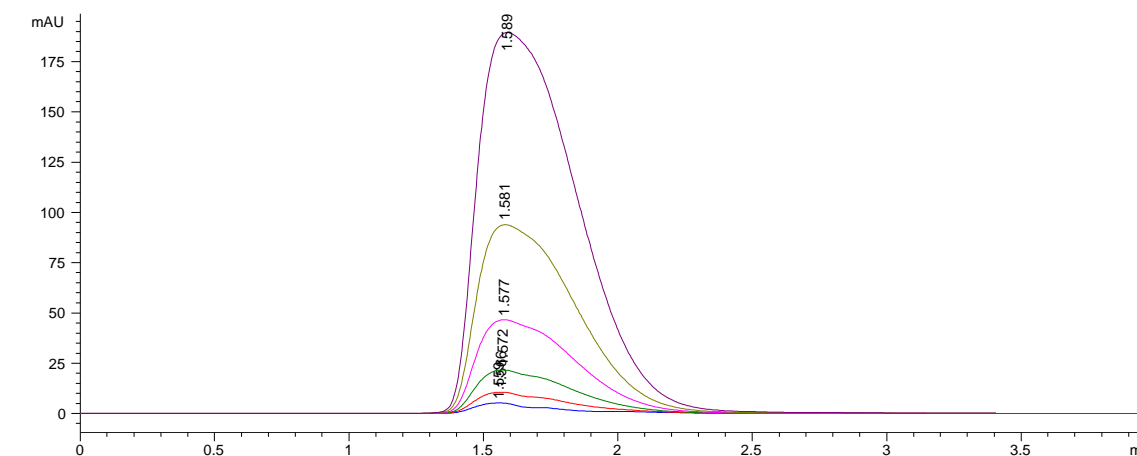
Figure 6.5. HPLC analysis of GSH standards at 220 nm. Retention times ~ 1.58 min were demonstrated.
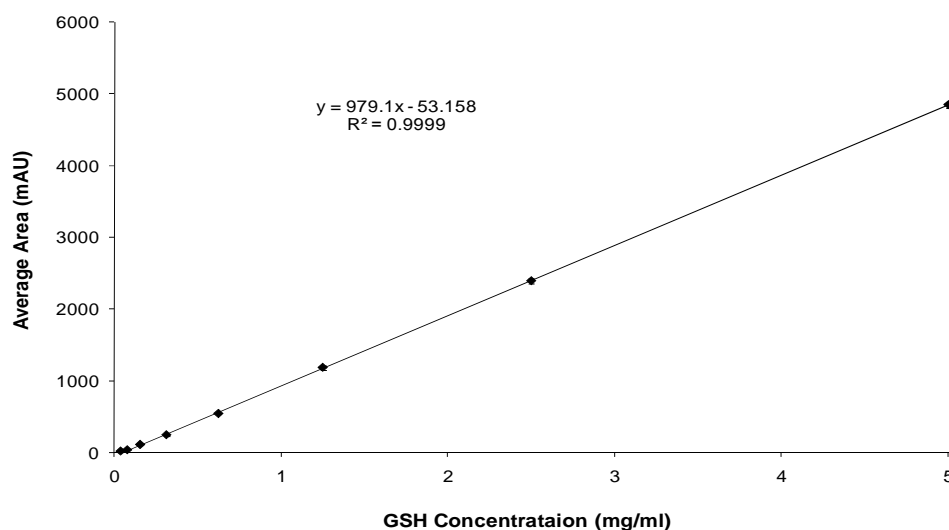
Figure 6.6. GSH Standard Curve at 220 nm.

6.4.2.2 HPLC Analysis of Crude Seaweed Protein Extracts

HPLC was untilised in the analysis of both crude extracts and gel filtration Peak 2 fractions. Tables 6.10 and 6.11 are summaries of results for crude protein extracts of *P. lanosa* samples exposed to 1 ppm Cd^{2+} for $0 - 72$ h. The retention time of the GSH standard was determined as 1.58 min. Analysis of crude extracts however demonstrated a main peak (A) at approximately 2.0 min for all samples, thereby confirming that this peak was not GSH.

Table 6.10. Analysis of HPLC Peak A Crude Extracts $0 - 72$ hours @ 220 nm.

Exposure Time *(hrs)*	*Peak A* *Retention Time* *(min)*	*Peak Area* *mAU*
Control	1.9	3694 ± 19.8
6	2.0	3676 ± 5.7
24	2.0	3855 ± 18.4
48	2.0	3080 ± 33.9
72	2.0	2458 ± 38.2

A similar trend was observed for Peak B of crude extracts. Retention times for all extracts were 3.4 min. Additionally, peak areas demonstrated similar trends. (Table 6.11).

Table 6.11. Analysis of HPLC Peak B Crude Extracts 0 – 72 hours @ 220 nm.

Exposure Time (hrs)	Peak B Retention Time (min)	Peak Area mAU
Control	3.4	228 ± 5.7
6	3.4	246 ± 4.2
24	3.4	223 ± 4.2
48	3.4	209 ± 8.5
72	3.4	192 ± 7.1

HPLC analysis of crude extracts was performed at two different wavelengths in order to determine the optimum wavelength (Figure 6.7). Results demonstrated an enhanced response at 220 nm.

220 nm   **280 nm**
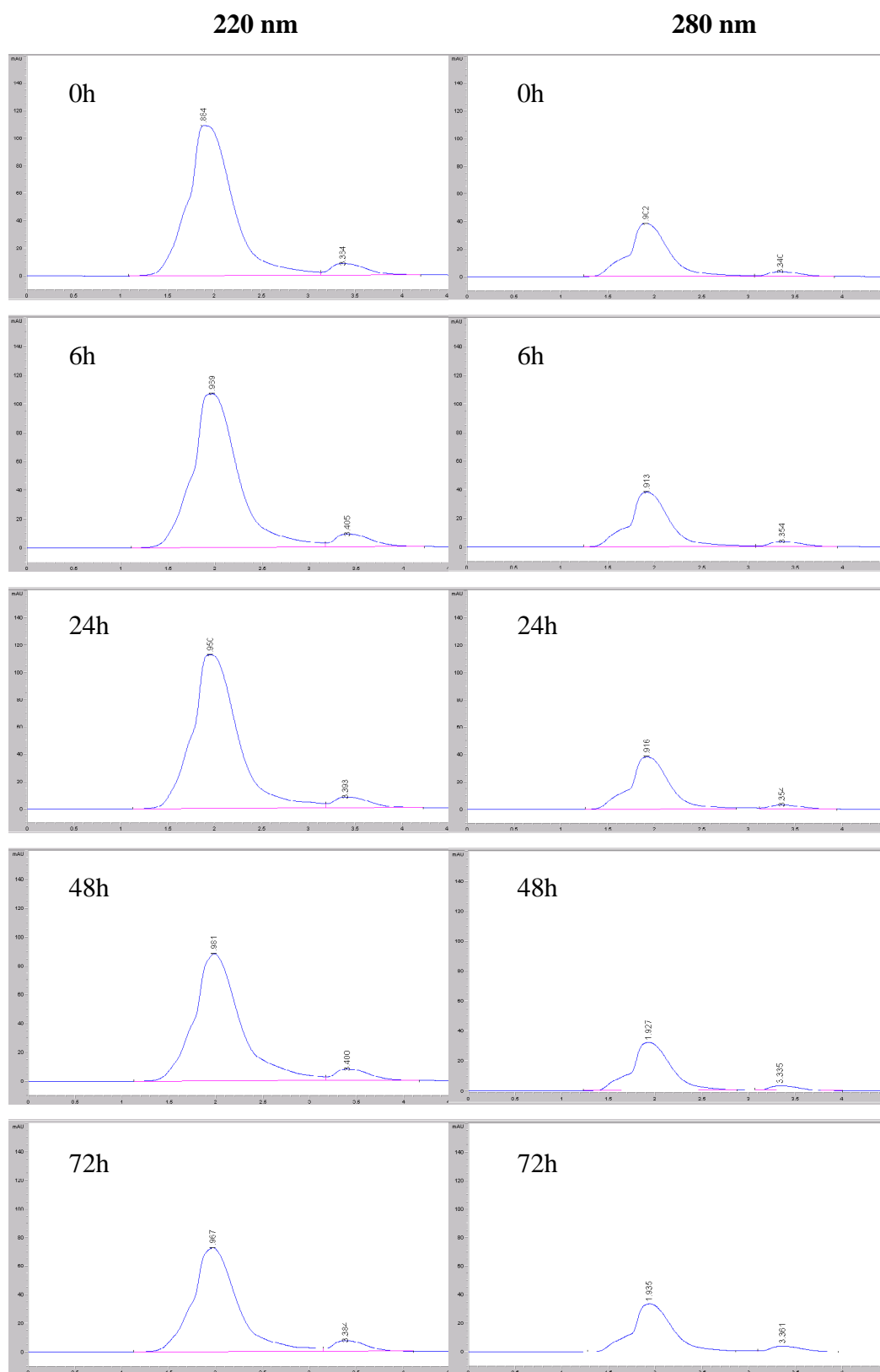
Figure 6.7. HPLC analysis of Crude Extracts (0 – 72h) at 220 and 280 nm. Analysis is based on triplicate runs.

6.4.2.3 HPLC Analysis of Peak Fractions from G-50 Gel Filtration Chromatography

The 2nd peak fractions derived from gel filtration chromatography could be related to PC. HPLC analysis of these fractions demonstrated that the 0 and 6h peaks eluted with the void volume at 1.5 mins (Table 6.12 and Figure 6.8). Changes to the HPLC profile were however observed for 24 – 72h peak fractions with significant peaks at a retention time of 2.0 mins (Table 6.12 and Figure 6.8).

Table 6.12. HPLC Analysis of Gel Filtration Peak Fraction 2 (0 – 72 hours @ 220 nm).

Exposure Time (hrs)	*Fraction No.*	*Retention Time (min) (HPLC Peak)*	*Peak Area mAU*
Control	18	1.5	9.4 ± 0.3
6	22	1.5	6.6 ± 0.1
24	21	2.0	270.8 ± 7.1
48	22	1.9	386.6 ± 22.6
72	20	2.0	264.4 ± 5.7
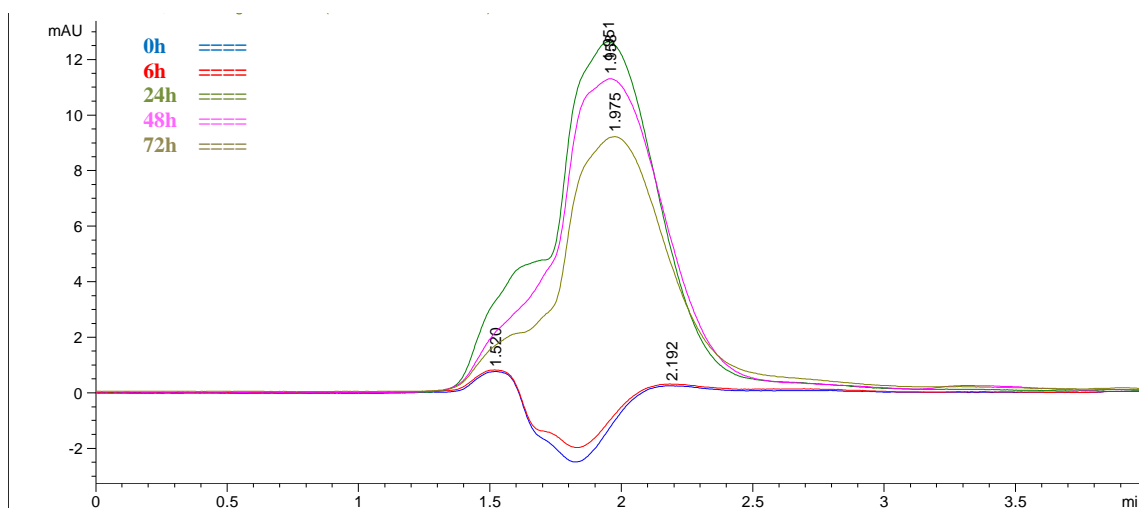
Figure 6.8. HPLC analysis of Peak 2 Fractions (Overlay of 0h to 72h) at 220 nm.

Co-elution of protein compounds was observed for 24 – 72 h fractions (Figure 6.8). It is possible that several proteins eluted at the same time. Further optimisation of the HPLC method is therefore required in order to separate the various proteins present.

6.4.3 SDS-PAGE and Silver Staining

6.4.3.1 Standard Curve Data

Calculations and data relating to the standard curve are shown in Table 6.13.

Table 6.13. SDS-PAGE standard curve data.

Average Distance protein travelled (cm)	*Distance to reference point (cm)*	*Average Relative Migration (Rf)*	*Log_{10} Mr*	*Mr (kDa)*
0.5	6.2	0.08 ± 0.00	1.99	97
1.03	6.2	0.17 ± 0.02	1.82	66
1.73	6.2	0.28 ± 0.01	1.65	45
2.35	6.2	0.38 ± 0.00	1.48	30
4.20	6.2	0.68 ± 0.01	1.30	20.1
5.33	6.2	0.86 ± 0.02	1.16	14.4

6.4.3.2 Standard Curve

The SDS-PAGE standard curve is demonstrated in Figure 6.9.   Standards and their corresponding molecular weights were as follows: Phosphorylase b (97 kDa), Albumin (66 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 kDa), Trypsin inhibitor (20.1 kDa) and α-Lactalbumin (14 kDa), (Figure 6.9).
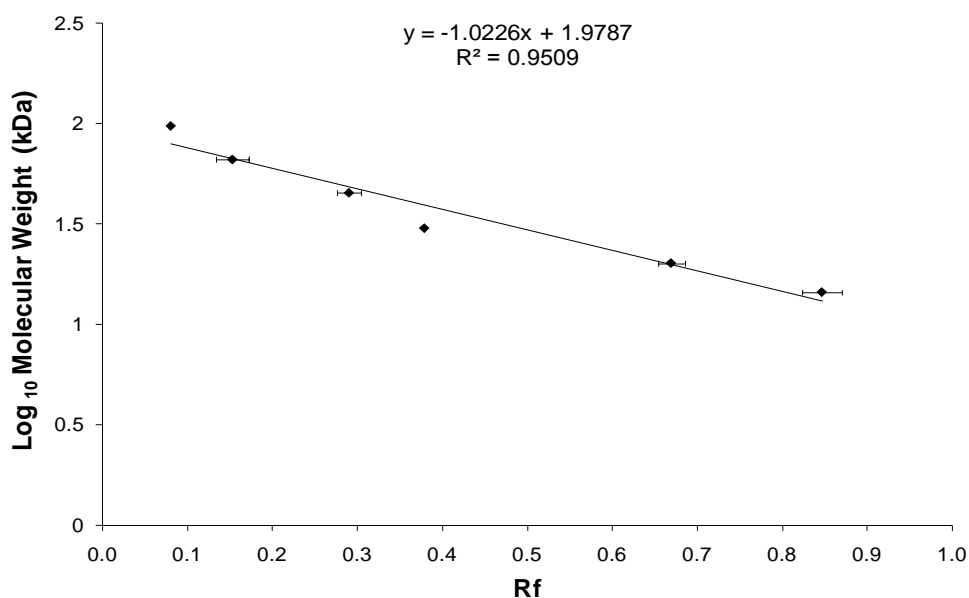
Figure 6.9. SDS-PAGE standard curve.

6.4.3.3 SDS-PAGE of Baseline P. lanosa Protein Extracts

The protein pattern of baseline *P. lanosa* was characterised by the presence of five main bands with molecular weights between 15.98 and 28.79 kDa (Figure 6.10).
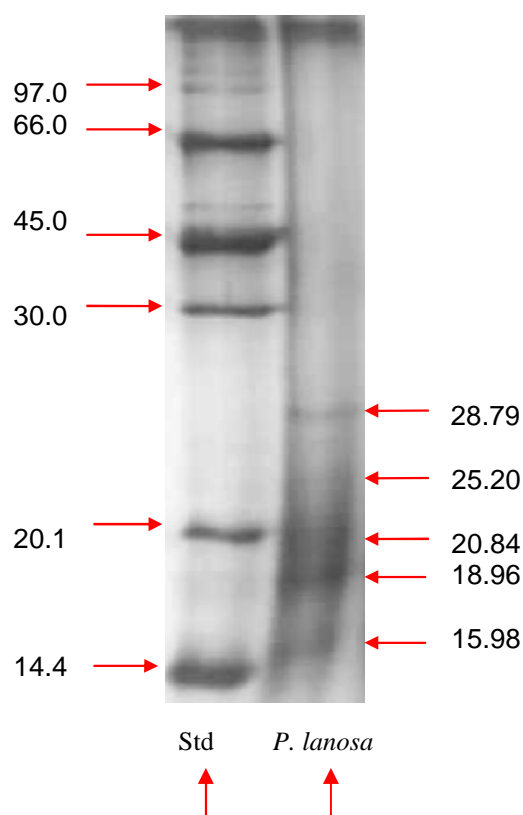
Figure 6.10. SDS-PAGE of baseline *P. lanosa* protein extracts. Analysis is based on duplicate samples with 95% confidence intervals.

6.4.3.4 SDS-PAGE of P. lanosa exposed to 1 ppm Cadmium

Following exposure, the protein pattern of *P. lanosa* changed and was characterised by the presence of six main bands with molecular weights between 16.0 and 44.55 kDa (Figure 6.11 and Table 6.14).
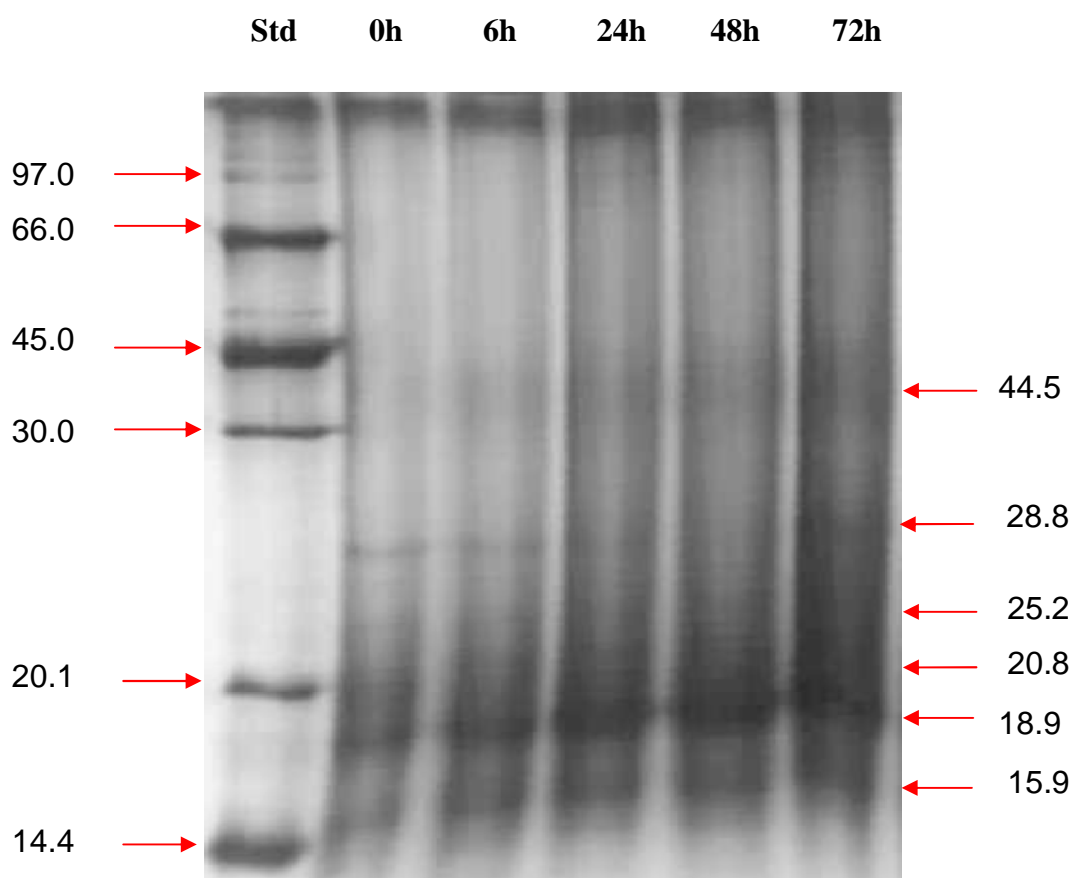
Figure 6.11. SDS-PAGE of protein extracts from *P. lanosa*, exposed to 1 ppm Cd for 72h. Analysis is based on duplicate samples with 95% confidence intervals.

Table 6.14. SDS-PAGE protein sample data. Following exposure to 1 ppm Cd, 6 protein bands were observed.

Average Distance protein travelled (*cm*)	Distance to Reference Point (*cm*)	Average Rf	Log $_{10}$	1/Log $_{10}$	MW kDa
2.0	6.2	0.32	1.65	44.55	44.5
3.15	6.2	0.51	1.46	28.79	28.8
3.5	6.2	0.56	1.40	25.2	25.2
4.0	6.2	0.65	1.32	20.84	20.8
4.25	6.2	0.69	1.28	18.96	19.0
4.7	6.2	0.76	1.20	15.98	16.0

6.5   Discussion

6.5.1   Sephadex G50 Gel Filtration Chromatography

In previous studies, the molecular weights of phytochelatin synthase (PCS) associated with plant and yeast cultures were 50 and ~46 kDa, respectively (Grill *et al.,* 1989; Clemens *et al.,* 1999).   In this study, the molecular weights of peak 1 fractions following gel filtration chromatography were in the range 48 − 52 kDa.   These values were therefore generally consistent with previous PCS values.   There is therefore some possible evidence of the presence of PCS in the *P. lanosa* extracts.   The presence of PCS is significant as these enzymes are only activated in the presence of heavy metals and are responsible for the synthesis of PC (Grill *et al*., 1989).

With respect to peak 2 fractions, the highest molecular weight was demonstrated by the 0h sample (PC n=5).   This sample was not exposed to Cd^{2+} experimentally however would have been exposed over long periods to background levels of heavy metals.   The system was therefore in equilibrium, resulting in synthesis of larger heavy metal defence molecules.

Results from this study demonstrated that following Cd^{2+} exposure for 72 h, the molecular weights of peak 2 fractions of the 6 − 72 h samples were 0.750 − 1.146 kDa. These molecular weight values correspond to PCs 2 − 4.   The 6 − 48h samples, which corresponded to PC n=2 − 3, demonstrated changes in their molecular weights (0.75 − 0.948 kDa) when compared to that of the 0 h sample (the 0 h sample was not exposed to Cd^{2+}).   However the corresponding value for the 72 h sample demonstrated an increase to 1.146 kDa.   This increase perhaps signifies a return to baseline/control indicating a possible return to equilibrium/steady state and the synthesis of longer heavy metal binding peptides over time.

Wu (1998) identified PCs as the main intracellular Cd^{2+} chelators in the marine macroalga *Kappaphycus alvarezii*.   Following exposure to 100μM $CdCl_2$ for 9 days, the PCs isolated from the seaweed demonstrated a less complex thiol profile than that found in yeast and higher plants.   Using the general PCn structure, the n value for *K. alvarezii* was < 3.   Furthermore, Malea *et al.* (2006) demonstrated the induction of PCs 2 and 3 in *E. prolifera* following exposure to Cd^{2+}.   Additionally, PC2 to PC4 synthesis in the

freshwater filamentous green alga *Stigeoclonium* sp., was also demonstrated by Pawlik-Skowrońska (2001), following exposure to 10μM Cd^{2+} for 17h.

Previous studies have shown biochemical changes in seaweed protein following heavy metals exposure however this is the first time that gel filtration chromatography has been used to demonstrate these changes in the red alga, *P. lanosa*. In order to further investigate changes in peptides, extracted from seaweed following exposure to heavy metals, HPLC analysis of crude extracts and gel filtration peak 2 fractions was performed.

6.5.2   HPLC Analysis

6.5.2.1 HPLC Analysis of Crude Seaweed Protein Extracts

HPLC analysis of the standard (GSH) demonstrated a retention time of 1.58 min. HPLC analysis of crude extracts however demonstrated a main peak (A) at approximately 2.0 mins for all samples, thereby confirming that this peak was not GSH. There was however evidence of an elution shoulder associated with peak A, extending into the GSH region (~1.53 minutes). This shoulder was therefore eluting at the GSH retention time. Results demonstrated co-elution of protein extracts therefore a change in chromatographic conditions is required in order to sufficiently separate proteins.

The average HPLC peak A area for 0 – 6h were generally the same. Following 48 and 72h exposure however, a decrease in peak area was observed. This decrease suggests possible peptide-Cd binding following intracellular uptake of the heavy metal. It was previously demonstrated in Chapter 3 that the highest proportion of Cd^{2+} associated with *P. lanosa* was intracellular (~90%). As *P. lanosa* is capable of bioaccumulating significant levels of Cd^{2+} intracellularly, sufficiently high levels of metal binding peptides are therefore required to combat heavy metal toxicity. This is perhaps consistent with results from Chapter 4 (4.4.2.2) whereby *P. lanosa* (collected in February, 2009), exposed to 1 ppm Cd^{2+}, demonstrated increases in extracted protein levels up to 72 h.

6.5.2.2 HPLC Analysis of Peak Fractions from G-50 Gel Filtration Chromatography

HPLC analysis of 2nd peak fractions demonstrated that the 0 and 6h peaks eluted with the void volume at 1.5 mins. Changes to the HPLC profile were however observed for 24 − 72h peak fractions with significant peaks at a retention time of 2.0 mins. An increase in retention time suggests the synthesis of less complex heavy metal defence peptides in response to heavy metal exposure. Significantly, a decrease in peak area at 72h was observed, indicating a return to equilibrium. A similar trend was also observed for the 2nd peak in gel filtration chromatography (Section 6.4.1). Furthermore, co-elution of proteins was observed for the 24 − 72h peak fractions. An elution shoulder, extending into the GSH retention time was also observed. Retention times for elution shoulders associated with 24, 48 and 72h peak fractions were 1.6, 1.54 and 1.53 minutes respectively. It is possible that several proteins eluted at the same time. Further optimisation of the HPLC method is therefore required in order to separate the various proteins present.

Many previous studies have investigated the changes in GSH/PC levels in higher plants (Wu *et al*., 2001; Alvarez-Legorreta, 2008), microalgae (Ahner *et al*., 1994; Scarano & Morelli, 2002), diatoms (Rijstenbil *et al*., 1998), seedlings (Rauser *et al*., 1991), etc., following heavy metal exposure. Fewer studies have monitored those changes in seaweeds. For example, Malea *et al.* (2006) demonstrated that PC induction in seaweeds varies from species to species. Following Cd^{2+} exposure variations in the ability of two *Enteromorpha* sps to combat heavy metal exposure were demonstrated. In *E. prolifera*, Cd^{2+} induced PC synthesis, whereas in *E. linza*, Cd^{2+} only induced oxidative stress following exposure.

This is the first time that changes in the protein profile of *P. lanosa* following heavy metal exposure have been monitored. Following HPLC analysis of protein extracts, changes in sample peak sizes in this study may correspond to changes in levels of metal binding peptides following exposure to Cd^{2+}. Further investigations however were required in order to determine the exact nature of the compounds present. SDS-PAGE was utilised in order to visualise the mass range of proteins produced by seaweed following heavy metal exposure.

6.5.3   SDS-PAGE and Silver Staining

6.5.3.1 SDS-PAGE of Baseline P. lanosa Protein Extracts

The baseline *P. lanosa* extract was characterised by the presence of five main bands with molecular weights between 15.98 and 28.79 kDa

Investigations involving the deciphering of protein patterns for many baseline species of algae have previously been carried out (Rouxel *et al.*, 2001, Price 1987, Fleurence *et al.*, 1995a, Galland-Irmouli *et al.*, 1999). Baseline protein bands in this study generally correspond to SDS bands for other red seaweeds. For many of those species however, an extra band with a high molecular weight (~50 – 60 kDa) has also been observed (Table 6.15). The SDS protein determination of baseline *P. lanosa* however has never previously been performed. Comparisons with previous work therefore demonstrate variation in protein patterns for red algae.

Table 6.15. Variation in SDS protein bands (kDa) of red seaweeds without metal exposure.

Band No.	P. lanosa *	P. palmata †	C. crispus †
1	28.79	59.6	49.3
2	25.2	48.3	46.2
3	20.84	32.7	43.2
4	18.96	25.9	19.8
5	15.98	20.3	17.2
6		15.2	16.4
7			15.2

* Present Study; † Rouxel *et al.* (2001).

6.5.3.2 SDS-PAGE of P. lanosa exposed to 1 ppm Cadmium

Following exposure, *P. lanosa* was characterised by the presence of six main bands with molecular weights between 16.0 and 44.55 kDa. The same 5 bands observed for 0 h samples were present but an additional band at 44.55 kDa was also present.

SDS gel electrophoresis of protein extracts of some red seaweeds have shown bands with molecular weights between 43 – 49 kDa. These particular protein bands were not present in baseline *P. lanosa* in the current study. Following Cd^{2+} exposure however, a

band with a molecular weight ~ 45 kDa appeared after 6h exposure and the intensity of the band further increased up to 72 h exposure.

In a previous study, Grill *et al.* (1989) discovered an enzyme from plant cell cultures that catalysed the synthesis of PCs. The enzyme, PCS demonstrated a molecular weight of 95 kDa (and its dimer, 50 kDa). Furthermore, in a later study, Clemens *et al.* (1999) determined that the PCS in yeast had a molecular weight of ~ 46 kDa. These values correspond generally to a protein band with an apparent molecular weight of ~ 45 kDa in this study that appear with increasing intensity following heavy metal exposure. This protein band also corresponded to a high molecular weight peak fraction obtained during gel filtration chromatography in this study.

Among all 6 protein bands, two with molecular weights of 19.0 and 16.0 kDa were dominant. Following exposure to 1 ppm Cd for 0 – 72 h, the intensity of these particular bands increased. Following exposure, seaweeds can accumulate metals using a variety of cellular mechanisms (Pawlik-Skowrónska *et al.*, 2007). A number of peptides containing the amino acid cysteine are important metal-binding cytosolic ligands (Rijstenbil *et al.*, 1998). These include glutathione (Grill *et al.*, 1987) and phytochelatins (PC). PC are enzymatically synthesised in the presence of heavy metal ions such as Cd^{2+} (Gekeler *et al.*, 1988). The molecular weights of PC in plants range from 3 – 10 kDa (Bayçu, 2002). These values are below the molecular weight range of standards in this study. SDS gel electrophoresis with lower molecular weight standards therefore is required for the analysis of PC. Protein bands with molecular weights of 16.0 and 19.0 kDa do not therefore represent PC. However protein bands with these molecular weights are represented in gel filtration chromatography analysis of protein extracts (as part of peak 1 between 61 and 68 ml).

Many previous studies have utilised electrophoretic techniques to determine baseline protein characteristics of various seaweeds (Price, 1987; Fleurence *et al.,* 1995a; Galland-Irmouli *et al.*, 1999; Rouxel *et al.*, 2001). In a unique study however, the baseline profile of protein extracted from the red seaweed *P. lanosa* is reported. Significantly, variations in the profile of proteins of *P. lanosa*, following heavy metal exposure are documented in this study for the first time.

6.6   Conclusions

Following exposure to 1 ppm Cd for 0 – 72 h, *P. lanosa* demonstrated variations in levels of total and extracted protein. This study monitored changes in the protein extracts by utilising various chromatographic techniques. Results from gel filtration chromatography demonstrated similar trends for each sample in that each formed two distinctive peaks. The first peak had a molecular weight of ~ 50 kDa. This value generally corresponds to previously determined PCS values for plant and yeast cells as 46 – 50 kDa (Grill *et al.,* 1989; Clemens *et al.,* 1999). In relation to the second peaks, the 0 h extract contained proteins with the highest molecular weight (1.507 kDa). Following exposure there was a subsequent decrease in the molecular weights of the 6 – 48 h extracts (these molecular weights generally corresponded to PCs n=3 and n=2). Following further exposure, the molecular weight for the 72 h sample increased to 1.146 kDa, equivalent to PC4. The initial decrease in molecular weight for 6 – 48 h may have corresponded to the synthesis of smaller heavy metal defence peptides in response to a sudden exposure to higher concentrations of $Cd^{2+.}$ The increase in molecular weight at 72h may have corresponded to a return to equilibrium and the synthesis of larger defence peptides. Further studies however are required in order to identify these molecules.

Reverse phase HPLC analysis of GSH resulted in a 1.58 min. Analysis of crude extracts however demonstrated a main peak at approximately 2.0 min for all samples. This therefore demonstrated that the elution peak was not GSH. An elution shoulder however, associated with this peak extended into the GSH region. Results also demonstrated co-elution of the protein extracts. Areas of the main HPLC peak for 0 – 6h were generally the same. An increase in area was observed for the 24 h sample. However, following 48 – 72h exposure, decreases in peak area were observed, suggesting biochemical changes upon intracellular uptake of the heavy metal.

The 2nd peak fractions derived from gel filtration chromatography were associated with molecular weights ranging from 0.75 – 1.5 kDa. HPLC analysis of these fractions established that the 0 and 6h peaks eluted with the void volume at 1.5 min. The 24 – 72h peak fractions however demonstrated increases in retention time at 2.0 min, suggesting the synthesis of less complex heavy metal defence peptides in response to Cd^{2+} exposure. Notably, a decrease in peak area at 72h was also observed, indicating

perhaps a return to baseline/control indicating a possible return to equilibrium/steady state. A similar trend was also observed for the 2nd peaks following gel filtration chromatography.

SDS PAGE of baseline samples demonstrated five bands with molecular weights between 16.0 and 28.8 kDa. Following exposure, a band with a molecular weight of 44.55 kDa was observed in the 6 – 72h samples. This band was not present for the 0 h sample. It may indicate the enhanced synthesis of PCS following Cd^{2+} exposure. Furthermore, a compound with a similar molecular weight was observed in this study using gel filtration.

The aim of this study was to develop methods capable of monitoring changes in the biochemical composition of *P. lanosa* following exposure to Cd^{2+}. Many techniques have previously been performed in order to determine such changes in higher plants (Wu *et al*., 2001), microalgae (Scarano & Morelli, 2002) and diatoms (Rijstenbil *et al*., 1998), following exposure to heavy metals. These include gel filtration chromatography (Grill *et al*., 1985), HPLC analysis (mBBr derivatisation) (Ahner *et al*., 1994; Clemens *et al*., 2001; Gawel *et al*., 2001; Ahner *et al*., 2002) and SDS PAGE (Grill *et al*., 1989; Clemens *et al*., 2001; Gorinova *et al*., 2007). In this novel study however, these various techniques were utilised to decipher the biochemical changes in the protein characteristics of the red seaweed *P. lanosa* following exposure to a heavy metal over 72 h. This work represents very initial characterisation studies and further analysis is warranted to explore changes and to verify the exact proteins being produced in response to heavy metal stress in algae. Changes to the molecular weight species and proteins in seaweeds following Cd^{2+} exposure have been observed and some suggestions as to the possible biochemical changes have been presented.

Chapter 7

Conclusions and Future Work

7.   Conclusions and Future Work

7.1   Conclusions

This comprehensive study contains the most up to date reference work relating to relating to the development & optimisation of seaweed protein & polyphenol quantitation methods, temporal variations in seaweeds and the biochemical mechanisms of seaweeds

In this study standard techniques that have previously been used to determine the biochemical composition of seaweeds were further developed and optimised. Furthermore temporal, interspecies and spatial variations in the levels of various metals associated with seaweeds were also demonstrated. Variations in the proportions of surface bound and intracellular levels of metals were observed. The location of metals within the seaweed varied according to both metal type and seaweed species. Additionally, seasonal variations in protein and polyphenol induction following heavy metal exposure were also established.

Methods for the extraction of *P. lanosa* protein and polyphenols were developed and optimised in this study. A method involving an initial extraction in deionised water at 4^oC followed by a pellet extraction with 0.1M NaOH in the presence of 0.5% β-mercaptoethanol was the chosen method of protein extraction. Furthermore, a method utilising a 70% methanol solvent extraction at room temperature was developed and optimised for polyphenol analysis. Single, optimised extraction protocols for all seaweed species under investigation were utilised in order facilitate an interspecies comparison throughout the study.

Significant spatial and interspecies variations in the baseline levels of total protein, extracted protein and polyphenols of the different seaweed species investigated were observed. *P. lanosa* contained the highest total protein and the brown species the lowest. A reverse in trends was observed for extracted protein whereby the brown seaweeds yielded the highest levels. Difficulties in protein extraction were attributed to tough cell wall polysaccharides of red and green seaweeds. The total protein levels in *F. vesiculosus* collected from the Suir Estuary and Fethard-on-Sea were ~ 48% higher than those of the Newfoundland sites. A positive correlation between total protein and

temperature was demonstrated. *F. vesiculosus* and *A. nodosum* yielded the highest levels of polyphenols. A downstream increase in polyphenol levels of seaweeds was observed and correlated with a downstream increase in seawater salinity.

Significant spatial and interspecies variations in total and intracellular metals were observed for all seaweeds. The highest proportions of most metals were found to occur intracellularly. Levels of Zn^{2+}, As^{3+}, Al^{3+} and Mn^{2+} were dominant for all seaweed species. Seaweeds have different affinities for different metals as a result of variations in metal binding potential and nutritional requirements. *P. lanosa* demonstrated the best overall metal bioaccumulation potential and this may be attributed to a greater surface area associated with the filamentous seaweed and enhanced metal binding capacity of red seaweeds. Mn^{2+} and Al^{3+} established the highest overall levels of seaweed bioaccumulation. Furthermore, levels of Mn^{2+} and Al^{3+} associated with *P. lanosa* in this study were significantly higher than those previously recorded by any other seaweed in the literature. Levels of Pb^{2+}, Cr^{3+} and Cu^{2+} in seaweed were highest for sites in Waterford City, a location in close proximity to industrial activity. Regression analysis demonstrated possible correlations between total protein and Pb^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} and also between extracted protein and intracellular As^{3+}, Co^{2+}, Cr^{3+} and Cu^{2+}.

Seasonal variations in the baseline levels of protein, polyphenols and metal levels seaweeds were also observed. Furthermore, seasonal variations in the levels of total protein, extracted protein and polyphenols of *P. lanosa* were also demonstrated. The highest levels of total protein were yielded in May, while February demonstrated the highest extracted protein and November, the lowest polyphenols. Increases in total and extracted protein levels following heavy metal exposure were observed for *P. lanosa* sampled in February only, as a result of possible induction of heavy metal defence peptides such as PC. Increases in polyphenol levels following Cd^{2+} exposure were only observed in the November samples. Results suggest the possible activation of two different modes of heavy metal defence in *P. lanosa* at different times of the year. Seasonal variations in baseline total, intracellular and surface bound Cd^{2+} levels of *P. lanosa* were demonstrated. Furthermore, seasonal variations in heavy metal uptake following Cd^{2+} exposure were also observed. The highest proportions of intracellular metals were observed for *P. lanosa* harvested in May (33%) and the highest fraction of surface bound metals were demonstrated for the February samples (83%). These variations were attributed to seasonal changes to the seaweed plasma membrane and cell

wall polysaccharides. Regression analysis demonstrated strong negative temporal variations for intracellular Cd^{2+} versus extracted protein levels and for total Cd^{2+} versus total protein in exposed samples.

Potentiometric titrations and FTIR analysis demonstrated for the first time, seasonal variations in the total and weak acid groups associated with *P. lanosa*. The highest proportions of total and weak acidic groups were associated with the February samples. These variations may be attributed to seasonal changes in the carrageenan levels of *P. lanosa* which therefore influence the metal binding potential of seaweeds. Seasonal changes in the carrageenan levels of several Rhodophyceae were demonstrated by Cossan *et al*. (1990). Furthermore, they established a positive correlation between carrageenan concentration and seaweed growth. Temporal studies demonstrated the induction of protein following heavy metal exposure only in *P. lanosa* samples collected in February, therefore further investigations of these protein extracts were performed.

P. lanosa, sampled in February, saw changes in its extracted protein levels following exposure to 1ppm Cd^{2+} over 72h. Results from gel filtration chromatography demonstrated two distinctive peaks for each of the protein extracts. The first peak had a molecular weight of ~ 50 kDa. This value generally corresponded to previously determined PCS values for plant and yeast cells (46 – 50 kDa), (Grill *et al.,* 1989; Clemens *et al.,* 1999). The molecular weight of the second peaks ranged from 0.750 – 1.507 kDa. An initial decrease in molecular weight for 6 – 48 h may have corresponded to the synthesis of smaller heavy metal defence peptides in response to a sudden exposure to higher concentrations of $Cd^{2+.}$ A subsequent increase in molecular weight at 72h may have corresponded to a return to steady state and the synthesis of larger defence peptides.

HPLC analysis of crude extracts demonstrated a main peak for all sample extracts at approximately 2.0 mins, thereby demonstrating that the elution peak was not GSH (retention time = 1.5 min). An elution shoulder however, associated with this peak extended into the GSH region. Following 24 – 72h exposure decreases in peak area were observed, suggesting possible peptide-Cd binding following intracellular uptake of the heavy metal or perhaps exudation of peptide-Cd complexes. HPLC analysis of the 2nd peak fractions from gel filtration chromatography demonstrated an increase in

retention time at 2 mins for the 24 – 72h peak fractions, suggesting the synthesis of less complex heavy metal defence peptides in response to Cd^{2+} exposure. Notably, a decrease in peak area at 72h was also observed, indicating perhaps a return to steady state. A similar trend was also observed for the 2nd peaks following gel filtration chromatography.

SDS PAGE of baseline samples demonstrated five bands with molecular weights between 15.98 and 28.79 kDa. Following exposure, an additional band with a molecular weight of 44.55 kDa was observed in the 6 – 72h samples. This band was not present for the 0 h sample and may perhaps indicate the enhanced synthesis of metal binding peptides following Cd^{2+} exposure. Furthermore a molecular weight of 44.55 kDa generally corresponded with values from gel filtration chromatography and also with values for PCS in previous studies.

In this unique study, the biochemical effects of heavy metal exposure on seaweeds were investigated. Various procedures were used to study the temporal, spatial and interspecies variations in total protein, extracted protein, polyphenols and metals levels in seaweeds. Additionally, interactions between seaweeds, heavy metals and physico-chemical parameters were also investigated. Outcomes from this research may have constructive economic implications due to the potential sustainable utilisation of our natural resources. Furthermore, results from this study will provide vital background knowledge to those involved in the development of new metal biosorbents, phycologists interested in the interactions of seaweeds and metals and environmental scientists interested in the use of seaweeds as bioindicators.

7.2   Future Work

Some potential areas of future work directly arising from the research in this thesis are detailed below.

Levels of Zn^{2+}, As^{3+} and Mn^{2+} were found to be significant for *P. lanosa, A. nodosum, F. vesiculosus* and *Ulva* sp. Evidence of competitive interactions between Mn^{2+} and Zn^{2+} was also demonstrated.   Further detailed studies therefore, involving the interactions of these metals with each other and with macroalgae are required. Sunda & Huntsman (1996) previously studied the antagonistic interactions between the toxic metals Cd^{2+} and Zn^{2+} and the micronutrient Mn^{2+} in diatoms.

Further development of the reverse phase HPLC chromatographic conditions in order to enhance protein separation is required. In this study, proteins eluted quickly (~1.5 minutes) and there was also evidence of co-elution. By making the mobile phase more non-polar the retention times of individual proteins should increase and therefore, separation enhanced. HPLC methods could also be developed in order to quantify glutathione and phytochelatins following heavy metal exposure.

Non-protein reduced sulphur like cysteine, glutathione and phytochelatins might be major thiol compounds involved in the detoxification of intracellular Cd^{2+} in macroalgae (García Ríos *et al*., 2007). Monobromobimane (mBBr), one of the most commonly used sulphur-specific fluorescent tags, has previously been reported for the derivatisation of PCs produced by phytoplankton, with subsequent fluorescence detection (Kawakami *et al*., 2006). Furthermore, previous studies have also utilised fluorescent tags for the derivatisation of thiol compounds in land plants (Grill *et al*., 1987; de Knecht *et al*., 1994), trees (Gawel *et al*., 2001) and nematodes (roundworm), (Clement *et al*., 2001).   mBBr derivatisation methods for thiol compounds in macroalgae can therefore be potentially developed.

Following gel filtration chromatography, fractionated protein extracts specifically associated with heavy metal induction could be subjected to HPLC analysis in order to identify and quantify heavy metal binding peptides. Grill *et al.* (1987) previously identified PCs in fractionated extracts of red cabbage following Cd^{2+} exposure and the proportion of metal associated with this peak fraction was established.

Potentiometric titrations and FTIR investigations could be extended to encompass the study of both non-protonated and exposed seaweeds. These analytical techniques could potentially be utilised to monitor kinetic changes in the metal binding potential of seaweeds following heavy metal exposure.

Two-dimensional electrophoresis could be employed to identify specific proteins from seaweed extracts. The first dimensional stage separates proteins according to their isoelectric point. The second dimensional stage separates proteins according to molecular weight. Protein spots can then be excised and analysed using mass spectrometry techniques (Aina *et al.,* 2007). Aina *et al.,* 2007 used this technique to identify specific proteins that were induced in Cd-treated rice roots.

By the use of ultra low molecular weight standards (1.060 – 26.6 kDa), SDS PAGE of samples can be used to identify phytochelatins which have molecular weights $\leq$ 10 kDa. Furthermore, confirmation of the presence of PC could be achieved through the use of mass spectroscopic techniques. Hartley-Whitaker *et al.* (2001) previously utilised this technology to identify PC in higher plants. Additionally, following amino acid sequencing, western blotting, which utilises specific antibodies, could be used for the identification of heavy metal induced phytochelatin synthase and phytochelatins.

References

References

Abdala-Díaz R, Cabello-Pasini A, Pérez-Rodríguez E, Álvarez R, & Figueroa F (2006) Daily and seasonal variations of optimum quantum yield and phenolic compounds in *Cystoseira tamariscifolia* (Phaeophyta). *Marine Biology* 148, 459-465.

Ahner BA, Price NM & Morel FM (1994) Phytochelatin production by marine phytoplankton at low free metal ion concentrations: laboratory studies and field data from Massachusetts Bay. *Proceedings of the National Academy of Sciences of the United States of America* 91, 8433-8436.

Ahner BA, Kong AS & Morel FMM (1995) Phytochelatin production in marine algae. I. An interspecies comparison. *Limnology and Oceanography* 40, 649-657.

Ahner BA & Morel FMM (1995) Phytochelatin production in marine algae. II. Induction by various metals. *Limnology and Oceanography* 40, 658-665.

Ahner BA, Wei L, Oleson JR & Ogura N (2002) Glutathione & other low molecular weight thiols in marine phytoplankton under metal stress. *Marine Ecology progress Series* 232,93–103.

Aina R, Labra M, Fumagalli P, Vannini C, Marsoni M, Cucchi U, Bracale M, Sgorbati S & Citterio S (2007) Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa L.* roots. *Environmental and Experimental Botany* 59, 381-392.

Algaebase. www.algaebase.org (May, 2009/2010).

Alvarez-Legorreta T, Mendoza-Cozatl D, Moreno-Sanchez R & Gold-Bouchot G (2008) Thiol peptides induction in the seagrass *Thalassia testudinum* (Banks ex König) in response to cadmium exposure. *Aquatic Toxicology* 86, 12-19.

Amado Filho GM, Andrade LR, Karez CS, Farina M & Pfeiffer WC (1999) Brown algae species as biomonitors of Zinc and Cadmium at Sepetiba Bay, Rio de Janeiro, Brazil. *Marine Environmental Research* 48, 213-224.

Andrad S, Contreras L, Moffett JW & Correa JA (2006) Kinetics of copper accumulation in *Lessonia nigrescens* (Phaeophyceae) under conditions of environmental oxidative stress. *Aquatic Toxicology* 78, 398–401.

Antolovich M, Prenzler P, Robards K & Ryan D (2000) Sample preparation in the determination of phenolic compounds in fruits. *Analyst.* 125, 989-1009.

Arnold TM, Tanner CE & Hatch WI (1995) Phenotypic variation in polyphenolic content of the tropical brown alga *Lobophora variegata* as a function of nitrogen availability. *Marine Ecology Progress Series* 123, 177-183.

Arslan G & Pehlivan E (2008) Uptake of Cr^{3+} from aqueous solution by lignite-based humic acids. *Bioresource Technology* 99, 7597-7605.

Association of Official Analytical Chemists (AOAC) (1995).

Atkins CA, Paterson BD & Graham D (1972) Plant carbonic anhydrases. 1. Distribution of types among species. *Plant Physiology* 50, 214-217.

ATSDR (2008) Agency for Toxic Substances and Disease Registry, U.S. Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry. http://www.atsdr.cdc.gov/ (May, 2010).

Azlim Almey AA, Ahmed Jalal Khan C, Syed Zahir I, Mustapha Suleiman K, Aisyah MR & Kamarul Rahim K (2010) Total phenolic content and primary antioxidant activity of methanolic

and ethanolic extracts of aromatic plants' leaves. *International Food Research Journal* 17, 59-65.

Backor M, Gibalová A, Budová J, Mikes J & Solár P (2006) Cadmium-induced stimulation of stress-protein hsp70 in lichen photobiont Trebouxia erici. *Plant Growth Regulation* 50, 159–164.

Badger MR & Price GD (1994) The Role of Carbonic Anhydrase in Photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 45, 369-392.

Bailey SE, Olin TJ, Bricka RM & Adrian DD (1999) A review of potentially low-cost sorbents for heavy metals. *Water Research* 33, 2469-2479.

Bakir A, McLoughlin P, Tofail SAM & Fitzgerald E (2009) Competitive Sorption of Antimony with Zinc, Nickel, and Aluminum in a Seaweed Based Fixed-bed Sorption Column. *Clean-Soil, Air, Water* 37, 712-719.

Barbarino E & Lourenco S (2005) An evaluation of methods for extraction and quantification of protein from marine macro- and microalgae. *Journal of Applied Phycology* 17, 447-460.

Barrett J & Anderson JM (1977) Thylakoid membrane fragments with different chlorophyll A, chlorophyll C and fucoxanthin compositions isolated from the brown seaweed Ecklonia radiata. *Plant Science Letters* 9, 275-283.

Battistoni A & Rotilio G (1995) Isolation of an active and heat-stable monomeric form of Cu,Zn superoxide dismutase from the periplasmic space of *Escherichia coli*. *FEBS Letters* 374, 199-202.

Bayçu G (2002) Phytochelatin biosynthesis and cadmium detoxification. *Journal of Cell and Molecular Biology* 1, 45-55.

Bidwell RGS (1979) Plant Physiology, second edition. Macmillan, New York.

Bierkens J, Maes,J & Plaetse FV (1998) Dose-dependent induction of heat shock protein 70 synthesis in (*Raphidocelis subcapitata*) following exposure to different classes of environmental pollutants. *Environmental Pollution* 101, 91–97.

Black WAP & Dewar ET (1949) Correlation of Some of the Physical and Chemical Properties of the Sea With the Chemical Constitution of the Algae. *Journal of the Marine Biological Association of the UK* 28, 673-699.

Black WAP (1953) Constituents of the marine algae. *Chemical Society London.* 150, (*Annual Reports on the Progress of Chemistry*) 322–35.

Bold HC & Wynne MJ (1985) Introduction to the algae: Structure and Reproduction, Prentice-Hall, Englewood Cliffs, NJ, 516.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248-254.

Brown MT & Depledge MH (1998) Trace metal contamination of marine ecosystems: In, Metal metabolism in aquatic environments; Edited by WJ Langston & MJ Bebianno. Chapman & Hall, London, 185 – 218.

Brown MT, Hodgkinson WM & Hurd CL (1999) Spatial and temporal variations in the copper and zinc concentrations of two green seaweeds from Otago Harbour, New Zealand. *Marine Environmental Research* 47, 175-184.

Bruland KW, Donat JR & Hutchins DA (1991) Interactive influences of bioactive trace metals on biological production in oceanic waters. *Limnology and Oceanography*. 36, 1555-1577.

Burdon RH (1986) Heat shock and the heat shock proteins. *Biochemical Journal* 240, 313–324.

Burtin P (2003) Nutritional Value of Seaweeds. *Electronic Journal of Environmental,*

Agricultural and Food Chemistry 1579-4377.

Canadian Census, 2006. www.statcan.gc.ca/ (June, 2010).

Castro R, Zarra I & Lamas J (2004) Water-soluble seaweed extracts modulate the respiratory burst activity of turbot phagocytes. *Aquaculture* 229, 67-78.

Central Statistics Office (Ireland) 2006. www.cso.ie/statistics/ (June, 2010)

Chan JCC, Cheung PCK &Ang PO (1997) Comparative Studies on the Effect of Three Drying Methods on the Nutritional Composition of Seaweed *Sargassum hemiphyllum* (Turn.) C. Ag. *Journal of Agricultural and Food Chemistry* 45, 3056-3059.

Chen L, Yang L & Wang Q (2009) In vivo phytochelatins and Hg-phytochelatin complexes in Hg-stressed. *Brassica chinensis L. Metallomics* 1, 101-106.

Cheng L, Zou D, Zheng Q, Liu Z, Li, Jiang H (2010) Effects of temperature and light intensity on the nitrate uptake kinetics of nitrogen starved and replete *Ulva lactuca. Chinese Journal of Ecology* 29: 939

Chew YL, Lim YY, Omar M & Khoo KS (2008) Antioxidant activity of three edible seaweeds from two areas in South East Asia. *LWT - Food Science and Technology* 41, 1067-1072.

Clemens S, Kim EJ, Neumann D & Schroeder JI (1999) Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO Journal* 18, 3325-3333.

Clemens S, Schroeder JI & Degenkolb T (2001) *Caenorhabditis elegans* expresses a functional phytochelatin synthase. *European Journal of Biochemistry* 268, 3640-3643.

Clothup NB, Daly LH & Wiberley SE (1990) Introduction to infrared and raman spectroscopy, 3rd Edition, *Academic Press*, London.

Cobbett CS (2000) Phytochelatins and Their Roles in Heavy Metal Detoxification. *Plant Physiology* 123, 825-832.

Collén J & Davison IR (1999) Stress tolerance and reactive oxygen metabolism in the intertidal red seaweeds *Mastocarpus stellatus & Chondrus crispus. Plant, Cell & Environment* 22, 1143–1151.

Connan S, Goulard F, Stiger V, Deslandes E & Ar Gall E (2004) Interspecific and temporal variation in phlorotannin levels in an assemblage of brown algae. *Botanica Marina* 47, 410-416.

Connan S, Deslandes E & Gall EA (2007) Influence of day-night and tidal cycles on phenol content and antioxidant capacity in three temperate intertidal brown seaweeds. *Journal of Experimental Marine Biology and Ecology* 349, 359-369.

Connan S & Stengel D (2009) Macroalgal Biomonitoring - Applying Phenolic Compounds as Biomarkers for Metal Uptake Characteristics in Irish Coastal Environments. STRIVE Environmental Protection Agency Programme 2007-2013. Report Series No.42.

Cosson J, Cosson E, Deslandes & Braud JP (1990) Preliminary approach to the characterization and seasonal variation of carrageenans from four Rhodophyceae on the Normandy coast (France). *Hydrobiologia* 204/205, 539-544.

Cowan MM (1999) Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews* 12, 564-582.

Cox S, Abu-Ghannam N & Gupta S (2010) An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds International. *Food Research Journal* 17: 205-220.

Cronin G & Hay ME (1996) Effects of light and nutrient availability on the growth, secondary chemistry, and resistance to herbivory of two brown seaweeds. *Oikos* 77: 93–106.

Cullinane JP & Whelan PM (1982) Copper, cadmium and zinc in seaweeds from the south coast of Ireland. *Marine Pollution Bulletin* 13, 205-208.

Davis TA, Volesky B & Vieira RHSF (2000) *Sargassum* seaweed as biosorbent for heavy metals. *Water Research* 34, 4270-4278.

Davis TA, Llanes F, Volesky B & Mucci A (2003b) Metal Selectivity of *Sargassum* spp. and Their Alginates in Relation to Their α-L-Guluronic Acid Content and Conformation. *Environmental Science & Technology* 37, 261-267.

Davis TA, Volesky B & Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research* 37, 4311-4330.

Dawczynski C, Schubert R & Jahreis G (2007) Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chemistry* 103, 891-899.

Dawes CJ, Lawrence JM, Cheney DP & Mathieson AC (1974) Ecological Studies of Floridian Eucheuma (Rhodophyta, Gigartinales). III. Seasonal Variation of Carrageenan, Total Carbohydrate, Protein, and Lipid. *Bulletin of Marine Science* 24, 286-299.

DeBoer JA (1981) Nutrients. In: Lobban CS, Wynne MJ (eds) The biology of seaweeds. *Blackwell Scientific Publications,* London.

de Knecht JA, van Dillen M, Koevoets PLM, Schat H, Verkleij JAC & Ernst WHO (1994) Phytochelatins in Cadmium-Sensitive and Cadmium-Tolerant *Silene vulgaris* (Chain Length Distribution and Sulfide Incorporation). *Plant Physiology* 104, 255-261.

de Knecht JA, van Baren N, Ten Bookum WM, Wong Fong Sang HW, Koevoets PLM & Schat H, Verkleij JAC (1995) Synthesis and degradation of phytochelatins in Cadmium-sensitive and Cadmium-tolerant *Silene vulgaris. Plant Science* 106, 9-18.

De Vos CHR, Vonk MJ, Vooijs R & Schat H (1992) Glutathione Depletion Due to Copper-Induced Phytochelatin Synthesis Causes Oxidative Stress in *Silene cucubalus. Plant Physiol*ogy 98, 853-858.

Dere S, Dalkýran N, Karacaoðlu D, Yildiz G & Dere E (2003) The determination of total protein, total soluble carbohydrate and pigment contents of some macroalgae collected from Gemlik-Karacaali (Bursa) and Erdek-Ormanlý (Balýkesir) in the Sea of Marmara, Turkey. *Oceanologia* 45, 453-471.

Devi K, Suganthy N, Kesika P & Pandian S (2008) Bioprotective properties of seaweeds: In vitro evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. *BMC Complementary and Alternative Medicine* 8, 38.

Dhargalkar VK & Pereira N (2005) Seaweed: Promising plant of the millennium. *Science and Culture* 60 – 66.

Dillon M, White R & Power D (2004) Tailings storage at Lisheen Mine, Ireland. *Minerals Engineering* 17, 123-130.

Dominguez-Solis JR, Gutierrez-Alcala G, Romero LC & Gotor C (2001) The Cytosolic O-Acetylserine(thiol)lyase Gene Is Regulated by Heavy Metals and Can Function in Cadmium Tolerance. *Journal of Biological Chemistry* 276, 9297-9302.

Downs CA, Rayan SL & Heckathorn SA (1999) The chloroplast small heat-shock protein: evidence for a general role in protecting photosystem II against oxidative stress and

photoinhibtion. *Journal of Plant Physiology* 115, 488–496.

Dummermuth AL, Karsten U, Fisch KM, König GM & Wiencke C (2003) Responses of marine macroalgae to hydrogen-peroxide stress. *Journal of Experimental Marine Biology and Ecology* 289, 103–121.

Dring MJ (1992) The Biology of Marine Plants. *Cambridge University Press*.

Edwards EA, Rawsthorne S & Mullineaux PM (1990) Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (*Pisum sativum L.*). *Planta* 180, 278-284.

Ekvall L & Greger M (2003) Effects of environmental biomass-producing factors on Cd uptake in two Swedish ecotypes of *Pinus sylvestris*. *Environmental Pollution* 121, 401-411.

Elbay-Poulichet F, Martin JM, Huang WW & Zhu IX (1987) Dissolved Cd behaviour in some selected French and Chinese estuaries. Consequences on Cd supply to the ocean. *Marine Chemistry* 22:125-136.

Environmental Protection Agency, USA (1998) Method 2051A, Microwave assisted acid digestion of sediments, sludges, soils and oils.

Environmental Protection Agency, Ireland. www.epa.ie/downloads/ (June, 2010).

Escribano-Bailon MT & Santos-Buelga C (2003) Polyphenol extraction from foods. In: C. Santos-Buelga and G. Williamson, Editors, Methods in Polyphenol Analysis, *Royal Society of Chemistry, Cambridge, United Kingdom*, pp. 1–16.

Esposito A, Pagnanelli F & Vegliò F (2002) pH-related equilibria models for biosorption in single metal systems. *Chemical Engineering Science* 57, 307-313.

EU Directive 80/778/EEC. The Drinking Water Directive (DWD). http://eur-lex.europa.eu/ (May, 2010).

EU Directive 98/83/EU. The Drinking Water Directive (DWD). http://eur-lex.europa.eu/ (May, 2010).

Faure G (2001) Origin of Igneous Rocks, The Isotopic Evidence. *Springer*.

Fathi AA & M.A.Al-Omair (2006) Effects of pH on toxicity of cadmium, cobalt and copper to *Scenedesmus bijuga*. *Protistology* 4, 221-226.

Feder ME & Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology* 61, 243–282.

Fehrmann C & Pohl P (1993) Cadmium adsorption by the non-living biomass of microalgae grown in axenic mass culture. *Journal of Applied Phycology* 5, 555-562.

Ferrat L, Pergent-Martini C & Romeo M (2003) Assessment of the use of biomarkers in aquatic plants for the evaluation of environmental quality: application to seagrasses. *Aquatic Toxicology* 65, 187-204.

Fitzgerald EJ, Caffrey JM, Nesaratnam ST & McLoughlin P (2003) Copper and lead concentrations in salt marsh plants on the Suir Estuary, Ireland. *Environmental Pollution* 123, 67-74.

Fleurence J, Coeur C, Mabeau S, Maurice M & Landrein A (1995a) Comparison of different extractive procedures for proteins from the edible seaweeds *Ulva rigida* and *Ulva rotundata*. *Journal of Applied Phycology* 7, 577-582.

Fleurence J, Massiani L, Guyader O & Serge S (1995b) Use of enzymatic cell wall degradation for improvement of protein extraction from *Chondrus crispus, Gracilaria verrucosa* and *Palmaria palmata*. *Journal of Applied Phycology* 7, 393-397.

Fleurence J (1999a) Seaweed proteins: biochemical, nutritional aspects and potential uses. *Trends in Food Science & Technology* 10, 25-28.

Fleurence J (1999b) The enzymatic degradation of algal cell walls: a useful approach for improving protein accessibility? *Journal of Applied Phycology* 1, 313-314.

Food Safety Authority or Ireland, Toxicology Factsheet, Issue 1, 2009.

Forstner U (1979) Metal transfer between solid and aqueous phases. In: Forstner, U., Wittman, G.T.W. (Eds.), *Metal Pollution in the Aquatic Environment*. Springer-Verlag, Berlin, 197-270.

Foster P (1976) Concentrations and concentration factors of heavy metals in brown algae. *Environmental Pollution* 10, 45–53.

Fourest E & Volesky B (1996) Contribution of Sulfonate Groups and Alginate to Heavy Metal Biosorption by the Dry Biomass of *Sargassum fluitans*. *Environmental Science & Technology* 30, 277-282.

Fritioff Å, Kautsky L & Greger M (2005) Influence of temperature and salinity on heavy metal uptake by submersed plants. *Environmental Pollution* 133, 265-274.

Fuge R & James KH (1974). Trace metal concentrations in *Fucus* from the Bristol Channel. *Marine Pollution Bulletin* 5, 9–12.

Galland-Irmouli AV, Fleurence J, Lamghari R, LuçonM, Rouxel C, Barbaroux O, Bronowicki JP, Villaume C & GuéantJL (1999) Nutritional value of proteins from edible seaweed *Palmaria palmata* (dulse). *The Journal of Nutritional Biochemistry* 10, 353-359.

Galland-Irmouli AV, Pons L, Luτon M, Villaume C, Mrabet NT, Guéant JL, & Fleurence J (2000) One-step purification of R-phycoerythrin from the red macroalga Palmaria palmata using preparative polyacrylamide gel electrophoresis. *Journal of Chromatography B: Biomedical Sciences and Applications* 739, 117-123.

Ganesan P, Kumar CS & Bhaskar N (2008) Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. *Bioresource Technology* 99, 2717-2723.

Garcia E & Salazar R (2003) Cadmium-Binding Peptide in Whole Cells of *Acetabularia calyculus*. *Bulletin of Environmental Contamination and Toxicology* 71, 0098-0105.

García-Ríos V, Freile-Pelegrín Y, Robledo D, Mendoza-Cózatl D, Moreno-Sánchez R & Gold-Bouchot G (2007) Cell wall composition affects Cd^{2+} accumulation and intracellular thiol peptides in marine red algae. *Aquatic Toxicology* 81, 65-72.

Gardea-Torresdey JL, Tiemann KJ, Armendariz V, Bess-Oberto L, Chianelli RR, Rios J, Parsons JG & Gamez G (2000). Characterization of Cr(VI) binding and reduction to Cr(III) by the agricultural byproducts of Avena monida (Oat) biomass. *Journal of Hazardous Materials* 80, 175-188.

Garnham GW, Codd GA & Gadd GM (1992) Kinetics of uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *Chlorella salina*. *Applied Microbiology and Biotechnology* 37, 270-276.

Gawel JE, Trick CG & Morel FMM (2001) Phytochelatins Are Bioindicators of Atmospheric Metal Exposure via Direct Foliar Uptake in Trees near Sudbury, Ontario, Canada. *Environmental*

Science & Technology 35, 2108-2113.

Gekeler W, Grill E, Winnacker EL & Zenk MH (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. *Archives of Microbiology* 150, 197-202.

Giusti L (2001) Heavy metal contamination of brown seaweed and sediments from the UK coastline between the Wear River and the Tees River. *Environment International* 26, 275-286.

Glazer AN (1984) Phycobilisome a macromolecular complex optimized for light energy transfer. *Biochimica et Biophysica Acta (BBA) - Reviews on Bioenergetics* 768, 29-51.

Glazer AW & Nikaido H (1995) Microbial Biotechnology: fundamentals of applied microbiology. San Francisco: WH Freeman, p. 340. ISBN 0-71672608-4.

Gonzalez-Davila M, Santana-Casiano JM, Perez-Pena J & Millero FJ (1995) Binding of Cu(II) to the Surface and Exudates of the Alga *Dunaliella tertiolecta* in Seawater. *Environmental Science & Technology* 29, 289-301.

Gordillo FJL, Aguilera J & Jimenez C (2006) The response of nutrient assimilation and biochemical composition of Arctic seaweeds to a nutrient input in summer. *Journal of Experimental Botany* 57, 2661-2671.

Gorinova N, Nedkovska M, Todorovska E, Simova-Stoilova L, Stoyanova Z, Georgieva K, mirevska-Kepova K, Atanassov A & Herzig R (2007) Improved phytoaccumulation of cadmium by genetically modified tobacco plants (*Nicotiana tabacum L.*). Physiological and biochemical response of the transformants to cadmium toxicity. *Environmental Pollution* 145, 161-170.

Greger M, Kautsky L & Sandberg T (1995) A tentative model of Cd uptake in *Potamogeton pectinatus* in relation to salinity. *Environmental and Experimental Botany* 35, 215-225.

Grill E, Winnacker EL & Zenk MH (1985) Phytochelatins: The Principal Heavy-Metal Complexing Peptides of Higher Plants. *Science* 230, 674-676.

Grill E, Gekeler W, Winnacker EL & Zenk HH (1986) Homo-phytochelatins are heavy metal-binding peptides of homo-glutathione containing *Fabales*. *FEBS Letters* 205, 47-50.

Grill E, Winnacker EL & Zenk MH (1987) Phytochelatins, a class of heavy metal-binding peptides from plants are functionally analogous to metallothioneins. *Proceedings of the National Academy of Sciences* 84, 439-443.

Grill E, Loeffler S, Winnacker EL & Zenk MH (1989) Phytochelatins, the heavy-metal-binding peptides of plants, are synthesized from glutathione by a specific gamma-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthase). *Proceedings of the National Academy of Sciences of the United States of America* 86, 6838-6842.

Gromova & Celis JE (2004) Protein Detection in Gels by Silver Staining: A Procedure Compatible with Mass-Spectrometry. *Elsevier. Academic Press Cell Biology:* A Laboratory Handbook. 3rd Edition. v.4 Eds: Celis, J.E., Carter N., Hunter T., Simons K., Small J.V. and Shotton D.

Gunning AP, Cairns P, Kirby AR, Round AN, Bixler HJ & Morris VJ (1998) Characterising semi-refined iota-carrageenan networks by atomic force microscopy. *Carbohydrate Polymers* 36 (1), 67-72.

Haritonidis S & Malea P (1995) Seasonal and local variation of Cr, Ni and Co concentrations in *Ulva rigida* C. Agardh and *Enteromorpha linza* (Linnaeus) from Thermaikos Gulf, Greece. *Environmental Pollution* 89, 319.

Haritonidis S & Malea P (1999) Bioaccumulation of metals by the green alga *Ulva rigida* from

Thermaikos Gulf, Greece. *Environmental Pollution* 104, 365-372.

Hartley-Whitaker J, Ainsworth G, Vooijs R, Bookum WT, Schat H & Meharg AA (2001) Phytochelatins Are Involved in Differential Arsenate Tolerance in Holcus lanatus. *Plant Physiology* 126, 299-306.

Hashim MA & Chu KH (2004) Biosorption of Cadmium by brown, green, and red seaweeds. *Chemical Engineering Journal* 97, 249-255.

Haug A (1961) The affinity of some divalent metals to different types of alginates. *Acta Chemica Scandinavica* 15, 1794–5.

Haug A, Myklestad S, Larsen B, Smidsrød O (1967) Correlation between chemical structure and physical properties of alginates *Acta Chemica Scandinavica* 21, 768–78.

Hay ME & Fenical W (1988) Marine Plant-Herbivore Interactions: The Ecology of Chemical Defense. *Annual Review of Ecology and Systematics* 19, 111-145.

Heckathorn SA, Mueller JK, Laguidice S, Zhu B, Barrett T, Blair B & Dong Y (2004) Chloroplast small heat-shock proteins protect photosynthesis during heavy metal stress. 1312. *American Journal of Botany* 91, 1312–1318.

Hernández-Allica J, Garbisu C, Becerril JM, Barrutia O, García-Plazaola JI, Zhao FJ & McGrath SP (2006) Synthesis of low molecular weight thiols in response to Cd exposure in *Thlaspi caerulescens. Plant, Cell & Environment* 29, 1422–1429.

Herrero R, Cordero B, Lodeiro P, Rey-Castro C & Sastre de Vicente ME (2006) Interactions of Cadmium(II) and protons with dead biomass of marine algae *Fucus sp. Marine Chemistry* 99, 106-116.

Herrero R, Lodeiro P, Rojo R, Ciorba A, Rodríguez P & Sastre de Vicente ME (2008) The efficiency of the red alga *Mastocarpus stellatus* for remediation of cadmium pollution. *Bioresource Technology* 99, 4138-4146.

Hider RC, Liu ZD & Khodr HH (2001) Metal chelation of polyphenols. *Methods in Enzymology* 335, 190–203.

Hirata K, Tsujimoto Y, Namba T, Ohta T, Hirayanagi N, Miyasaka H, Zenk MH & Miyamoto K (2001) Strong induction of phytochelatin synthesis by zinc in marine green alga, *Dunaliella tertiolecta. Journal of Bioscience and Bioengineering* 92, 24-29.

Holiman PCH, Hertog MGL & Katan MB (1996) Analysis and health effects of flavonoids. *Food Chemistry* 57, 43-46.

Holmes MA, Brown MT, Loutit MW & Ryan K (1991) The involvement of epiphytic bacteria in zinc concentration by the red alga Gracilaria sordida. *Marine Environmental Research* 31, 55-67.

Honya M, Kinoshita T, Ishikawa M, Mori H & Nisizawa K (1994) Seasonal variation in the lipid content of cultured *Laminaria japonica*: fatty acids, sterols, β-carotene and tocopherol. *Journal of Applied Phycology* 6, 25-29.

Hou X & Yan X (1998) Study on the concentration and seasonal variation of inorganic elements in 35 species of marine algae. *The Science of The Total Environment* 222, 141

Howarth JR, Domínguez-Solís JR, Gutiérrez-Alcalá G, Wray JL, Romero LC & Gotor C (2003) The serine acetyltransferase gene family in *Arabidopsis thaliana* and the regulation of its expression by Cadmium. *Plant Molecular Biology* 51, 589-598.

Hu S & Wu M (1998) Cadmium sequestration in the marine macroalga *Kappaphycus alvarezii*.

Molecular Marine Biology and Biotechnology 7, 97-104.

Huang CS, He W, Meister A & Anderson ME (1995) Amino acid sequence of rat kidney glutathione synthetase. *Proceedings of the National Academy of Sciences of the United States of America* 92, 1232-1236.

Huerta-Diaz MA, Delgadillo-Hinojosa F, Hernández-Ayón M, Segovia-Zavala JA, García-Esquivel Zl, López-Zárate H, Siqueiros-Valencia A & Galindo-Bect S (2008) Diagnosis of trace metal contamination in sediments: The example of Ensenada and El Sauzal, two harbors in Baja California, Mexico. *Marine Environmental Research* 66, 345-358.

Ikawa M, Schaper TD, Dollard CA & Sasner JJ (2003) Utilization of Folin-Ciocalteu phenol reagent for the detection of certain nitrogen compounds. *Journal of Agricultural and Food Chemistry* 51 (7), 1811–5.

Inouhe M (2005) Phytochelatins. *Brazilian Journal of Plant Physiology* 17, 65-78.

Ireland HE, Harding S, Bonwick G, Jones M, Smith C & Williams J (2004) Evaulation of heat shock protein 70 as a biomarker of environmental stress in *Fucus seratus* and *Lemna minor*. *Biomarkers* 9, 139–155.

Iyer A, Mody K & Jha B (2004) Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloaceae*. *Marine Pollution Bulletin* 49, 974-977.

Jadeja RN & Tewari A (2007) Effect of soda ash industry effluent on bioaccumulation of metals by seaweeds of coastal region of Gujarat, India. *Journal of Hazardous Materials* 147, 148-154.

Jarup L (2002) Cadmium overload and toxicity. *Nephrol Dial Transplant* 17, 35-39.

Jayasekera R & Rossbach M (1996) Use of seaweeds for monitoring trace elements in coastal waters. *Environmental Geochemistry and Health* 18, 63-68.

Jervis L, Rees-Naesborg R & Brown M (1997) Biochemical responses of the marine macroalgae *Ulva lactuca* and *Fucus vesiculosus* to cadmium and copper from sequestration to oxidative stress. *Biochemical Society Transaction* 25, 63S.

Jones DB (1931) Factors for converting percentages of nitrogen in foods and feeds into percentages of protein. *US Department of Agriculture Circular* 183, 1–21.

Jones WE & Williams R (1966) The seaweeds of Dale. *Field Studies* 2, 303-330.

Joselow MM, Tobias E, Koehler R, Coleman S, Bogden J & Gause D (1978) Manganese pollution in the city environment and its relationship to traffic density. *American Journal of Public Health* 68, 557-560.

Joubert Y & Fleurence Jl (2008) Simultaneous extraction of proteins and DNA by an enzymatic treatment of the cell wall of *Palmaria palmata* (Rhodophyta). *Journal of Applied Phycology* 20, 55-61.

Kaczmarska I & Dowe LL (1997) Reproductive Biology of the red alga *Polysiphonia lanosa* (Ceramiales) in the Bay of Fundy, Canada. *Marine Biology* 128, 695 – 703.

Kaimoussi A, Chafik A, Mouzdahir A & Bakkas S (2002) Diagnosis on the state of healthiness, quality of the coast and biological resources `case of the Moroccan Atlantic coast' (City of El Jadida). *Comptes Rendus Biologies* 325, 253-260.

Kamiya M, Nishio T, Yokoyama A, Yatsuya K, Nishigaki T, Yoshikawa S & Ohki K (2010) Seasonal variation of phlorotannin in sargassacean species from the coast of the Sea of Japan. *Phycological Research* 58, 53–61.

Kangwe JW, Hellblom F, Semesi AK, Mtolera MSP & Bjork, M (2001) Heavy metal inhibition of calcification and photosynthetic rates of the geniculate calcareous algae *Amphiroa tribulus*. *Marine Science Development in Tanzania and Eastern Africa*. WIOMSA Book Series. No. 1, 147-157.

Kawakami SK, Gledhill M & Achterberg EP (2006) Determination of phytochelatins and glutathione in phytoplankton from natural waters using HPLC with fluorescence detection. *Trends in Analytical Chemistry* 25 (2), 133-142.

Kawashima CG, Berkowitz O, Hell R, Noji M & Saito K (2005) Characterization and Expression Analysis of a Serine Acetyltransferase Gene Family Involved in a Key Step of the Sulfur Assimilation Pathway in Arabidopsis. *Plant Physiology* 137, 220-230.

Koivikko R, Loponen J, Honkanen T & Jormalainen V (2005) Contents of soluble, cell-wallbound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *Journal of Chemical Ecology* 31, 195–212.

Kramer KJM (1994) Biomonitoring of Coastal Waters and Estuaries. *CRC Press, Boca Raton, FL.*

Kratochvil D & Volesky B (1998) Biosorption of Cu from ferruginous wastewater by algal biomass. *Water Research* 32, 2760-276.

Kreger DR (1962) In: Lewin RA, editor. Cell walls, in physiology and biochemistry of algae. *New York: Academic Press* 315-335.

Kumar HD & Singh HN (1979) A textbook on algae. *The Macmillan Press Ltd., London-Basingstoke 38*-48.

Kumar M, Kumari P, Gupta V, Anisha P, Reddy C & Jha B (2010) Differential responses to cadmium induced oxidative stress in marine macroalga *Ulva lactuca* (Ulvales, Chlorophyta). *BioMetals* 23, 315-325.

Labconco (2009) A Guide To Kjeldahl Nitrogen Determination Methods and Apparatus. An Industry Service Publication.

Lacerda LD, Teixeira VL & Guimaraes JRD (1985) Seasonal variation of heavy metals in seaweeds from Conceiçao de Jacareí (R. J.), *Brazil. Botanica Marina* 28, 339–343.

Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 227, 680-685.

Landberg T & Greger M (2004) No phytochelatin (PC2 and PC3) detected in *Salix viminalis*. *Physiologia Plantarum* 121, 481–487.

Lapornik B, Prosek M & Golc Wondra A (2005) Comparison of extracts prepared from plant by-products using different solvents and extraction time. *Journal of Food Engineering* 71, 214-222.

Lares ML, Flores-Muñoz G & Lara-Lara R (2002) Temporal variability of bioavailable Cd, Hg, Zn, Mn and Al in an upwelling regime. *Environmental Pollution* 120, 595-608.

Lavid N, Schwartz A, Yarden O & Tel-Or E (2001) The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the waterlily (*Nymphaeaceae*). *Planta* 212, 323-331.

Law RJ, Jones BR, Baker JR, Kennedy S, Milne R & Morris RJ (1992) Trace metals in the livers of marine mammals from the Welsh coast and the Irish Sea. *Marine Pollution Bulletin* 24, 296-304.

Lee T F (1986) The Seaweed Handbook. *Dover Publications Inc*, *New York* 43-210.

Lee TM, Chang YC & Lin YH (1999) Differences in physiological responses between winter and summer *Gracilaria tenuistupitata* (Gigartinales, Rhodophyta) to varying temperature *Botanical Bulletin of Academia Sinica* 49, 93– 100.

Lee WY & Wang WX (2001) Metal accumulation in the green macroalga *Ulva fasciata*: effects of nitrate, ammonium and phosphate. *The Science of The Total Environment* 278,11-22.

Levine HG (1984) The use of seaweeds for monitoring coastal waters. In: L.E. Shubert (ed.), Algae as Ecological Indicators, 188-210. Academic Press, London, New York.

Lewis S, Donkin ME & Depledge MH (2001) Hsp70 expression in (*Enteromorpha intestinalis*) (Chlorophyta) exposed to environmental stressors. *Aquatic Toxicology* 51, 277–291.

Lignell .A, Roomans GM & Pedersen M (1982) Localization of absorhed cadmium In *Fucus vcsjculosus* L. by x-ray microanalysis. *Zeitschrift Fur Pflanzenphysiologie* 105:103-109.

Lincoln RA, Strupinski K & Walker JM (1991) Bioactive compounds from algae. *Life Chemistry Reports* 8, 97–183.

Littler MM, Littler DS & Taylor PR (1983) Evolutionary strategies in a tropical barrier reef system: Functional-form groups of marine macroalgae1. *Journal of Phycology* 19, 229-237.

Lobban CS & Harrison PJ (1997) Seaweed Ecology and Physiology. *Cambridge University Press*, Cambridge, UK.

Lodeiro P, Cordero B, Barriada JL, Herrero R & Sastre de Vicente ME (2005) Biosorption of Cadmium by biomass of brown marine macroalgae. *Bioresource Technology* 96, 1796-1803.

Lodeiro P, Barriada JL, Herrero R & Sastre de Vicente ME (2006) The marine macroalga *Cystoseira baccata* as biosorbent for Cadmium(II) and lead(II) removal: Kinetic and equilibrium studies. *Environmental Pollution* 142, 264-273.

Loeffler S, Hochberger A, Grill E, Winnacker EL & Zenk MH (1989) Termination of the phytochelatin synthase reaction through sequestration of heavy metals by the reaction product. *FEB Letters*. 258, 42-46.

Lundgren L, Olsson K & Theander O (1979) Synthesis of some bromophenols present in red algae. *Acta Chemica Scandinavica* 33, 105–108.

Lourenço S O. Barbarino E, C. De-Paula J , da S. Pereira L O & Lanfer Marquez U M (2002) Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. *Phycological Research* 50, 233–241.

Lowry OH, Rosebrough NJ, Farr AL & Randall RJ (1951) Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193, 265-275.

Lozzi I, Pucci A, Pantani OL, D'Acqui LP & Calamai L (2008) Interferences of suspended clay fraction in protein quantitation by several determination methods. *Analytical Biochemistry* 376, 108-114.

Luoma SN, Bryan GM & Langston L (1982) Scavenging of heavy metals from particulates by brown seaweeds. *Marine Pollution Bulletin* 13:394–6.

Luoma SN (1983) Bioavailability of trace metals to aquatic organisms. A review. *Science of the Total Environment* 28, 1–22.

Luoma SN (1989) Can we determine the biological availability of sediment-bound trace elements? *Hydrobiologia* 176-177, 379-396.

Lynch DV & Steponkus PL (1987) Plasma Membrane Lipid Alterations Associated with Cold Acclimation of Winter Rye Seedlings (*Secale cereale L. cv Puma*). *Plant Physiology* 83, 761-767.

MacArtain P, Gill CIR, Brooks M, Campbell R, & Rowland IR (2007) Nutritional Value of Edible Seaweeds. *Nutrition Reviews* 65, 535-543.

Mackie W & Preston RD (1974) Cell wall and intercellular region polysaccharides. In: Stewart WDP, editor. Algal physiology and biochemistry. *Oxford, UK: Blackwell Scientific Publications*. 58–64.

Mageswaran R & Sivasubramaniam S (1984) Mineral and protein contents of some marine algae from the coastal areas of Northern Sri Lanka. *Journal of National Science, Sri Lanka* 12, 179-189.

Malea P, Haritonidis S & Kevrekidis T (1995) Metal content of some green and brown seaweeds from Antikyra Gulf (Greece). *Hydrobiologia* 310, 19-31.

Malea P, Rijstenbil JW & Haritonidis S (2006) Effects of cadmium, zinc and nitrogen status on non-protein thiols in the macroalgae *Enteromorpha* spp. from the Scheldt Estuary (SW Netherlands, Belgium) and Thermaikos Gulf (N Aegean Sea, Greece). *Marine Environmental Research* 62, 45-60.

Malik A (2004) Metal bioremediation through growing cells. *Environment International* 30, 261-278.

Mantoura RFC, Dickson A & Riley JP (1978) The complexation of metals with humic materials in natural waters. *Estuarine and Coastal Marine Science* 6: 387-408.

Marinho-Soriano E, Fonseca PC, Carneiro MAA & Moreira WSC (2006) Seasonal variation in the chemical composition of two tropical seaweeds. *Bioresource Technology* 97, 2403-2406.

Marschner H. (1995) 'Mineral Nutrition of Higher Plants.' 2nd edn. *Academic Press*, London.

Marsham S, Scott GW & Tobin ML (2007) Comparison of nutritive chemistry of a range of temperate seaweeds. *Food Chemistry* 100, 1331-1336.

Martínez AJ (2003) Marine life of the North Atlantic: Canada to New England. *Aqua Quest Publications*, New York.

Martinez B & Rico JM (2002) Seasonal variation of P content and major N pools in *Palmaria palmata* (Rhodophyta). *Journal of Phycology* 38, 1082-1089.

Matanjun P, Mohamed S, Mustapha N, Muhammad K & Ming C (2008) Antioxidant activities and phenolics content of eight species of seaweeds from north Borneo. *Journal of Applied Phycology* 20, 367-373.

McDonald M, Mila I & Scalbert A (1996) Precipitation of metal ions by plant polyphenols: optimal conditions and origin of precipitation. *Journal of Agricultural and Food Chemistry* 44, 599-606.

McLusky DS, Bryant V & Campbell R (1986) The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. *Oceanography and Marine Biology: An Annual Review* 24: 481-520.

Merrifield ME, Ngu T & Stillman MJ (2004) Arsenic binding to *Fucus vesiculosus* metallothionein. *Biochemical and Biophysical Research Communications* 324, 127-132.

Mishra S, Srivastava S, Tripathi RD, Kumar R, Seth CS & Gupta DK (2006) Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatins and antioxidant system in response to its accumulation. *Chemosphere* 65, 1027-1039.

Morris CA, Nicolaus B, Sampson V, Harwood JL & Kille P (1999) Identification and characterization of a recombinant metallothionein protein from a marine alga, *Fucus vesiculosus*. *Biochemical Journal* 338, 553–560.

Morrison L, Baumann HA & Stengel DB (2008) An assessment of metal contamination along the Irish coast using the seaweed *Ascophyllum nodosum* (Fucales, Phaeophyceae). *Environmental Pollution* 152, 293-303.

Mountouris A, Voutsas E & Tassios D (2002) Bioconcentration of heavy metals in aquatic environments: the importance of bioavailability. *Marine Pollution Bulletin* 44, 1136-1141.

Mucha AP, Bordalo AA & Vasconcelos M (2004) Sediment quality in the Douro river estuary based on trace metal contents, macrobenthic community and elutriate sediment toxicity test (ESTT). *Journal of Environmental Monitoring* 6, 585-592.

Munda IM & Kremer BP (1977) Chemical composition and physiological properties of fucoids under conditions of reduced salinity. *Marine Biology* 42, 9-15.

Munda IM (1979) Temperature Dependence of Zinc Uptake in *Fucus virsoides* (Don.) J. Ag. and *Enteromorpha prolifera* (O. F. Mull.) J. Ag. from the Adriatic Sea. *Botanica Marina* 22, 149-152.

Munda IM & Hudnik V (1988) The effects of Zn, Mn, and Co accumulation on growth and chemical composition of *Fucus vesiculosus* L. under different temperature and salinity conditions. *Marine Ecology*. 9, 213-225.

Munksgaard NC & Parry DL (2001) Trace metals, arsenic and lead isotopes in dissolved and particulate phases of North Australian coastal and estuarine seawater. *Marine Chemistry* 75, 165-184.

Murphy V, Hughes H & McLoughlin P (2007) Cu(II) binding by dried biomass of red, green and brown macroalgae. *Water Research* 41, 731-740.

Murphy V, Hughes H & McLoughlin P (2008) Comparative study of Cr biosorption by red, green and brown seaweed biomass. *Chemosphere* 70, 1128-1134.

Murugan K & Harish SR (2007) Antioxidant modulation in response to heavy metal induced oxidative stress in *Cladophora glomerata*. *Indian Journal of Experimental Biology* 45, 980-983.

Muse JO, Stripeikis JD, Fernández FM, d'Huicque L, Tudino MB, Carducci CN & Troccoli OE (1999) Seaweeds in the assessment of heavy metal pollution in the Gulf San Jorge, Argentina. *Environmental Pollution* 104, 315-322.

Muse JO, Carducci CN, Stripeikis JD, Tudino MB & Fernandez FM (2006) A link between lead and cadmium kinetic speciation in seawater and accumulation by the green alga *Ulva lactuca*. *Environmental Pollution* 141, 126-130.

Nagalakshmi N & Prasad MNV (2001) Responses of glutathione cycle enzymes and glutathione metabolism to copper stress in *Scenedesmus bijugatus*. *Plant Science* 160, 291-299.

Nakano Y & Asada K (1987) Purification of Ascorbate Peroxidase in Spinach Chloroplasts; Its Inactivation in Ascorbate-Depleted Medium and Reactivation by Monodehydroascorbate Radical. *Plant and Cell Physiology* 28, 131-140.

National Geographical Survey of Ireland, 2010.

Nicholson BE & Brightman F (1979) The illustrated book of flowerless plants. *Peerage Books, Oxford University Press*.

Noraho N & Gaur JP (1995) Effect of cations, including heavy metals, on cadmium uptake by

Lemna polyrhiza L. *BioMetals* 8, 95-98.

Office of Public Works (OPW), Ireland 2010.

Okamoto OK & Colepicolo P (1998) Response of Superoxide Dismutase to Pollutant Metal Stress in the Marine Dinoflagellate *Gonyaulax polyedra*. Comparative Biochemistry and Physiology Part C: *Pharmacology, Toxicology and Endocrinology* 119, 67-73.

O'Leary C & Breen J (1997) Metal levels in seven species of molluscs and in seaweeds from the Shannon Estuary. *Biology and Environment* 97B, 121–32.

Olivares E (2003) The effect of lead on the phytochemistry of *Tithonia diversifolia* exposed to roadside automotive pollution or grown in pots of Pb-supplemented soil. *Brazilian Journal of Plant Physiology* 15, 149-158.

O'Sullivan MP, Nixon ER, McLaughlin D, O'Sullivan MI & O. O'Sullivan D (1991) Chemical contaminants in the estuarine and coastal waters 1978 to 1988. *Fisheries Bulletin, Dublin* 10, 34.

Pagnanelli F, Beolchini F, Esposito A, Toro L & Vegliò F (2003) Mechanistic modeling of heavy metal biosorption in batch and membrane reactor systems. *Hydrometallurgy* 71, 201-208.

Paucot H & Wollast R (1997) Transport and transformation of trace metals in the scheldt estuary. *Marine Chemistry* 58, 229-244.

Pavia H, Cervin G, Lindgren A & Aberg P (1997) Effects of UVB radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum. Marine Ecology Progress Series* 157, 139-146.

Pavia H, Toth G & Åberg P (1999) Trade-offs between phlorotannin production and annual growth in natural populations of the brown seaweed *Ascophyllum nodosum. Journal of Ecology* 87, 761-771.

Pavia H & Brock E (2000) Extrinsic factors influencing phlorotannin production in the brown alga *Ascophyllum nodosum. Marine Ecology Progress Series* 193, 285–294.

Pawlik-Skowrońska B (2000) Relationships between acid-soluble thiol peptides and accumulated Pb in the green alga *Stichococcus bacillaris. Aquatic Toxicology* 50, 221-230.

Pawlik-Skowronska B (2001) Phytochelatin production in freshwater algae *Stigeoclonium* in response to heavy metals contained in mining water; effects of some environmental factors. *Aquatic Toxicology* 52, 241-249.

Pawlik-Skowrońska B, Pirszel J & Brown MT (2007) Concentrations of phytochelatins and glutathione found in natural assemblages of seaweeds depend on species and metal concentrations of the habitat. *Aquatic Toxicology* 83, 190-199.

Peckol P, Krane JM & Yates JL (1996) Interactive effects of inducible defense and resource availability on phlorotannins in the North Atlantic brown alga *Fucus vesiculosus. Marine Ecology Progress Series* 138, 209–217.

Pedersen A (1984) Studies on phenol content and heavy metal uptake in fucoids. *Hydrobiologia* 116-117, 498-504.

Percival EGV & McDowell, RH (1967) Chemistry and Enzymology of Marine Algal Polysaccharides. *Academic Press, London, UK*. 99–126.

Percival E (1979) The polysaccharides of green, red and brown seaweeds: Their basic structure, biosynthesis and function. *British Phycological Journal* 14, 103-117.

Pérez AA, Farías SS, Strobl AM, Pérez LB, López CM, Piñeiro A, Roses O & Fajardo MA (2007)

Levels of essential and toxic elements in *Porphyra columbina* and *Ulva sp*. from San Jorge Gulf, Patagonia Argentina. *Science of The Total Environment* 376, 51-59.

Perez-Rama M, Lopez CH, Alonso JA & Vaamonde ET (2001) Class III metallothioneins in response to Cadmium toxicity in the marine microalga *Tetraselmis Suecica* (*Kylin*) *Butch*. *Environmental Toxicology and Chemistry* 20, 2061-2066.

Pérez-Rama M, Abalde Alonso J, Herrero López C & Torres Vaamonde E (2002) Cadmium removal by living cells of the marine microalga *Tetraselmis suecica*. *Bioresource Technology* 84, 265-270.

Pérez-Rodríguez E, Aguilera J & Figueroa FL (2003) Tissular localization of coumarins in the green alga *Dasycladus vermicularis* (Scopoli) Krasser: a photoprotective role? *Journal of Experimental Botany* 54, 1093-1100.

Phaneuf D, Côté I, Dumas P, Ferron L A & LeBlanc A (1999) Evaluation of the Contamination of Marine Algae (Seaweed) from the St. Lawrence River and Likely to Be Consumed by Humans. *Academic Press* 80, 175 -182.

Pickering IJ, Prince RC, George GN, Rauser WE, Wickramasinghe WA, Watson AA, Dameron CT, Dance IG, Fairlie DP & Salt DE (1999) X-ray absorption spectroscopy of Cadmium phytochelatin & model systems. *Biochimica et Biophysica Acta* (*BBA*) 1429,35-364.

Plouguerné E, Le Lann K, Connan S, Jechoux G, Deslandes E & Stiger-Pouvreau V (2006) Spatial and seasonal variation in density, reproductive status, length and phenolic content of the invasive brown macroalga *Sargassum muticum* (Yendo) Fensholt along the coast of Western Brittany (France). *Aquatic Botany* 85, 337-344.

Pocker Y & Ng JSY (1974) Plant carbonic anhydrase. Hydrase activity and its reversible inhibition. *Biochemistry* 13, 5116-5120.

Porath J & Flodin P (1959) Gel filtration: a method for desalting and group separation. *Nature* 183, 1657-9.

Preston A, Jeffries DF, Dutton JWR, Harvey BR & Steele AK (1972) British Isles coastal waters: The concentrations of selected heavy metals in sea water, suspended matter and biological indicators -- A pilot survey. *Environmental Pollution* (*1970)* 3, 69-82.

Price JH, Pettitt JM & Russell S (1987) Indications of species status from total protein signatures in *Callithamnion* (Ceramiaceae). *Hydrobiologia* 151-152, 213-220.

Price NM & Morel FMM (1990) Cadmium and cobalt substitution for zinc in a marine diatom. *Nature* 344, 658-660.

Qi H, Zhang Q, Zhao T, Hu R, Zhang K & Li Z (2006). In vitro antioxidant activity of acetylated and benzoylated derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). *Bioorganic & Medicinal Chemistry Letters* 16, 2441-2445.

Radi R, Turrens JF, Chang LY, Bush KM, Crapo JD & Freeman BA (1991) Detection of catalase in rat heart mitochondria. *Journal of Biological Chemistry* 266, 22028-22034.

Ragan MA & Jensen A (1978) Quantitative studies on brown algal phenols. II. Seasonal variation in polyphenol content of *Ascophyllum nodosum* (L.) Le Jol. and *Fucus vesiculosus* (L.). *Journal of Experimental Marine Biology and Ecology* 34, 245-258.

Ragan MA, Smidsrød O & Larsen B (1979) Chelation of divalent metal ions by brown algal polyphenols. *Marine Chemistry* 7, 265-271.

Ragan MA & Glombitza KW (1986) Phlorotannins, brown algal polyphenols. *In Progress in*

phycological research, Round & Chapman, Bristol: Biopress Ltd 4, 129–241.

Ratkevicius N, Correa JA & Moenne A (2003) Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev. (Chlorophyta) from heavy metal-enriched environments in northern Chile. *Plant Cell & Environment* 26,1599-1608.

Rauser WE, Schupp R & Rennenberg H (1991) Cysteine, gamma-Glutamylcysteine, and Glutathione Levels in Maize Seedlings : Distribution and Translocation in Normal and Cadmium-Exposed Plants. *Plant Physiology* 97, 128-138.

Reese RN, White CA & Winge DR (1992) Cadmium-Sulfide Crystallites in Cadmium- (γEC)nG Peptide Complexes from Tomato. *Plant Physiology* 98, 225-229.

Reinfelder JR & Fisher NS (1994) The assimilation of elements ingested by marine planktonic bivalve larvae. *Limnology and Oceanography* 39, 12-20.

Rice DL & Lapointe BE (1981) Experimental outdoor studies with *Ulva fasciata Delile*.II. Trace metal chemistry. *Journal of Experimental Marine Biology and Ecology* 54, 1–11.

Riget F, Johansen P & Asmund G (1997) Baseline levels and natural variability of elements in three seaweed species from West Greenland. *Marine Pollution Bulletin* 34, 171-176.

Rijstenbil JW, Dehairs F, Ehrlich R & Wijnholds JA (1998a) Effect of the nitrogen status on copper accumulation and pools of metal-binding peptides in the planktonic diatom *Thalassiosira pseudonana. Aquatic Toxicology* 42, 187-209.

Rijstenbil JW, Haritonidis S, Malea P, Seferlis M & Wijnholds JA (1998b) Thiol pools and glutathione redox ratios as possible indicators of copper toxicity in the green macroalgae *Enteromorpha* spp. from the Scheldt Estuary (SW Netherlands, Belgium) and Thermaikos Gulf (Greece, N Aegean Sea). *Hydrobiologia* 385, 171-181.

Rioux LE, Turgeon SL & Beaulieu M (2010) Structural characterization of laminaran and galactofucan extracted from the brown seaweed *Saccharina longicruris*. *Phytochemistry* 71, 1586-1595.

Robinson N (1989) Algal metallothioneins: secondary metabolites and proteins. *Journal of Applied Phycology* 1, 5-18.

Romera E, González F, Ballester A, Blázquez ML & Muñoz JA (2007) Comparative study of biosorption of heavy metals using different types of algae. *Bioresource Technology* 98, 3344-3353.

Rose M, Lewis J, Langford N, Baxter M, Origgi S, Barber M, MacBain H & Thomas K (2007) Arsenic in seaweed--Forms, concentration and dietary exposure. *Food and Chemical Toxicology* 45, 1263-1267.

Rossell KG & Srivastava LM (1985) Seasonal variations in total nitrogen, carbon and amino acids in *Macrocystis integrifolia* and *Nereocystis luetkeana* (Phaeophyta). *Journal of Phycology* 21, 304 – 309.

Rouxel C, Daniel A, Jqróme M, Etienne M & Fleurence J (2001) Species identification by SDS-PAGE of red algae used as seafood or a food ingredient. *Food Chemistry* 74, 349-353.

Royer TC (1979) On the Effect of Precipitation and Runoff on Coastal Circulation in the Gulf of Alaska. *Journal of Physical Oceanography* 9, 555-563.

Ruegsegger A & Brunold C (1993) Localization of [γ]-Glutamylcysteine Synthetase and Glutathione Synthetase Activity in Maize Seedlings. *Plant Physiology*. 101, 561-566.

Sadiq M (1992) Toxic Metal Chemistry in Marine Environments, Marcel Dekker, New York.

Sakagami H, Kushida T, Oizumi T, Nakashima H, & Makino T (2010) Distribution of lignin-carbohydrate complex in plant kingdom and its functionality as alternative medicine. *Pharmacology & Therapeutics* 128, 91-105.

Salgado LT, Cinelli LP, Viana NB, De Carvalho RT, de Souza Mourão PA, Teixeira VL, Farina M & Amado Filho GM (2009) A vanadium bromoperoxidase catalyses the formation of highmolecular-weight complexes between brown algal phenolic substances and alginates. *Journal of Phycology* 45 193–202.

Salomons W & Förtsner U (1984) Metals in the Hydrocycle. *Springer-Verlag, Berlin.*

Salvador N, Gómez Garreta A, Lavelli L, Ribera M (2007) Antimicrobial activity of Iberian macroalgae. *Scientia Marina* 71, 101-113.

Samiullah Y (1990) Prediction of the environmental fate of chemicals. London: Elsevier.

Sánchez-Machado D I, López-Cervantes J, López-Hernández J & Paseiro-Losada P (2004) Fatty acids, total lipid, protein and ash contents of processed edible seaweeds. *Food Chemistry* 85, 439-444.

Sánchez-Rodríguez I, Huerta-Diaz MA, Choumiline E, Holguín-Quiñones O & Zertuche-González JA (2001) Elemental concentrations in different species of seaweeds from Loreto Bay, Baja California Sur, Mexico: implications for the geochemical control of metals in algal tissue. *Environmental Pollution* 114, 145-160.

Sanders BM, Jenkins KD, Sunda WG & Costlow JD (1983) Free Cupric Ion Activity in Seawater: Effects on Metallothionein and Growth in Crab Larvae. *Science* 222, 53-55.

Sanita di Toppi L, Vurro E, Rossi L, Marabottini R, Musetti R, Careri M, Maffini M, Mucchino C, Corradini C & Badiani M (2007) Different compensatory mechanisms in two metal-accumulating aquatic macrophytes exposed to acute cadmium stress in outdoor artificial lakes. *Chemosphere* 68, 769-780.

Sawidis T, Brown MT, Zachariadis G & Sratis I (2001) Trace metal concentrations in marine macroalgae from different biotopes in the Aegean Sea. *Environment International* 27, 43-47.

Scarano G & Morelli E (2002) Characterization of cadmium- & lead- phytochelatin complexes formed in a marine microalga in response to metal exposure. *BioMetals* 15, 145-151.

Schat H & Kalff MMA (1992) Are Phytochelatins Involved in Differential Metal Tolerance or Do They Merely Reflect Metal-Imposed Strain? *Plant Physiol*ogy 99, 1475-1480.

Schat H, Llugany M, Vooijs R, Hartley-Whitaker J & Bleeker PM (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumulator and non-hyperaccumulator metallophytes. *Journal of Experimental Botany* 53, 2381-2392.

Schoenwaelder MEA (2002) The occurrence and cellular significance of physodes in brown algae. *Phycologia* 41, 125–139.

Schiewer S & Volesky B (2000) Biosorption by marine algae. In: Valdes JJ, editor. Remediation. Dordrecht, *The Netherlands: Kluwer Academic Publishers*, 139–69

Schmoger MEV, Oven M & Grill E (2000) Detoxification of Arsenic by Phytochelatins in Plants. *Plant Physiology* 122, 793-802.

Schroda M, Vallon O, Wollman FA & Beck CF (1999) A chloroplast-targeted heat shock protein 70 (HSP70) contributes to the photoprotection and repair of photosystem II during and after

photoinhibition. *Plant Cell* 11, 1165–1178.

SeeligerU & Edwards P (1977) Correlation coefficients and concentration factors of copper and lead in seawater and benthic algae. *Marine Pollution Bulletin* 8, 16-19.

Seeliger U & Edwards P (1979) Fate of biologically accumulated copper in growing and decomposing thalli of two benthic red marine algae. *Journal of the Marine Biological Association of the United Kingdom* 59, 227– 38.

Sharp GJ, Samant HS & Vaidya OC (1988) Selected metal levels of commercially valuable seaweeds adjacent to and distant from point sources of contamination in Nova Scotia and New Brunswick. *Bulletin of Environmental Contamination and Toxicology* 40, 724-730.

Sheng PX, Ting YP, Chen JP & Hong L (2004) Sorption of lead, copper, cadmium, zinc, and nickel by marine algal biomass: characterization of biosorptive capacity and investigation of mechanisms. *Journal of Colloid and Interface Science* 275, 131-141.

Silva N, Haro J & Prego R (2009) Metals background and enrichment in the Chiloé Interior Sea sediments (Chile). Is there any segregation between fjords, channels and sounds? *Estuarine, Coastal and Shelf Science* 82, 469-476.

Skipnes 0, Roald T & Haug A (1975) Uptake of zinc and strontium by brown algae. *Physiologia Plantarum* 34, 314-320.

Skowroñski T, De Knecht JA, Simons J & Verkleij JAC (1998) Phytochelatin synthesis in response to Cadmium uptake in *Vaucheria* (Xanthophyceae). *European Journal of Phycology* 33, 87-91.

Smidsrød O & Draget KI (1996) Chemistry and physical properties of alginates. *Carbohydrates in Europe* 14, 6-13.

Smit AJ (2004) Medicinal and pharmaceutical uses of seaweed natural products: A review. *Journal of Applied Phycology* 16, 245-262.

Spijkerman E, Barua D, Gerloff-Elias A, Kern J, Gaedke U & Heckathorn SA (2007) Stress responses and metal tolerance of (*Chlamydomonas acidophila*) in metal-enriched lake water and artificial medium. *Extremophiles* 11, 551–562.

Stengel DB & Dring MJ (1997) Morphology and in situ growth rates of plants of *Ascophyllum nodosum* (Phaeophyta) from different shore levels and responses of plants to vertical transplantation. *European Journal of Phycology. Cambridge University Press.* 32, 193-202.

Stengel DB, Macken A, Morrison L & Morley N (2004) Zinc concentrations in marine macroalgae and a lichen from western Ireland in relation to phylogenetic grouping, habitat and morphology. *Marine Pollution Bulletin* 48, 902-909.

Stengel DB, McGrath H & Morrison LJ (2005) Tissue Cu, Fe and Mn concentrations in different-aged and different functional thallus regions of three brown algae from western Ireland. *Estuarine, Coastal and Shelf Science* 65, 687-696.

Stirk WA, Reinecke DA & van Staden J (2007) Seasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds. *Journal of Applied Phycology.* 19, 271 – 276.

Stuessy TF (1990) Plant taxonomy: The systematic evaluation of comparative data. New York: *Columbia University Press*.

Sueur S, van den Berg CMG & Riley JP (1982) Measurement of the metal complexing ability of exudates of marine macroalgae. *Limnology and Oceanography* 27, 3, 536-543.

Suh HJ, Kim SR, Lee KS, Park S, & Kang SC (2010) Antioxidant activity of various solvent extracts from *Allomyrina dichotoma* (Arthropoda: Insecta) larvae. *Journal of Photochemistry and Photobiology B: Biology* 99, 67-73.

Sunda WG & Huntsman SA (1998a) Interactive effects of external manganese, the toxic metals copper and zinc, and light in controlling cellular manganese and growth in a coastal diatom. *Limnology and Oceanography* 43, 1467–1475.

Sunda WG & Huntsman SA (1998b) Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems. *Science of The Total Environment* 219, 165-181.

Tang D, Shafer MM, Karner DA & Armstrong DE (2005) Response of nonprotein thiols to copper stress and extracellular release of glutathione in the diatom *Thalassiosira weissflogii. American Society of Limnology and Oceanography, Inc.* 50, 516–525.

Targett N & Arnold T (1998) (Minireview) Predicting the effects of brown algal phlorotannins on herbivores in tropical and temperate oceans. *Journal of Phycology* 34, 195–205.

Targett NM & Arnold TM (2001) Effects of secondary metabolites on digestion in marine herbivores. In: Marine Chemical Ecology, edited by James B. McClintoch & Bill J. Baker. *CRC Press LLC* 391-411.

Taylor R, Sotka E & Hay M (2002) Tissue-specific induction of herbivore resistance: seaweed response to amphipod grazing. *Oecologia* 132, 68-76.

Thangavel P, Long S & Minocha R (2007) Changes in phytochelatins and their biosynthetic intermediates in red spruce (*Picea rubens Sarg*.) cell suspension cultures under cadmium and zinc stress. *Plant Cell, Tissue and Organ Culture* 88 (2), 201-216.

Thermo Fisher Scientific, 2009.

Thévenot DR, Moilleron R, Lestel L, Gromaire MC, Rocher V, Cambier P, Bonte P, Colin JL, de Ponteves C & Meybeck M (2007) Critical budget of metal sources and pathways in the Seine River basin (1994-2003) for Cd, Cr, Cu, Hg, Ni, Pb and Zn. *Science of The Total Environment* 375, 180-203.

Thornber JP (1975) Chlorophyll-Proteins: Light-Harvesting and Reaction Center Components of Plants. *Annual Review of Plant Physiology* 26, 127-158.

Tomlinson D, Wilson J, Harris C & Jeffrey D (1980) Problems in the assessment of heavy-metal levels in estuaries and the formation of a pollution index. *Helgoland Marine Research* 33, 566-575.

Topinka JA, Robbins JV (1976) Effects of nitrate and ammonium enrichment on growth and nitrogen physiology in *Fucus spiralis. Limnology and Oceanography* 21:659–664.

Torres E, Cid A, Fidalgo P, Herrero C & Abalde J (1997) Long-chain class III metallothioneins as a mechanism of Cadmium tolerance in the marine diatom *Phaeodactylum tricornutum Bohlin. Aquatic Toxicology* 39, 231-246.

Toth G & Pavia H (2000) Lack of phlorotannin induction in the brown seaweed *Ascophyllum nodosum* in response to increased copper concentrations. *Marine Ecology Progress Series* 192, 119-126.

Tsuji N, Hirayanagi N, Iwabe O, Namba T, Tagawa M, Miyamoto S, Miyasaka H, Takagi M, Hirata K & Miyamoto K (2003) Regulation of phytochelatin synthesis by Zinc and Cadmium in marine green alga, *Dunaliella tertiolecta. Phytochemistry* 62, 453-459.

United States Environmental Protection Agency. www.epa.gov/ (2009).

USA Department of Health and Human Services, Public Health Service Agency for Toxic Substances and Disease Registry. www.atsdr.cdc.gov/ (May, 2009).

USA Food and Drug Administration. www.fda.gov (May, 2009).

USA OHSA. USA Occupational Health and Safety Administration. www.osha.gov/ (May, 2009).

Van Den Hoek C, Mann DG & Jahns HM (1995) Algae: An Introduction to Phycology. *Cambridge: Cambridge University Press.* ISBN 0-521-31687-1.

van Netten C, Hoption Cann SA, Morley DR & van Netten JP (2000) Elemental and radioactive analysis of commercially available seaweed. *The Science of the Total Environment.* 255, 169-175.

Vasconcelos MTSD & Leal MFC (2001) Antagonistic interactions of Pb and Cd on Cu uptake, growth inhibition and chelator release in the marine algae *Emiliania huxleyi. Marine Chemistry* 75, 123-139.

Vasconcelos MT & Leal MF (2001b). Seasonal variability in the kinetics of Cu, Pb, Cd and Hg accumulation by macroalgae. *Marine Chemistry* 74, 65-85.

Vasconcelos MT & Leal MF (2008) Exudates of different marine algae promote growth and mediate trace metal binding in *Phaeodactylum tricornutum. Marine Environmental Research* 66, 499-507.

Vatamaniuk OK, Mari Sp, Lu YP & Rea PA (1999) AtPCS1, a phytochelatin synthase from *Arabidopsis*: Isolation and in vitro reconstitution. *Proceedings of the National Academy of Sciences of the United States of America* 96, 7110-7115.

Vattem DA, Ghaedian R & Shetty K (2005) Enhancing health benefits of berries through phenolic antioxidant enrichment: focus on cranberry. *Asia Pacific Journal of Clinical Nutrition* 14, 120–30.

Veinott G, Sylvester P, Hamoutene D, Robin Anderson M, Meade J & Payne J (2003) State of the marine environment at Little Bay Arm, Newfoundland and Labrador, Canada, 10 years a "do nothing" response to a mine tailings spill. *The Royal Society of Chemistry* 5, 626-634.

Vergara J, Bird KT & Niell FX (1995) Nitrogen assimilation following NH_4^+ pulses in the red alga *Gracilariopsis lemaneiformis*: effect on C metabolism. *Marine Ecology Progress Series* 122, 253–263.

Villares R, Puente X & Carballeira A (2002) Seasonal variation and background levels of heavy metals in two green seaweeds. *Environmental Pollution* 119, 79-90.

Vinson J, Zubik L, Bose P, Samman N & Proch J (2005) Dried fruits: excellent in vitro and in vivo antioxidants. *Journal of the American College of Nutrition* 24 (1), 44–50.

Volesky B & Holan ZR (1995) Biosorption of heavy metals. *Biotechnology Progress* 11,235-250.

Volesky B (2001) Detoxification of metal-bearing effluents: biosorption for the next century. *Hydrometallurgy* 59, 203-216.

Wallenstein FM, Couto RP, Amaral AS, Wilkinson M, Neto AI & Rodrigues AS (2009) Baseline metal concentrations in marine algae from São Miguel (Azores) under different ecological conditions - Urban proximity and shallow water hydrothermal activity. *Marine Pollution Bulletin* 58, 438-443.

Wang CL & Oliver DJ (1997) Glutathione synthetase : similarities of the proteins from

Schizosaccharomyces pombe and *Arabidopsis thaliana. Biochemical Journal* 326, 563–566.

Wang T, Jónsdóttir R & Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. *Food Chemistry* 116, 240-248.

Wang WX & Dei RCH (1999) Kinetic measurements of metal accumulation in two marine macroalgae. *Marine Biology* 135, 11-23.

Warrand J (2006) Healthy Polysaccharides The Next Chapter in Food Products. *Food Technology and Biotechnology* 44, 355-370.

Whitfield FB, Helidoniotis F, Shaw KJ & Svoronos D (1999) Distribution of Bromophenols in Species of Marine Algae from Eastern Australia. *Journal of Agricultural and Food Chemistry* 47, 2367-2373.

Waterford County Council. www.waterfordcoco.ie (May, 2010)

Water Framework Directive 2000/60/EC. http://ec.europa.eu/environment/ (May, 2009)

Wikfors GH, Neeman A & Jackson PJ (1991) Cadmium-binding polypeptides in microalgal strains with laboratory-induced cadmium tolerance. *Marine Ecology Progress Series* 79, 163 – 170.

Wong KH & Cheung PCK (2001) Nutritional evaluation of some subtropical red and green seaweeds Part II. In vitro protein digestibility and amino acid profiles of protein concentrates. *Food Chemistry* 72, 11-17.

World Health Organization, Geneva (1996) Guidelines for drinking-water quality, 2nd edition. Vol. 2. Health criteria and other supporting information.

Wright P & Mason CF (1999) Spatial and seasonal variation in heavy metals in the sediments and biota of two adjacent estuaries, the Orwell and the Stour, in eastern England. *The Science of The Total Environment* 226, 139-156.

Wu S, Zu Y & Wu M (2001) Cadmium response of the hairy root culture of the endangered species *Adenophora lobophylla. Plant Science* 160, 551-562.

Yagawa Y, Shiraiwa Y & Miyachi S (1984) Carbonic Anhydrase from the Blue-Green Alga (Cyanobacterium) *Anabaena variabilis. Plant and Cell Physiology* 25, 775-783.

Yoshie-Stark Y, Hsieh YP & Suzuki T (2003) Distribution of flavonoids and related compounds from seaweeds in Japan. *Journal of Tokyo University of Fisheries* 89, 1-6.

Young SD, Bache BW, Welch D & Anderson HA (2006) Analysis of the potentiometric titration of natural and synthetic polycarboxylates. *European Journal of Soil Science* 32 (4) 579-592.

Zenk MH (1996) Heavy metal detoxification in higher plants - a review. *Gene* 179, 21-30.

Zhang Q, Zhang J, Shen J, Silva A, Dennis D & Barrow C (2006) A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. *Journal of Applied Phycology* 18, 445-450.

Żbikowski R, Szefer P & Lata A (2006) Distribution and relationships between selected chemical elements in green alga *Enteromorpha sp*. from the southern Baltic. *Environmental Pollution* 143, 435-448.

Zubia M, Payri CE, Deslandes E & Guezennec J (2003) Chemical Composition of Attached and Drift Specimens of *Sargassum mangarevense* and *Turbinaria ornata* (Phaeophyta: Fucales) from Tahiti, French Polynesia. *Botanica Marina* 46, 562–571.