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VOLTAMMETRIC CHARACTERIZATION OF MACROALGAE-EXUDED ORGANIC LIGANDS IN RESPONSE TO METAL EXPOSURE

Submitted by

Hollydawn Murray, MSc., HBSc.

Supervisors

Dr. Rachel R. Cave, Earth and Ocean Sciences, School of Natural Sciences, National University of Ireland Galway

Dr. Dagmar B. Stengel, Botany and Plant Science, School of Natural Sciences, National University of Ireland Galway

A thesis submitted for the degree of

Doctor of Philosophy

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Declaration

I, Hollydawn Murray, certify that the Thesis is all my own work and that I have not obtained a degree in this University or elsewhere on the basis of any of this work.

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Abstract

Trace metal availability, and hence toxicity, is largely dependent on metal speciation. It is well established that in seawater complexation by organic ligands dominates the speciation of most metals including Cd, Cu, Pb and Zn. Recent marine studies on microorganisms suggest ligands are biologically produced. There are few studies on macroalgal ligand production.

The aim of this thesis was to investigate seaweeds as a source of naturally occurring organic ligands and to investigate metals as stimuli of their release. Voltammetric techniques were employed to measure the release of metal-complexing ligands by three brown macroalgae common in Irish waters. *Ascophyllum nodosum*, *Fucus vesiculosus* and *Laminaria hyperborea* were cultured under environmentally relevant levels of Cd (0, 0.89, 8.89 nM), Cu (0, 7.86, 15.70 nM), Pb (0, 1.40, 4.83 nM), or Zn (0, 15.20, 91.70 nM) exposure. Cultures were monitored for seaweed photochemical quantum yield (F_v/F_m), metal speciation, complexing ligands (L_M), conditional stability constants ($logK'L_M$), glutathione (GSH), cysteine (Cys), and seaweed metal content on days 0, 1, 4 and 7. All macroalgae released ligands in response to Cd, Cu, Pb, and Zn establishing seaweeds as a relevant source of L. These ligands generally reduced labile metal concentration (M_L) in cultures, potentially alleviating toxicity. Significantly higher L_M concentrations in cultures with added metals compared to the controls indicate that all metals act as stimuli for L. Similarities were identified between the $logK'L_M$ and structural groups of L_{Cd} and sulfides, and L_{Pb} and humic substances. By contrast, L_{Cu} and L_{Zn} did not compare well to those compounds already identified as ligands in seawater. Similar techniques were also employed to examine the release of L by *F. vesiculosus* in response to a quaternary metal mixture (Cd, Cu, Pb, Zn). In comparison to the single metal cultures, we observed: increases in $[Cd]_L$ and $[Pb]_L$; decreases in L_{Cu}, L_{Zn}, and L_{Zn2}; and the appearance of a 2^{nd} L_{Cu}. Evidence from a field study on the Lough Furnace estuary confirmed the existence of L *in-situ*. Metal speciation, $logK'L_M$, and seaweed metal contents point to macroalgae as a potential source of L_{Zn} in surface seawater, whilst the L_{Cd} pool is dominated by fulvic acids. In all cases, lack of correlation between thiol and total ligand concentrations suggest that GSH and Cys do not contribute substantially to those ligands exuded by macroalgae.

Overall, the results of this thesis demonstrate that macroalgae-exuded ligands modulate metal speciation in seawater. To the best of our knowledge, the work herein is the first to report the exudation of L_{Cd}, L_{Pb}, and L_{Zn} by macroalgae. In comparison to previous literature, particular merits of this work include the use of more environmentally relevant metal concentrations, the incorporation of photophysical measurements, and the determination of specific potential ligands. These studies highlight macroalgae as a source of L and Cd, Cu, Pb and Zn as environmental stimuli that induce seaweed ligand production, although further work is required to elucidate ligand identity.

List of Articles

This thesis is based on the following peer-reviewed journal publications, which are referred to in text by their citation.

A. Murray, H., Meunier, G., van den Berg, C.M.G., Cave, R., and Stengel, D.B. (a). Voltammetric characterization of macroalgae-exuded organic ligands (L) in response to Cu and Zn: a source and stimuli for L. *Environmental Chemistry (Available online*).

B. Murray, H., Meunier, G., van den Berg, C.M.G., Cave, R., and Stengel, D.B. (b). Voltammetric characterization of macroalgae-exuded organic ligands (L) in response to Cd and Pb: expanding the source and stimuli for L. *Marine Chemistry* (*Submitted September 12 2013*).

C. Murray, H., Meunier, G., Cave, R., and Stengel, D.B. (c). No metal stands alone: the voltammetric characterization of macroalgae-exuded organic ligands (L) in response to a quaternary metal mixture. *Chemosphere* (*Submitted November 27 2013*).

D. Murray, H., Meunier, G., Stengel, D.B., and Cave, R. (d). Metal complexation by organic ligands (L) in near-pristine estuarine waters: evidence for the identity of L. *Environmental Chemistry* (*In press*).

Personal contributions to aforementioned peer-reviewed journal publications

A. Led the planning and performed all laboratory work, with the exception of the ICP-OES analyses. Analyzed all data, composed the manuscript, and acted as the corresponding author. Method development was largely completed by G. Meunier.

B. Led the planning and performed all laboratory work, with the exception of the ICP-OES analyses. Analyzed all data, composed the manuscript, and acted as the corresponding author. Method development was largely completed by G. Meunier.

C. Led the planning and performed all laboratory work, with the exception of the ICP-OES analyses. Analyzed all data, composed the manuscript, and acted as the corresponding author. Method development was largely completed by G. Meunier.

D. Participated in the planning and performed all laboratory work, with the exception of the ICP-OES analyses. Fieldwork was assisted by G. Meunier. Analyzed all data, composed the manuscript, and acted as the corresponding author.

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Nomenclature

Superscripts

$^{2+}$	Ionic charge

Subscripts

$_D$	Total dissolved
$_L$	Lability parameter
$_M$	Metal ion

Photophysiology

F_v	Variable fluorescence, defined as the difference between minimal and maximal fluorescence measured in the dark-adapted state
F_m	Maximal fluorescence measured in the dark-adapted state
F_v/F_m	Photochemical quantum yield; efficiency of photosystem II (i.e.: the maximum change in the quantum yield of fluorescence)

Metal speciation

K	Thermodynamic equilibrium constant
$K'L_M$	Conditional equilibrium constant for ML_M formation
L	Simple ligand
L_M	Metal-specific simple ligand
M	Metal ion
ML	Complex form of M with simple ligand L
α	Side reaction coefficient

Abbreviations and acronyms

AAS	Atomic absorption spectroscopy
ASV	Anodic stripping voltammetry
BLM	Biotic ligand model
CASS	Canadian Arctic Seawater Standard
CLE	Competitive ligand exchange
CRM	Certified reference material
CSV	Cathodic stripping voltammetry
Cys	Cysteine
DGT	Diffusive gradient thin films
DNA	Deoxyribonucleic acid
DOM	Dissolved organic matter
DP	Differential pulse
FA	Fulvic acid
FF	Fresh film
GC	Gas chromatography
GFAAS	Graphite furnace atomic absorption spectrometry
GSH	Glutathione
HA	Humic acid

HDPE	High density polyethylene
HMDE	Hanging mercury drop electrode
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IMAC	Immobilized metal ion affinity chromatography
ISE	Ion selective electrode
LLE	Liquid liquid extraction
MT	Metallothionein
MQ	Milli-Q
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NIES	National Institute of Environmental Studies
PC	Phytochelatin
RNA	Ribonucleic acid
RDE	Rotating disk electrode
RGCDE	Rotating glassy carbon disk electrode
SAS	Surface active substance
SMDE	Static mercury drop electrode
TMF	Thin mercury film
WFD	Water Framework Directive

Chapter 1 General Introduction

Chapter 1 General Introduction

Coastal waters are strongly affected by human activities and are often enriched in contaminants. Marine environments are generally the final repositories of contamination derived from industrial, urban and rural sources. Metals are of particular interest due to their toxicity, ubiquity and variety of environmental sources. Unlike organic pollutants, metals cannot be degraded or removed using current technology, making metal pollution a lasting problem. Metals present in coastal waters are derived from natural and/or anthropogenic sources and range in concentration from pico- to nano-molar (Bruland 1980; Morel and Price 2003). Most metals are naturally localized in rock formations; however, anthropogenic metal inputs to water have essentially overwhelmed the natural biogeochemical cycles (Zenk 1996). Predominant anthropogenic sources of metals in the environment include mining, smelting and refining, manufacturing processes, energy production, waste disposal, and transportation (Butler 1978). Normally, seawater contains low concentrations of trace elements such as Zn and Cu, which are required micronutrients for ocean life (Vrapsir and Butler 2009). Elements of primary concern are those not required, such as Cd and Pb, whose presence in coastal areas may decrease primary productivity and affect community structure (Philips and Rainbow 1998; Crathorne et al. 2001; Butler 1978).

Metal bioavailability is determined by the physiochemical state of a metal (Bryan et al. 1985). In the marine environment metals exist in either the dissolved or particulate form. Dissolved forms include free hydrated ions as well as complex ions (chelated with organic or inorganic ligands) while particulate forms include colloid aggregates, adsorbed particles and precipitated metal coatings. The majority of metals in seawater are complexed by organic matter (Coale and Bruland 1990; Kozelka and Bruland 1998; Ellwood and van den Berg 2001; Laglera and van den Berg 2003). Of course, the physicochemical properties of water greatly influence chemical form and hence metal bioavailability. Metal solubility generally increases as water pH decreases resulting in increased metal bioavailability. By the same token, increasing water pH results in the formation of low solubility compounds and the precipitation of some metals as insoluble hydroxides, oxides, carbonates or phosphates, thus decreasing bioavailability (Bryan et al. 1985). Metal availability to algae is also inversely related to salinity (Munda 1984).

Several trace metals, including Cd, Cu, Pb and Zn, occur dominantly bound to organic ligands (L) in seawater (Kozelka and Bruland 1998). As such, it is widely accepted that dissolved organic matter (DOM) plays an important role in aquatic systems via the binding of

metals. Such binding influences metal speciation and decreases bioavailability. By decreasing the concentration of the free metal ion in water, DOM is thought to protect aquatic communities from metal stress. The importance of biogenic ligands in the control of metal speciation, and hence bioavailability, is well acknowledged in studies on ocean speciation. Although seaweeds are essential components of coastal ecosystems and known producers of L, much of the fundamental insight into algae-exuded organic ligands is derived from experiments employing only microalgae or metal concentrations much greater than those of natural systems. In this context, our attention is focused on the current evidence for macroalgae-exuded metal-binding ligands. A discussion of macroalgal responses to metal exposure, metal speciation, marine ligands, and voltammetry is included. The metals and macroalgae considered are mainly those that have been studied most intensely in Murray et al. a, b, c, and d (Cd, Cu, Pb, Zn; *Ascophyllum nodosum*, *Fucus vesiculosus*, *Laminaria hyperborea*).

1.1 Macroalgal responses to metal exposure

Macroalgae are known to accumulate metals from seawater. Several metals act as essential micronutrients for marine organisms, including macroalgae (DeBoer 1981; Lobban and Harrison 1996). Copper, for example, is required in trace amounts by seaweeds for several enzymatic processes including photosynthetic electron transport, plastocyanin and respiratory electron transport, and cytochrome oxidase (Genter 1996; Gledhill et al. 1999; Vinit-Dunand et al. 2002; Andrade et al. 2004). Similarly, Zn is a necessary component of algal enzymes and redox proteins such as DNA and RNA polymerase, carbonic anhydrase, and alkaline phosphatase (Sunda et al. 1987). Other metals, including Cd and Pb, are generally considered non-essential. While Pb has no known biological function and is only toxic at high concentrations, Cd is toxic even in trace amounts. Regardless of their requirement, elevated metal concentrations are generally toxic due to non-specific binding of molecular coordination sites, the production of reactive oxygen species, or the overall reduction of antioxidant capacity (Rai et al. 1981; Pinto et al. 2003). Such metal toxicity may inhibit photosynthesis as a result of substitution of the central Mg^{2+} atom in chlorophyll and subsequent interference with the light harvesting complex, oxygen evolution complex, cytochrome complex, plastoquinone, plastocyanin, ferrodoxin, and $NADP^{+}$ (Prasad and Strzałka 1999; Küpper et al. 2002). Nevertheless, macroalgae have developed intra- and extra-cellular mechanisms of tolerance that allow them to accumulate metals and thrive in

waters impacted by high metal concentrations (Clemens 2002). Macroalgae acquire metals entirely from water borne exposure, and though accumulation rates differ among metals, the general order of metal toxicity is Cu>Cd>Pb>Zn (Lobban and Harrison 1996). In seaweeds, metals are taken up in their ionic form via active and passive transport (Eide et al. 1980). As with terrestrial plants the exact mechanism is not entirely clear, but consists of three major steps: adsorption, translocation, and storage. Because adsorption processes largely control the accumulation of metals in seaweeds their study is of utmost importance in understanding how metals are transferred from water to algae. A number of physiological variables influence the amount of metal taken up and translocated within a seaweed such as compartmentalization of metals in cell walls or vacuoles, which could restrict metal translocation (Clemens 2001); species-specific and tissue-specific differences in the affinity of membrane-bound transport proteins for metal ions (Davis et al. 2003); and species-specific and metal-specific patterns of metal transport within the thalli (Munda and Hudnik 1988, Stengel and Dring 2000).

While the mechanisms by which macroalgae simultaneously tolerate and accumulate metal are not entirely understood, the cell wall is the first instance of metal-algae interaction. For brown algae, the cell wall is comprised of an inner fibrallar skeleton and an outer amorphous-embedding matrix. The embedding matrix, predominantly composed of alginic acid and to a lesser extent fucoidan, imparts the majority of the adsorption potential of the algal cell wall (**Figure 1.1**). Here, metal ions may bind free sites or, alternatively, bind to sites already occupied by ions derived from seawater (such as $Ca^{2+,}$ K^{+}, Mg^{2+}, Na^{+}). These processes are termed sorption and ion exchange, respectively. As established by Haug et al. (1961), the affinity of alginates for Cd, Cu, Pb, and Zn differs with the mannuronic to guluronic acid ratios of different species of brown algae. In any case, the adsorption capacity of the alginate polymer is directly related to presence of the carboxyl functional group. Similarly, the adsorption capacity of fucoidan is directly related to the presence of sulfonic functional groups and, to a lesser extent, carboxyl groups. Recently, the complexing capacity of alginates and fucoidans was confirmed via the electrochemical analysis of crude fractions isolated from *Dictyota dichotoma*. As expected, both polysaccharides comprised binding sites for Cu, each with a conditional stability constant (logK') of 6.0 (Karavoltsos et al. 2013).

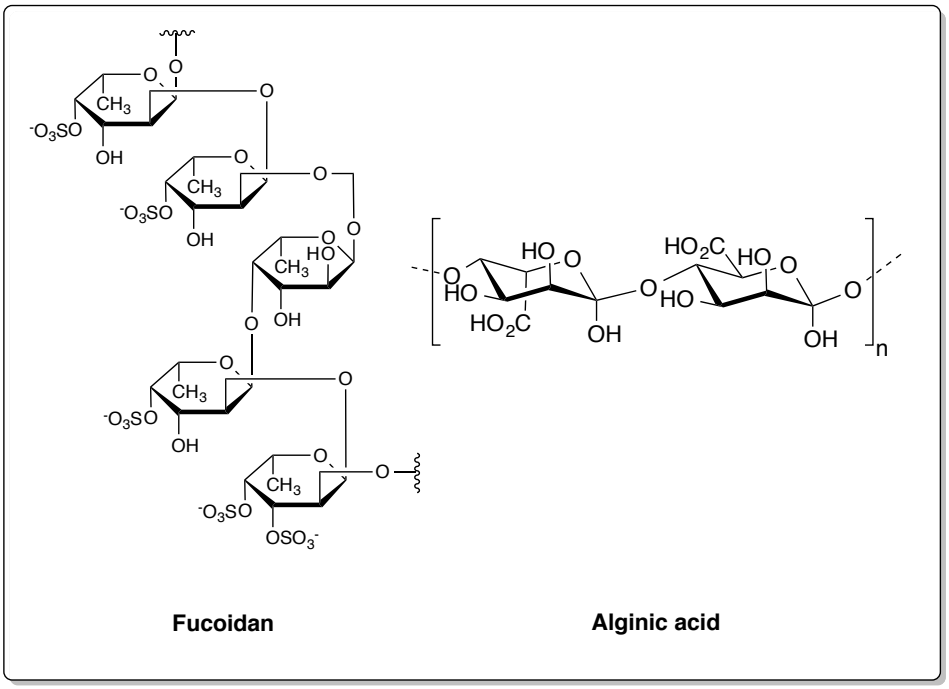

Figure 1.1. The structural components of the embedding matrix of the macroalgal cell wall which impart metal-binding capacity.

Whether bound through sorption or ion-exchange, metals may enter macroalgal cells via energy dependent transport across the plasma membrane (Pawlik-Skowronska 2000). Intracellularly, metals are usually bound as a result of their high reactivity and low solubility. Several compounds have been identified in macroalgae as intracellular metal complexants including thiol peptides, glutathione-rich phytochelatins (PCs), and cysteine-rich metallothioneins (MT)(Malea et al. 2006; Pawlik-Skowronska et al. 2007). Previous field studies on natural assemblages of *Fucus vesiculosus* have already identified a direct positive correlation between seawater metal concentration and total seaweed PC and glutathione (GSH) concentrations (Pawlik-Skowronska et al. 2007). Similarly, the presence of gene-encoded MTs and their subsequent induction on Cu exposure has been reported for *F. vesiculosus* (Morris et al. 1999).

1.2 A focus on Phaeophyceae

Given the recorded ability of *F. vesiculosus* to stimulate a defence or exclusion mechanism, it is not surprising that brown algae generally dominate metal-contaminated coastal environments (Nielsen and Nielsen 2005). Despite their common occurrence on Irish shores, only a handful of studies providing data on the content of metals in brown algae exist (**Table**

1.1). In recognition of this deficiency, Murray et al. (d) expands this dataset indicating that, even in near-pristine Irish waters, brown seaweeds accumulated Cd, Cu, Pb and Zn. Concentrations of Cd, Cu, Pb and Zn in tips of *F. vesiculosus* were 0.45, 1.17, 0.76, and 38.08 μg g^{-1} DW respectively, and generally greater than those of *A. nodosum*. Species-specific differences were attributed to ion interactions at the algal surface, in agreement with Connan et al. (2011a). Brown algae detoxify metals or increase metal tolerance much like other seaweeds: via incorporation into non-toxic organometallic compounds, intracellular precipitation, and binding at the cell wall. In addition to these mechanisms, metal tolerance of brown algae is also attributed to phlorotannin concentration and composition (Ragan and Jensen 1979; Hider et al. 2001).

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Table 1.1. A comparison of published values (μg g^{-1} DW) for the mean concentrations of metals in brown macroalgae from estuarine and coastal locations in Ireland.

Location	Species	Cd	Cu	Pb	Zn	Method	Reference
Lough Furnace estuary	*A. nodosum*	0.072-0.32	0.23-0.89	0.21-0.30	57.10-108.65	ICP-OES	(Murray et al. d)
	Fucus ceranoides	0.34-0.43	1.91-2.33	0.58-0.61	36.25-44.32		
	Fucus spiralis	0.34-1.379	1.28-1.79	0.30-0.51	70.34-156.76		
	F. vesiculosus	0.45-0.66	1.13-1.17	0.19-0.76	39.08-72.68		
Irish coast (Ballyconeely, Galway Docks, Killimer, Cork Harbour, Dun Laoghaire, Carlingford Lough)	*A. nodosum*	0.11-0.60	-[A]	0.12-2.11	-	AAS	(Morrison et al. 2008)
Western Irish coast (Spiddal, Clarinbridge)	*A. nodosum*	-	-	-	50.00-90.00*[B]	AAS	(Stengel et al. 2004)
	F. spiralis	-	-	-	20.00-140.00*		
	F. vesiculosus	-	-	-	80.00-110.00*		
	Polysiphonia lanosa	-	-	-	90.00-185.00*		
Strangford Lough	*A. nodosum*	-	13.27-72.60	-	-	ICP-AES	(Stengel and Dring 2000)

- represents data not provided in the literature.
* represents values taken from graphs.

Phlorotannins are polyphenolic, long-chained, complex secondary metabolites that occur at high concentrations and are restricted to brown algae (Steinberg and de Nys 2002). They are mainly found in specialized vacuoles termed 'physodes' but may also be free in the cytoplasm or linked to the cell wall (Ragan and Glombitza 1986; Bond et al. 1999; Salgado et al. 2009). Though mainly regarded as a defence mechanism against herbivory (Yates and Peckol 1993), phlorotannins appear to have several ecological functions (Pavia et al. 1997). Phlorotannin production has been shown to increase in response to pathogenic organisms, antifouling agents (Sieburth and Conover 1965), solar UV radiation (Pavia et al. 1997), and most recently metal concentration (Connan and Stengel 2011a). Connan et al. (2011b) demonstrated that both *F. vesiculosus* and *A. nodosum* increased cell wall phlorotannins under elevated Cu exposure. In the same study, phlorotannins isolated from *A. nodosum* were shown to complex Cu.

1.3 Metal Speciation

Adsorption and intracellular uptake are not the only prerequisites involved in the accumulation of metals by macroalgae. In fact, both processes are entirely dependent on metal speciation (Munda and Hudnik 1986; Davis et al. 2003). Metal speciation refers to the distribution of an element among specific forms defined by isotopic composition, conformation, electronic or oxidation state, and/or complex or molecular structure (Templeton et al. 2000). The chemical species employed in this thesis and the resulting publications are based largely on complex structure and are classified in the dissolved fraction as follows:

M_D	total metal
M_L	labile metal
ML	complexed metal

The chemical speciation of trace metals in seawater has been studied since the 1970s (Boyle et al. 1976; Bruland 1980). Early research focused on establishing the concentration levels of trace metals in seawater as well as their interaction with inorganic ligands. The development of bioinorganic chemistry spurred the identification of strong metal-organic complexes in aquatic systems (Barber and Ryther 1969). Since then, numerous chemical speciation studies have concluded that metal speciation in seawater is dominated by organic

complexes (as reviewed by Luther et al. 2001). Such complexes are best defined by their conditional stability constants (logK') and the associated ligand (L_M) concentrations. The conditional stability constant refers to how tightly a ligand coordinates a metal ion, a value that is thermodynamically unique for each ion-ligand pair for a given ionic strength and pH.

It is well-established that the most important chemical species in the acquisition of metals by macroalgae is M_L, as predicted by the biotic ligand model model (BLM, Di Toro et al. 2001). This model describes equilibrium between M_L and those metal ions bound to the physiologically active sites of biotic ligands, such as ion transporters in the cell wall of brown macroalgae. Thus, the bioavailability and toxicity of metals in coastal systems is thought to be a function of $[M]_L$ in seawater, although this is not always confirmed. However it is important to note that both natural and anthropogenic changes in the environment can influence the distribution, mobility, bioavailability and toxicity of metals.

1.3.1 Organic complexation

Metal concentrations, $[L_M]$, and logK'L_Ms for Cd, Cu, Pb and Zn in various aquatic systems are presented in **Table 1.2**. As indicated, chemical speciation studies revealed that >99% of Cu is complexed with logK'L_{Cu} ranging from 9-15. Similarly, >70% of Zn is complexed with logK'L_{Zn} ranging from 7.5-11. Lower logK's for Zn in comparison to Cu are logical given consideration of the Irvin-Williams series. Organic complexation of Cd and Pb, though evident, is lower than that of both Cu and Zn with average complexation of 70% and 50-70%, respectively. However, a discussion of logK'L_{Cd} and LogK'L_{Pb} is limited by the scarcity of data in the literature.

Table 1.2. A comparison of current literature values for the speciation of dissolved metals in various oceanic surface waters (< 100 m).

Location	$[M]_D$ (nM)	$[M]_L$ (nM)	ML (%)	$[L_M]$ (nM)	$LogK'L_M$	Method	Reference
Cd							
Bay of Biscay, France	0.28	0.26	2	-[A]	-	ICP-MS	(Point et al. 2007)
Antarctic seawater	0.93	0.31	67	0.31	14.40	AAS	(Biesuz et al. 2006)
South Pacific	0.004-0.05	-	-	0.97-2.13	9.82-10.93	ASV, CSV	(Ellwood 2004)
Manila Bay, Philippines	0.40-2.90	0.70-0.99	96-100	-	-	DPASV	(Velasquez et al. 2002)
Firth of Clyde, Scotland	0.13-0.31	0.06-0.45	<40	0.13-2.60	9.74-10.39	DPASV	(Muller 1999)
Southern England coast	0.12-0.21	0.00145-0.00295	20-80	1.4-6.5	10.00-10.49	DPASV	(Muller 1996b)
Ross Sea, Antarctica	0.18	0.085	48	0.38	5.00	DPASV	(Scarponi et al. 1995)
North Adriatic	0.26	-	-	1.2	9.9	ASV	(Capodaglio et al. 1995)
Central North Pacific	0.00007	0.00002	70	0.1	12.0	DPASV	(Bruland 1992)
Western North Atlantic Gulf Stream	0.073	< 0.010	33-92	-	-	DPASV	(Sakamoto-Arnold et al. 1987)
Cu							
Bay of Biscay, France	24.49	3.14	89	-	-	ICP-MS	(Point et al. 2007)
Antarctic	4.00	0.16	96	3.92	19.60	Resin titration	(Biesuz et al. 2006)
Gullmar Fjord, Sweden	3.0-13.1	-	> 99.8	5.0-115	10.5-13.8	CLE-CSV	(Croot 2003)
Eel Pond Harbour, San Diego Harbour, Vineyard Sound, USA	6.2-80	0.12-5.2	93.5-99.8	-	-	DGT, CLE-ACSV	(Twiss and Moffett 2002)
Manila Bay, Philippines	0.32-45.65	0.00-15.24	0.00-100	-	-	DPCSV	(Velasquez et al. 2002)
Western Black Sea	4.0-21.0	0.01-0.76	93-99.8	3-70	9.4-13.9	DPASV	(Muller et al. 2001)
Firth of Clyde, Scotland	3.00-9.00	0.18-1.54	> 82	1.30-69.00	9.06-15.62	DPASV	(Muller 1999)
Cape Cod, USA	3.8-117	-	-	11-135.7	9.94-14.15	CLE-CSV	(Moffett et al. 1997)
Atlantic coast	-	-	-	7, 36	10.95, 7.5	IMAC-ASV	(Donat et al. 1997)

Table 1.2 continued

Location	$[M]_D$ (nM)	$[M]_L$ (nM)	ML (%)	$[L_M]$ (nM)	$LogK'L_M$	Method	Reference
Chesapeake Bay, USA	-	-	-	15, 75	11.0, 8.1	IMAC-ASV	(Donat et al. 1997)
Equatorial Pacific	-	-	-	0.5-15	7.08-8.93	ISE	(Midorikawa and Tanoue 1996)
Gran Canaria, Spain	-	-	-	214	8.16	ASV	(Gonzalez-Davila and Santana-Casiano 1995)
North Adriatic	7.2	-	-	2.3, 41	16, 8.1	ASV	(Capodaglio et al. 1995)
Sargasso Sea	1.16	-	-	0.8	$\geq$ 14.2	CLE/LLE	(Miller and Bruland 1994)
San Francisco Bay, USA	49-50.7	-	-	40-119	11.6-13.4	CLE/LLE	(Miller and Bruland 1994)
Central Pacific	0.24-0.37	-	-	1-15	10.2-15.0	CLE/LLE	(Miller and Bruland 1994)
Muroto Cape, Japan	0.49	-	-	130, 578	9.26, 7.40	CLE-CSV	(Midorikawa and Tanoue 1994)
San Francisco Bay, USA	45.4	-	-	12.8-104	8.-13.8	CLE-CSV, ASV, GFAAS	(Donat et al. 1994)
North Sea	3.7	-	-	13.04-13.74	12.79-12.99	CLE-CSV	(Campos and van den Berg 1994)
Mediterranean Sea	3.1	-	-	3.2-14.5	13.08-14.8	CLE-CSV	(Campos and van den Berg 1994)
Northeast Atlantic	1.55	-	-	4.9-12.8	12.2-13.1	CLE-CSV	(Campos and van den Berg 1994)
Tyrrhenian Sea, Italy	6.2	-	-	< 5	13.1	CLE-ASV	(Scarano et al. 1992)
Shimizu Port, Japan	4.12	-	-	9.4, 49	9.12, 7.05	ISE	(Midorikawa et al. 1992)
Indian Ocean	1.96-3.24	-	-	4.13-16.2	12.4-15.6	CLE-CSV, ASV	(Donat and van den Berg 1992)
Sargasso Sea	0.99	-	-	3.5-44	10.5-12.4	CLE-CSV	(van den Berg and Donat 1992)
North Sea	3.2	-	-	1.2-21	12.2-15.6	CLE-CSV	(van den Berg and Donat 1992)
Sargasso Sea	1.4	-	-	70	9.4	CLE-CSV	(Moffett et al. 1990)
Suruga Bay, Japan	1.37	-	-	8.6, 19	8.61, 7.25	ISE	(Midorikawa et al. 1990)
Japan Sea	0.33	-	-	1.2, 6.5	9.60, 7.57	ISE	(Midorikawa et al. 1990)
NW Pacific	2.7	-	-	2.2, 7.3	8.89, 7.09	ISE	(Midorikawa et al. 1990)
NE Pacific	0.47-1.46	-	-	0.84-9.68	8.06-12.92	ASV	(Coale and Bruland 1990)

Table 1.2 continued

Location	$[M]_D$ (nM)	$[M]_L$ (nM)	ML (%)	$[L_M]$ (nM)	$LogK'L_M$	Method	Reference
Sargasso Sea	1.0-1.2	-	-	4.3-7.9	9.86-10.08	ASV, RPLC	(Hanson et al. 1988)
NE Pacific	0.58-0.59	-	-	1.1-7.23	8.53-10.92	ASV	(Coale and Bruland 1988)
Narragansett Bay, USA	19-27	0.5-1	94-98	22-100	10.1-12.4	CLE-RPLC	(Sunda and Hanson 1987)
North Atlantic	1.6-2.9	-	-	41-59	7.87-8.28	ASV	(Kramer 1986)
North Atlantic	3-6.6	-	-	12-1780	8.0-11.9	CLE-CSV, ASV, MnO2	(Buckley and van den Berg 1986)
Guanabara Bay, Brazil	2.2-26	-	-	42-490	9.6-12.38	CLE-CSV	(van den Berg and De Luca Rebello 1986)
South Atlantic	2.3	-	-	11.1, 33.1	12.2, 10.2	CLE-CSV	(van den Berg 1984)
Northwest Pacific	24	-	-	7, 21	13.8, 11.1	CLE-XAD-2	(Hirose et al. 1982)
Pb							
Celtic Sea	0.032	0.022	32	-	16	Pseudo pology raphy	(Bi et al. 2013)
Irish Sea	0.22	0.048	78	-	15	Psuedo pology raphy	(Bi et al. 2013)
Bay of Biscay, France	0.97	0.63	34	-	-	ICP-MS	(Point et al. 2007)
Firth of Clyde, Scotland	0.03-0.15	0.02-0.08	> 75	0.02-3.53	9.5-12.3	DPASV	(Muller 1999)
Gran Canaria, Spain	-	-	-	120	8.36	ASV	(Santana-Casiano et al. 1995)
North Adriatic	0.2	-	-	6.1	9.2	ASV	(Scarponi et al. 1995)
Northeast Pacific	0.017-0.049	0.0001-0.0009	> 95	0.22-0.56	9.4-10.0	ASV	(Capodaglio et al. 1990)
Ross Sea, Antarctica	0.025-0.114	-	-	0.27-0.91	9.3-9.9	ASV	(Capodaglio and Toscano 1989)
Zn							
Southern Ocean	0.76-2.17	0.092-0.57	> 72	1.4-5.3	9.1-10.4	DPASV	(Baars and Croot 2011)
North Atlantic	-	-	-	0.6-1.5	9.8-11.3	FF-ASV, CSV	(Jakuba et al. 2008)
Bay of Biscay, France	44.84	27.36	39	-	-	ICP-MS	(Point et al. 2007)
North Pacific	0.069	0.0018	97	0.72	10.5	CSV	(Lohan et al. 2005)
South Pacific	0.006-0.960	-	-	1.28-2.46	9.82-10.93	CSV, ASV	(Ellwood 2004)
Manila Bay, Philippines	2.48-147.43	0.02-14.68	0.00-99.9	-	-	DPCSV	(Velasquez et al. 2002)

Table 1.2 continued

Location	$[M]_D$ (nM)	$[M]_L$ (nM)	ML (%)	$[L_M]$ (nM)	$LogK'L_M$	Method	Reference
Northeast Atlantic	0.17-0.32	0.007-0.0098	96-99	1.13-1.79	7.0-9.8	CLE-CSV	(Ellwood and van den Berg 2001)
Western Black Sea, Turkey	1.6-7.2	0.08-0.39	82-97	8-23	9.4-10.2	DPASV	(Muller et al. 2001)
Narragansett Bay, USA	16.3-71.5	0.3-13.3	75-99	10.6-48.2	$\geq$ 9.0	ASV	(Kozelka and Bruland 1998)
Chesapeake Bay, USA	4-20	0.01-0.3	99	8-200	9.5-10.0	CSV, ASV	(Henry and Donat 1996)
Delaware Bay, USA	3.47	-	-	15.25	$\geq$ 10.9	ASV	(Lewis et al. 1995)
Narragansett Bay, USA	-	-	-	49	7.85	ASV	(Muller and Kester 1991)
Northeast Pacific	0.12	-	-	1.60-1.76	10.30-11.20	CLE-CSV, ASV	(Donat and Bruland 1990)
Central North Pacific	0.30-0.35	-	-	0.94-1.34	10.6-10.7	ASV	(Bruland 1989)
Irish Sea	-	-	-	26-62	7.52-8.36	CLE-CSV	(M G Van den Berg 1985)
South Atlantic	5	1.8	64	30	7.4	MnO2	(van den Berg and Dharmvanij 1984)
Northwest Pacific	26	-	-	5-8	9.3	CLE-CSV	(Hirose et al. 1982)

- represents data not provided in the literature.
* represents values taken from graphs.

1.4 The ligand pool

The significance of the ligand pool in metal speciation in aquatic environments has been reported by several groups. But despite their obvious presence in seawater, the source, stimuli, and structure of the metal-complexing organic ligands remains unknown. Difficulties encountered in describing the ligand pool are largely the result of the inherent physicochemical complexity of the ligands themselves. Coastal waters contain a diverse range of metal complexers derived from both terrestrial and aquatic sources (**Figure 1.2**). Several aquatic organisms have been identified as producers of marine ligands including cyanobacteria (Moffet and Brand 1996), dinoflagellates (Croot et al. 2000), coccolithophores (Leal et al. 1999) and macroalgae (Andrade et al. 2010). In addition to the dynamic contribution of living organisms, processes (decay and decomposition) of dead organisms also constitute an important source of L as do sediments (Chapman et al. 2009). Thus, it is postulated that a continuum of binding sites with varying metal complexing capacity makeup the ligand pool (Town and Filella 2002). It is generally agreed that given the continuum the strongest and least labile sites will be the first occupied by metals, followed by those with a lower affinity until all sites are full (Luther et al. 2001).

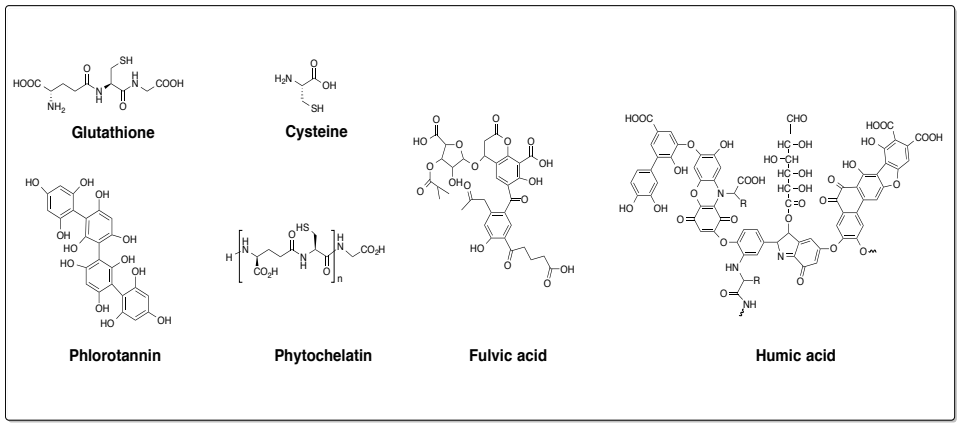

Figure 1.2. Structures of ligands known to bind trace metals in seawater.

While the chemical structures of a handful of marine siderophores have been identified, the identity of the remaining ligand pool remains unknown. Recently discussed candidates for the chemical nature of L include humic substances (Ytreberg et al. 2011) and thiols (Leal and van den Berg 1998). Inspection of the literature reveals several important

features in regards to ligand identity. Most notably, metal-binding curves established for natural waters lie between those of humic substances and biota-derived compounds, assuming that the humic logK' is always less than that of the biotic logK' for a given metal (Town and Filella 2002). This highlights the importance of biota-derived ligands in coastal systems and indicates that a further understanding of speciation may be gained once the significance of biota-derived ligands is discerned.

1.4.1 Macroalgae-exuded organic ligands

Macroalgae have often been proposed as the primary source of biogenic ligands in natural systems. In a recent study by Andrade et al. (2010), four of five macroalgae tested were found to release Cu complexing ligands in response to 157nM Cu exposure. **Table 1.3** shows that similar results have been reported for *F. vesiculosus*, *Porphyra* spp, and *Enteromorpha* spp (*Ulva*) (i.e. representing brown, red, and green species, respectively). Likewise, Karavoltsos et al. (2013) examined the release of Cu-complexing ligands by 24 macroalgae common to the Mediterranean. They found that all species released ligands, even if the complexing contribution per mass unit of organic matter was limited. To our knowledge, no similar data exist on the release of L_{Cd}, L_{Pb}, or L_{Zn} by macroalgae.

Table 1.3. A comparison of current literature values for the concentration and logK' of metal complexing ligands exuded by macroalgae. Adapted from Murray et al. (a).

Macroalgae	Medium	$[L_M]$ (nM)	$LogK'L_M$	Method	Reference
Cd					
A. nodosum, *F. vesiculosus, L. hyperborea*	Artificial seawater, + 0.89 or 8.89 nM Cd	5.13-32.52	8.17-10.11	DPASV	(Murray et al. b)
Cu					
A. nodosum, *F. vesiculosus*, *L. hyperborea*	Artificial seawater, + 7.86 or 15.70 nM Cu	2-72	8.96-11.77	CLE-CSV	(Murray et al. a)
Asparagopsis taxiformis, *Cystoseira corniculata, Caulerpa prolifera*, *Lophocladia lallemandii*, *Laurencia majuscula*, *Laurencia microcladia, Laurencia obtusa, Pterocladiella capilacea*, *Plocamium cartilagineum*, *Predaea ollivieri* (Rhodophyta)					
Cystoseira barbata, *Cystoseira compressa*, *Caulerpa racemosa*, *Flabellia petiolata*, *Halimeda tuna*, *Penicillus capitatus, Posidonia oceanica*, *Ulva rigida*, *Cystoseira foeniculacea*, *Colpomenia sinuosa* (Chlorophyta)	Artificial seawater	109-744	7.00-8.60	DPASV	(Karavoltsos et al. 2013)
Dictyota dichotoma, *Dictyopteris polypodioides*, *Halopetris scoparia*, *Predaea ollivieri*, *Padina pavonica* (Phaeophyceae)					
Chaeotomorpha firma, *Ulva lactuca*, *Porphyra columbina*, *Gelidium lingulatum*, *Lessonia nigrescens*	Artificial seawater, + 157 nM Cu	42-117	7.60-8.90	ASV	(Andrade et al. 2010)
Porphyra spp., *Enteromorpha* spp.	Filtered natural seawater, containing 6.80 nM Cu	77-123	12.07-12.65	C(A)SV	(Vasconcelos and Leal 2008)
Porphyra spp., *Enteromorpha* spp.	Filtered natural seawater, containing 29 nM Cu	92-127	12.40-12.49	C(A)SV	(Vasconcelos et al. 2002)

Table 1.3 continued

Macroalgae	Medium	$[L_M]$ (nM)	$LogK'L_M$	Method	Reference
F. vesiculosus	Modified Aquil, + 2-1000 nM Cu	146-793	10.20-12.50	CSV	(Gledhill et al. 1999)
Ectocarpus siliculosus, *Avicularia purpurea*, *Antithamnionella spirographidis*	Filtered, UV treated natural seawater	270-1010	9.77-10.15	MnO_2 exchange	(Sueur et al. 1982)
Pb					
A. nodosum, *F. vesiculosus*, *L. hyperborea*	Artificial seawater, + 1.40 or 4.83 nM Pb	5.05-18.16	8.66-10.14	DPASV	(Murray et al. b)
Zn					
A. nodosum, *F. vesiculosus*, *L. hyperborea*	Artificial seawater, + 15.20 or 91.70 nM Zn	6.59-45.51	11.54-14.99	CLE-CSV	(Murray et al. a)

1.4.2 Humic and fulvic acid

It is estimated that 23-62% of macroalgae net production is exuded as DOM (Abdullah and Fredriksen 2004). DOM is composed of several molecules and is functionally defined as that which can pass through a 0.45 μm filter (Stevenson 1994). Up to 70% of marine DOM is comprised of humic (HA) and fulvic (FA) acids (Abbt-Braun et al. 2004). Both acids have a low molecular weight and carboxyl groups which contribute binding capacity, where the maximum binding capacity of the humic substance for any metal ion is approximately equal to the content of acidic functional groups (Stevenson 1994). Accordingly, both HA and FA have a large capacity to complex dissolved metal species including metal ions, and that complexation could alter the solubility of both the ligand and the bound species.

1.4.3 Glutathione and cysteine

Macroalgae are also known to exude low molecular weight thiols. As outlined in **Table 1.4**, field studies show that both GSH and Cys are present in natural marine and fresh waters. Their omnipresence is likely to be a function of their presence in all eukaryotes (Toppi et al. 2003). In the same manner as the carboxyl groups of HA and FA, thiols have a large capacity to complex metal ions via sulphydryl coordination (Scott et al. 1993). Factors influencing the quantity and proportion of metal ions bound include pH, type and number of thiol groups, ionic strength and molecular weight (Krezel and Bal 1999). It has already been demonstrated that Cd, Cu, Pb, and Zn bind GSH preferentially at the thiol moiety (Krezel and Bal 1999). Accordingly, thiols may contribute to the natural pool of L, and thus influence metal bioavailability in seawater as well as metal toxicity in macroalgae.

Glutathione synthesis in macroalgae is a physiological function of sulfur metabolism and defence. At the cellular level, GSH has several roles; acting as an antioxidant (Noctor et al. 1998), a precursor for PCs (Ahner and Morel 1995), and a ligand capable of metal complexation (Le Gall and van den Berg 1998). An analysis of intracellular GSH in natural assemblages of *F. vesiculosus* revealed concentrations varied with metal concentration of the habitat but were often as high as 3969 nM g^{-1} DW (Pawlik-Skowronska et al. 2007). However, data on the release of these compounds into the surrounding media are scarce. Vasconcelos and Leal (2001) found that *Porphyra* and *Enteropmorpha* (*Ulva*) spp. exuded GSH and Cys following 29.40 nM Cu exposure, Cys only following 10.30 nM Pb exposure, and GSH only

following 10.40 nM Cd exposure. Whilst their study supports the promotion of thiol exudation in the presence of metals, extension of the findings to coastal areas is difficult due to the high concentrations of metals employed, compared to natural conditions. To the best of our knowledge, the exudation of GSH and Cys has not been previously observed for brown algae and/or at metal concentrations low enough to be realistic for natural systems.

Table 1.4. A comparison of current literature values for the concentration of GSH and Cys in natural waters. Adapted from Murray et al. (d).

Location	Water type	[GSH] (nM)	[Cys] (nM)	DOC (μM)	Method	Reference
Lough Furnace, Ireland	Estuary	2.29-3.85	2.83-3.11	219-641	CSV	(Murray et al. d)
Tamar Estuary, UK	Estuary	19-250*	-	-	HPLC	(Kawakami and Achterberg 2012)
Linsley Pond, USA	Freshwater lake	0-11	-	0.25-0.45	Chromatography	(Hu et al. 2006)
Pacific Ocean	Seawater	0-0.84	0.003-0.085	-	HPLC	Dupont et al. 2006
Venice Lagoon, Italy	Porewater	57-154	-	-	CSV	(Chapman et al. 2009)
Elizabeth River, USA	Estuary	1-178*	-	-	HPLC	Dryden et al. 2007)
Eel Pond, USA	Freshwater lake	33-74*	-	-	HPLC	(Wei et al. 2003)
Elizabeth River, USA	Estuary	14.7-25.3	21.1-35	-[A]	HPLC	(Dryden et al. 2007)
Norfolk Estuary, UK	Estuary	0.37-0.45	-	2.4-3.5	HPLC	(Tang et al. 2004)
San Diego Bay (1), USA	Estuary	0.17-0.52	-	0.86-1.6	HPLC	(Tang et al. 2004)
San Diego Bay (2), USA	Estuary	0.23-0.79	-	0.80-1.7	HPLC	(Tang et al. 2004)
Cape Fear Estuary, USA	Estuary	0.08-0.13	-	2.6-10.4	HPLC	(Tang et al. 2004)
Galveston Bay, USA	Estuary	0.23-6.1	-	1.7-5.5	HPLC	(Tang et al. 2000)
Mersey Estuary, UK	Estuary	20-300	-	-	CSV (with and without Cu addition)	(Le Gall and van den Berg 1993)
South California, USA	Coastal waters	100-250*	-	-	HPLC	(Matrai and Vetter 1988)

Table 1.4 continued

Location	Water type	[GSH] (nM)	[Cys] (nM)	DOC (µM)	Method	Reference
Saanich Inlet, USA	Anoxic fjord	28-200*	-	-	HPLC	(Matrai and Vetter 1988)

- represents data not provided in the literature.
* represents values in units of µmol g^{-1} chl a

1.5 Analytical techniques

The techniques and calculations most frequently employed for trace metal speciation, including organic complexation, have been extensively reviewed (Ure et al. 1995). In developing and/or choosing a speciation technique the most prominent concerns remain sample integrity and methodological implications. Maintenance of sample integrity is critical in speciation studies as both contamination and perturbation can affect the total metal concentration and its distribution amongst species. In general, minimal sample handling, storage and pretreatment combined with *in-situ* measurements best ensure accurate speciation. Currently M_D, M_L, L_M and $\log K'L_M$ are most commonly measured via the electrochemical titration techniques, with or without ligand competition, described below.

1.6 Voltammetry

Voltammetry is widely used for trace metal speciation and distribution analyses in seawater. It is based on the measurement of a current flowing through an electrochemical cell in response to potential applied at the electrode. Extremely low detections limits, high sensitivity, minimal sample manipulation, and the simultaneous determinations of several elements have made voltammetry very popular. Modern voltammetric instruments are relatively simple and low-cost, comprising only a three-electrode cell, a potentiostat (applies potential and measures current) and a computer for automated data acquisition (**Figure 1.3**).

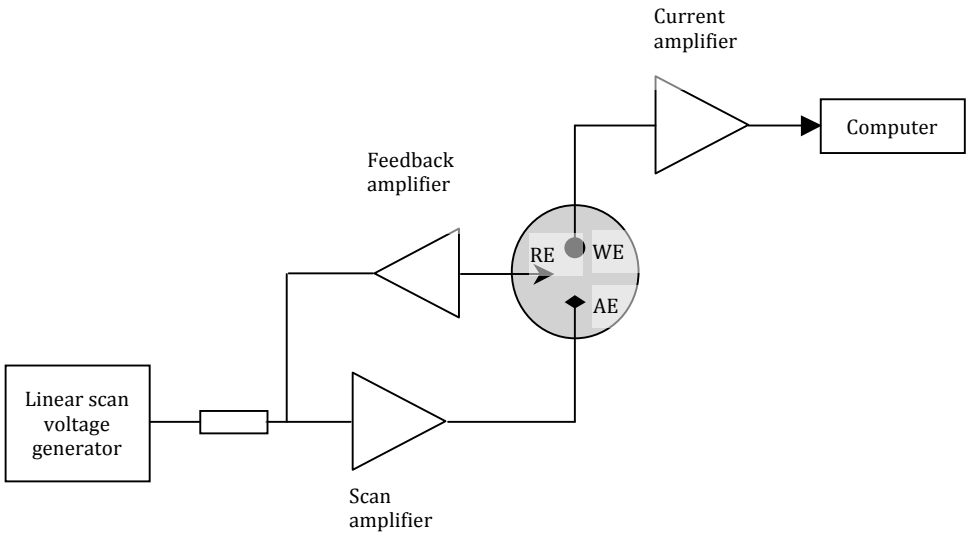

Figure 1.3. Schematic representation of a modern voltammetric instrument. RE: reference electrode; WE: working electrode; AE: auxillary electrode. Adapted from G. Meunier (Thesis, in prep).

The most common working, reference, and counter electrodes used in voltammetry for trace metal analysis are the hanging mercury drop (HMDE), Ag/AgCl/KCl (**Figure 1.4**), and Pt wire, respectively. Popularity of the HMDE is due largely to its reliability, where each new drop yields a new, reproducible electrode surface. Significant advances in voltammetry over the past 25 years include the development of microelectrodes, automation of analyses and instrument portability.

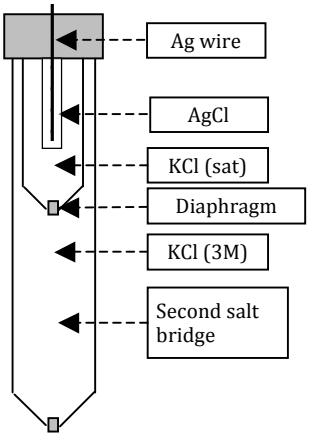

Figure 1.4: Schematic representation of the Ag/AgCl/KCl reference electrode and salt bridge. Adapted from G. Meunier (Thesis, in prep)

1.6.1 Anodic stripping voltammetry (ASV)

Anodic stripping voltammetry (ASV) is based on the preconcentration of analyte on the electrode prior to a stripping step. This method allows for the determination of the total amount and the 'labile' fraction of metal, as well as the ligand concentration and logK' following mathematical treatment (e.g.: Ruzic-van den Berg linearization, Scratchard linearization, Langmuir model, discrete ligand model, etc.) of the experimental data. It has been applied successfully in the past for trace metal measurements of Cd, Cu, Pb and Zn (**Table 1.5**) and is useful for the determination of any analyte that reacts directly with the electrode and is soluble in mercury.

_____

Table 1.5. Selected methods for the determination of trace metal complexation by ASV present in recent literature.

Element	Sample handling	Electrode	Ligand (nM)	Data treatment	M_L LOD (nM)	Reference
Cd	Frozen 0.45 µm	HMDE	None	Ruzic-van den Berg linearization	0.1	(Murray et al. b)
	-80°C 0.45 µm	HMDE	None	Ruzic-van den Berg linearization	-	(Sander et al. 2007)
	4°C 0.45 µm	HMDE	Ethylenediamine(0.2-1 mM)	Scratchard linearization	0.05	(Xue and Sigg 1998)
	4°C 0.45 µm	HMDE	None	Discrete 1 ligand model	0.05	(Knauer et al. 1998)
Cu	- 0.45 µm	SMDE	None	Ruzic-van den Berg linearization	-	(Karavoltsos et al. 2013)
	- 0.45 µm	TMF-RGCDE	None	Ruzic-van den Berg linearization	-	(Andrade et al. 2010)
	4°C 0.45 µm	SMDE	None	Discrete 1 or 2 ligand model	0.2	(Louis et al. 2009)
	- 0.4 µm	RGCDE	Ethylenediamine(0.007-1)	Langmuir model	0.2	(Muller et al. 2005)
Pb	Frozen 0.45 µm	HMDE	None	Ruzic-van den Berg linearization		(Murray et al. b)
	- 0.4 µm	RGCDE	None	Ruzic-van den Berg linearization	-	(Meylan et al. 2004)
	4°C 0.4 µm	RGCDE	Ethylenediamine -	Ruzic-van den Berg linearization	0.05	(Muller 1999)
	4°C 0.4 µm	RGCDE	Ethylenediamine (0.008-1)	Ruzic-van den Berg linearization	-	(Muller 1996a; 1999)
Zn	- 0.2 µm	TFME-GC-RDE	None	Ruzic-van den Berg linearization	-	(Baars and Croot 2011)
	Frozen 0.4 µm	GC-RDE	None	Discrete 1 ligand model	0.03	(Jakuba et al. 2008)
	Frozen 0.4 µm or 3kDa	HMDE	None	Ruzic-van den Berg linearization	-	(Hoffmann et al. 2007)
	- 0.4 µm	GCE	None	Langmuir model	0.4	(Muller et al. 2005)

- represents data not provided in the literature.

During analyses, the application of a sufficient negative potential (i.e. a potential more negative than the reduction potential of the element being measured) concentrates metal ions in the sample solution onto the electrode. The result is a reduction of the metal ions and the subsequent formation of an amalgam with mercury. The actual stripping process occurs when the potential is scanned in the positive direction and the metals in the amalgam are oxidized out of the Hg (**Figure 1.5**A). Peak currents, located at the specific position of the peak potential of each metal, are proportional to the concentration of each metal in the sample (**Figure 1.5**B). Subsequent measurements following standard additions of the desired analytes allow for the calculation of the initial sample concentration.

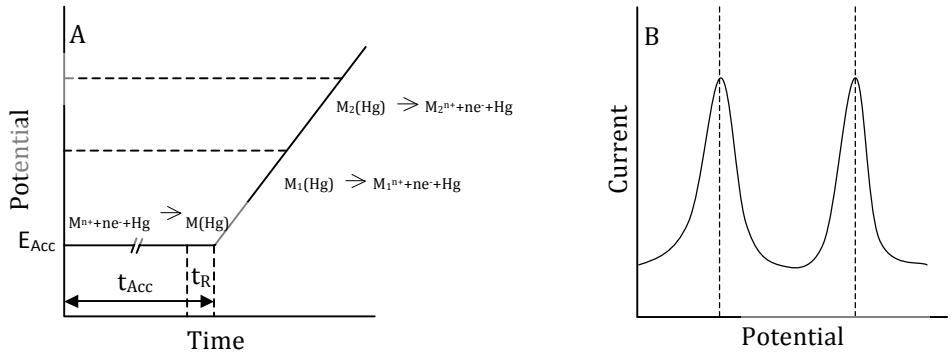

Figure 1.5. Evolution of potential as a function of time during ASV analysis (A) and the resulting voltammogram (B). E_{Acc}: accumulation potential; t_{Acc}: accumulation time; t_R: rest time; M_1: metal one; M_2:metal 2; ne^-:number of electrons. Dashed lines correspond to half-wave potentials. Adapted from G. Meunier (Thesis, in prep).

Differential pulse refers to the mode in which the potential is modulated and scanned. DPASV scans the potential in a series of fixed, small pulses. The difference between current measurements made just prior to the application of each pulse and immediately following are plotted against the base potential, yielding the final voltammogram. In addition to total and labile metal measurements, DPASV is useful for the study of metal ligands in seawater. The parameters related to metal complexation, including ligand concentration and logK', are derived from the titration of the unknown ligands in the sample solution by multiple, discrete metal additions. The reduction peak remains suppressed until the excess ligand is bound by the added metal. Raw titration data combined with an independent measurement of total metal concentration allow for the elucidation of the complexation parameters via

linearization of the least squares analysis, according to the following equation (van den Berg 1982):

$$[M]_L/[ML]=[M]_L/[L_M] + \alpha/(K'_{ML}L_M)$$

Where a single ligand is present, the linearization plot for the titration data shows a single straight line with concentration equal to the slope and logK' derived from the y-intercept. Where more than one ligand is present, the linearization plot for the titration data is curved and concentrations of L_M and $logK'L_M$ are obtained from the linearization of the first few data points. Subsequent ligand parameters are derived from a second linear least squares regression of the remaining titration data (minus the contribution of L_{M1}).

1.6.2 Cathodic stripping voltammetry (CSV)

Like ASV, CSV is based on the preconcentration of analyte on the electrode prior to a stripping step and allows for the determination of the total amount and the 'labile' fraction of metal, as well as the ligand concentration and logK' following mathematical treatment of the experimental data. CSV differs from ASV in that the element is reduced, rather than oxidized, during the potential scan.

In most cases, CSV speciation involves the addition of a known ligand prior to metal determination. Those ligands used most widely are presented in **Figure 1.6**. Competitive ligand-exchange CSV (CLE-CSV) is applicable to any electroactive absorbable complex with a metal of interest. Thus, CSV is particularly useful in quantifying those elements that are not sufficiently soluble in mercury (i.e. Fe and Co); although it is also frequently employed for Cu and Zn speciation analyses. **Table 1.6** gives examples of studies where CLE-CSV is employed for metal speciation, with particular reference to the metals discussed in this thesis (Cd, Cu, Pb, Zn).

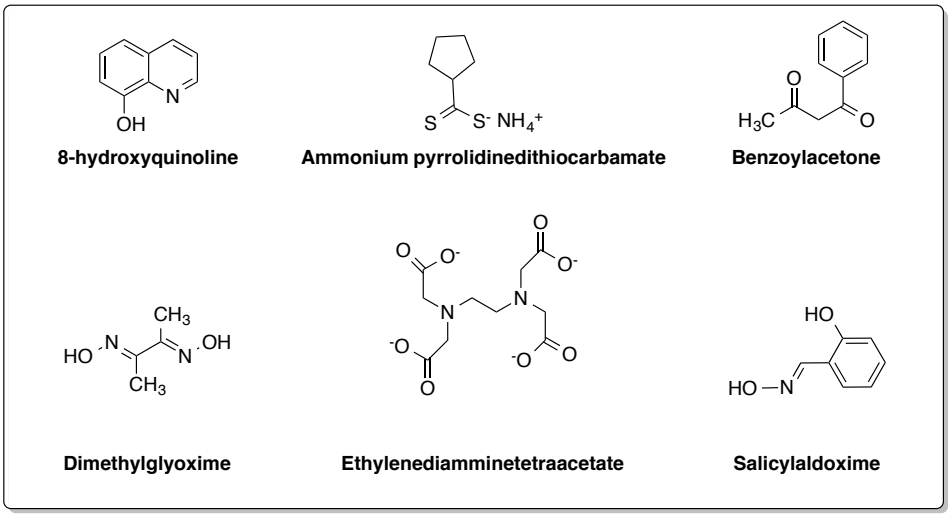

Figure 1.6. The structures of the ligands most commonly used in CLE-CSV analyses.

Table 1.6. Selected methods for the determination of trace metal complexation by CSV present in recent literature.

Element	Sample handling	Electrode	Ligand (nM)	Data treatment	M_L LOD (nM)	Reference
Cu	Frozen 0.45 µm	HMDE	SA	Ruzic-van den Berg linearisation	0.1	(Murray et al. a)
	- 0.45 µm	HMDE	SA (500-5000)	Ruzic-van den Berg linearisation	-	(Ytreberg et al. 2011)
	Frozen 0.2 µm	HMDE	SA (2000)	Ruzic-van den Berg linearisation	-	(Chapman et al. 2009)
	Frozen 0.4 µm or 3kDa	HMDE	SA or 8HQ	Ruzic-van den Berg linearisation	-	(Hoffmann et al. 2007)
Pb	Frozen 0.45 µm	HMDE	Calcein-Blue (500)	Ruzic-van den Berg linearisation	-	(Ploger et al. 2005)
	Frozen 0.45 µm	HMDE	Calcein-Blue (500)	Ruzic-van den Berg linearisation	0.1	(Fischer and van den Berg 2001)
Zn	Frozen 0.45 µm	HMDE	APDC	Ruzic-van den Berg linearisation	0.07	(Murray et al. a)
	Frozen 0.45 µm	HMDE	APDC (60000)	Ruzic-van den Berg linearisation	-	(Sander et al. 2007)
	Frozen 0.4 µm or 3kDa	HMDE	APDC	Ruzic-van den Berg linearisation	-	(Hoffmann et al. 2007)
	Frozen 0.22 µm	HMDE	APDC (200)	Ruzic-van den Berg linearisation	0.03	(Lohan et al. 2005)

- represents data not provided in the literature.

During CLE-CSV analyses, the added ligand-element complex is preconcentrated on the electrode via the application of an oxidizing potential and is quantified via a potential scan in the negative direction, resulting in the reduction of the metal in the adsorbed complex. Thus, the labile metal concentration is equal to the concentration of metal complexed to the added ligand. As in ASV, the labile metal concentration is directly related to the reduction current and subsequent measurements following standard additions of the desired analyte (but not the ligand) allow for the calculation of the initial sample concentration. Here, the 'labile' fraction includes the free metal ion, inorganic complexes, and those organic complexes with a logK' less than that of the added ligand.

CLE-CSV is also commonly employed for the determination of ligand concentrations and logK' (**Table 1.6**). The analysis involves serial additions of metal to discrete samples containing a constant amount of competitive ligand and sufficient equilibration time. Here, the oxidation peak is initially suppressed until the competitive ligand is fully saturated by added metal, and again suppressed following the eventual saturation of the natural ligand. The resulting voltammogram and linearization of the raw data can be used to calculate the logK' of the metal-natural ligand complex and the total natural ligand concentration, given that the binding parameters of the added ligand are known (van den Berg 1982).

Each technique outlined above satisfies the trace metal speciation requirements concerning sensitivity, selectivity, and reliability. Further benefits include subnanomolar detection limits, low risk of contamination and the maintenance of original sample composition throughout analysis. Nevertheless, each method has its own limitations. For both ASV and CSV, it is important to note that the results are strictly dependent on the measurement conditions (Buck and Bruland 2005). As such, the use of alternate conditions including changes to the stirring rate, drop size or deposition time, could result in dissimilar values. The major difficulty encountered in the determination of metal complexing parameters is the detection window. In ASV, the detection window is a function of the added metal range and is generally 6 logK units (Luther et al. 2001). The detection window in CSV is significantly lower, at only 2 logK units, and can only be altered by changing the concentration of the added ligand (Luther et al. 2001). However, choice of the detection window entirely determines the character of the ligands that are detectable. This is particularly limiting given

the broad diversity of ligands in natural samples and must be kept in mind when interpreting data or comparing ligand parameters across studies.

Voltammetric speciation measurements may also be impeded by the presence of compounds with electroactive or surface-active properties. Such compounds may result in peaks that overlap or interfere with those of the desired analyte, making quantification difficult. Whenever possible, measurements should be made so as to exclude known interferences. One such example is the adsorption of organic matter on the electrode surface during ASV. In samples containing organic matter, the adsorption of surface-active substances (SAS) greatly affects the electrochemical measurement of Cu (Plavsic et al. 1994). This issue is resolved by using CLE-CSV where adsorbed SAS occurs at more negative potentials, allowing for the measurement of well-resolved copper peaks (Scarano et al. 1992).

1.7 Motivation and Objectives

While most studies on trace metal-seaweed interaction have focused on accumulation and subsequent toxicity, the release of complexing ligands as a potential defence mechanism in addition to cell-wall binding and intracellular compartmentalization, should not be ignored. The production of ligands with metal-binding ability is significant as the speciation of several metals in seawater is dominated by organic complexation. Thus, the macroalgal biosynthesis of ligands could constitute an important portion of the ligand pool in seawater.

In recent years, the majority of headway in this field has concerned the characterization of Fe and Cu specific ligands. From the work on Fe alone, it is apparent that *in-situ* biogenic ligands influence bioavailability (as reviewed by Vraspir and Butler 2009). Several marine siderophores have been isolated and characterized from natural seawater (Gledhill et al. 2004) and porphyrins (intracellular molecules thought to be released into seawater by marine organisms, including algae) have been detected in seawater at low-nM concentrations (Vong et al. 2007). Rue and Bruland (2001) have also shown that Domoic acid, an algal toxin produced by *Pseudo-nitzschia*, is capable of Fe complexation. Still, we are only beginning to understand the function of algae-derived ligands in aquatic systems. Much of the fundamental insight into algae-exuded organic ligands comes from experiments employing extremely high metal concentrations or using microalgae. For most seaweeds and metals other than Fe, significant questions remain to be answered regarding the source, structure, and stimuli of the exuded compounds.

The aim of this thesis was to investigate seaweeds as a source of naturally occurring organic ligands and metals as stimuli of their release. Recent advances in voltammetry were exploited in order to measure the production of Cd, Cu, Pb, and Zn ligands by brown macroalgae. In the absence of direct identification, comparison of measured logK' values to known compounds provided evidence on the identity of L. In contrast to previous literature, the attempts to determine metal-complexing substances via laboratory culture experiments here were carried out at environmentally relevant metal concentrations. The rationale and specific objectives for each chapter are outlined below. As a cohesive unit, the end goal of the thesis was to assess the potential contribution of extracellular material released by macroalgae to the overall biological control of the ligand pool in coastal areas.

1.7.1 Chapter 3

Understanding the source and stimuli responsible for organic ligands in seawater is crucial to understanding trace metal bioavailability. Voltammetric techniques were employed to characterize the water chemistry of seaweed cultures exposed to low levels of Cu or Zn over 7 days. The main objective was to assess seaweeds as a potential source of L, and Cu and Zn as stimuli of macroalgal ligand production.

This chapter corresponds to the peer-reviewed journal publication entitled 'Voltammetric characterization of macroalgae-exuded organic ligands (L) in response to Cu and Zn: a source and stimuli for L' (Murray et al. a) and has been accepted for publication in Environmental Chemistry.

1.7.2 Chapter 4

Although seaweeds are known producers of L, their ability to exude Cd- or Pb- complexing ligands has not yet been studied. Again, voltammetric techniques were employed to characterize the water chemistry of seaweed cultures exposed to low levels of Cd or Pb over 7 days. The main objective was to establish if seaweeds produce L in response to Cd and Pb and what effects, if any, macroalgal exudates have on metal speciation in seawater. To the best of our knowledge, no such data exist for macroalgae.

This chapter corresponds to the manuscript entitled 'Voltammetric characterization of macroalgae-exuded organic ligands (L) in response to Cd and Pb: expanding the source and

stimuli for L' (Murray et al. b) which been submitted for peer-review and publication in Marine Chemistry.

1.7.3 Chapter 5

Recent evidence suggests that metals may behave synergistically with respect to speciation. Peers et al. (2005) found that Fe availability depends of Cu availability, while the bioavailability of both metals is governed by organic ligands. However, so far most estimates of macroalgal ligand production have been based on data obtained from single-metal experiments. Voltammetric techniques were employed to examine the release of metal-specific complexing ligands in response to a quaternary metal mixture. The main objective of this study was to investigate whether the presence of multiple metals influenced macroalgal ligand exudation, and subsequent metal speciation. A comparison of the results to the single metal systems was used to determine whether macroalgae-exuded ligands are metal- and/or non-metal specific.

This chapter corresponds to the manuscript entitled 'No metal stands alone: the voltammetric characterization of macroalgae-exuded organic ligands (L) in response to a quaternary metal mixture' (Murray et al. c) which been submitted for peer-review and publication in Environmental Science and Technology.

1.7.4 Chapter 6

Trace metal toxicity to marine organisms is largely controlled by organic ligands though the source, structure and stimuli of these ligands remain mostly unknown. In this case study, trends in Cd and Zn complexing ligands in a near –pristine estuary in western Ireland were studied in order to gain clues on the identity of L. The main objective was to confirm the existence of macroalgal ligands *in-situ* while simultaneously establishing a relationship between salinity, L, and metal speciation.

This chapter corresponds to the peer-reviewed journal publication entitled 'Metal complexation by organic ligands (L) in near-pristine estuarine waters: evidence for the identity of L' (Murray et al. d) and has been accepted for publication in Environmental Chemistry.

Chapter 2 Materials and Methods

Chapter 2 Materials and Methods

In order to examine seaweeds as a source of naturally occurring organic ligands and metals as stimuli of their release, experiments were designed to investigate key factors responsible for macroalgal ligand production including ambient metal concentration, duration of exposure, and species-specific response.

The general experimental design used for the identification, quantification, and characterization of macroalgae-exuded organic ligands is outlined in **Figure 2.1**. For the most part, each experiment consisted of 6 stages: macroalgal collection, macroalgal culture, analysis of macroalgal tissue, analysis of culture solutions, quality assurance, and data analysis. Summaries of the most commonly employed methods such as maintenance of sample integrity and quality assurance are provided here, whereas complete and more detailed descriptions of all analyses employed can be found in the relevant Methods sections of Chapters 3-6.

Seaweeds were exposed to Cd, Cu, Pb and Zn in single and quaternary mixtures in the laboratory. Seaweed tissues were analyzed for metal content and culture media were analyzed for metal speciation, metal ligands, complexing capacity, GSH, and Cys. A case study conducted in the field also involved the analyses of seaweed tissues for metal content and water samples for metal speciation, metal ligands, complexing capacity, GSH, and Cys.

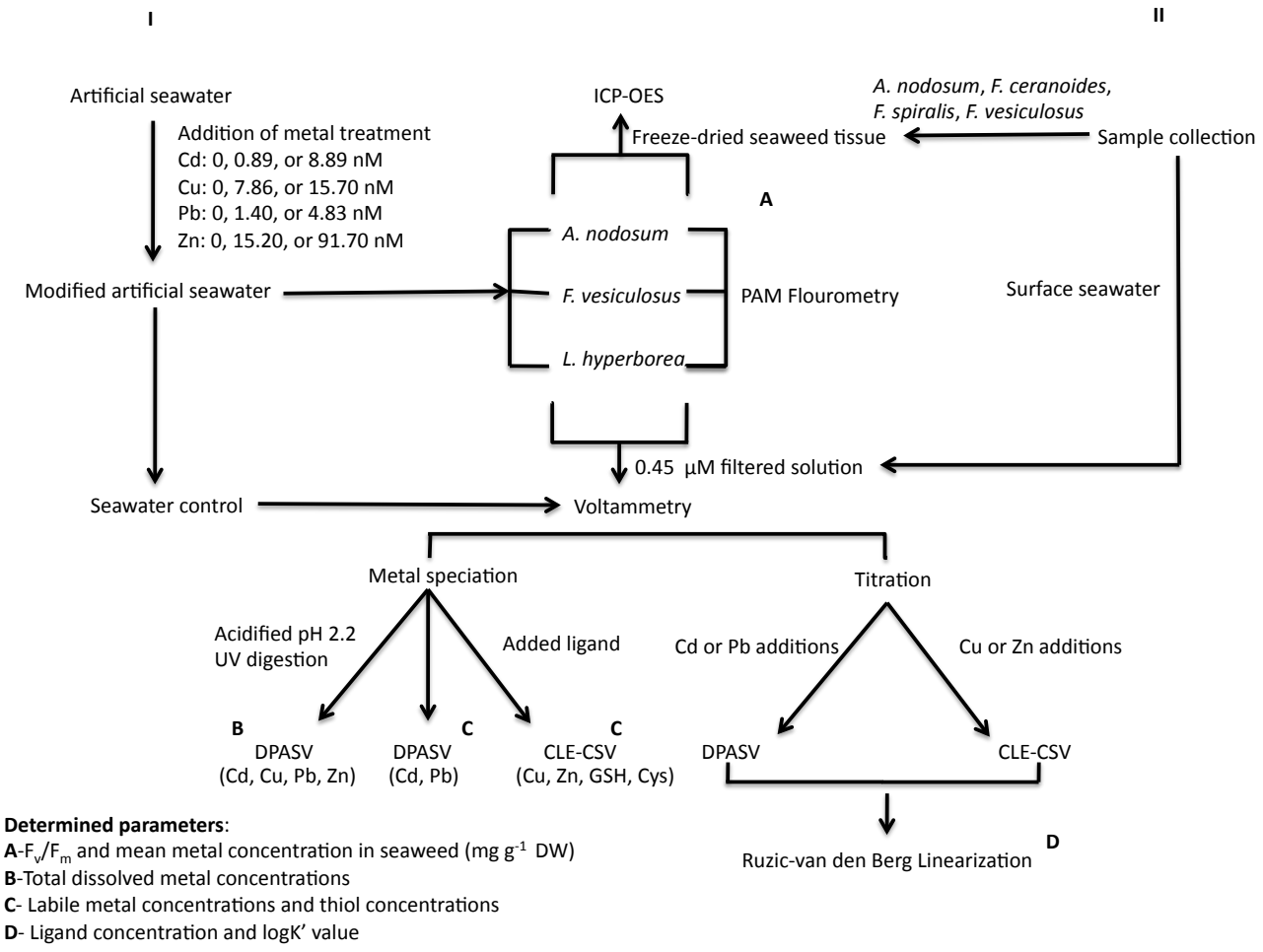

Figure 2.1. Experimental design of the studies discussed in this thesis. Three sets of experiments (Murray et al. a, b, c) were carried out in the laboratory where the determined parameters were measured on days 0, 1, 4, and 7 (I). Another experiment (Murray et al. d) was conducted in the field (II).

2.1 Description of sampling sites and macroalgal species

2.1.1 Finavarra

Macroalgae used in laboratory cultures were collected from Finavarra (53◦ 09′ 25′′ N; 09◦ 06′ 58′′ W), Co. Clare, western Ireland. According to the coastal typologies set out by the Water Framework Directive (WFD; 2000/60/EC), this site is characterized as euhaline, mesotidal and moderately exposed. Much like the remainder of the Irish coast, this site has very low levels of metal pollution (National Summary Report 2005) and is surrounded by only a few small residential settlements. As such, it is considered near pristine. On each sampling occasion, water temperature was between 7-10˚C, pH ranged from 8.1-8.6, and salinity was always greater than 33. While nutrient analysis was not conducted, Connan et al. (2011b) previously found water sampled from the same site to be nutrient-poor.

2.1.2 Lough Furnace

A second site was used for the field study detailed in Murray et al. (d). The Lough Furnace Estuary (9˚ 34’ 20” W, 53˚ 55’ 22” N) located in Co. Mayo, western Ireland, has a meromictic lagoon lake, strong tidal currents, and saline intrusions from the nearby Newport Bay (**Figure 6.1**). It was chosen for its range from fresh to full salinity water.

2.1.3 Macroalgae

The macroalgae employed in this thesis are restricted to the Phaeophyceae (brown algae). All three species are common perennial seaweeds of European temperate rocky shores, but can be separated based on their morphology and/or shore level. *Ascophyllum nodosum* and *Fucus vesiculosus* are both mid-shore species characterized by apical growth. According to Littler’s Form-Function model, the former has coarsely branched thalli while the later has thick sheets (Littler and Littler 1980). *Laminaria hyperborea*, by contrast, is a low-shore species that has leathery thalli. These species were chosen based on their common occurrence on Irish shores and their known ability to both accumulate and detoxify metals (Cullinane and Whelan 1982; Stengel and Dring 2000; Stengel et al. 2004; Morrison et al. 2008). In addition, *Fucus ceranoides* and *Fucus spiralis* were also examined in the case study (Murray et al. d).

2.2 Maintenance of sample integrity

Most problems in trace metal analyses are related to sample contamination or displacement of chemical equilibria. Therefore, several techniques were employed throughout this work to ensure the maintenance of sample integrity. Sample collection, treatment, and storage procedures outlined here were tested on surface water samples taken from Lough Furnace and showed maintenance of sample integrity.

In order to avoid direct contamination of samples, gloves were always worn in the laboratory and whenever possible, work was carried out in a laminar flow hood. Any equipment used was first soaked for 1 week in 1 M HCl then rinsed 3 times with MQ and allowed to air dry in a laminar flow hood. Prior to this, those lab-materials commonly in contact with organic matter were soaked for 1 week in 5% Decon. When not in use, all cleaned and dried glassware was stored in double-sealed bags (where size allowed).

In an effort to minimize loss of metal due to adsorption, all containers (including culture jars, HDPE storage bottles, titration tubes, and voltammetric cells) were preconditioned by rinsing twice with artificial seawater containing the metal of interest at a concentration similar to that expected in the sample. Alternatively, where sample volume allowed, containers were rinsed twice with the sample itself. In both instances, the rinse solution was discarded and replaced by new sample. In order to minimize the constraints of the conditioning requirement, Sterilin tubes were rinsed with MQ between titrations and used again in the same order to maintain conditioning for the sample replicates. Preliminary experiments showed that metal losses were insignificant using this method.

Given that the voltammetric methods employed here rely on relatively slow processes, most water samples could not be analyzed immediately but required storage. Although few examples of storage validation experiments are available in the literature, laboratory tests confirmed the best procedure for storage and later analysis was rapid freezing of samples followed by a slow thaw prior to use. Therefore, any samples that could not be analyzed on the day were stored at -8˚C until required for analyses, but never more than 3 months. The re-thawing procedure involved storage overnight at 4˚C and gentle, yet sufficient, mixing to ensure the dissolution of particulates. The liquid-solid-liquid phase transitions and consequent rehomogenization of the sample did not change the metal species pattern of interest (Murray et al. d).

For all methods used it was important to minimize sample perturbation, although some perturbation was required in order to obtain the appropriate analytical signal. Here,

samples required 0.45 µm filtration in order to determine the dissolved metal speciation. Validation of this method is well documented for samples pre-freeze (Capodaglio et al. 1995). Further to this, it has been shown that conservation of filtered samples by freezing does not alter metal concentration or speciation (Capodaglio et al. 1995). Accordingly, samples were generally filtered prior to freezing. However, this method was slightly altered in Murray et al. (d) since samples were frozen whole and filtered post-thaw. Data illustrating the effects of these changes on sample integrity are provided in the Supplementary Information of Chapter 6. In general, filtering pre- or post-freeze was species-retaining for Cd but resulted in higher concentrations of Zn_L.

2.3 Voltammetric speciation and titration

Table 2.1/2.2 and Table 2.3 outline the details of the procedures used for the voltammetric speciations and titrations, respectively. All voltammetric conditions were optimized prior to experimental analyses (see **Appendix 1**).

Table 2.1. Instrumental parameters and reagent concentrations used for the total dissolved metal determinations using DPASV.

	Cd	Cu	Pb	Zn
Ligand	-	-	-	-
Buffer	Acetate, 0.07 M	Acetate, 0.01 M	Acetate, 0.07 M	Acetate, 0.01 M
pH	5.0	5.0	5.0	5.0
LOD (nM)	0.1	0.3	0.2, 0.024	0.6
Purge with N_2 (s)	300	300	300	300
Deposition Potential (V)	-1.15	-1.15	-1.15	-1.15
Deposition Time (s)	60	60	60, 300	60
Waiting Time (s)	10	10	10	10

	Cd	Cu	Pb	Zn
Initial Potential (V)	-1.15	-1.15	-1.15	-1.15
End Potential (V)	0.05	0.05	0.05	0.05
Step Potential (V)	0.05	0.05	0.05	0.05
Peak Potential (V)	-0.6	-0.1	-0.4	-1
Drop Size (Metrohm)	5	5	5	5
Stir Rate (rpm)	2000	2000	2000	2000

Table 2.2. Instrumental parameters and reagent concentrations used for the dissolved labile metal determinations via DPASV or CLE-CSV.

	Cd	Cu	Pb	Zn
Voltammetric Method	DPASV	CLE-CSV	DPASV	CLE-CSV
Ligand	-	SA, 5 μM	-	APDC, 100 μM
Buffer	HEPPS, 0.01 M	HEPPS, 0.01 M	HEPPS, 0.01 M	HEPPS, 0.01 M
pH	8.1	8.1	8.1	8.1
LOD (nM)	0.10	0.30	0.20	0.60
Purge with N_2 (s)	300	300	300	300
Deposition Potential (V)	-0.80	-0.20	-0.60	-0.80
Deposition Time (s)	60	60	60	60
Waiting Time (s)	10	10	10	10
Initial Potential (V)	-0.80	-0.15	-1.15	-0.80
End Potential (V)	0.05	-0.90	0.05	-1.20
Step Potential (V)	0.01	0.05	0.05	0.02
Peak Potential (V)	-0.60	-0.20	-0.40	-1.00
Drop Size (Metrohm)	5	5	5	5
Stir Rate (rpm)	2000	2000	2000	2000

Table 2.3. Typical titration parameters employed for the voltammetric determination of [L_M] and log$K'L_M$

Method	Metal	Ligand	Titration Points (nM)	Equilibriation Time (h)
DPASV	Cd	-	0, 0.5, 1, 2, 5, 10, 20, 30, 40, 60	12-18
	Pb	-	0, 1, 5, 10, 20, 30, 40, 60, 80, 90, 100, 120	12-18
CLE-CSV	Cu	SA, 5 μM	0, 5, 10, 15, 20, 30, 40, 50, 60, 80, 100, 120	12-18
	Zn	APDC, 100 μM	0, 5, 10, 20, 30, 40, 50, 60, 80, 90, 100, 120	12-18

For all metal species, quantification was achieved by two standard metal additions, each scanned twice. It should be noted that in some cases small changes in sample pH resulted in slight shifts in the potential of the metal peaks. Where this occurred, standard additions were monitored to confirm the peak identity and the scan range was subsequently expanded to include the shifted peak. GSH and Cys were also quantified using CSV, where detected.

For titrations, the points provided in **Table 2.3** are only an example of those typically used. Titration schemes employed were sample-specific and their range pended on the total metal concentration of the sample. Thus, many titrations were performed at points somewhat different from those listed here. Regardless, all titrations performed were forward multi-cup with 10-12 aliquots equilibrated approximately 16 h.

2.4 Voltammetric titration modeling

Figure 2.2 shows the results of representative ASV and CLE-CSV titrations. As illustrated, the titrant metal is not fully detected at low concentrations due to binding with the natural ligand, however, after the analyte ligand is saturated, the titrant metal is detected resulting in the expected linear range.

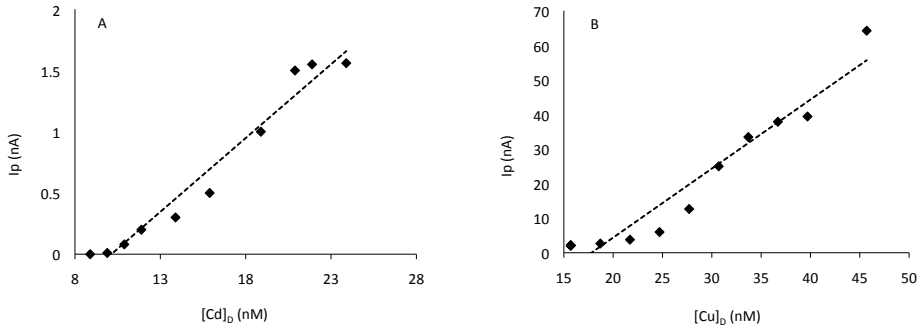

Figure 2.2. Representative titrations of: Cd using ASV (A) and Cu using CLE-CSV (B). Adapted from Murray et al. (a, b).

Modeling methods for the determination of [L] and logK' values from this data have been fully developed in literature and generally involve a linearization procedure. Here, voltammetric titration data was modeled using the Ruzic-van den Berg linearization as follows (van den Berg 1982):

$$[M]_L/[ML]=[M]_L/[L_M] + \alpha/(K'_{ML}L_M)$$

As derived from this equation, plots of $[M]_L/[ML]$ versus $[M]_L$ were linearly transformed (**Figure 2.2**). A straight line was indicative of a single ligand and a curved line of more than one ligand. In all instances, the concentrations of L_M and $logK'L_M$ were determined from the slope and y-intercept of a least squares regression of such titration curves.

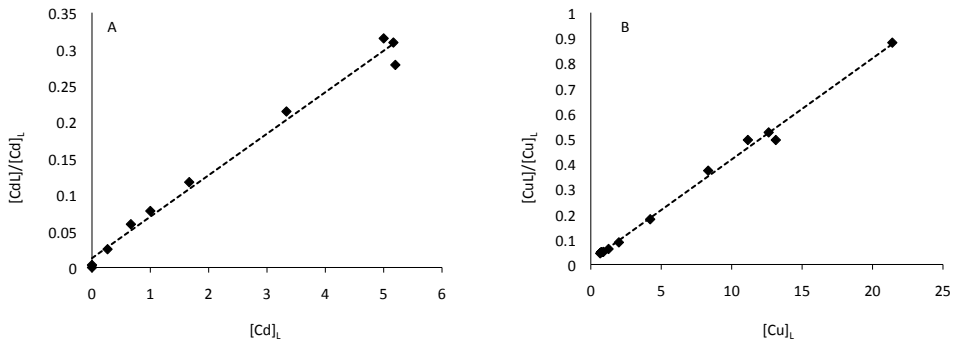

Figure 2.3. Linearisation of the representative titration data presented in Fig. 2.3 for: Cd using ASV (A) and Cu using CLE-CSV (B). Adapted from Murray et al. (a, b).

Where more than one ligand was detected, concentrations of L_M and $logK'L_M$ were obtained using an iterative fitting method (van den Berg 1984). Initial estimates of L_{M1} and $logK'L_{M1}$ were taken from the linearization of the first few data points. A 2nd linear least

squares regression of the remaining titration data (minus the contribution of L_{M1}) was used to calculate L_{M2} and $\log K'L_{M2}$. This method was then repeated for the first few data points, now subtracting the contribution of L_{M2} to obtain a better estimate of L_{M1} and $\log K'L_{M1}$.

Calculation of sensitivity was modified from the Ruzic-van den Berg method as per Turoczy and Sherwood (1997). The convential Ruzic-van den Berg method (a linear least squares regression of the peak heights of the final four titration points, which refer to the free metal response) was applied to obtain a first estimate of S', C_L', and K'. These values were then used to generate model titration data (ie: peak heights for each metal addition). The sensitivity of the final 4 points of the model data (S'') was then used to adjust the original estimate of S as follows:

$$S_{adj} = S' * S'/S''$$

A reapplication of the Ruzic-van den Berg method to the original titration data using S_{adj}, yielded a new C_L' and K'. The adjustment process was typically iterated 20 times of in order to improve the model accuracy. The advantage of this method is that ligands need not be fully saturated and so, large concentrations of metals do not need to be introduced into the cell. This decreases the likelihood of contamination and removes emphasis from the maximum titrant concentration. More detailed instructions for the calculations applied in Excel are listed in **Appendix 2**.

2.5 Quality assurance

The techniques employed were regularly tested for accuracy by analyzing Certified Reference Materials (CRMs) for seaweed metal content (NIES no. 9) and seawater metal content (CASS 5). For seaweed metal content, the metal concentrations determined were never significantly different than the expected values ('t'-test, $p>0.05$). For seawater metal content, analyses of samples were only ever carried out after measurements on the reference material were not significantly different ($p>0.05$) than the expected values using a 't'-test. Further to this, a laboratory internal standard of coastal seawater was analyzed daily to ensure day-day constancy.

The most common reasons for failed accuracy of measurement were sample contamination and/or the presence of SAS on the working electrode. The former was generally resolved by re-cleaning of all materials whereas the latter required changing the capillary or rinsing it thoroughly in methanol.

Chapter 3 Murray et al. (a)

Voltammetric characterization of macroalgae-exuded organic ligands (L) in response to Cu and Zn: a source and stimuli for L

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Hollydawn Murray[A,C*], Guillaume Meunier[A], Constant M.G. van den Berg[B], Rachel R. Cave[A], and Dagmar B. Stengel[C]

[A]Earth and Ocean Sciences, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

[B]Oceanography Laboratory, Department of Earth Science, University of Liverpool, Liverpool, United Kingdom

[C]Botany and Plant Science, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

3.1 Abstract

It is widely accepted that organic ligands control metal speciation in seawater (Vraspir and Butler 2009), although little is known about their source, stimuli, or identity. To gain insight on the possible environmental controls of metal complexing ligands (L), 3 brown macroalgae common in Irish waters (*Ascophyllum nodosum, Fucus vesiculosus* and *Laminaria hyperborea*) were cultured under low levels of Cu (0, 7.86, or 15.7 nM) or Zn (0, 15.2, or 91.7 nM) exposure. Seaweed photochemical quantum yield (F_v/F_m), metal speciation (Cu, Zn), complexing ligands (L_M), conditional stability constants ($logK'L_M$), glutathione (GSH), cysteine (Cys) and seaweed metal contents were monitored over 7 days. Although there was no effect on the internal seaweed metal concentrations, Cu and Zn additions significantly altered the water chemistry of each culture. Metal additions increased the total dissolved metal concentrations for all 3 species. Significantly higher [L_M] values in cultures with added metals than the relevant controls point to both metals as stimuli of L production. All species released ligands in response to Cu or Zn exposure, indicating each seaweed is a relevant source of L. Comparison of logK's to those of known ligands provides little evidence that the ligands reported here belong to the compounds identified as L in previous literature.

3.2 Introduction

Copper (Cu) and zinc (Zn) are essential micronutrients for marine macroalgae (DeBoer 1981; Lobban and Harrison 1996). Copper is used in trace amounts for electron transport in photosynthesis and enzyme systems such as amine oxidase (Burkhead and Reynolds 2009). Likewise, Zn is a component of several enzymes and proteins (Vallee and Auld 1990; Clemens 2001) including DNA and RNA polymerase, carbonic anhydrase and alkaline polymerase (Sunda et al. 1987). However, the presence of excess Cu or Zn in algal growing waters has toxic effects. Excess Cu has been previously shown to inhibit photosynthesis (Küpper et al. 2002), disrupt electron transport in photosystem II (Nielsen and Nielsen 2008), decrease pigment concentrations (Pinto et al. 2003) and have negative consequences for macroalgae growth and development (Bond et al. 1999; Nielsen and Nielsen 2005). A surplus of Zn, although less studied than that of Cu, has been shown to inhibit growth in six species of brown algae (Baumann et al. 2009). Whilst both metals are toxic, a study by Rai et al. (1981) suggests that Cu toxicity is greater than that of Zn. In either case, overall biological toxicity depends on the form in which the metal occurs, also known as its 'speciation'.

The current literature of metal speciation in seawater is dominated by the concept of ligands. Cu and Zn (to a lesser extent) are known to be bound by organic ligands in ocean waters (van den Berg 1984; Buckley and van Den Berg 1986; Coale and Bruland 1988; Bruland 1989; Donat and Bruland 1990; Moffett 1995; Leal and van den Berg 1998; Ellwood and van den Berg 2000). The concentration of Cu complexing ligands is estimated to be greater than 100 nM in coastal waters (van den Berg 1984; Manping et al. 1990) and 10-20 nM in open ocean waters (Buckley and van den Berg 1986; Coale and Bruland 1988). Although very little is known about their source or structure, it is speculated that these ligands are of a biogenic nature (Buffle and Chalmers 1988; Hedges et al. 2000).

Macroalgae are known to affect speciation via metal uptake (Vasconcelos 2001), surface adsorption (Crist et al. 1990; Manley et al. 2009), and the production of organic ligands with metal complexing capacity (Sueur et al. 1982; Gledhill et al. 1999; Vasconcelos 2001; Andrade et al. 2010). Andrade et al. (2010) found that, in the presence of copper, *Chaetomorpha firma* (Chlorophyta), *Ulva lactuca* (Chlorophyta), *Porphyra columbina* (Rhodophyta), *Gelidium lingulatum* (Rhodophyta) and *Lessonia nigrescens* (Phaeophyta) all release copper complexing ligands, despite belonging to different groups (red, green, or brown algae). There is evidence in the literature that thiols form metal complexes and are a

major constituent of the metal complexing ligand pool (Leal and van den Berg 1998; Leal et al. 1999; Tang et al. 2001; Laglera and van den Berg 2003). Vasconcelos et al. (2002) identified and quantified several thiol compounds in algal exudates. They found exudates of *Enteromorpha* species to contain both cysteine and glutathione, while exudates of *Porphyra* species were rich only in glutathione, indicating species-specific exudation patterns. These studies confirm the importance of algae-exuded organic ligands in metal speciation. Unfortunately, no such information exists for Zn or brown algae, with the exception of *Fucus vesiculosus* germlings (Gledhill et al. 1999) and *Lessonia nigrescens* (Andrade et al. 2010).

Over the last three decades, voltammetry has been widely employed for metal speciation of seawater (Town and Filella 2000). First introduced by van den Berg (1984) competitive ligand exchange-cathodic stripping voltammetry (CLE-CSV) is now commonly used to determine seawater complexing capacity and the associated stability constants (Boye and van den Berg 2000; Tian et al. 2006). This method is uniquely suited as it is the only technique available for determination of complexing capacity at sub-nM levels (Pesavento et al. 2009).

In this work, we investigated the production of organic ligands (including thiols) by three brown macroalgae in response to Cu or Zn exposure in cultures. The aim of the study was to describe the relationship, if any, that exists between metal exposure, ligand production, and metal bioavailability.

3.3 Materials and Methods

3.3.1 Reagents

Water used for dilutions and preparation of modified artificial seawater was Milli-q (MQ) and all reagents were analytical-grade. Stock solutions of Cu and Zn were prepared in 0.01 M HCl, by dissolution of atomic absorption standard solutions (Fluka). Thiol working standards of glutathione, cysteine, and thioactemide (all Aldrich) were prepared prior to use via dissolution in MQ water. Stock solutions of 0.01 M salicylaldoxime (SA, Aldrich) and 0.025 M ammonium pyrrolidinedithiocarbamate (APDC, Aldrich) were prepared monthly in 0.1 M HCl and MQ water, respectively. One molar 3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulfonic acid (HEPPS, Aldrich) and 0.55 M NaOH (Aldrich) were used in voltammetric determinations where the addition of 70 µL buffer to 10 mL sample yielded a pH of 8.1. While

all experiments were conducted at room temperature, when not in use, all metal, ligand, and buffer solutions were stored at 4°C. Artificial seawater was prepared according to Kester et al. (1967), however, modified to eliminate trace metals (**Table S1**). This water was further supplemented with 176 µmol nitrate (as sodium nitrate, Aldrich) and 7.26 µmol phosphate (as sodium dihyrdogenophosphate, Aldrich).

3.3.2 Cleaning of laboratory materials

All equipment including flasks, culture jars, bottles, filters and plastics were soaked for 1 week in 1 M HCl then rinsed 3 times with MQ and allowed to air dry in a laminar flow hood. Where possible, clean equipment was then stored in doubled sealed bags. Prior to this, culture jars and bottles were soaked for 1 week in detergent (5% Decon). Gloves were worn throughout and sample manipulations were always carried out in a laminar flow hood.

3.3.3 Macroalgae collection

The areas of 'new growth' of *Ascophyllum nodosum* (Linnaeus) Le Jolis, *Fucus vesiculosus* (Linnaeus), and *Laminaria hyperborea* (Gunnerus) were collected manually during low tide at Finavarra (53◦ 09′ 25′′ N; 09◦ 06′ 58′′ W), Co. Clare, western Ireland. For *A. nodosum* and *F. vesiculosus*, the apical parts (tips) were collected from the longest fronds of different thalli. For *L. hyperborea*, the basal meristem was sampled from the thallus, just above the stipe. Collections were made on different sampling dates between January and May 2011 corresponding to two separate laboratory experiments. On each occasion, macroalgae were placed in acid-cleaned plastic bags with seawater from the site, stored at 4°C and transported to the laboratory within an hour of collection. Seaweeds were stored at 10°C, under 60 ± 5 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400-700 nm) on a 12 h:12 h light: dark cycle in filtered seawater for a maximum of two days.

3.3.4 Macroalgal cultures

Seaweeds were removed from the filtered seawater and rinsed 3 times with the modified artificial seawater. Following this, single tips (*A. nodosum* and *F. vesiculosus*) or meristems (*L. hyperborea*) of approximately 6 cm in length of each species were placed in 500 mL polycarbonate culture jars containing 300 mL of modified artificial seawater enriched with either Cu (at 0, 7.86 or 15.70 nM) or Zn (at 0, 15.20, or 91.70 nM). Each was triplicated (n=3)

and cultures without seaweed were included for determination of background metal and ligand levels. Seaweeds were grown for 7 days at 10°C, under 60 ± 5 μmol m^{-2} s^{-1} PAR on a 12 h:12 h light: dark cycle, with constant bubbling with air.

3.3.5 Photosynthesis

Fluorescence measurements were taken using a Pulse Amplitude Modulated (PAM) fluorometer (PAM 2000, Heinz Walz GmbH, Effeltrich, Germany) on day 0, 1, 4, and 7, following dark adaptation of tips or meristems for 20 min. Measurements were carried out at 10°C, on samples just barely submerged in their corresponding culture media. Maximum photosystem II efficiency (F_v/F_m) was measured by applying a saturating pulse to the dark adapted tips in order to determine minimum (F_0) and maximum fluorescence (F_m) (Kitajima and Butler 1975), where:

$$F_v/F_m = (F_m - F_0)/F_m$$

Here, F_v/F_m is expressed as a percent of the day 0 control values in each experiment so as to account for any decreases in yield following harvest and to allow for easier comparison of results across the 3 species.

3.3.6 Internal metal contents of macroalgae after culture

Immediately following culture, algae were rinsed twice with 1:9 ethanol/seawater (Gledhill et al. 1999), placed in acid cleaned bags, and frozen. Within 6 months, tips were freeze-dried to a constant weight then ground using an agate mortar and pestle. Approximately 1 g of the powdered biomass was dry-ashed at 450°C then brought up to 10 mL volume using 20% nitric acid. Cadmium, copper, lead and zinc contents were measured using inductively coupled plasma optical emission spectrometry (ICP-OES), with the respective limits of detection as follows: Cd 0.1 ng g^{-1}, Cu 0. 2 ng g^{-1}, Pb 5 ng g^{-1}, Zn 0.2 ng g^{-1}.

3.3.7 Analysis of culture solutions

Culture solutions were removed for analyses on day 0, 1, 4 and 7. When culture solutions could not be analyzed within 24 h, they were transferred to 500 mL HDPE bottles and frozen immediately for analysis within 2 months. In such cases, samples were thawed overnight at 4°C and thoroughly mixed to ensure the dissolution of particulates formed due to freezing as per Laglera and van den Berg (2003).

3.3.8 Voltammetric instrumentation

The voltammeter used here was a Metrohm 757 VA Computrace controlled by a computer (PC). The working electrode was a hanging mercury drop (HMDE) with a drop size of approximately 0.52 mm^2. The reference electrode was a double junction Ag/saturated AgCl, KCl (3 M), with a KCl-filled salt bridge (3 M). The counter electrode was a platinum wire. A polytetrafluoroethylene (PTFE) stirrer, rotating at 2 000 rpm, was employed during the adsorption step.

3.3.9 Metal speciation

The total dissolved metal concentrations ($[M]_D$) were analyzed simultaneously using differential pulse anodic stripping voltammetry (DPASV). Thawed seawater samples were filtered through 0.45 µm glass-fiber filters and acidified to pH 2.2 using 6 M HCl, prior to UV digestion in PTFE-capped quartz silica tubes for 2 h. Ten mL aliquots of sample were pipetted into the voltammetric cell. Acetate buffer (100 µL, final concentration 0.01 M) was added and the solution was purged for 5 min using nitrogen. The voltammetric parameters were: deposition 60 s at -1.15 V whilst stirring; 10 s quiescence; potential scan using the differential pulse modulation: pulse height 0.05 V, scan from -1.15 to 0.05 V. Initial concentration was determined via two standard metal additions, each replicated twice. Peaks for Cu and Zn occurred at –0.1 V and –1 V with the respective limits of detection at 0.3 nM and 0.6 nM.

The labile dissolved metal concentrations ($[M]_L$) of Cu and Zn were determined using CSV with ligand competition as previously described by Leal et al. (1999), Campos and van den Berg (1994) and van den Berg (1985). For these determinations, the pH was fixed at 8.1 using HEPPS and SA ligand (5 µM) for Cu, or APDC ligand (100 µM) for Zn was added immediately before measurements. In all cases, initial concentration was determined via two standard metal additions, each replicated twice. Here, the goal was to measure the actual free metal and that which is very weakly complexed and so, only the 5 min purging time was allowed for equilibriation to prevent organic complexation of the added metal in the case that uncomplexed organic ligands remain.

3.3.10 Metal complexing-capacities

Titration of filtered seawater with individual metal ions was used to determine the concentration of metal-specific complexing ligand (L_M) and the associated conditional stability constant ($logK'L_M$). As outlined for $[M]_L$, Cu and Zn measurements were made using CSV with ligand competition. HEPPS (0.01 M) and appropriate ligand (5 µM SA for Cu, or 100 µM APDC for Zn) were added to 120 mL of filtered seawater. Ten mL aliquots were then pipetted into 10-20 mL polystyrene vials (Sterilin) containing metal additions in approximately equal increments (where the actual range depends on the initial metal concentration). Solutions were left to equilibrate overnight (12-15 h). The fully-equilibriated $[M]_L$ in each vial was then measured as previously outlined.

3.3.11 Identification and quantification of thiol compounds

Thiols were both identified and quantified using CSV at pH 8.1 (0.01 M HEPPS), according to Leal et al. (1999), where cysteine, glutathione and thioactemide peaks present at -0.47 V, -0.58 V and -0.68 V respectively. The voltammetric parameters were: deposition 60 s at -0.25 V whilst stirring; potential scan using the square wave modulation: scan rate $20mVs^{-1}$, modulation amplitude 25 MV, and frequency 50 Hz. Initial concentration was determined via two standard thiol additions (cysteine, glutathione, or thioacetamide), each replicated twice.

3.3.12 Complexing-capacity calculations

A modified van den Berg linearization (van den berg 1982) was used to determine the complexing ligand concentration and corresponding conditional stability constant for each metal based on the following relationship:

$$[M]_L/[ML]=[M]_L/L_M + \alpha/(K'_{ML}L_M)$$

Where [ML] is calculated as the difference between $[M]_D$ and $[M]_L$, while the α-coefficient represents the overall side reactions producing inorganic complexes for each metal. Values of α were calculated as described previously by Vasconcelos et al. (2002) using the following values: α_{Cu}=36 (Turner et al. 1981), α_{Zn}=2.1 (Turner et al. 1981), $logK'_{CuSA}$=9.22 (Campos and van den Berg 1994), $log\beta'_{Cu(SA)2}$=9.52 (Campos and van den Berg 1994), and $logK'_{ZnAPDC}$=4.4.(van den Berg 1985; Donat and Bruland 1990). Linearly transformed plots of $[M]_L/[ML]$ versus $[M]_L$ were used to elucidate the number of ligands present wherein a linear plot was indicative of a single ligand and a curved plot of more than one ligand. The

concentrations of L_M and $logK'L_M$ were determined from the slope and y-intercept of a least squares regression of this titration curve. Where plots showed curvature, concentrations of L_M and $logK'L_M$ were obtained using an iterative fitting method (van den Berg 1984). Initial estimates of L_{M1} and $logK'L_{M1}$ were taken from the linearization of the first few data points. A 2nd linear least squares regression of the remaining titration data (minus the contribution of L_{M1}) was used to calculate L_{M2} and $logK'L_{M2}$. This method was then repeated for the first few data points, now subtracting the contribution of L_{M2} to obtain a better estimate of L_{M1} and $logK'L_{M1}$. Example titration and linearization plots (**Figures S1, S2**) are presented in the Supplementary Information.

The calculation of sensitivity was modified as per Turoczy and Sherwood (1997) using multiple iteration of the final four titration points in order to avoid underestimation (see **Section 2.4**)

3.3.13 Quality assurance

Certified reference materials (CRM) for trace metals from the National Institute for Environmental Studies, Japan Environment Agency algal standard (NIES no. 9) and the National Research Council of Canada Reference standard seawater (CASS 5) for coastal seawater were measured to ensure accuracy of analysis of seaweed metal contents and seawater metal concentrations, respectively. The metal concentrations determined were never significantly different than the expected values ('t' test, $p>0.05$), showing a good accuracy of measurement (**Table S2**).

3.3.14 Data analysis

Significant differences in seaweed metal contents as well as seawater metal contents, complexing ligands, conditional stability constants, and thiol concentrations among the experimental treatments were tested using a one-way between subjects analysis of variance (ANOVA). Data were also compared among species, between sites, and over time using multiple analyses of variance (MANOVA). In each case, where significant differences were identified, a post hoc Tukey's multiple comparison was employed. Pearson-product moment correlation coefficients were used to identify and measure the relationships between complexing ligands and the remaining variables. All tests were performed using SPSS version 15.0 or 20.0.

3.4 Results

3.4.1 Effects on photosynthesis

Photochemical quantum yield (F_v/F_m) values of seaweeds exposed to metals at defined concentrations are presented in

Figure 3.1. In general, F_v/F_m decreased in the order: day 0 >1 > 4 >7; treatment control > low metal addition > high metal addition; species *A. nodosum* > *F. vesiculosus* > *L. hyperborea*.

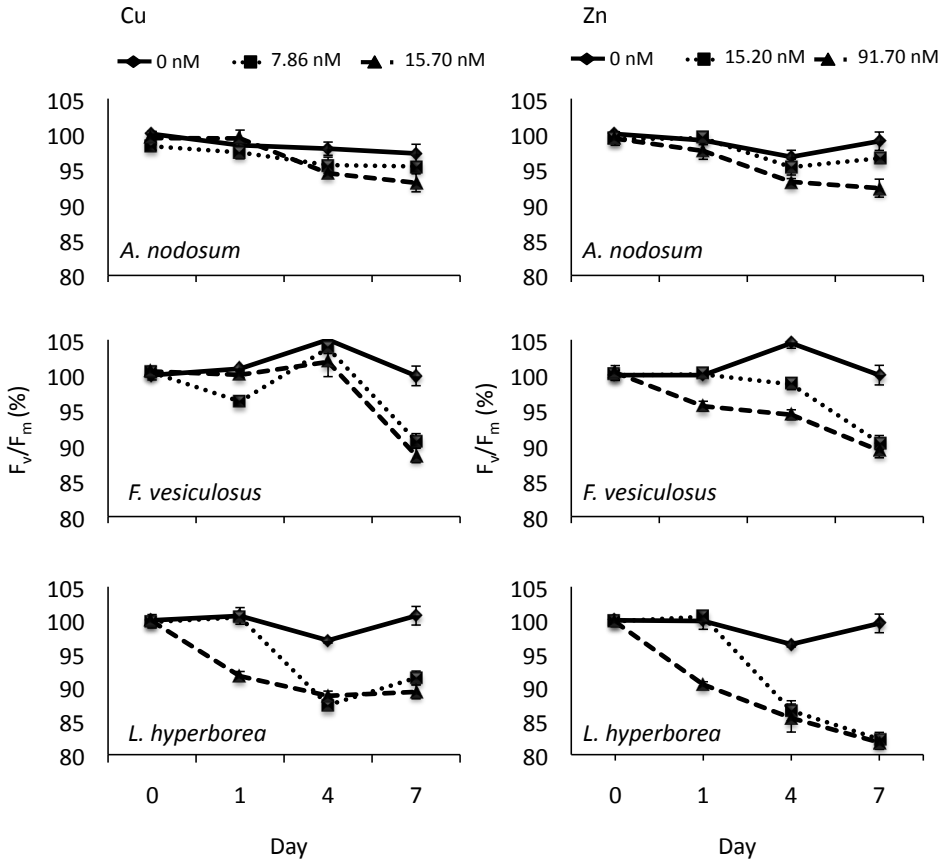

Figure 3.1. Photochemical quantum yield of three brown macroalgae cultured in artificial seawater for 7 days at 10°C, under 60 ± 5 µmol m^{-2} s^{-1} PAR on a 12 h:12 h light: dark cycle, in different concentrations of Cu (0 nM, 7.86 nM, 15.70 nM) or Zn (0 nM, 15.20 nM, 91.70 nM). Data are expressed as a % of the corresponding day 0 control value; *n*=3. Error bars represent the standard error of triplicate cultures.

As shown, temporal variations in photochemical quantum yield were highly dependant on species yet independent of metal. There was no significant effect of any Cu treatment on the F_v/F_m of *A. nodosum* or *F. vesiculosus* ($p>0.05$). By contrast, 15.70 nM of Cu significantly reduced the F_v/F_m of *L. hyperborea* after only 1 day ($F_{treatment}=22.09$, $p<0.05$; $F_{time}=8.88$, $p<0.05$). A significant decrease in the F_v/F_m of *F. vesiculosus* observed on day 7 was the result of time, not treatment ($F_{time}=19.53$, $p<0.05$).

For Zn, there was no significant effect of treatment on the F_v/F_m of *A. nodosum* or *F. vesiculosus* over 7 days ($p<0.05$). As with Cu, the highest Zn treatment (91.70 nM) significantly reduced the F_v/F_m of *L. hyperborea* but only after day 4 ($F_{treatment}=13.17$, $p<0.05$; $F_{time}=5.71$, $p<0.05$).

3.4.2 Seaweed metal contents

Both Cu and Zn were present in all seaweeds analyzed (**Figure 3.2**). Zn concentrations were consistently higher than those of Cu. Multiple analyses of variance (MANOVA), including both elements, showed no significant effects of time, treatment, or species on the mean vectors of the tissue metal concentrations. However, in general the internal metal content increased in the order *A. nodosum* ~ *F. vesiculosus* > *L. hyperborea*.

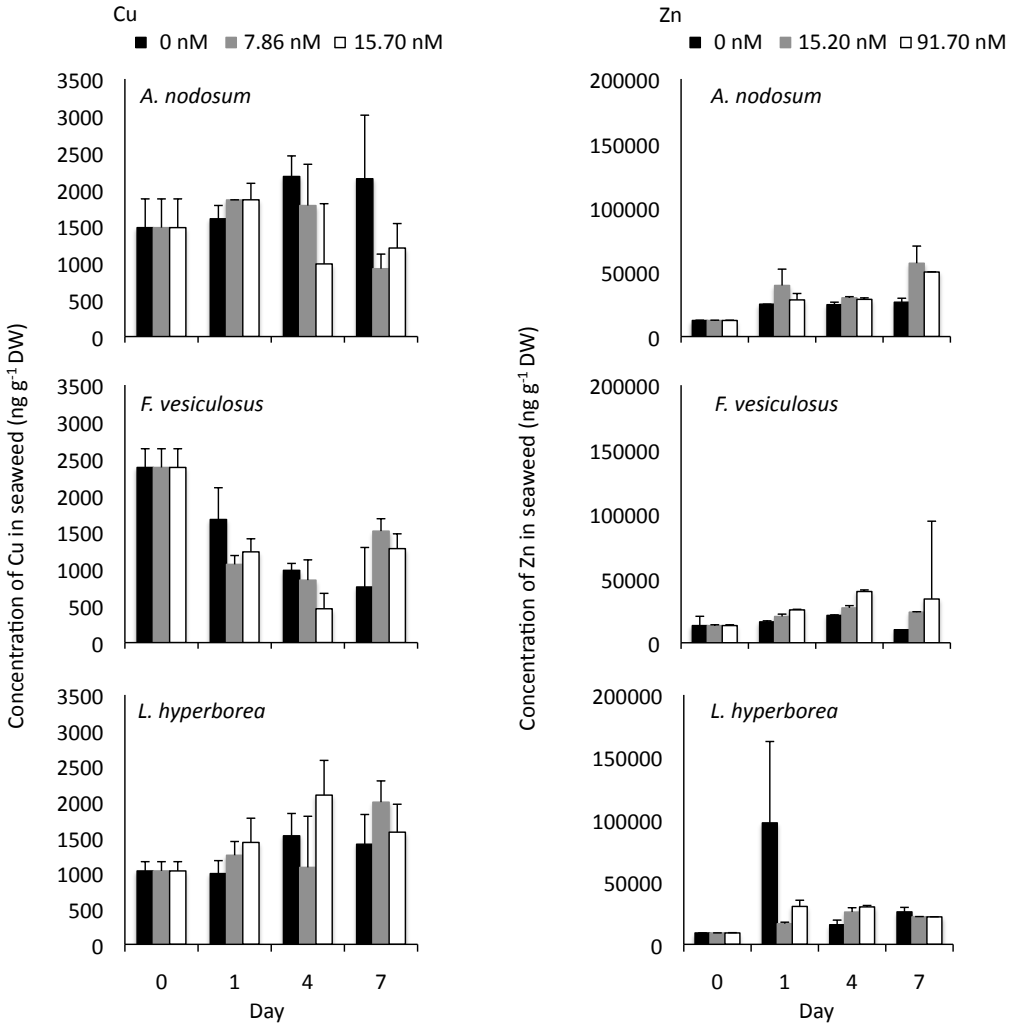

Figure 3.2. Concentration of metals in three seaweed species cultured for 7 d in different concentrations of Cu (0 nM, 7.86 nM, 15.70 nM) or Zn (0 nM, 15.20 nM, 91.70 nM); *n*=3. For other culture conditions, see fig. 1. Error bars represent the standard error of triplicate cultures. Note the Y-axes have different scales.

3.4.3 Metal speciation

Table 3.1 and **Table 3.2** summarize the results of the speciation analyses for Cu and Zn, respectively. As expected, metal additions significantly increased the total dissolved metal concentrations in all cultures ($F=209.35$, $p<0.05$; $F=12426.44$, $p<0.05$). In each case, $[M]_D$ increased with time. Despite large differences in the initial concentrations, $[Cu]_L$ showed a very narrow range of 0.12-0.85 nM on day 7. In general, $[Cu]_L$ decreased in the order *L. hyperborea* > *F. vesiculosus* > *A. nodosum* whilst $[Zn]_L$ decreased in the order *L. hyperborea* > *A. nodosum* > *F. vesiculosus*. Unfortunately, not all added metal was recovered. This may be the result of adsorption on the container walls.

The concentration of bound Cu covaried with $[Cu]_D$ with a maximum $[L_{Cu}]$ of 14.42 nM measured in the 15.70 nM *A. nodosum* culture on day 4. The maximum [CuL] did not correspond to the culture with the greatest proportion of bound Cu. Rather, the maximum percent bound Cu (98.89%) occurred in the 15.7 nM *A. nodosum* culture on day 7. Labile Cu ranged from 0.15-0.62, 0.16-0.57, and 0.21-0.85 nM for *A. nodosum*, *F. vesiculosus*, and *L. hyperborea,* respectively. In contrast to the other metal species, the maximum $[Cu]_L$ was measured in the 0 nM *L. hyperborea* culture on day 7.

Table 3.1 continued

Table 3.1. A summary of the CLE-CSV speciation measured for macroalgal cultures under Cu exposure, mean ± standard error, *n=3*. AN: *Ascophyllum nodosum*; FV: *Fucus vesiculosus*; LH: *Laminaria hyperborea.*

Species	Treatment (nM)	Day	$[Cu]_D$ (nM)	$[Cu]_L$ (nM)	[CuL] (nM)	$[L_{Cu}]$ (nM)	$LogK'L_{Cu}$
AN	0	0	-[A]	-	-	-	-
		1	0.15 ± 0.013	0.15 ± 0.12	0.0030	6.05 ± 1.18	10.52 ± 0.41
		4	0.67 ± 0.88	0.19 ± 0.23	0.48	4.86 ± 1.41	9.49 ± 0.41
		7	1.45 ± 0.15	0.36 ± 0.34	1.09	6.65 ± 0.12	9.95 ± 0.58
	7.86	0	7.69 ± 0.45	7.94 ± 1.82	-	-	-
		1	5.23 ± 0.58	0.25 ± 0.31	4.97	10.14 ± 0.87	10.76 ± 0.31
		4	6.06 ± 3.72	0.33 ± 0.18	5.73	13.28 ± 0.65	9.22 ± 0.27
		7	6.72 ± 0.33	0.62 ± 0.12	6.10	9.50 ± 0.37	10.78 ± 0.99
	15.70	0	16.03 ± 0.74	15.82 ± 0.85	-	-	-
		1	11.78 ± 1.59	0.36 ± 0.14	11.47	9.52 ± 0.20	11.22 ± 0.021
		4	14.67 ± 5.87	0.25 ± 0.18	14.42	11.52 ± 1.13	10.76 ± 0.037
		7	11.37 ± 0.28	0.12 ± 0.036	11.24	39.90 ± 7.31	10.43 ± 0.82
FV	0	0	-	-	-	-	-
		1	0.16 ± 0.05	0.16 ± 0.12	0.00	9.91 ± 1.98	9.19 ± 0.087
		4	0.67 ± 0.85	0.28 ± 0.15	0.39	5.60 ± 3.33	9.50 ± 0.177
		7	1.64 ± 0.18	0.42 ± 0.03	1.22	2.22 ± 0.56	9.57 ± 1.01
	7.86	0	7.69 ± 0.45	7.43 ± 0.69	-	-	-
		1	4.93 ± 0.96	0.57 ± 0.35	4.36	9.04 ± 1.88	8.96 ± 0.21
		4	9.09 ± 0.18	0.45 ± 0.34	8.64	9.85 ± 0.80	9.54 ± 0.122
		7	6.90 ± 0.59	0.53 ± 0.07	6.36	13.65 ± 1.02	9.92 ± 0.89
	15.70	0	16.03 ± 0.74	15.82 ± 0.85	-	-	-
		1	10.82 ± 0.89	0.54 ± 0.07	10.28	10.41 ± 3.81	9.02 ± 0.09
		4	14.40 ± 1.18	0.34 ± 0.22	24.06	58.95 ± 5.84	10.04 ± 0.022
		7	11.09 ± 0.29	0.22 ± 0.15	10.87	72.45 ± 9.84	9.27 ± 0.83
LH	0	0	-	-	-	-	-
		1	0.16 ± 0.013	0.20 ± 0.0042	0.00	9.91 ± 1.98	10.03 ± 0.049

Table 3.1 continued

Species	Treatment (nM)	Day	$[Cu]_D$ (nM)	$[Cu]_L$ (nM)	[CuL] (nM)	$[L_{Cu}]$ (nM)	$LogK'L_{Cu}$
		4	0.025 ± 0.026	0.36 ± 0.10	0.00	6.46 ± 0.71	10.38 ± 0.31
		7	1.56 ± 0.21	0.85 ± 0.051	0.71	8.61 ± 0.21	9.64 ± 0.59
	7.86	0	7.69 ± 0.45	7.54 ± 1.03	-	-	-
		1	3.83 ± 0.90	0.59 ± 0.047	3.23	10.38± 1.56	9.75 ± 0.43
		4	10.84 ± 2.22	0.55 ±0.56	10.28	9.62 ± 1.30	9.94 ± 0.012
		7	6.03 ± 1.77	0.47 ± 0.13	5.55	12.20 ± 0.78	10.35 ± 0.68
	15.70	0	16.03 ± 0.74	15.82 ± 0.85	-	-	-
		1	11.15 ± 1.90	0.40 ± 0.011	10.75	24.96 ± 1.98	11.51 ± 0.038
		4	11.19 ± 0.75	0.78 ± 0.034	10.32	25.69 ± 2.12	11.38 ± 0.41
		7	9.05 ± 0.71	0.36 ± 0.35	8.69	23.64 ± 9.50	11.77 ± 0.27

- represents concentrations below the detection limits.

Table 3.1 continued

Speciation of Zn showed less variation; $[Zn]_L$ ranged from 0.75-8.21, 0.75-8.74, and 0.81-8.05 nM for *A. nodosum*, *F. vesiculosus*, and *L. hyperborea,* respectively. As with Cu, the concentration of bound Zn covaried with $[Zn]_D$ but the maximum [ZnL] did not correspond to the culture with the greatest proportion of bound metal. In this case, maximum [ZnL] and percentage bound occurred in the 91.70 nM *L. hyperborea* culture on day 7 and the 91.70 nM *F. vesiculosus* culture on day 7, respectively.

Table 3.2: A summary of the CLE-CSV speciation measured for macroalgal cultures under Zn exposure, mean ± standard error, *n=3*. AN: *Ascophyllum nodosum*; FV: *Fucus vesiculosus*; LH: *Laminaria hyperborea.*

Species	Treatment (nM)	Day	$[Zn]_D$ (nM)	$[Zn]_L$ (nM)	[ZnL] (nM)	$[L_{Zn}]$ (nM)	$LogK'L_{Zn}$	$[L_{Zn2}]$ (nM)	$LogK'L_{Zn2}$
AN	0	0	-	-	-	-	-	-	-
		1	2.49 ± 0.515	0.98 ± 0.0075	1.50	8.14 ± 0.26	11.82 ± 2.68	-	-
		4	2.70 ± 0.87	1.84 ± 0.38	0.85	7.55 ± 1.88	11.78 ± 0.097	-	-
		7	2.27 ± 0.059	0.75 ± 0.33	1.52	15.31 ± 1.23	11.82 ± 0.071	-	-
	15.20	0	16.43 ± 0.89	15.32 ± 0.93	-	-	-	-	-
		1	10.99 ± 0.14	1.16 ± 0.057	9.82	12.61 ± 1.61	12.15 ± 1.61	6.59 ± 0.0058	13.91 ± 0.063
		4	11.02 ± 0.58	3.16 ± 1.13	7.86	12.86 ± 1.66	11.27 ± 0.00	6.95 ± 0.36	13.95 ± 0.075
		7	10.52 ± 0.20	3.91 ± 0.45	6.61	13.71 ± 2.44	12.15 ± 0.22	7.12 ± 0.00	13.91 ± 0.021
	91.70	0	96.82 ± 3.34	89.49 ± 1.32	-	-	-	-	-
		1	43.21 ± 0.010	8.21 ± 1.81	35.00	40.47 ± 4.49	12.035 ± 4.49	45.5 ± 0.11	14.92 ± 0.18
		4	41.84 ± 0.89	7.13 ± 0.94	34.71	41.19 ± 0.24	11.92 ± 0.019	45.41 ± 0.07	14.99 ± 0.09
		7	42.11 ± 0.77	6.22 ± 0.52	34.87	40.62 ± 3.95	12.035 ± 0.077	45.51 ± 0.056	14.92 ± 0.00
FV	0	0	-	-	-	-	-	-	-
		1	2.58 ± 0.73	0.75 ± 0.085	0.56	18.26 ± 0	11.54 ± 0	-	-

Species	Treatment (nM)	Day	$[Zn]_D$ (nM)	$[Zn]_L$ (nM)	[ZnL] (nM)	$[L_{Zn}]$ (nM)	$LogK'L_{Zn}$	$[L_{Zn2}]$ (nM)	$LogK'L_{Zn2}$
		4	2.53 ± 0.63	2.3 ± 0.78	0.01	19.26 ± 0.051	11.53 ± 0.0089	-	-
		7	1.88 ± 0.078	1.18 ± 1.13	0	16.41 ± 1.66	11.61 ± 0.035	-	-
	15.20	0	16.43 ± 0.89	14.89 1.17	-	-	-	-	-
		1	11.21 ± 0.14	1.11 ± 0.44	9.62	19.21 ± 4.77	11.75 ± 0.29	7.04 ± 0.0022	13.92 ± 0.046
		4	10.56 ± 1.11	3.36 ± 1.64	7.44	22.21 ± 1.78	11.52 ± 0.30	7.02 ± 0.16	13.91 ± 0.022
		7	10.20 ± 0.39	4.03 ± 0.085	8.02	16.31 ± 10.88	12.035 ± 0.021	7.077 ± 0.077	13.91 ± 0.014
	91.70	0	96.82 ± 3.34	95.23 ± 5.87	-	-	-	-	-
		1	43.27 ± 1.04	8.74 ± 1.56	40.66	29.15 ± 2.17	12.35 ± 0.46	45.24 ± 0.37	14.85 ± 0.085
		4	40.42 ± 1.60	6.44 ± 1.18	39.14	28.72 ± 2.42	12.26 ± 0.46	45.02 ± 0.44	14.99 ± 0.12
		7	43.33 ± 0.44	7.76 ± 0.18	41.97	27.45 ± 2.77	12.81 ± 0.021	45.27 ± 0.32	14.89 ± 0.049
	0	0	-	-		-	-	-	-
		1	1.66 ± 0.099	0.82 ± 0.019	0.14	11.67 ± 9.01	11.69 ± 0.21	-	-
		4	2.19 ± 0.63	2.94 ± 0.29	0.00	11.66 ± 6.64	11.64 ± 0.13	-	-
		7	1.64 ± 0.23	1.45 ± 1.21	0	10.95 ± 8.87	11.89 ± 0.0010	-	-
		0	16.43 ± 0.89	15.47 ± 0.49	-	-	-	-	-
		1	11.02 ± 0.89	0.88 ± 0.24	9.25	13.94 ± 3.81	11.79 ± 0.41	6.86 ± 0.35	13.91 ± 0.017
		4	10.66 ± 2.53	3.55 ± 1.43	7.13	16.0044 ± 1.63	11.61 ± 0.08	6.65 ± 0.28	13.94 ± 0.031
		4	10.66 ± 2.53	3.55 ± 1.43	7.13	16.0044 ± 1.63	11.61 ± 0.08	6.65 ± 0.28	13.94 ± 0.031
		7	9.76 ± 0.12	3.97 ± 0.35	7.51	13.31 ± 3.76	11.78 ± 0.042	6.98 ± 0.31	13.91 ± 0.0010

Species	Treatment (nM)	Day	$[Zn]_D$ (nM)	$[Zn]_L$ (nM)	[ZnL] (nM)	$[L_{Zn}]$ (nM)	$LogK'L_{Zn}$	$[L_{Zn2}]$ (nM)	$LogK'L_{Zn2}$
		0	96.82 ± 3.34	97.32 ± 7.68		-	-	-	-
		1	43.74 ± 1.12	7.35 ± 1.84	39.81	21.31 ± 0.49	12.32 ± 0.21	45.08 ± 0.018	14.84 ± 0.014
	91.70	4	46.74 ± 0.24	6.89 ± 0.58	45.08	20.34 ± 0.41	12.56 ± 0.45	45.02 ± 0.061	14.86 ± 0.015
		7	44.53 ± 1.67	8.05 ± 0.54	43.04	21.46 ± 1.37	12.41 ± 0.38	45.18 ± 0.098	14.77 ± 0.0010

- represents concentrations below the detection limits.

Table 3.2 continued

3.4.4 Organic ligands and conditional stability constants

As evident in **Table 3.1** and **Table 3.2**, the presence of algae enhanced the culture medium with ligands. Metal additions stimulated the release of ligands as indicated, with significantly higher [L_M] values in cultures with added metals than the relevant controls (F_{Cu}=903, $p<0.05$; F_{Zn}= 80, $p<0.05$).

Copper titrations were found to fit the presence of a single complexing ligand, meaning that other ligands make only a minor contribution to the speciation. Both the maximum and minimum [L_M] were measured in the *F. vesiculosus* cultures on day 7. The maximum occurred at 15.70 nM Cu addition and the minimum at 0 nM Cu. Multiple analyses of variance (MANOVA) showed significant time and species effects on the mean vectors of the [L_{Cu}] (F_{Time}=245; $p<0.05$; $F_{Species}$=180, $p<0.05$). Complexing ligand increased with time, from day 0 to 7, and *F. vesiculosus* cultures consistently contained less ligand than the other 2 species.

With the exception of the 0 nM cultures, the Zn titration data was not linear but curved and, therefore, was best modeled by 2 complexing ligands. The first ligand ranged from 7.55-41.19, 16.31-29.15, and 10.94-21.46 nM in cultures of *A. nodosum*, *F. vesiculosus*, and *L. hyperborea*, respectively. The second ligand, although not detectable in the 0 nM cultures, showed much higher maximum concentrations: 45.51, 45.24 and 45 .18 nM in *A. nodosum*, *F. vesiculosus* and *L. hyperborea* cultures, respectively. In each instance, [L_{Zn}] was significantly lower in cultures of *L. hyperborea* than those of the other 2 species (F_{LZn1}=8.569, $p<0.05$; F_{LZn2}=18.92, $p<0.05$). In the case of both ligands and as with Cu, [L_{Zn}] was always greater than $[Zn]_D$. In contrast to Cu, the effect of time was observed only for L_{Zn2}, with a significant increase in concentration from day 4 to day 7 ($F=6.62$, $p<0.05$). Again, MANOVA showed significant species effects on the mean vectors of the [L_{Zn}]. Here, *L. hyperborea* cultures consistently contained significantly less L_{Zn1} and L_{Zn2} than cultures of *A. nodosum* or *F. vesiculosus* (F_{LZn1}=8.569, $p<0.05$; F_{LZn2}=18.92, $p<0.05$).

The conditional stability constants of the 3 ligands detected showed much less variation than their respective concentrations, although MANOVA revealed significant treatment and species effects for each. In the case of Cu, logK' increased significantly in the order 0 nM < 7.86 nM < 15.70 nM Cu addition, and was significantly lower in the *F. vesiculosus* cultures ($F_{Treatment}$=13.93, $p<0.05$; $F_{Species}$=45.21, $p<0.05$). For Zn, logK' was lowest in *F. vesiculosus* cultures in direct contrast to Cu (F_{LZn1}=6.38, $p<0.05$; F_{LZn2}=5.21, $p<0.05$). In any case, both L_{Zn} logKs were highest in cultures with 91.70 nM Zn addition.

A Pearson correlation applied to the ligand concentrations showed several significant correlations ($p<0.05$, $n=162$). For Cu, $[L_{Cu}]$ was positively correlated with $[Cu]_D$, $[Cu]_L$ and GSH. Similarly, both Zn ligands were positively correlated with each metal species, but negatively correlated with [GSH].

3.4.5 Glutathione and Cysteine

Glutathione was the only thiol species present in all cultures, although there were very few significant differences in [GSH] amongst time, treatment, or species (**Figure 3.3**). Glutathione increased significantly with both Cu and Zn treatment for *L. hyperborea* cultures on day 1. For Zn, the lowest [GSH] was always measured in the 91.70 nM cultures on day 7 whilst the highest [GSH] was always measured in the corresponding control cultures. A similar trend did not exist for Cu. Rather, the highest and lowest [GSH] was measured on day 1 in the 15.70 nM Cu cultures of *L. hyperborea* and A. nodosum, respectively. Cysteine was detected only in the 15.70 nM Cu *L. hyperborea* culture on day 7 at a concentration of 0.16 nM.

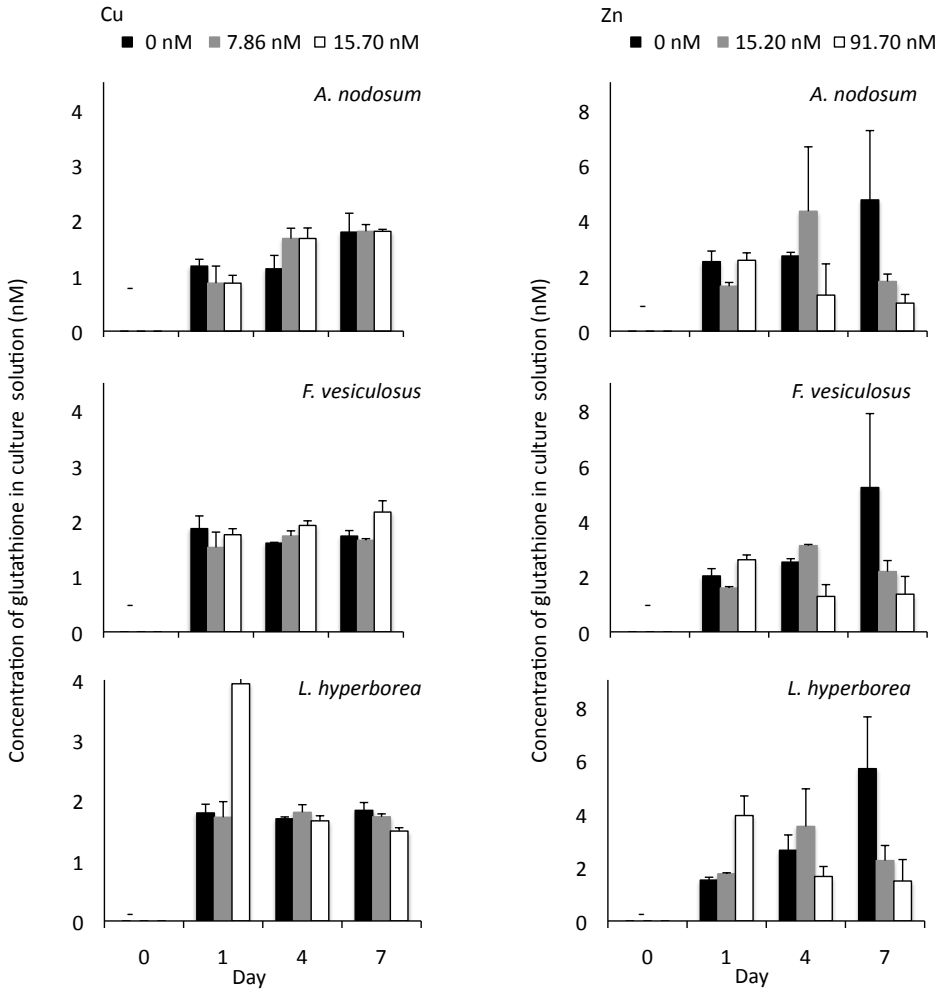

Figure 3.3. Glutathione concentrations measured for macroalgal cultures under different concentrations of Cu (0 nM, 7.86 nM, 15.70 nM) or Zn (0 nM, 15.20 nM, 91.70 nM); *n*=3. For other culture conditions, see fig. 1. Error bars represent the standard error of triplicate cultures. Note the Y-axes have different scales. Dash represents concentrations not detected.

3.5 Discussion

3.5.1 Macroalgal responses to metal exposure

An assessment of the physiological responses of macroalgae to metal treatment is an aspect that is generally excluded from most ligand studies (Vasconcelos and Leal 2008; Andrade et al. 2010), however, exposure to metals can be expected to influence ligand release (Connan and Stengel 2011b). Chlorophyll *a* parameters can be used as valuable endpoints to assess metal impact. Pena-Vazequez et al. (2010) tested the sensitivity of several end points in microalgae using PAM fluorometry and found the fluorescence yield to be the most

appropriate parameter for use in Cu bioassays. Because most detailed investigations on this subject are based on studies using microalgae there is a general lack of data for macroalgae, although reduced yields in response to metal exposure have been reported for macroalgae in previous literature; for example, *G. longissima* yield was significantly reduced by 30% on exposure to 3925 nM Cu (Brown and Newman 2003). However, the present results of photochemical quantum yield indicate that low level Cu and Zn exposure do not disrupt photosynthesis of *A. nodosum* or *F. vesiculosus*. This apparent discrepancy is likely the result of differences in exposure levels where previously published toxicological studies generally employ metal concentrations in excess of natural conditions. Given that Baumann et al. (2009) found no significant effect of 10000 nM Cu exposure on the yield of *A. nodosum* and *F. vesiculosus*, indicating these species are very Cu tolerant, it is not surprising our low nanomolar treatments had little impact.

By contrast, *L. hyperborea* showed significant sensitivity to both metals as indicated by decreased yield following exposure to 15.70 nM Cu and 91.70 nM Zn. These findings suggest photosynthetic effects are both metal- and seaweed- specific. For Cu and Zn, differences in response are likely the result of differences between the inhibition mechanisms of the metals. The lower threshold for Cu in comparison to Zn has been previously described (Rai et al. 1981), although there has been no adequate explanation reported. Similarly, seaweed-specific differences are not yet well-defined although may be attributed to genetics, morphology, metal-binding capacity, or other allometric parameters influencing metal-algae interactions.

Despite being recognized as efficient metal accumulators, the brown seaweeds examined here showed no uptake of Cu or Zn over 7 days, from any metal additions. The treatment effect on uptake was likely masked by high original tissue concentrations. Although comparable to natural systems, the spike concentrations used here were minute relative to background levels in seaweeds (**Figure 3.2**).

3.5.2 Metal speciation

Despite there being no evidence of metal uptake by seaweeds, additions of Cu and Zn significantly affected the water chemistry. Strong positive correlations for the pairs treatment-[ML] and [ML]-[L_M] suggest that total dissolved metal concentration modulates the concentration of metal-binding ligands, which, in turn, control the distribution of chemical species for Cu and Zn. Copper behavior in the cultures resembled the general characteristics

of Cu in coastal waters, as evidenced by predominant organic complexation. Similar to seawater, our speciation studies indicate that >98.9% of Cu was complexed by algae-derived organic ligands. Lowest dissolved Cu concentrations measured on day 7 were within the range reported for the northeast Atlantic (Campos and van den Berg 1994), yet the highest dissolved concentrations were significantly less than the more extensively impacted San Francisco Bay (Hurst and Bruland 2008). Similarly, the behavior of Zn in culture solutions appeared to be dominated by organic ligand complexation with >63% of Zn bound on day 7. These results compare well with those reported for Zn in estuarine and coastal waters by Kozelka and Bruland (1998) who reported 50-97% of Zn to be complexed by ligands. The lower percentage of Zn complexation in comparison to Cu was the result of lower [L_{Zn1}] and a lower associated logK'. In terms of ecotoxicological implications, perhaps the most noteworthy change in metal speciation due to algae-exuded ligands was the considerable decrease in $[M]_L$ in the presence of increased [L_M]. For example, on day 7 the culture of *L. hyperborea* treated with the highest concentration of Cu contained 42% less M_L than the control despite having been spiked with an excess of 15.70 nM of Cu. The lowering of the concentration of labile Cu was most likely due to complexation with ligands exuded by the algae.

3.5.3 A novel source of L for Zn (L_{Zn})

This study provides the first direct laboratory evidence for the production of strong L_{Zn} by macroalgae, and supports previous studies on the production of L_{Cu}. Current literature values for macroalgae-exuded ligands are presented in **Table 3.3**. Our estimates of L_{Cu} were mostly below the range (42-117 nM) reported by Andrade et al. (2010) for five species of brown macroalgae. Only the highest measured concentration (72 nM) in this study is comparable, with the second highest concentration (26 nM) being 39% lower than their lowest reported value. Comparisons with other studies show similar leanings: L_{Cu} exuded by *Porphyra* (Rhodophyta) and *Enteromorpha* (*Ulva*, Chlorophyta) after 24 hours were generally 4 times greater than the values reported here (Vasconcelos et al. 2002). However, relating the results obtained in other studies is difficult largely due to differences in methodology and, where CSV is employed, differences in the detection window. Further differences in type of water (salinity, nutrients, filtration, UV-treatment, etc.), culture conditions, and species only compound this problem. Nonetheless, the most plausible explanation for these new findings is the range of metal concentrations employed. The concentration of Cu used by Andrade et

al. (2010) (157 nM) is an order of magnitude higher than the highest Cu spike used here. In each case, the concentration of ligand increases in proportion to the total dissolved Cu. Thus, it is only logical that large Cu additions (Andrade et al. 2010) showed greater ligand concentrations than those measured in this study, where Cu additions of 7.86 and 15.70 nM were within the range of natural values.

Despite differences in ligand concentrations, logK' values here are within the range (7.0-12.5) for macroalgae in previous literature (**Table 3.3**). This is not surprising given that logK is thought to remain unchanged by Cu exposure (Andrade et al. 2010). No previous studies exist on the exudation of L_{Zn} by macroalgae, making these results the first. In any case, similar logK' values among species, treatments, and even studies indicate that the exuded ligands have a similar chemical structure, independent of the amount of ligand released.

Table 3.3: A comparison of current literature values for the concentration and logK' of Cu complexing ligands exuded by macroalgae, including this study.

Macroalgae	Medium	$[L_{Cu}]$ (nM)	$LogK'L_{Cu}$	Method	Reference
A. nodosum, *F. vesiculosus*, *L. hyperborea*	Artificial seawater, + 7.86 or 15.70 nM Cu	2-72	8.96-11.77	CLE-CSV	This study
A. taxiformis, *C. corniculata*, *C. prolifera*, *L. lallemandii*, *L. majuscula*, *L. microcladia*, *L. obtusa*, *P. capilacea*, *P. cartilagineum*, *P. ollivieri* (Rhodophyta)					
C. barbata, *C. compressa*, *C. racemosa*, *F. petiolata*, *H. tuna*, *P. capitatus, P. oceanica, U. rigida, C. foeniculacea*, *C. sinuosa* (Chlorophyta)	Artificial seawater	109-744	7.0-8.60	DPASV	(Karavoltsos et al. 2013)
D. dichotoma, *D. polypodioides*, *H. scoparia*, *P. ollivieri*, *P. pavonica* (Phaeophyta)					
C. firma, *U. lactuca*, *P. columbina*, *G. lingulatum, L. nigrescens*	Artificial seawater, + 157 nM Cu	42-117	7.60-8.90	ASV	(Andrade et al. 2010)
Porphyra spp., *Enteromorpha* spp.	Filtered natural seawater, containing 6.80 nM Cu	77-123	12.07-12.65	C(A)SV	(Vasconcelos and Leal 2008)
Porphyra spp., *Enteromorpha* spp.	Filtered natural seawater, containing 29 nM Cu	92-127	12.40-12.49	C(A)SV	(Vasconcelos et al. 2002)
F. vesiculosus	Modified Aquil, + 2-1000 nM Cu	146-793	10.20-12.50	CSV	(Gledhill et al. 1999)
E. siliculosus, *A. purpurea*, *A. spirographidis*	Filtered, UV treated natural seawater	270-1010	9.77-10.15	MnO_2 exchange	(Sueur et al. 1982)

Both the concentration and logK' of L_{Zn} determined in this study are within the range of the ambient ligands recently found in Lough Furnace (Murray et al. d) suggesting there is a direct relationship between the quantity and quality of ligands identified in natural systems and those described here for macroalgal cultures. Likewise, the culture concentrations of L_{Cu} and the associated logK' values are comparable in magnitude to those measured in the Clyde Sea, on the west coast of Scotland (Muller et al. 1999). Such similarities between the concentration and logK' of exuded ligands in cultures and natural systems provide evidence that algal exudates account for an important portion of organic ligands in seawater.

Evidence from the wide ligand liberation also points to algae-exuded GSH as a potential Cu ligand source in marine systems. In agreement with a microalgae study carried out by Vasconcelos and Leal (2002), statistically significant correlations between L_{Cu} and GSH suggest that this thiol contributes to the algae-exuded ligand pool. However, in contrast to the above study, the portion of exuded ligand comprised by GSH is exceptionally small. Whilst GSH accounted for 87% of total dissolved ligand in the previous study, here it accounted for only 37%. In contrast to Cu, there was a significant negative correlation between GSH and L_{Zn}, and this relationship was consistent for all three brown algae. Although we did not experimentally evaluate the presence of phytochelatin, the decrease in GSH with increased ligand exudation may reflect the consumption of GSH for phytochelatin production. Similarly, the absence of Cys from the majority of cultures is not surprising given that Cys is a precursor for GSH, and GSH is a known precursor for phytochelatin (Zenk 1996). The fact that Cys was exuded by *L. hyperborea* under only the highest metal addition, and that this coincided with the lowest measured F_v/F_m, suggests that in this particular species Cys may be produced in response to metal-induced photosynthetic damage. Alternatively, metal-induced photosynthetic damage may have impaired seaweed capacity for phytochelatin production, resulting in an unused pool of Cys. Confirmation of either scenario would require further research.

3.5.4 Two stimuli for exudation of L_{Cu} and L_{Zn}

Based on both the laboratory and field studies described so far, it appears that most algal ligand production is a function of $[M]_D$ at naturally occurring concentrations. In fact, metal additions significantly enhanced ligand exudation in all cultures. Thus, both Cu and Zn can be

considered stimulants of brown macroalgae-exuded organic ligands. While this response is not yet fully understood, it is generally ascribed to one of two mechanisms: (1) a feedback mechanism wherein ligand production is stimulated by free metal concentration so as to lower metal bioavailability (Moffett and Brand 1996) or (2) an alternative mechanism where metals are complexed by ligands intracellularly and exuded as bound complexes (Lee et al. 1996). Although both mechanisms account for ligand production as a result of metal stimulation, the latter fails to account for the significant excess of ligand over total metal. This lack of stoichiometry, also common in surface seawater, supports the first mechanism by providing tangible evidence against the second. This is not surprising given that the presence of an increased $[L_M]$ corresponds with a decrease in $[M]_L$.

Current uncertainties remain regarding the role of exuded ligands. Not only is it plausible that ligands are produced to reduce metal bioavailability but also to enhance nutrient acquisition. A hypothesis first put forward by Barber and Ryther (1969) proposes that microalgae alter seawater to create conditions of optimal growth. This hypothesis seems appropriate for the present study given that both Cu and Zn are required micronutrients for seaweeds. However, the control cultures did not show evidence of superior ligand production despite being void of Cu or Zn. As a result, it seems more likely that ligands are released as a defence mechanism. Thus, we propose the metal-stimulated ligand release reported here is the result of a ligand-mediated mechanism of metal-tolerance in seaweeds rather than nutrient deficiency.

It should be emphasized that ligands may have multiple functions, as previously illustrated for phlorotannins. Phlorotannins have been shown to function not only in trace metal availability but also as a defence against fouling, pathogens, UV radiation, and herbivory (Sieburth and Conover 1965; Lau and Qian 1997; Pavia et al. 1997). Likewise, environmental stimuli of algae-exuded ligands may not be limited solely to Cu and Zn. Even in our cultures, the release of DOM may be at least partially due to damage of the algal body upon removal of the holdfast from the substrate (Moebus et al. 1974.) That said, ligands likely function in response to numerous stimuli. This further lends to the possibility that joint occurrence of more than one stimulus may form synergistic, additive, or antagonistic effects on ligand production.

3.5.5 Deciphering the nature of the Cu and Zn binding ligands

Various metal-binding compounds have been identified in seawater, including humic substances and thiols. In the absence of direct identification, logK's are useful in deducing the potential identity of the ligands where similar logK's are indicative of similar chemical structure. The relatively high logK's reported here for L_{Zn} rule out both humic and fulvic acids ($logK_{HAZn}$=9.59; $logK_{FAZn}$=8.79; Yang and Van Den Berg 2009), as well as sulfides ($logK_{sZn}$=5.76; Al-Farawati and van den Berg 1999) as candidates for the identity of L. Similarly, Cu logKs for humic substances ($logK_{HACu}$=9.26; $logK_{FACu}$=8.58; Yang and Van Den Berg 2009) and sulfides ($logK_{sCu}$=11.5; Al-Farawati and van den Berg 1999) in seawater are in poor agreement with the values determined here for macroalgae-exuded organic ligands. However, there is some evidence for humic complexation of Cu in seawater with conditional stability constants (logK=10-13) closer to the range determined here. Thus humic and fulvic acids can not be ruled out entirely based on differences in logK's, given the range of values evident in the literature, and extreme caution should be taken when attempting to identify ligands by comparisons of conditional stability constants. While these substances may not be explicitly released by macroalgae, the breakdown of released compounds by bacteria and the subsequent aggregation of these smaller compounds into humic-like substances is one possibility. Nevertheless, even the seaweed metabolites ($logK_{app}$=6.0) most recently characterized by Karavoltsos et al. (2013) do not appear to contribute to the identity of L. While we found only very weak evidence that the strong ligands reported here belong to the list of compounds outlined above, the L_{Cu} detected in this study had binding properties similar to the brown-algae exuded organic ligands previously described by Andrade et al (2010). This is significant as most studies previous to this work have employed much higher concentrations of metal additions to culture solutions, reflecting contaminated rather than natural conditions.

3.5.6 Environmental implications

It is well established that strong ligands, such as those determined in this study, control metal bioavailability (Vraspir and Butler 2009). Nevertheless, weaker ligands are known to exist in natural systems. Unfortunately, the relatively high detection window employed in this study did not permit the measurement of this group of ligands. Further to this, any attempt to fit the titration data to a subsequent weaker ligand resulted in extremely large errors in both [L_M] and logK'. In hindsight, this lack of data needs specific attention given that recent studies

have revealed that weak ligand-metal complexes are bioavailable. Aristilde et al. (2012) have shown that weak organic ligands increase Zn and Cd availability to phytoplankton. Therefore, metals complexed by weak ligands cannot be ignored as potentially bioavailable metal species, and require greater attention in speciation studies. Here, speciation of culture solutions would benefit from measurements at multiple, lower concentrations of competitive ligand (and hence, a broader and lower detection window).

To circumvent sampling complexities and to facilitate environmental monitoring, speciation models are often employed to estimate free and inorganic metal concentrations in natural waters. However, large differences between actual and predicted free metal concentrations have been observed for even the most popular models: WHAM, Nica-Donnan, and SHM. Meylan et al. (2004) highlighted this phenomenon with model predictions reported to overestimate Cu and Zn for dynamic river waters and mesocosm experiments by 3 and 1 order(s) of magnitude, respectively. In both cases, discrepancies can be easily explained by differences in the logK's where those employed in all three models are significantly lower than the group of strong ligands identified in natural waters as well as this study. In previous work, we found binding constants measured in a pristine estuary to be 33% greater than even the highest humic binding constants employed by WHAM and SHM (Muller 1999). This results in an underestimation of metal binding by strong ligands and consequently an overestimation of labile metal concentration. As evidenced by Ndungu (2012), this problem can only be rectified if model databases are parameterized with logK's of real marine ligands measured. Accordingly, the logK's determined here for macroalgae-exuded organic ligands are valuable in advancing chemical-equilibrium models.

3.6 Conclusions

On the whole, the voltammetric characterization of algae-exuded ligands under Cu and Zn exposure has highlighted a source and stimuli of ligands common in natural systems. Our observations confirm the conclusions of previous studies; strong ligands of biological origin considerably reduce labile metal concentrations in marine systems. They further show how macroalgae respond to naturally occurring trace metal levels. Although a number of possible explanations exist, we propose that exudation is the result of a ligand-mediated mechanism of metal tolerance in seaweeds. It has been demonstrated here that ligands liberated by seaweeds have important implications for the availability and modeling of Cu and Zn. While a similar scenario is likely for other metals and seaweed species, it remains to be proven.

From these results, we also infer that trace metals in culture solutions condition the concentration, but not the nature, of macroalgal exudates. As such, Cu and Zn are recognized as two of several possible environmental stimuli that induce seaweed ligand production. While studies such as ours provide a good foundation for understanding the role of naturally occurring organic ligands in metal bioavailability, further studies are required to elucidate ligand identity. As previous qualitative studies on algae-exuded ligand structure have been performed only for hydroxamate siderophores, this leaves the remainder of algae-exuded ligands molecularly uncharacterized. By employing a multi-disciplinary approach combining LS-ESI-MS, NMR and voltammetric techniques, future studies will allow for the full characterization of marine ligands.

3.7 Acknowledgements

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3.8 Supplementary Information

Table S1. Artificial seawater used for cultures, prepared according to Kester et al. (1967).

Salt	Concentration in solution
Sodium chloride	23.926 g kg^{-1}
Sodium sulfate	4.008 g kg^{-1}
Potassium chloride	0.677 g kg^{-1}
Sodium bicarbonate	0.196 g kg^{-1}
Potassium bromide	0.098 g kg^{-1}
Boric acid	0.026 g kg^{-1}
Sodium fluoride	0.003 g kg^{-1}
Magnesium chloride	0.0532 mol kg^{-1}
Calcium chloride	0.01033 mol kg^{-1}
Strontium chloride	0.00009mol kg^{-1}

Table S2. Accuracy of the analytical procedures employed for seaweed metal concentration and seawater total dissolved metal concentration (mean ± s.d., *n*=3). The algal CRM (NIES no. 9) was certified by the National Institute of Environment Studies, Japan Environment Agency. The near-shore seawater CRM (CASS-5) was certified by the National Research Council of Canada.

Element	NIES no. 9			CASS-5		
	Measured (µg g^{-1} DW)	RSD (%)	Certified (µg g^{-1} DW)	Measured (nM)	RSD (%)	Certified (nM)
Cu	5.001 ± 0.58	11.59	4.9 ± 0.20	5.21 ± 0.19	3.64	5.97 ± 0.44
Zn	16.13 ± 0.21	1.31	15.6 ± 1.20	16.36 ± 0.25	1.52	10.92 ± 1.034

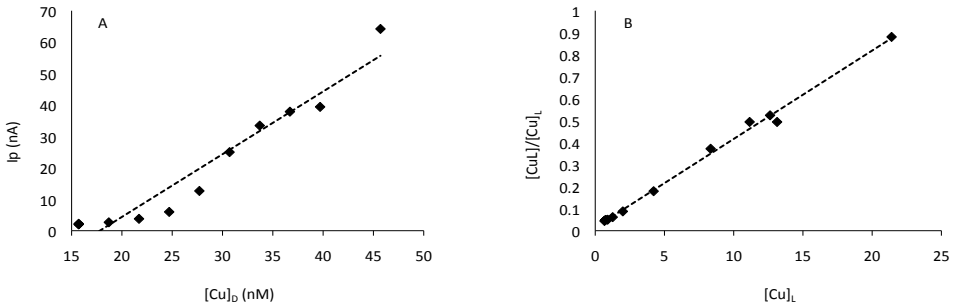

Figure S1. Representative titration curve for the day 1, 15.70 nM Cu treated *L. hyperborea* culture: (a) the current (nA) plotted versus the total amount of Cu (nM), and (b) the linear least squares fit using the van den Berg method (1982).

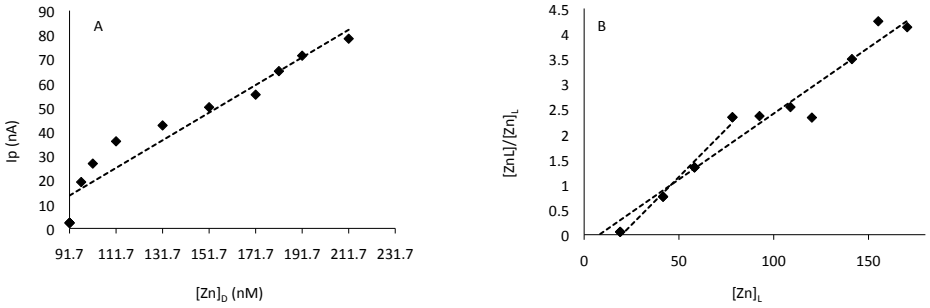

Figure S2. Representative titration curve for the day 1, 91.70 nM Zn treated *L. hyperborea* culture: (a) the current (nA) plotted versus the total amount of Zn (nM), and (b) the linear least squares fit using the van den Berg method (1982).

Chapter 4 Murray et al. (b)

Voltammetric characterization of macroalgae-exuded organic ligands (L) in response to Cd and Pb: expanding the source and stimuli for L

Marine Chemistry, *submitted September 12 2013*.

Hollydawn Murray[A,C*], Guillaume Meunier[A], Constant M.G. van den Berg[B], Rachel R. Cave[A], and Dagmar B. Stengel[C]

[A]Earth and Ocean Science, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, Ireland

[B]Oceanography Laboratory, Department of Earth Science, University of Liverpool, Liverpool, United Kingdom

[C]Botany and Plant Science, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, Ireland

4.1 Abstract

Given that the majority of metals in seawater occur bound to organic ligands (L), identifying the source, stimuli, and structure of L is crucial in fully understanding marine trace metal availability. Although seaweeds are known producers of L, their ability to exude Cd- or Pb-complexing ligands has been largely ignored until now. Here, voltammetric techniques were employed to examine the release of Cd- and Pb-complexing ligands by 3 brown macroalgae common in Irish waters. *Ascophyllum nodosum, Fucus vesiculosus* and *Laminaria hyperborea* were cultured under low levels of Cd (0, 0.89, or 8.89 nM) or Pb (0, 1.40, or 4.83 nM) exposure, representative of natural background levels in uncontaminated seawater. For each culture we monitored seaweed photochemical quantum yield (F_v/F_m), metal speciation (Cd, Pb), complexing ligands (L_M), conditional stability constants ($logK'L_M$), glutathione (GSH), cysteine (Cys) and seaweed metal contents over 7 days. The selected algae released ligands in response to Cd and Pb with concentrations ranging from 5.13-32.52 nM, consequently confirming seaweeds as a source of L. Significantly higher [L_M] values in cultures with added metals compared to the relevant controls indicate that both metals act as stimuli for L. Similarities in magnitude of the conditional stability constants were identified between L_{Cd} and sulfides, and L_{Pb} and humic substances, giving possible clues on their chemical structure. In any case, our results demonstrated that macroalgae-exuded organic ligands are not necessarily the sole or the principal contributors to the L pool in natural systems though their clear ability to modulate metal speciation means they can no longer be ignored.

4.2 Introduction

Cadmium (Cd) and lead (Pb) have no clear biological function in macroalgae (Stewart 1977; Leborans and Novillo 1996; Pawlik-Skowronska 2000; Lamaia et al. 2005). In fact, both metals are listed as Dangerous Substances (76/464/EEC) and are of concern to aquatic ecosystems due to their inherent toxicity (Phillips and Rainbow 1998; Crathorne et al. 2001). At elevated concentrations, Cd has been shown to reduce chlorophyll fluorescence, cause a loss of pigmentation and inhibit growth in macroalgae (Markham et al. 1980; Strömgren 1980; Baumann et al. 2009). In comparison to Cd, Pb is not overly toxic. Of a suite of 5 metals, Pb was the only metal that did not induce a reduction in chlorophyll *a* fluorescence for 7 species (Baumann et al. 2009). However, extremely high Pb concentrations have been shown to negatively affect algal morphology, growth and photosynthesis (Strömgren 1980; Pawlik-Skowroska 2002).

Metal speciation refers to the distribution of metals among possible forms. In seawater, these forms include the labile fraction (free ionic plus labile-bound) and those strongly bound to organic ligands. A number of studies have illustrated that only the labile species are bioavailable (Campbell et al. 2002; Kola and Wilkinson 2005; Vigneault and Campbell 2005). Likewise, metal complexation is important because it is thought to reduce metal bioavailability. In ocean surface waters it is estimated that 70% of dissolved Cd and 50-70% of dissolved Pb is strongly bound by organic ligands (Bruland 1992; Capodaglio et al. 1998). The source and structure of these ligands remains unclear.

Several marine organisms have been identified as producers of metal-complexing ligands. These include cyanobacteria, dinoflagellates, coccolithophores and macroalgae (Moffett and Brand 1996; Leal et al. 1999; Croot et al. 2000; Andrade et al. 2010). In a recent study, Karavoltsos et al. (2013) examined the release of copper-complexing ligands by 24 macroalgal species and found all algal species produced Cu ligands in the range of 109-744 nM. Similarly, in a parallel study we demonstrated that, in response to low levels of Cu or Zn exposure, *Ascophyllum nodosum*, *Fucus vesiculosus*, and *Laminaria hyperborea* affect metal speciation via the production of organic ligands with metal complexing capacity (Murray et al. a). Glutathione and cysteine were also present in some cases. These thiols are common in natural waters and likely constitute a major portion of complexing ligands in seawater (Ahner et al. 1994; 1995; 1998; Leal and van den Berg 1998; Leal et al. 1999; Tang et al. 2000; Laglera and van den Berg 2003). These papers further emphasize the importance of macroalgae in

metal speciation studies. Unfortunately, no such information exists regarding the production of complexing ligands by macroalgae in response to Cd or Pb.

A large variety of techniques are currently used to determine metal speciation (van Leeuwen et al. 2005; Sigg et al. 2006). For complexation studies, equilibrium-approach voltammetric methods appear to be the most common and offer many advantages (reviewed by Buffle and Chalmers 1988). Here, differential pulse anodic stripping voltammetry (DPASV) is employed as it has adequate sensitivity and selectivity. DPASV has been used successfully in the past by Rozan et al. (2000) and Capodaglio (1995).

The objective of this study was to investigate the effect of Cd or Pb exposure on photosynthesis and ligand production of 3 brown macroalgae, under controlled laboratory conditions. The overall aim was to establish a quantitative link between the physiological response of the algae to metal exposure, extracellular ligand release, and the subsequent effect on metal speciation.

4.3 Materials and Methods

4.3.1 Reagents

All reagents were analytical-grade and water used for both dilutions and the preparation of artificial seawater was Milli-Q (MQ). Stock solutions of Cd and Pb were prepared in 0.01 M HCl, by dissolution of atomic absorption standard solutions (Fluka). Thiol working standards of glutathione, cysteine, and thioactemide (all Aldrich) were prepared prior to use by dissolution in MQ water. One molar 3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulfonic acid (HEPPS, Aldrich) and 0.55 M NaOH (Aldrich) were used in voltammetric determinations. A pH of 8.1 was achieved via the addition of 70 µL buffer to 10 mL sample. All experiments were conducted at room temperature, and when not in use, all metal, ligand, and buffer solutions were kept at 4°C. Artificial seawater was prepared according to Kester et al. (1967) and tested using voltammetry to ensure the absence of trace metals. This water was further supplemented with 176 µmol nitrate (as sodium nitrate, Aldrich) and 7.26 µmol phosphate (as sodium dihyrdogen phosphate, Aldrich).

4.3.2 Cleaning of materials

All flasks, culture jars, filters and plastics were soaked for 1 week in 1 M HCl then rinsed 3 times with MQ and allowed to air dry in a laminar flow hood. Whenever possible, clean equipment was then stored in doubled sealed bags. Culture jars were soaked for 1 week in detergent (5% Decon) prior to this. Gloves were worn throughout and sample manipulations were always carried out in a laminar flow hood.

4.3.3 Seaweed collection

The areas of 'new growth' of *Ascophyllum nodosum* (Linnaeus) Le Jolis, *Fucus vesiculosus* (Linnaeus), and *Laminaria hyperborea* (Gunnerus) were collected as described previously (Murray et al. a), manually during low tide at Finavarra (53◦ 09′ 25″ N; 09◦ 06′ 58″ W), Co. Clare, western Ireland. For *A. nodosum* and *F. vesiculosus*, the apical parts (tips) were collected from the longest fronds of different individual thalli. For *L. hyperborea*, the basal meristem was sampled from the blade section, just above the stipe. Collections were made on different sampling dates between September and October 2011 corresponding to 2 separate laboratory experiments. On each occasion, tips were placed in acid-cleaned plastic bags with seawater from the site, stored at 4°C and transported to the laboratory within an hour of collection. Seaweeds were stored at 10 °C, under 60 ± 5 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400-700 nm) on a 12 h:12 h light: dark cycle in filtered seawater for a maximum of 2 days.

4.3.4 Seaweed cultures

Seaweeds were removed from the filtered seawater and rinsed 3 times with the modified artificial seawater. Single tips (*A. nodosum* and *F. vesiculosus*) or meristems (*L. hyperborea*) of approximately 6 cm in length of each species were then placed in 500 mL polycarbonate culture jars containing 300 mL of modified artificial seawater enriched with either Cd (at 0, 0.89 or 8.89 nM) or Pb (at 0, 1.40 or 4.83 nM). Each was reproduced in triplicate (n=3) and cultures without seaweed were included for determination of background metal and ligand levels. Seaweeds were grown for 7 days at 10 °C, under 60 ± 5 µmol m^{-2} s^{-1} PAR on a 12 h:12 h light: dark cycle, with constant bubbling with air.

4.3.5 Photosynthesis measurements

Fluorescence measurements were taken using a Pulse Amplitude Modulated (PAM) fluorometer (PAM 2000, Heinz Walz GmbH, Effeltrich, Germany) on day 0, 1, 4, and 7, following dark adaptation of tips or mersitems for 20 min. Maximum photosystem II efficiency (F_v/F_m) was measured by applying a saturating pulse to the dark adapted tips in order to determine minimum (F_0) and maximum fluorescence (F_m) (Kitajima and Butler 1975), where:

$$F_v/F_m = (F_m - F_0)/F_m$$

Here, F_v/F_m is expressed as a percent of the day 0 control values in each experiment in order to account for any decreases in yield following harvest and for easier comparison of results across species.

4.3.6 Internal metal contents of macroalgae after culture

Algae were rinsed twice with 1:9 ethanol/seawater (Gledhill et al. 1999), placed in acid cleaned bags, and frozen immediately following culture. Tips were freeze-dried to a constant weight then ground using an agate mortar and pestle within 6 months. Approximately 1 g of the powdered biomass was dry-ashed at 450°C then brought up to 10 mL volume using 20% nitric acid. Cadmium and lead contents were measured using inductively coupled plasma optical emission spectrometry (ICP-OES), with the respective limits of detection as follows: Cd 0.1 ng g^{-1} and Pb 5 ng g^{-1}. To ensure quality of methodology, experimental blanks and the brown seaweed Certified Reference Material (CRM NIES No9, National Institute for Environmental Studies, Japan; *Sargassum fulvellum*) were analyzed.

4.3.7 Analysis of culture media

Culture media were analyzed on day 0, 1, 4 and 7. When culture solutions could not be analyzed within 24 h, they were filtered, transferred to 500 mL HDPE bottles and frozen immediately for analysis within 2 months. In these instances, samples were thawed overnight at 4°C and thoroughly mixed to ensure the dissolution of particulates formed due to freezing as per Laglera and van den Berg (2003).

4.3.8 Instrumentation

The voltammeter used here was either a Metrohm 757-VA Computrace or Metrohm 797 Computrace controlled by a computer (PC). The working electrode was a hanging mercury drop (HMDE) with a drop size of approximately 0.52 mm^2. The reference electrode was a double junction Ag/saturated AgCl, KCl (3 M), with a KCl-filled salt bridge (3 M). The counter electrode was a platinum wire. A polytetrafluoroethylene (PTFE) stirrer, rotating at 2 000 rpm, was employed during the adsorption step.

4.3.9 Metal speciation

The total dissolved metal concentrations ($[M]_D$) were measured simultaneously using differential pulse anodic stripping voltammetry (DPASV). Seawater samples were filtered through 0.45 µm glass-fiber filters and acidified to pH 2.2 using 6 M HCl, prior to UV digestion in PTFE-capped quartz silica tubes for 2 h. Ten mL aliquots of sample were pipetted into the voltammetric cell. Acetate buffer (70 µL, final concentration 0.07 M) was added and the solution was purged for 5 minutes using nitrogen. The voltammetric parameters were: deposition 60 s at -1.15 V whilst stirring; 10 s quiescence; potential scan using the differential pulse modulation: pulse height 0.05 V, scan from -1.15 to 0.05 V. Initial concentration was determined via two standard metal additions, each replicated twice. Peaks for cadmium and lead occurred at –0.6 V and –0.4 V with the respective limits of detection at 0.1 nM and 0.2 nM. In those cases where Pb could not be quantified, the deposition time was increased to 5 min, lowering the detection limit to 0.024 nM.

The labile dissolved metal concentrations ($[M]_L$) of Cd and Pb were determined using ASV as described above for $[M]_D$ but without acidification and UV digestion. For these determinations, the pH was fixed at 8.1 using HEPPS. In all cases, initial concentration was determined via two standard metal additions, each replicated twice.

4.3.10 Metal complexing-capacities

To determine the concentration of metal-specific complexing ligand (L_M) and the associated conditional stability constant ($\log K'L_M$), titration of filtered seawater with individual metal ions was used. As outlined for $[M]_L$, Cd and Pb measurements were made using ASV. HEPPS (0.01 M) was added to 120 mL of filtered seawater. Ten mL aliquots were then pipetted into 10-20 mL pre-conditioned polystyrene vials (Sterilin) containing metal additions in

approximately equal increments (where the actual range depends on the initial metal concentration). Solutions were left to equilibrate overnight (12-15 h). The fully-equilibrated $[M]_L$ of each vial was then measured as previously outlined.

4.3.11 Identification and quantification of thiol compounds

Thiols were both identified and quantified using CSV at pH 8.1 (0.01 M HEPPS), according to Leal et al. (Leal et al. 1999). Cysteine, glutathione and thioactemide peaks present at -0.47 V, -0.58 V and -0.68 V respectively, and the voltammetric parameters were: deposition 60 s at -0.25 V whilst stirring; potential scan using the square wave modulation: scan rate 20mV/s, modulation amplitude 25 MV, and frequency 50 Hz. Two standard thiol additions (cysteine, glutathione, or thioactemide), each replicated twice, were used to determine initial concentrations.

4.3.12 Calculations

As described previously for Cu and Zn (Murray et al. a), a modified van den Berg-linearization (1982) was used to determine the complexing ligand concentration and corresponding conditional stability constant for each metal based on the following relationship:

$$[M]_L/[ML]=[M]_L/C_L + \alpha/(K'_{ML}L_M)$$

Where [ML] is calculated as the difference between $[M]_d$ and $[M]_L$ while the α-coefficient represents the overall side reactions producing inorganic complexes for each metal. Values of α were calculated as described previously by Vasconcelos et al. (2002) using the following values: α_{Cd}=35 and α_{Pb}=34 (Turner et al. 1981). Linearly transformed plots of $[M]_L/[ML]$ versus $[M]_L$ were used to elucidate the number of ligands present wherein a linear plot was indicative of a single ligand. The concentrations of L_M and $\log K'L_M$ were determined from the slope and y-intercept of a least squares regression of this titration curve. Example titration and linearization plots for both metals (**Figures S1, S2**) are presented in the Supplementary Information.

The sensitivity was corrected for under-saturation of the ligand by multiple iteration of the final four titration points (Turoczy and Sherwood 1997).

4.3.13 Quality assurance

Certified reference materials (CRM) for trace metals from the National Institute for Environmental Studies, Japan Environment Agency algal standard (NIES no. 9) and the National Research Council of Canada Reference standard seawater (CASS 5) for coastal seawater were also analyzed to determine the accuracy of analysis of seaweed metal contents and seawater metal concentrations, respectively. Showing a good accuracy of measurement, the metal concentrations determined were never significantly different than the expected values ('t' test, $p>0.05$) (**Table S2**).

4.3.14 Data analysis

Seaweed and seawater metal concentrations, complexing ligands, conditional stability constants, and thiol concentrations in the experimental treatments were tested for significant differences using a one-way between subjects ANOVA. Multiple analyses of variance (MANOVA) were also used to compare data among species, between sites, and over time. For both ANOVA and MANOVA, a *post hoc* Tukey's multiple comparison was employed where significant differences were identified. Pearson-product moment correlation coefficients were used to identify and measure the relationships between complexing ligands and the other variables. All tests were performed using SPSS version 20.0.

4.4 Results

4.4.1 Effects on photosynthesis

Photochemical quantum yield (F_v/F_m) values of seaweeds exposed to Cd and Pb are presented in **Figure 4.1**. Overall, F_v/F_m decreased in the order: day 0 > 1 > 4 > 7; treatment control > low metal addition > high metal addition; species *A. nodosum* ~ *F. vesiculosus* > *L. hyperborea.*

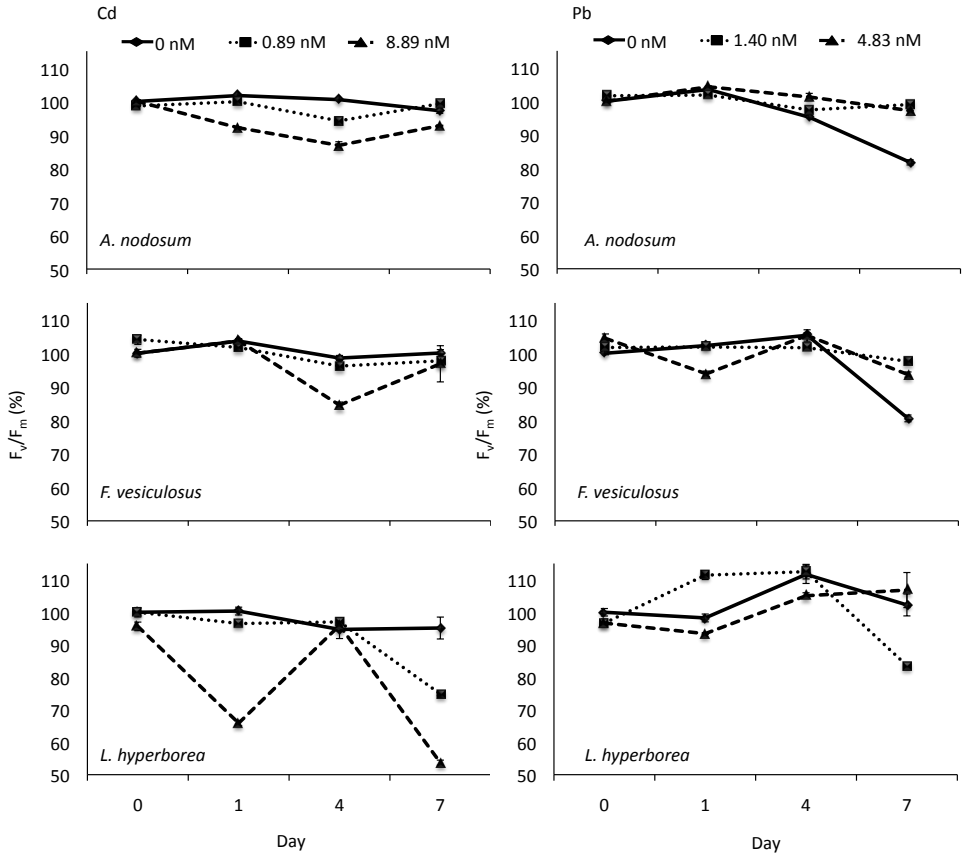

Figure 4.1. Photochemical quantum yield (F_v/F_m) of three brown macroalgae cultured in artificial seawater for 7 days at 10°C, under 60 ± 5 µmol m^{-2} s^{-1} PAR on a 12 h:12 h light: dark cycle, in different concentrations of Cd (0 nM, 0.89 nM, 8.89 nM) or Pb (0 nM, 1.40 nM, 4.83 nM). Data are expressed as a % of the corresponding day 0 control value; *n*=3. Error bars represent the standard error of triplicate cultures.

For Cd, there was no significant effect of treatment on the F_v/F_m of *F. vesiculosus* or *L. hyperborea* over 7 days ($p>0.05$). By contrast, 8.89 nM of Cd significantly reduced the F_v/F_m of *A. nodosum* from day 4 onwards ($F_{Treatment}=21.45$, $p<0.05$; $F_{Time}=3.11$, $p<0.05$). Although there was no significant effect of time on *F. vesiculosus* , F_v/F_m of *L. hyperborea* had decreased significantly on days 1 and 7 ($F=21.78$, $p<0.05$).

There was no significant effect of any Pb treatment on the F_v/F_m of *A. nodosum*, *F. vesiculosus*, or *L. hyperborea* over 7 days ($P>0.05$). A significant decrease in the F_v/F_m of all 3 species observed on day 7 was the result of time, not treatment (*A. nodosum*: $F=2.011$, $p<0.05$; *F. vesiculosus*: $F=4.46$, $p<0.05$; *L. hyperborea*: $F=4.52$, $p<0.05$).

4.4.2 Seaweed metal contents

All seaweeds analyzed contained both Cd and Pb (**Figure 4.2**). Cd concentrations were generally higher than those of Pb. Multiple analyses of variance (MANOVA) for Cd showed no significant effects of time, treatment, or species on the mean vectors of the tissue metal concentrations. By contrast, for Pb, the internal metal content of *L. hyperborea* was significantly lower than that of the other two species ($F=6.72$, $p<0.05$), but no effects of time or treatment were observed.

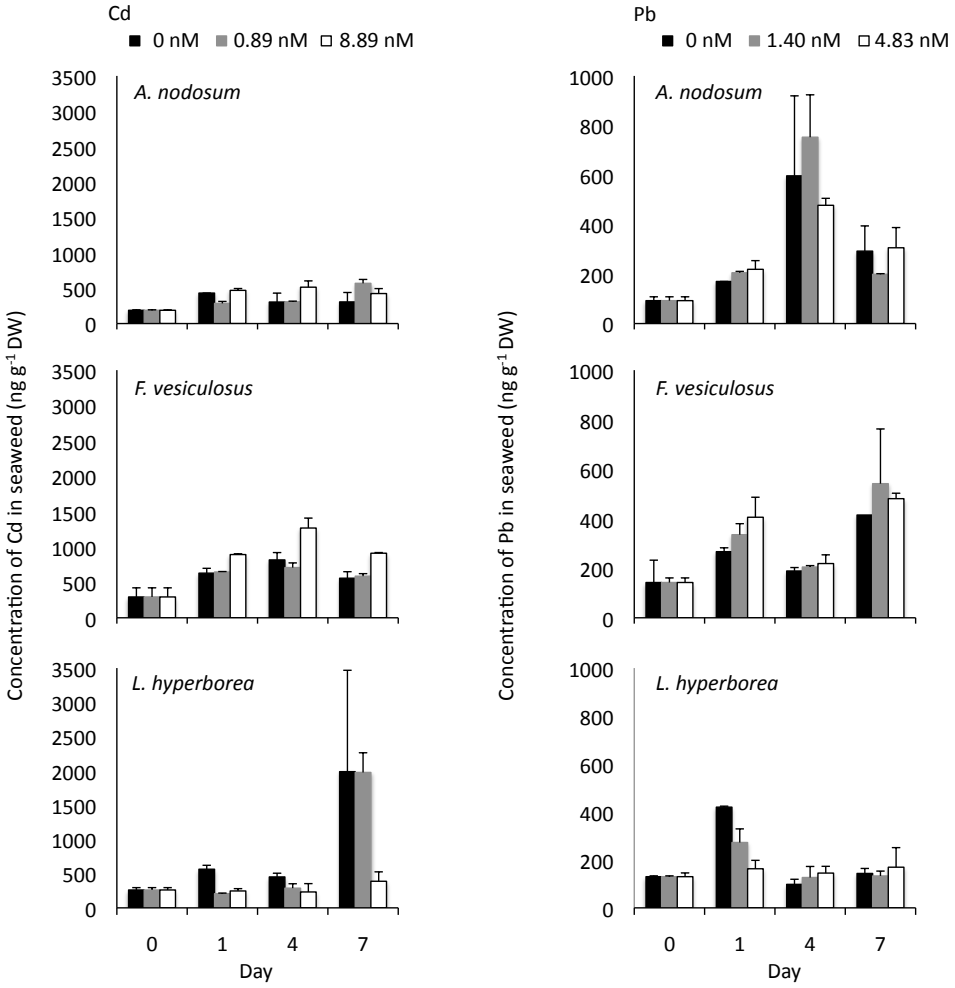

Figure 4.2. Concentration of metals in three seaweed species cultured for 7 d in different concentrations of Cd (0 nM, 0.89 nM, 8.89 nM) or Pb (0 nM, 1.40 nM, 4.83 nM); *n*=3. For other culture conditions, see fig. 1. Error bars represent the standard error of triplicate cultures. Note the Y-axes have different scales for Cd and Pb.

4.4.3 Metal speciation

The results of the speciation analyses for Cd and Pb are presented in **Table 4.1** and **Table 4.2**, respectively. In all cultures, metal additions significantly increased the total dissolved metal concentration (F_{Cd}=4810.44, $p<0.05$; F_{Pb}=2690.08, $p<0.05$). In general, $[Cd]_L$ decreased in the order *L. hyperborea* > *F. vesiculosus* > *A. nodosum,* whilst $[Pb]_L$ showed the opposite trend and increased in the order *L. hyperborea* < *F. vesiculosus* < *A. nodosum*. In each case, $[M]_D$ was highest on day 4.

Labile Cd ranged from 0.15-7.32 nM (*A. nodosum*), 0.21-7.01nM (*F. vesiculosus*), and 0.27-8.09 nM (*L. hyperborea*). For all species, the maximum $[Cd]_L$ was measured in the 8.89 nM culture on day 1 whilst the minimum $[Cd]_L$ was measured in the 0.89 nM culture on day 7. The concentration of bound Cd covaried with $[Cd]_D$ and $[Cd]_L$ with a maximum $[L_{Cd}]$ of 32.52 nM measured in the 8.89 nM *L. hyperborea* culture on day 7. However, the maximum $[L_{Cd}]$ did not correspond to the culture with the highest [CdL] nor the greatest proportion of bound Cd. Rather, the maximum [CdL] (0.89 nM) occurred in the 8.89 nM *A. nodosum* culture on day 7, and the greatest proportion bound (81.12%) occurred in the 0.89 nM *A. nodosum* culture on day 7.

Table 4.1 continued

Table 4.1. A summary of the DPASV speciation for macroalgal cultures under low-level Cd exposure, mean ± standard error, *n=3*. AN: *Ascophyllum nodosum*; FV: *Fucus vesiculosus*; LH: *Laminaria hyperborea.*

Species	Treatment (nM)	Day	$[Cd]_D$ (nM)	$[Cd]_L$ (nM)	[CdL] (nM)	$[L_{Cd}]$ (nM)	$LogK'L_{Cd}$
AN	0	0	-	-	-	-	-
		1	-	-	-	5.23 ± 0.56	9.87 ± 0.22
		4	-	-	-	5.29 ± 0.018	9.71 ± 0.58
		7	-	-	-	5.13 ± 0.47	9.68 ± 0.24
	0.89	0	0.74 ± 0.091	0.79 ± 0.032	-	-	-
		1	0.67 ± 0.16	0.92 ± 0.41	0.00	8.28 ± 0.99	9.72 ± 0.073
		4	1.09 ± 0.12	0.89 ± 0.090	0.20	10.63 ± 0.80	9.51 ± 0.14
		7	0.78 ± 0.29	0.15 ± 0.016	0.63	13.28 ± 0.65	9.44 ± 0.13
	8.89	0	7.41 ± 0.11	7.32 ± 0.43	-	-	-
		1	4.29 ± 0.086	5.42 ± 0.83	0.00	15.51 ± 0.15	8.52 ± 0.095
		4	4.33 ± 0.16	4.14 ± 0.29	0.20	18.67 ± 0.38	8.57 ± 0.012
		7	4.18 ± 00.17	2.39 ± 0.40	1.79	24.60 ± 3.41	8.37 ± 0.049
FV	0	0	-	-	-	-	-
		1	-	-	-	6.03 ± 0.64	9.72 ± 0.095
		4	-	-	-	5.51 ± 0.60	9.67 ± 0.48
		7	-	-	-	5.34 ± 0.037	10.11 ± 0.51
	0.89	0	0.91 ± 0.067	0.96 ± 0.021	-	-	-
		1	0.55 ± 0.25	1.48 ± 0.47	0.00	8.82 ± 1.39	9.63 ± 0.21
		4	0.66 ± 0.17	0.71 ± 0.13	0.00	10.55 ± 0.42	9.50 ± 0.073
		7	0.47 ± 0.022	0.21 ± 0.055	0.27	11.71 ± 1.37	9.35 ± 0.046
	8.89	0	7.21 ± 0.14	7.01 ± 0.19	0.00	-	-
		1	4.17 ±	5.19 ±	0.00	15.64 ±	8.37 ± 0.012

Table 4.1 continued

Species	Treatment (nM)	Day	$[Cd]_D$ (nM)	$[Cd]_L$ (nM)	[CdL] (nM)	$[L_{Cd}]$ (nM)	$LogK'L_{Cd}$
			0.13	0.0.83		0.73	
		4	4.69 ± 0.23	3.98 ± 0.55	0.72	23.021 ± 2.17	8.45 ± 0.058
		7	4.34 ± 0.11	2.90 ± 0.47	1.44	27.095 ± 1.18	8.36 ± 0.11
LH	0	0	-	-	-	-	-
		1	-	-	-	6.21 ± 0.80	9.67 ± 0.019
		4	-	-	-	6.19 ± 0.31	9.59 ± 0.33
		7	-	-	-	5.88 ± 0.18	10.059 ± 0.12
	0.89	0	0.78 ± 0.051	0.69 ± 0.19	-	-	-
		1	0.81 ± 0.057	1.07 ± 0.22	0.00	10.23 ± 0.52	9.49 ± 0.0092
		4	0.69 ± 0.071	0.64 ± 0.19	0.045	10.80 ± 0.095	9.50 ± 0.022
		7	0.76 ± 0.15	0.27 ± 0.16	0.49	13.44 ± 1.24	9.30 ± 0.018
	8.89	0	8.03 ± 0.91	8.09 ± 0.75	-	-	-
		1	4.11 ± 0.13	6.13 ± 0.35	0.00	16.095 ± 1.82	8.19 ± 0.17
		4	4.10 ± 0.0044	5.39 ± 0.34	0.00	21.78 ± 0.68	8.55 ± 0.021
		7	4.54 ± 0.27	2.35± 0.56	1.21	32.52 ± 2.53	8.17 ± 0.24

- represents concentrations below the detection limits.

As with Cd, the concentration of bound Pb covaried with $[Pb]_D$. The maximum $[L_{Pb}]$ (18.16 nM) was measured in the 4.83 nM *L. hyperborea* culture on day 7. Again, the maximum [PbL] did not correspond to the culture with the greatest proportion of bound Pb. Rather, the maximum percent bound Pb (98.59%) and the maximum [PbL] (1.59 nM) occurred in the 1.40 nM *L. hyperborea* culture on day 7 and the 4.83 nM *L. hyperborea* culture on day 4, respectively. Labile Pb ranged from 0.034-5.92 nM (*A. nodosum*), 0.042-4.72 nM (*F. vesiculosus*), and 0.031-4.42 nM (*L. hyperborea*). For all species, the highest $[Pb]_L$ was measured in cultures under the highest metal treatment, although the time when this occurred varied.

Table 4.2. A summary of the DPASV speciation for macroalgal cultures under low-level Pb exposure, mean ± standard error, *n=3*. AN: *Ascophyllum nodosum*; FV: *Fucus vesiculosus*; LH: *Laminaria hyperborea.*

Species	Treatment (nM)	Day	$[Pb]_D$ (nM)	$[Pb]_L$ (nM)	[PbL] (nM)	$[L_{Pb}]$ (nM)	$LogK'L_{Pb}$
AN	0	0	-	-	-	-	-
		1	-	-	-	5.71 ± 0.13	10.0087 ± 0.042
		4	0.32 ± 0.19	**0.18 ± 0.16**	0.14	5.97 ± 0.12	10.00 ± 0.14
		7	0.39 ± 0.11	**0.040 ± 0.039**	0.36	8.87 ± 1.14	9.11 ± 0.022
	1.40	0	1.63 ± 0.28	1.68 ± 0.21	-	-	-
		1	2.069 ± 0.15	**0.11 ± 0.019**	1.96	8.71 ± 0.52	10.0031 ± 0.12
		4	2.24 ± 0.018	**0.034 ± 0.0023**	2.21	9.32 ± 0.52	10.030 ± 0.044
		7	2.34 ± 0.082	**0.062 ± 0.008**	2.27	9.73 ± 0.11	9.00 ± 0.13
	4.83	0	4.46 ± 0.29	4.32 ± 0.019	-	-	-
		1	4.95 ± 0.058	4.37 ± 0.39	0.59	11.81 ± 0.42	9.94 ± 0.036
		4	5.82 ± 0.59	5.92 ± 0.67	0.00	9.044 ± 1.21	8.66 ± 0.085
		7	5.35 ± 0.21	3.91 ± 0.84	1.44	12.77 ± 0.72	8.31 ± 0.039
FV	0	0	-	-	-	-	-
		1	-	-	-	5.33 ± 0.95	9.51 ± 0.036
		4	**0.028 ± 0.022**	**0.042 ± 0.058**	0.00	5.058 ± 0.21	9.40 ± 0.058
		7	**0.039 ± 0.15**	**0.051 ± 0.047**	0.00	7.53 ± 1.35	9.12 ± 0.22
	1.40	0	1.63 ± 0.39	1.71 ± 0.019	-	-	-
		1	2.015 ± 0.0.41	**0.085 ± 0.0095**	1.93	10.47 ± 0.63	9.28 ± 0.12
		4	2.060± 1.40	**0.060 ± 0.022**	2.00	9.97 ± 0.21	9.30 ± 0.040
		7	2.25 ± 0.0079	**0.042 ± 0.044**	2.21	11.30 ± 1.11	9.24 ± 0.15
	4.83	0	4.69 ± 0.015	4.72 ± 0.13	-	-	-
		1	5.032 ± 0.22	3.51 ± 0.23	1.53	12.083 ± 2.15	10.14 ± 0.31
		4	5.27 ± 0.048	3.38 ± 0.012	1.89	11.37 ± 0.21	9.79 ± 0.39

Table 4.2 continued

Species	Treatment (nM)	Day	$[Pb]_D$ (nM)	$[Pb]_L$ (nM)	[PbL] (nM)	$[L_{Pb}]$ (nM)	$LogK'L_{Pb}$
		7	4.77 ± 0.61	3.038 ± 0.16	1.72	11.35 ± 0.66	9.77 ± 0.10
LH	0	0	-	-	-	-	-
		1	-	-	-	8.67 ± 0.71	9.014 ± 0.22
		4	0.43± 0.099	**0.037 ± 0.021**	0.39	9.096 ± 0.59	9.19 ± 0.29
		7	0.32± 0.12	**0.064 ± 0.0062**	0.25	8.81 ± 0.34	9.27 ± 0.032
	1.40	0	1.79 ± 0.068	1.33 ± 0.29	-	-	-
		1	1.96 ± 0.057	**0.082± 0.030**	1.88	10.66 ± 2.20	9.25 ± 0.19
		4	2.78 ± 0.19	**0.054 ± 0.015**	2.72	13.42 ± 0.34	8.91 ± 0.28
		7	2.26 ± 0.20	**0.031 ± 0.022**	2.23	14.085 ± 0.80	8.96 ± 0.18
	4.83	0	4.98 ± 0.16	4.42 ± 0.78	-	-	-
		1	5.51± 0.23	3.45± 0.78	2.061	11.39 ± 1.11	8.97 ± 0.044
		4	6.21 ± 0.12	3.036 ± 0.82	3.18	16.62 ± 0.89	8.81 ± 0.021
		7	5.14 ± 0.090	2.97 ± 0.33	2.16	18.16 ± 0.71	8.73 ± 0.014

- represents concentrations below the detection limits.
Bold represents those measurements where deposition time was increased from 1 to 5 min.

4.4.4 Organic ligands and conditional stability constants

The average complexation parameters of Cd and Pb in the cultures are listed in **Table 4.1** and **Table 4.2**. The addition of either Cd or Pb stimulated the release of ligands as evidenced by significantly higher [L_M] values in cultures with added metals than the relevant controls (F_{Cd}=872.42, p<0.05; F_{Pb}=167.85, p<0.05). In the case of both metals, titrations were found to fit the presence of a single complexing ligand, meaning that other ligands make only a minor contribution to metal speciation. Multiple analyses of variance showed significant time and species effects on the mean vectors of [L_M] ($F_{Time(Cd)=}$77.64, p<0.05; $F_{Time(Pb)=}$22.21, p<0.05; $F_{Species(Cd)=}$15.11, p<0.05; $F_{Species(Pb)=}$68.19, p<0.05): complexing ligand increased from day 0 to 7, and *L. hyperborea* cultures consistently contained more ligand than the other two species. In each instance, the [L_M] was greater than $[M]_D$.

The concentration of L_{Cd} ranged from 5.13-32.52 nM. For each species, both the maximum and minimum [L_{Cd}] were measured on day 7. The maximum occurred at 8.89 nM Cd addition and the minimum at 0 nM. A Pearson correlation applied to the ligand concentrations revealed several significant correlations (p>0.05; *n*=81): [L_{Cd}] was positively correlated with $[Cd]_D$, $[Cd]_L$ and [CdL], but negatively correlated with [GSH].

The concentration of L_{Pb} ranged from 5.71-12.77 nM (*A. nodosum*), 5.06-12.08nM (*F. vesiculosus*), and 8.67-18.05 nM (*L. hyperborea)*. In contrast to Cd, the cultures with the maximum and minimum [L_{Pb}] differed with species. Again, a Pearson correlation showed significant positive correlations of [L_{Pb}] with each metal species and a negative correlation of [L_{Pb}] with [GSH]. In addition, [L_{Pb}] was positively correlated with [Cys].

In contrast to the ligand concentrations, the conditional stability constants of both ligands detected were relatively constant. Still, MANOVA revealed significant treatment, time, and species effects on the mean vectors of [L_{Cd}]. In this case, logK increased significantly with increasing Cd addition, decreased with time, and was significantly lower in the *L. hyperborea* cultures ($F_{Treatment}$=4.97, p<0.05; F_{Day}=46.38, p>0.05; $F_{Species}$=51.23, p<0.05). For Pb, logK' was significantly higher in the 4.83 nM cultures, ranging from 8.31-10.14 nM (F=206.55, p<0.05), but no effects of time nor species were observed.

4.4.5 Glutathione and Cysteine

Glutathione was present in all cultures, while cysteine was detected only in the 4.83 nM Pb cultures (**Figure 4.3**). For each metal, MANOVA revealed significant treatment and time effects on the mean vectors of the GSH concentrations. Glutathione decreased significantly with increasing metal treatment and increased significantly with time ($F_{Treatment(Cd)}$=131.01; $F_{Time(Cd)}$=24.39; $F_{Treatment(Pb)}$=151.80, $p<0.05$; $F_{Time(Pb)}$=9.55, $p<0.05$). For Pb, Cys increased significantly with time, whilst concentrations measured in the *F. vesiculosus* cultures were significantly lower than for the other two species (F_{Time}=18.97, $p<0.05$; $F_{Species}$=19.12, $p<0.05$).

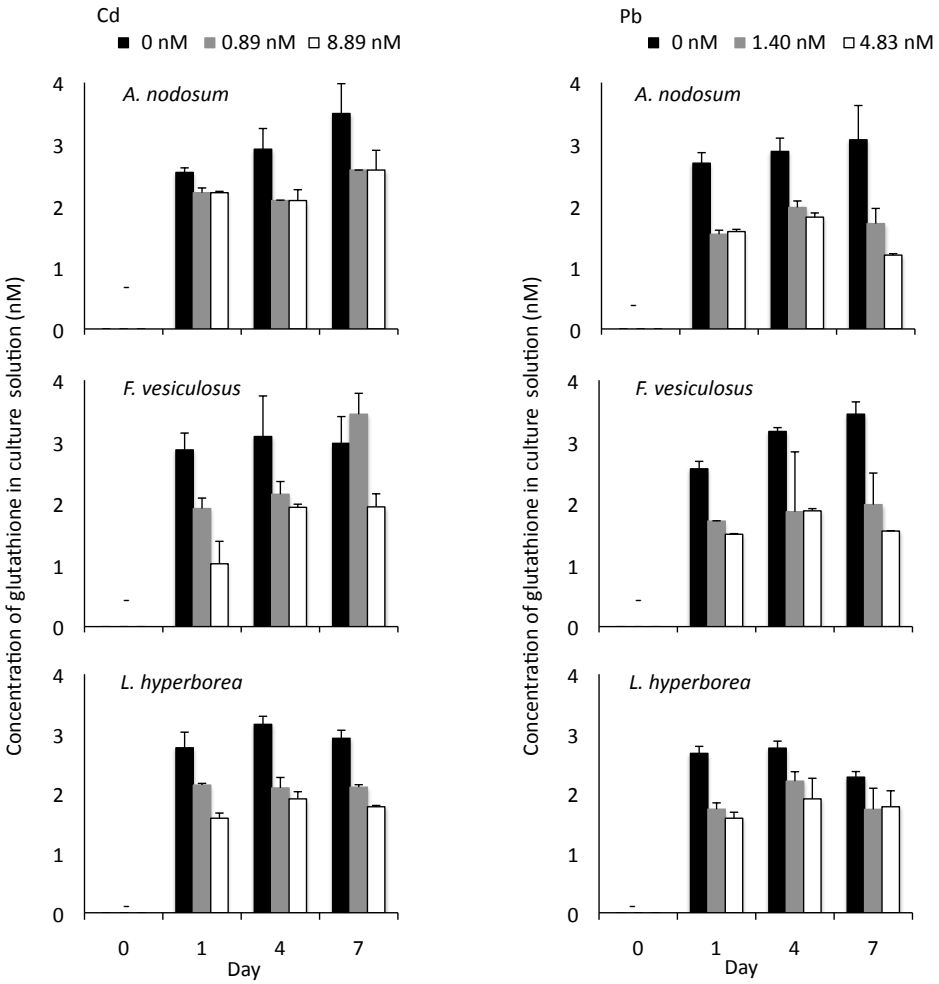

Figure 4.3. Glutathione concentrations measured for macroalgal cultures under different concentrations of Cd (0 nM, 0.89 nM, 8.89 nM) or Pb (0 nM, 1.40 nM, 4.83 nM); *n*=3. For other culture conditions, see fig. 4.1. Error bars represent the standard error of 3 (triplicate) cultures. Dash represents concentrations not detected.

4.5 Discussion

4.5.1 Macroalgal responses to metal exposure

Despite being generally excluded from most ligand studies, the physiological response of seaweeds to metal exposure has been previously reported in literature by means of chlorophyll *a* fluorescence. Parameters such as F_v/F_m are widely used as indicators of 'health' for photosynthetically active organisms and as such, are useful endpoints in assessing metal effects. Baumann et al. (2009) tested the sensitivity of seven macroalgae to five metals using PAM fluorometry and, as in our study, Pb did not induce a reduction of chlorophyll fluorescence in any species despite being present in the seaweed tissue. By contrast, in our study, *A. nodosum* showed significant sensitivity to Cd as indicated by decreased yield following exposure to 8.89 nM Cd. Comparison of the values of the photochemical quantum yield for both Cd and Pb treatments suggests that *A. nodosum* is more sensitive to metal exposure, compared to *F. vesiculosus* and *L. hyperborea*, within the metal concentrations used in this work. Thus, this study supports our previous finding that macroalgal response to low-level metal exposure is both species- and metal-specific (Murray et al. a).

Although Cd reduced the photochemical quantum yield of *A. nodosum*, this was not always the species that contained the most Cd. Instead, the internal [Cd] for *A. nodosum* under the highest Cd treatment was comparable to the other two species, which had no observed effects. This decoupling of response and uptake was likely the result of a tolerance mechanism present in *F. vesiculosus* and *L. hyperborea*, but absent or less functional in *A. nodosum*. As described previously for *F. serratus* (Nielsen and Nielsen 2010) it is possible that the more tolerant species regulated intracellular Cd uptake such that metals remained externally bound where they did not affect photosynthetic activity, yet still contributed to the total tissue metal burden. Inter-species differences may be further explained by variations in genetics, morphology, metal-binding capacity, or any other parameter that influences metal-algae interactions. For example, Connan et al. (2011) attributed species-specific differences in accumulation of metals to interspecies differences in interactions at the algal surface. Both intracellular Cd and Pb concentrations changed very little within the range of metal treatments applied.

Metal accumulation by all three species was always greater for Cd than Pb. This is in contradiction to some previous studies on metal accumulation by brown seaweeds; for example, an assessment of metal contamination along the Irish coast revealed that the mean

concentrations of metals in *A. nodosum* were in the order Pb>Cr>Co>Cd (Morrison et al. 2008). Such differences in the elemental composition of seaweeds may be attributed to several factors, both biological and environmental, but were mostly likely the result of differences in metal binding to cell wall constituents, which were included in our accumulation analyses but not in that of Morrison et al. (2008). According to data reviewed by Davis et al. (2003), the extent of metal binding (mmol g^{-1} DW) by *A. nodosum* was greater for Cd (1.91; Holan et al. 1993) than Pb (1.31; Holan and Volesky 1994), suggesting that the properties of the cell wall constituents, such as alginates and fucoidans, are responsible for the metal-specific differences in accumulation described here.

4.5.2 Metal speciation

Although no differences in seaweed internal metal concentrations were detected, the presence of Cd or Pb significantly altered the chemistry of the culture solutions. Speciation results showed both metals were predominantly bound as organic chelates. In the culture solutions, 26-81% of Cd was bound on day 7. These results are near the range (33-92%) previously reported for Cd complexation in the surface waters of the North Atlantic (Sakamoto-Arnold et al. 1987), and comparable to the average concentration of bound Cd (70%) calculated for the North Pacific (Bruland 1992). Measurements of Pb complexation in the culture solutions on day 7 ranged from 26-98% and were also consistent with the average Pb complexation (75%) in the North Pacific. General agreement of our speciation data with those of natural seawater suggests that the macroalgae exuded ligands measured here are likely to contribute to the pool of compounds which complex metals *in-situ*.

Significant positive correlations between Cd treatment, [CdL], and $[Cd]_L$ suggest that these three factors control the chemical distribution of Cd. In addition, initial total metal concentration presents a very good correlation with subsequent ligand concentration, emphasizing the correspondence between the rapid increase in $[L_{Cd}]$ and the depletion of $[Cd]_L$ with time. This direct relationship between initial $[Cd]_L$ and final $[L_{Cd}]$ indicates that ligands are released in response to a specific metal, where increased ligand production is likely the result of a resistance mechanism. While the release of exudates with metal complexing capacity could also be a macroalgal strategy to increase bioavailable metal, this hypothesis is not supported by our speciation results where the lowest $[L_M]$ occurs in the control cultures. Analogous results were obtained by extending the correlation analysis to the Pb speciation data. Such similarities between L_{Cd} and L_{Pb} in the culture solutions indicate that

similar phenomena may control their production and release by macroalgae. The existence of a parallel biochemical pathway for Cd and Pb would not be surprising given that both elements are non-essential divalent cations.

4.5.3 Seaweeds confirmed as a source of L

This work was the first to demonstrate the direct production of L_{Cd} and L_{Pb} by macroalgae, and supports our previous studies that confirmed seaweeds as a source of L (Murray et al. a). Exudation of ligands by *A. nodosum*, *F. vesiculosus*, and *L. hyperborea* in response to Cu and Zn has already been described (Murray et al. a). Although the importance of organic complexation of Cd and Pb has been demonstrated, the liberation of Cd and Pb complexing ligands was substantially less than that determined previously for Cu or Zn in comparable cultures. For example, total concentrations of trace metal-specific ligands for Cu, Zn, Cd, and Pb exuded by *A. nodosum* on day 7 in response to the highest metal additions (Cu, 15.7 nM; Zn, 91.70 nM; Cd, 8.89 nM; Pb, 4.83 nM) were 39.90 nM, 81.60 nM, 24.59 nM, and 12.76 nM, respectively. However, this comparison may be inaccurate given that seaweeds for the Cu and Zn determinations were collected in a different season than those for Cd and Pb; it is possible that seasonal variations in physiology affected the capacity of the seaweed for ligand production. The only relevant previous study on the biological production of ligands capable of complexing these metals also found the concentration of Cd and Pb ligands exuded by the microalga *E. huxleyi* to be an order of magnitude less than Cu and Zn ligands. Unfortunately, further discussion is limited by the fact that only a small number of macroalgae have been studied as sources of metal complexing ligands.

Despite the fairly high and variable concentrations of L_{Cd} and L_{Pb} in the culture solutions, the concentrations of GSH were relatively low and stable. Analyses of the culture solutions indicated that [GSH] was independent of macroalgal species, yet dependent on metal treatment. A comparison of the correlation coefficients between thiols and the other water chemistry parameters showed the strongest correlation with initial metal treatment. For each species, the [GSH] in the culture solutions decreased with increased metal addition. This is not surprising given that GSH is consumed by metal-induced oxidative stress (Contreras et al. 2005). Nevertheless, in previous studies thiols have been considered suitable candidates for the identity of macroalgae-exuded organic ligands based on their ability to complex Cu, their involvement in seaweed secondary metabolism, and their ubiquitous presence in shelf and coastal waters. However, a lack of significant correlation

between [L_M] and [GSH] in our cultures suggests that, in this case, GSH does not constitute a significant portion of the pool of macroalgae exuded L_{Cd} or $L_{Pb.}$ However, relating the results obtained in other studies is difficult due to differences in methodology, algal species, and metal concentrations employed. For example, previous studies that reported the promotion of GSH exudation in the presence of Cd used abnormally high levels of Cd (Vasconcelos et al. 2008) , compared to the natural levels used in this study. Accordingly, there are few studies in which GSH exudation has been studied at metal concentrations low enough to be realistic for natural conditions. Thus, the determinations of GSH exudation by macroalgae in this study improves our understanding of ligand behavior in natural systems.

In contrast to GSH, Cys was only detected in the 4.83 nM Pb culture solutions. Comparisons with previous studies on microalgae show similar trends: Cys was measured in exudates of *E. huxeyli* and *Enteromorpha* in response to Pb, but not Cd, exposure (Vasconcelos et al. 2002). In this study, exudation of Cys by both *F. vesiculosus* and *L. hyperborea* under the highest Pb addition coincided with the lowest measured initial F_v/F_m. These results reinforce previous conclusions in previous works that indicated Cys may be produced in response to metal-induced photosynthetic damage. This theory is not applicable to *A. nodosum* and/or any other species beyond day 1. Of course, the complex genetic and biochemical regulation of thiol production means that deciphering the relationship between metal stress and thiol production is not straightforward but likely to be influenced by several stressors including metal exposure, oxidative stress, and sulfur and nitrogen deficiency (Cózatl and Tavera 2005).

4.5.4 Expanding the stimuli for the exudation L

In addition to confirming seaweeds as a source of L, our results demonstrate that low levels of both Cd and Pb stimulate ligand exudation by macroalgae in seawater. We have shown previously (Murray et al. a) that macroalgae release ligands in response to low levels of Cu and Zn and so, this work expands and strengthens our prior conclusion: algal ligand production is a function of $[M]_D$ at naturally occurring concentrations. Possibly the most striking feature of this work is that metal binding intensity depended on metal loading conditions. This is especially important given that macroalgae are usually exposed to a broad spectrum of metals in the environment, not simply a single compound as employed in this work. Further studies involving multiple metal exposures would reveal whether ligand exudation is additive, synergistic, or antagonistic.

4.5.5 Deciphering the nature of Cd and Pb binding ligands

Published data for Cd- and Pb-biota-derived ligand equilibrium remain scarce. Most previous binding studies focused on microalgae, Cu and/or employed concentrations not relevant for natural systems. As such, a comparison of the binding parameters reported here with those of known compounds in seawater provides the only evidence on the identity of L_{Cd} and L_{Pb}. For L_{Cd}, the measured conditional stability constants on day 1 were lower than those recently reported for the near-pristine Lough Furnace estuary in western Ireland ($logK'L_{Cd}$=9.32-10.95; Murray et al. d) yet similar to those previously reported for sulfides in seawater (logK'CdS=8.38; Al-Farawati and van den Berg 1999). Sulfur has already been detected in macroalgal culture solutions and found to contribute to Cu complexing capacity via the formation of sulfides (Scoullos et al. 2004). Sulfide presence occurred due to the conversion of sulfates to sulfides by anaerobic bacteria in *U. rigida* culture solutions as a result of decreased dissolved oxygen due to macroalgal decomposition. It seems therefore possible that, in our cultures where F_v/F_m decreased as a function of metal treatment, sulfides may have been produced aerobically from sulfur-rich GSH. This provides yet another plausible explanation for the decreased [GSH] with increased metal treatment.

A comparison of the results obtained here with those observed recently in Lough Furnace (Murray et al. d) suggest that the primary L_{Cd} detected in near-pristine estuarine waters did not necessarily originate from macroalgae. Instead, both the [L_{Cd}] and the $logK'L_{Cd}$ reported for Lough Furnace were closer in value to fulvic acids than the ligands measured in this study. The existence of ligands other than those produced by macroalgae is not surprising given the known presence of various metal-binding compounds in seawater, such as humic substances and thiols, and the vast terrestrial and aquatic sources in coastal waters. It seems therefore that the extracellular organic ligands investigated in this study would likely not contribute significantly to the pool of Cd binding ligands found in temperate estuaries. By extension, the sulfur-like ligands biosynthesized by the 3 species of macroalgae are not expected to wholly regulate bioavailable Cd in coastal waters. This supports earlier work that suggests complexing material in natural waters is heterogeneous in terms of both structure and source.

Despite the parallel range of logK's for L_{Cd} and L_{Pb}, the findings presented here indicated a different identity for L_{Pb} compared with L_{Cd}. In contrast to L_{Cd}, the logK's determined here for Pb effectively rule out sulfides (logK'PbS=7.98; (Al-Farawati and van den Berg 1999) as a candidate for the identity of L. Instead, our conditional stability constants

were closest in nature to those reported for terrestrial humic substances (logK'PbHA=9.8; logK'PbFA=9.2; Bi et al. 2013). The similarity between the binding intensity of humic substances and macroalgae-exuded ligands suggests that the latter are likely best defined by carboxyl and phenolic functional groups. The formation of humic-like substances in the culture solutions may be due to the microbial breakdown of seaweed cellular constituents into small molecules that subsequently aggregate to form larger humic-like entities, rather than the direct exudation by macroalgae. Our results are in agreement with those of Karavoltsos et al. (2013) who found that most of the 24 macroalgae examined exuded Cu-complexing ligands with adsorption characteristics similar to FA.

4.5.6 Environmental implications

The aforementioned results demonstrate that the ability of macroalgae-exuded organic to influence metal behavior and subsequent bioavailability cannot be ignored, and may be of particular importance in the commercial culture of macroalgae, where external factors are controlled. We propose that the relative contribution of macroalgae-exuded ligands to metal speciation is dependent on the particular system. For example, the algae-exuded Cd ligands described here would be of particular importance in natural systems characterized by low humic substance content, or where $[Cd]_L > [L_{Cd}]$. In such systems, algae-exuded organic ligands would play a more significant role in the control of Cd bioavailability and hence toxicity, as a result of decreased [FA] or $[Cd]_L$ in excess of available FA binding sites. A similar hypothesis was put forth by Wangerksy (1986) to explain the importance of algal ligands in fresh waters with low organic matter. Accordingly, the function of algae-exuded organic ligands in metal speciation is conditioned not only by the presence of contaminants, but also by other environmental factors such as organic matter content.

While this study supports the well-established theory that strong ligands control metal bioavailability, it has not addressed the role, if any, of all particulate and some colloidal phase ligands in metal speciation. Until recently, this has been common practice given that the majority of metals are known to occur in the dissolved phase. However, a recent study by Karavoltsos et al. (2013) highlights the importance of measuring particulate and colloidal ligands, where ligands in these phases constituted up to 46% of the ligand total exuded by certain species of macroalgae. Accordingly, this study would benefit from ligand analyses of unfiltered culture solutions (and hence, the quantification of particulate and colloidal phase L). In contrast, size fractionation of river water revealed that conventional filtration (<0.45

μM) of samples containing a large fraction of organic colloids (such as humic substances) can significantly increase the number of ligands determined by DPASV titration resulting in an overestimation of [L], and a subsequent underestimation of $[M]_L$ (Rozan and Benoit 1999). This effect is reduced by ultrafiltration (<3000 MW). Thus, future studies should include the measurement of ligands in ultrafiltered, conventionally filtered and whole samples so as to provide a better estimation of actual ligand concentration. The relatively low detection window (determined by the deposition potential) employed here is sufficient to detect those ligands that form very weak complexes, resolving a major issue identified in our previous work (Murray et al. d). This is significant as recent evidence indicates weak organic ligands increase Cd availability to phytoplankton and the same may be true for macroalgae.

As with our previous studies, this work highlights the serious discrepancy between the binding constants used to model M-L interactions in speciation codes and those measured in real systems. At present, modeling trace metal speciation is a complicated task and, as a result, the current ligand databases are the product of gross simplifications based on binding experiments with only humic substances despite a recognized continuum of marine ligands. In fact, the binding constants measured here for Cd and Pb are higher than even the highest ligand binding constants employed by the WHAM and SHM models. Such differences may mean an underestimation of metal binding by strong ligands and consequently, an overestimation of labile metal concentration and hence, toxicity. Since chemical-equilibrium models are often employed in ecotoxicological studies, risk assessment evaluations, environmental monitoring, and the development of water quality guidelines it is important to broaden speciation models to include possible non-humic ligands known to complex metals in seawater. Accordingly, the conditional stability constants determined here for macroalgae-exuded organic ligands are valuable in the parameterization of model databases and the subsequent advancement of chemical-equilibrium models.

4.6 Conclusions

To our knowledge, this study is the first to report the exudation of L_{Cd} and L_{Pb} by macroalgae. The voltammetric characterization of algae-exuded organic ligands under environmentally relevant Cd and Pb exposures provided evidence on both the source and stimuli of L in natural systems. The results presented here confirm the conclusions of a comparable study using Cu and Zn; seaweeds produce strong ligands in response to low-level metal exposure, and these ligands greatly reduce labile metal concentrations in marine systems. In the

absence of direct identification, similarities in magnitude of the conditional stability constants provide insight on the nature of the algae-exuded ligands. Here, similarities were identified between L_{Cd} and sulfides, and L_{Pb} and humic substances. Regardless of identity, both Cd and Pb stimulated the production of L.

In the future, a molecular investigation on the exact chemical identity of macroalgae-exuded organic ligands is warranted. As evidenced by Stockdale et al. (2011), characterization of naturally occurring metal complexing ligands is necessary to improve current speciation models. Accordingly, this work emphasizes the need to incorporate real ligand parameters into chemical-equilibrium models in order to improve the prediction of metal speciation under natural conditions.

While this study successfully quantified the capacity of a given seaweed to produce L and determined the relative affinity of L for Cd and Pb, this was only the first step towards fully characterizing L. In addition to identification, analyses to elucidate the binding mechanisms are required. Only once this is complete we can determine the effects of varying environmental conditions on ligand source, structure, stimuli and function.

4.7 Acknowledgements

This research was funded by the Science Foundation Ireland (SFI) and the Irish Research Council for Science Engineering and Technology (IRCSET). The authors gratefully acknowledge the Agri-Food and Biosciences Institute (Belfast) for ICP-OES analyses.

4.8 Supplementary Information

Table S1. Artificial seawater used for cultures, prepared according to Kester et al. (1967).

Salt	Concentration in solution
Sodium chloride	23.926 g kg^{-1}
Sodium sulfate	4.008 g kg^{-1}
Potassium chloride	0.677 g kg^{-1}
Sodium bicarbonate	0.196 g kg^{-1}
Potassium bromide	0.098 g kg^{-1}
Boric acid	0.026 g kg^{-1}
Sodium fluoride	0.003 g kg^{-1}
Magnesium chloride	0.0532 mol kg^{-1}
Calcium chloride	0.01033 mol kg^{-1}
Strontium chloride	0.00009mol kg^{-1}

Table S2. Accuracy of the analytical procedures employed for seaweed metal concentration and seawater total dissolved metal concentration (mean ± s.d., *n*=3). The algal CRM (NIES no. 9) was certified by the National Institute of Environment Studies, Japan Environment Agency. The near-shore seawater CRM (CASS-5) was certified by the National Research Council of Canada.

Element	NIES no. 9			CASS-5		
	Measured (µg g^{-1} DW)	RSD (%)	Certified (µg g^{-1} DW)	Measured (nM)	RSD (%)	Certified (nM)
Cd	0.16 ± 0.034	21.25	0.15 ± 0.002	0.23 ± 0.026	11.30	0.19 ± 0.016
Pb	1.32 ± 0.059	4.47	1.35 ± 0.05	0.052 ± 0.0029[A]	5.58	0.053 ± 0.01

Bold represents those measurements where deposition time was increased from 1 to 5 min.

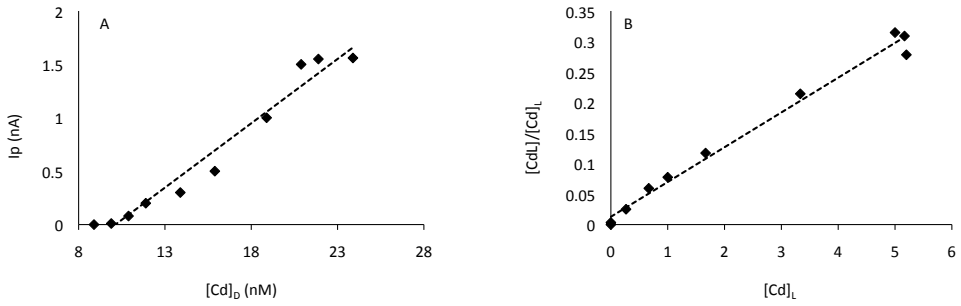

Figure S1. Representative titration curve for the day 1, 8.89 nM Cd treated *L. hyperborea* culture: (a) the current (nA) plotted versus the total amount of Cd (nM), and (b) the linear least squares fit using the van den Berg method (1982).

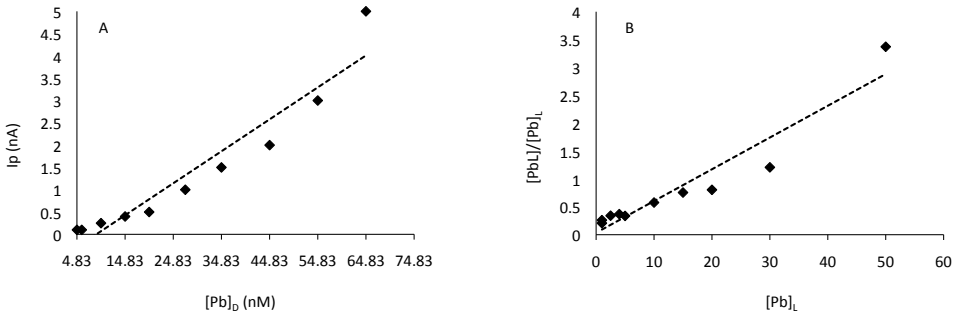

Figure S2. Representative titration curve for the day 1, 4.83 nM Pb treated *L. hyperborea* culture: (a) the current (nA) plotted versus the total amount of Pb (nM), and (b) the linear least squares fit using the van den Berg method (1982).

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No metal stand alone: the voltammetric characterization of macroalgae-exuded organic ligands (L) in response to a quaternary metal mixture

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Hollydawn Murray[A,B*], Guillaume Meunier[A], Rachel R. Cave[A], and Dagmar B. Stengel[B]

[A]Earth and Ocean Science, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, Ireland

[B]Botany and Plant Science, School of Natural Sciences, National University of Ireland Galway, University Road, Galway, Ireland

5.1 Abstract

Although seaweeds are known producers of metal-complexing ligands (L), their ability to exude L has only just recently been studied in detail. Until now, most estimates of L have been based on data obtained from laboratory tests employing single metals. Here, voltammetric techniques were employed to examine the release of 4 metal complexing ligands by a brown seaweed in response to a quaternary metal-mixture (Cd, Cu, Pb, Zn). *Fucus vesiculosus* was exposed to three different metal mixtures: control (Cd: 0 nM; Cu: 0 nM; Pb: 0 nM; Zn: 0 nM), low (Cd: 0.89 nM; Cu: 7.86 nM; Pb: 1.40 nM; Zn: 15.20 nM), and high (Cd: 8.89 nM; Cu: 15.70 nM; Pb: 4.83 nM; Zn: 91.70 nM), with both low and high mixtures being representative of natural conditions in coastal seawater. For each treatment we monitored seaweed photochemical quantum yield (F_v/F_m), metal speciation (Cd, Cu, Pb, Zn), complexing ligands (L_M), conditional stability constants ($logK'L_M$), glutathione (GSH), cysteine (Cys) and seaweed metal contents. Macroalgae released ligands in response to all metal mixtures confirming seaweeds as a source of L. Significantly higher [L_M] values in cultures with added metals than the relevant controls indicate that Cd, Cu, Pb and Zn stimulate L production. Comparisons of the metal speciation and complexation parameters obtained here with those determined previously for single metal cultures revealed significant increases in labile Cd and Pb, and decreases in L_{Cd}, L_{Zn}, and L_{Zn2}. In addition, a 2nd L_{Cu} was detected. These results demonstrate the macroalgal exudation of metal-specific ligands in response to Cu and Zn and non-metal specific ligands in response to Cd and Pb. The results also indicate that macroalgal responses to experimental work using single metals may not be representative of *in-situ* responses by macroalgae to natural mixtures of trace metals in the environment.

5.2 Introduction

Trace metals are ubiquitous in coastal areas due to both their natural and anthropogenic sources and to their biogeochemical cycling. Some metals, such as Cu and Zn, are required micronutrients for macroalgal growth while others, such as Cd and Pb, are mostly non-essential. In each case, elevated metal concentrations can be toxic to aquatic organisms regardless of their requirement. In macroalgae, trace metals generally exert toxicity via non-specific binding of coordination sites, the production of reactive oxygen species, or the reduction of antioxidant capacity (Prasad and Strzałka 1999; Pinto et al. 2003). Previous studies have shown that metal toxicity may inhibit photosynthesis through substitution of the central Mg^{2+} atom in the chlorophyll molecule resulting in interference with the light harvesting complex, oxygen evolution complex, cytochrome complex, plastoquinone, plastocyanin, ferrodoxin, and $NADP^{+}$ (Prasad and Strzałka 1999). In response, seaweeds have developed intra- and extracellular mechanisms by which they simultaneously tolerate and accumulate metals.

Macroalgal exudation of organic ligands with metal-binding capacity in response to metal contamination is of particular interest as biogenic ligands play an important role in controlling metal speciation and bioavailability in seawater. Recently, Karavoltsos et al. (2013) and Andrade et al. (2010) examined the release of copper-complexing ligands by 24 and 5 species of macroalgae, respectively. The former found all algal species produced Cu ligands in the range of 109-744 nM, while the latter measured ligands ranging from 42-117 nM released by 4 of the 5 species. Similarly, in parallel laboratory studies, we demonstrated that *A. nodosum*, *F. vesiculosus*, and *L. hyperborea* (all Phaeophyceae) affect metal speciation in seawater media via the production of organic ligands with metal complexing capacity in response to Cd, Cu, Pb, or Zn exposure (Murray et al. a, b).

Although macroalgae are usually exposed to a wide variety of metals in coastal environments, the current literature estimates of macroalgae-exuded organic ligands outlined above are based on data obtained in laboratory tests with single metals only. Previous experiments in this field, including our own, have neglected the realistic possibility of interactive metal effects. Previous laboratory culture studies investigating metal-microalgae interactions have clearly shown that metals can act both antagonistically and synergistically with reference to toxicity and organic ligand production. It was observed for *E. huxleyi* that 10 and 25 nM of Pb and 10 nM (but not 25 nM) of Cd slightly antagonized Cu uptake (Vasconcelos and Leal 2001). Further, the presence of Cd, but not Pb, enhanced the release of

copper-complexing ligands (Vasconcelos and Leal 2001). Thus, both microalgal uptake and ligand production cannot be described by a single metal concentration but rather, competition with other cations must be taken into account. Similar metal-mixture effects are likely to apply to macroalgae.

The objective of this study was to investigate the effect of a quaternary metal mixture on the photosynthesis and ligand production of *F. vesiculosus*, under controlled laboratory conditions. To the best of our knowledge, no such data exists for macroalgae. Until now, research on algae-exuded organic ligands has focused mainly on microalgae and/or single metal systems. The individual effects of Cd, Cu, Pb and Zn determined previously provide the groundwork for this next logical stage of study and are useful for comparison. Here, the end goal was to investigate whether the presence of multiple metals influenced the water chemistry of the *F. vesiculosus* culture solution in terms of: (1) metal speciation and (2) metal-specific ligand exudation.

5.3 Materials and methods

5.3.1 Reagents

Water used for the preparation of artificial seawater and other dilutions was Milli-q (MQ) and all reagents employed were analytical-grade. Stock solutions of Cd, Cu, Pb and Zn were prepared by dissolution of atomic absorption standard solutions (Fluka) in 0.01 M HCl. Glutathione, cysteine, and thioactemide working standards (all Aldrich) were prepared prior to use by dissolution in MQ water. Preparation and analyses were always conducted at room temperature, and all experimental solutions were kept at 4°C when not in use. Artificial seawater was prepared according to Kester et al. (1967), further supplemented with 176 µmol nitrate (as sodium nitrate, Aldrich) and 7.26 µmol phosphate (as sodium dihyrdogen phosphate, Aldrich), and tested to ensure the absence of trace metals.

5.3.2 Cleaning of materials

Glass flasks, culture vessels, filters and plastics were soaked for 1 week in 1 M HCl, rinsed 3 times with MQ, air-dried in a laminar flow hood, and stored in doubled sealed bags (where size allowed). Prior to this, culture vessels were soaked for 1 week in detergent (5% Decon).

Gloves were worn throughout and sample manipulations were always carried out in a laminar flow hood.

5.3.3 Seaweed collection

The sections of 'new growth' of *Fucus vesiculosus* (Linnaeus) Le Jolis were collected manually during low tide at Finavarra (53◦ 09′ 25′′ N; 09◦ 06′ 58′′ W), Co. Clare, western Ireland, as described previously (Murray et al. a, b) The apical parts (tips) were collected from the longest fronds of different thalli in August 2012. Tips were placed in acid-cleaned plastic bags with seawater from the site, stored at 4°C and transported to the laboratory within an hour of collection. Seaweeds were stored at 10 °C, under 60 ± 5 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR, 400-700 nm) on a 12 h:12 h light: dark cycle in filtered seawater for 1 day prior to experimentation.

5.3.4 Seaweed culture

Seaweeds were removed from the filtered seawater and rinsed 3 times with the modified artificial seawater. Single tips of approximately 6 cm were then placed in 500 mL polycarbonate culture jars containing 300 mL of modified artificial seawater enriched with either the control (Cd: 0 nM; Cu: 0 nM; Pb: 0 nM; Zn: 0 nM), low (Cd: 0.89 nM; Cu: 7.86 nM; Pb: 1.40 nM; Zn: 15.20 nM), or high (Cd: 8.89 nM; Cu: 15.70 nM; Pb: 4.83 nM; Zn: 91.70 nM) metal treatment. Note these concentrations were chosen to be environmentally relevant, i.e. representative of normal, uncontaminated coastal seawater. Each was triplicated (n=3) and cultures without seaweed were included for determination of background metal and ligand levels. Seaweeds were grown for 7 days at 10 °C, under 60 ± 5 μmol m^{-2} s^{-1} PAR on a 12 h:12 h light: dark cycle, with constant bubbling with air.

5.3.5 Photosynthesis

Fluorescence measurements were taken using a Pulse Amplitude Modulated (PAM) fluorometer (PAM 2000, Heinz Walz GmbH, Effeltrich, Germany) on day 0, 1, 4, and 7, following dark adaptation of tips in the culture medium at 10 °C for 20 min. Maximum photosystem II efficiency (F_v/F_m) was measured by applying a saturating pulse to the dark adapted tips in order to determine minimum (F_0) and maximum fluorescence (F_m) (Kitajima and Butler 1975), where:

$$F_v/F_m = (F_m - F_0)/F_m$$

Here, F_v/F_m is expressed as a percent of the day 0 control values in each experiment in order to account for any decreases in yield following harvest and for easier comparison of results across species.

5.3.6 Internal metal contents of macroalgae after culture

After rinsing twice with 1:9 ethanol/seawater (Gledhill et al. 1999), macroalgal tips were placed in acid cleaned bags and frozen immediately following culture. Within 6 months, tips were freeze-dried to a constant weight, then ground using an agate mortar and pestle. Approximately 1 g of the powdered biomass was dry-ashed at 450°C then brought up to 10 mL volume using 20% nitric acid. Cd, Cu, Pb, and Zn contents were measured using inductively coupled plasma mass spectrometry (ICP-OES), with the respective limits of detection as follows: Cd 0.1 ng g^{-1} , Cu, Pb 5 ng g^{-1} and Zn. Experimental blanks and the brown seaweed Certified Reference Material (CRM NIES No9, National Institute for Environmental Studies, Japan; *Sargassum fulvellum*) were also analyzed to ensure quality of methodology.

5.3.7 Analysis of culture solutions

Metals in culture solutions were analyzed on days 0, 1, 4, and 7. Ligands in culture solutions were analyzed on day 7. When culture solutions could not be analyzed within 24 h, they were filtered, transferred to 500 mL HDPE bottles and frozen immediately for analysis within 2 months. In these instances, samples were thawed overnight at 4°C and thoroughly mixed to ensure the dissolution of particulates formed due to freezing as per Laglera and van den Berg (2003).

5.3.8 Instrumentation

The voltammeter used here was either a Metrohm 757-VA Computrace or Metrohm 797 Computrace controlled by a computer (PC). The working electrode was a hanging mercury drop (HMDE) with a drop size of approximately 0.52 mm^2. The reference electrode was a double junction Ag/saturated AgCl, KCl (3 M), with a KCl-filled salt bridge (3 M). The counter electrode was a platinum wire. A polytetrafluoroethylene (PTFE) stirrer, rotating at 2 000 rpm, was employed during the adsorption step.

5.3.9 Metal speciation

The total dissolved metal concentrations ($[M]_D$) were measured simultaneously using differential pulse anodic stripping voltammetry (DPASV). Seawater samples were filtered through 0.45 μm glass-fiber filters and acidified to pH 2.2 using 6 M HCl, prior to UV digestion in PTFE-capped quartz silica tubes for 2 h. Ten mL aliquots of sample were pipetted into the voltammetric cell. Acetate buffer (70 μL, final concentration 0.07 M) was added and the solution was purged for 5 minutes using nitrogen. The voltammetric parameters were: deposition 60 s at -1.15 V whilst stirring; 10 s quiescence; potential scan using the differential pulse modulation: pulse height 0.05 V, scan from -1.15 to 0.05 V. Initial concentration was determined via two standard metal additions, each replicated twice. Peaks for Cd, Cu, Pb and Zn occurred at –0.6 V, -0.1V, –0.4 V, and -1V with the respective limits of detection at 0.1 nM, 0.3 nM, 0.2 nM and 0.6 nM. In those cases where Pb could not be quantified, the deposition time was increased to 5 min, lowering the detection limit to 0.024 nM.

The labile dissolved metal concentrations ($[M]_L$) of Cd and Pb were determined using ASV as described above for $[M]_D$ minus acidification and UV digestion. By contrast, $[Cu]_L$ and $[Zn]_L$ were determined using CLE-CSV. In all cases, the pH was fixed at 8.1 using HEPPS and initial concentration was determined via two standard metal additions, each replicated twice.

5.3.10 Metal complexing-capacities

Titration of filtered seawater with individual metal ions (Cd, Cu, Pb or Zn) was used to determine the concentration of metal-specific complexing ligand (L_M) and the associated conditional stability constant ($\log K'L_M$). Cd and Pb measurements were made using ASV whilst Cu and Zn measurements were made using CLE-CSV, as outlined for $[M]_L$. HEPPS (0.01 M) and the appropriate competitive ligand, if any, were added to 120 mL of filtered seawater. Ten mL aliquots were then pipetted into 10-20 mL pre-conditioned polystyrene vials (Sterilin) containing metal additions in approximately equal increments (where the actual range depends on the initial metal concentration). The $[M]_L$ of each Sterilin was measured as previously outlined, following overnight equilibration (12-15 h).

5.3.11 Identification and quantification of thiol compounds

CSV was employed for the identification and quantification of thiol compounds. Determinations were carried out at pH 8.1 (0.01 M HEPPS), according to Leal et al. (1999).

Cysteine, glutathione and thioactemide peaks present at -0.47 V, -0.58 V and -0.68 V respectively, and the voltammetric parameters were: deposition 60 s at -0.25 V whilst stirring; potential scan using the square wave modulation: scan rate 20mV/s, modulation amplitude 25 MV, and frequency 50 Hz. Two standard thiol additions (cysteine, glutathione, or thioactemide), each replicated twice, were used to determine initial concentrations.

5.3.12 Calculations

As described previously for Cd, Cu, Pb and Zn (Murray et al. a, b), a modified van den Berg linearization (1982) was used to determine the complexing ligand concentration and corresponding conditional stability constant for each metal based on the following relationship (van den Berg 1982):

$$[M]_L/[ML]=[M]_L/C_L + \alpha/(K'_{ML}L_M)$$

Where [ML] is calculated as the difference between $[M]_d$ and $[M]_L$ while the α-coefficient represents the overall side reactions producing inorganic complexes for each metal. Values of α were calculated as described previously by Vasconcelos et al. (2002) using the following values: α_{Cd}=35 (Turner et al. 1981), α_{Pb}=34 (Turner et al. 1981), α_{Cu}=36 (Turner et al. 1981), α_{Zn}=2.1(Turner et al. 1981), $\log K'_{CuSA}$=9.22 (Campos and van den Berg 1994), $\log \beta'_{Cu(SA)2}$=9.52 (Campos and van den Berg 1994), and $\log K'_{ZnAPDC}$=4.4 (van den Berg 1985; Donat and Bruland 1990). Linearly transformed plots of $[M]_L/[ML]$ versus $[M]_L$ were used to elucidate the number of ligands present wherein a linear plot was indicative of a single ligand. The concentrations of L_M and $\log K'L_M$ were determined from the slope and y-intercept of a least squares regression of this titration curve. Example titration and linearization plots for each metal (**Figures S1, S2, S3, S4**) are presented in the Supplementary Information.

In calculating sensitivity, multiple iteration of the final four titration points was used in order to avoid underestimation (Turoczy and Sherwood 1997).

5.3.13 Quality assurance

Certified reference materials (CRM) for trace metals from the National Institute for Environmental Studies, Japan Environment Agency algal standard (NIES no. 9) and the National Research Council of Canada Reference standard seawater (CASS 5) for coastal seawater were also analyzed to determine the accuracy of analysis of seaweed metal contents and seawater metal concentrations, respectively. Showing a good accuracy of measurement,

the metal concentrations determined were never significantly different than the expected values ('t' test, p>0.05) (**Table S2**).

5.3.14 Data analysis

Seaweed metal contents, seawater metal contents, complexing ligands, conditional stability constants, and thiol concentrations among the experimental treatments were tested for significant differences using a one-way between subjects ANOVA. Multiple analyses of variance (MANOVA) were also used to compare data over time and to prior findings for single metal experiments. For both ANOVA and MANOVA, a post hoc Tukey's multiple comparison was employed where significant differences were identified. Pearson-product moment correlation coefficients were used to identify and measure the relationships between complexing ligands and the other variables. All tests were performed using SPSS version 20.0.

5.4 Results

5.4.1 Effects on photosynthesis

Photochemical quantum yield (F_v/F_m) values of seaweeds exposed to quaternary mixtures of Cd, Cu, Pb and Zn decreased in the order: day 0 > 1 > 4 > 7; treatment control > low metal addition > high metal addition (**Figure 5.1**). However, for each metal addition, there was no significant effect of treatment on the F_v/F_m of *F. vesiculosus* over 7 days (p>0.05). This is in agreement with previous data published on single metal exposures where Cd, Cu, Pb, or Zn additions (Cd: 0.89 or 8.89 nM ; Cu: 7.86 or 15.70 nM ; Pb: 1.40 or 4.83 nM ; Zn: 15.20 or 91.70 nM) also showed no significant effect of treatment on photosynthesis (Murray et al. a, b). When current F_v/F_m values were compared to previous data for single metal cultures, no significant differences were found between the metal mixtures and the corresponding single metal effects.

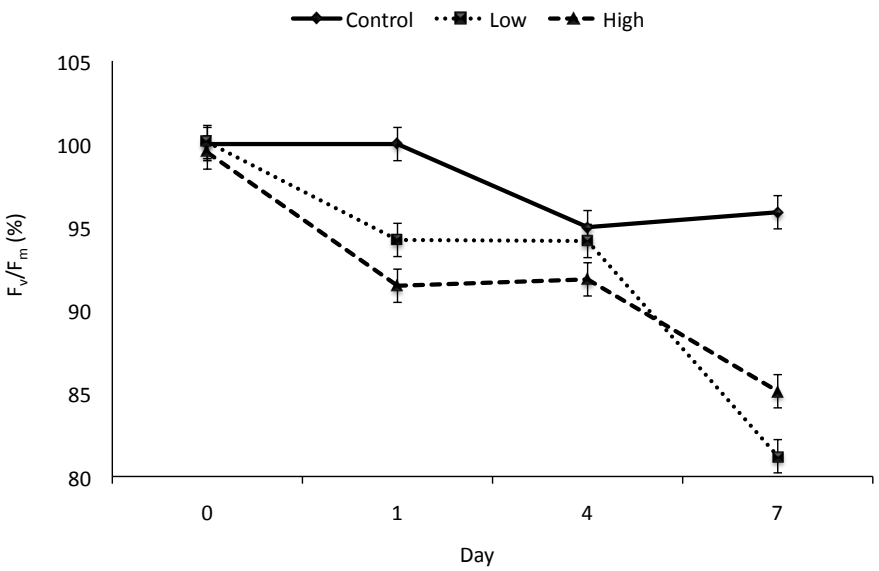

Figure 5.1. Photochemical quantum yield (F_v/F_m) of *F. vesiculosus* cultured in artificial seawater for 7 days at 10°C, under 60 ± 5 µmol m^{-2} s^{-1} PAR on a 12 h:12 h light: dark cycle, in different concentrations of a quaternary metal mixture: control (Cd: 0 nM; Cu: 0 nM; Pb: 0 nM; Zn: 0 nM), low (Cd: 0.89 nM; Cu: 7.86 nM; Pb: 1.40 nM; Zn: 15.20 nM), and high (Cd: 8.89 nM; Cu: 15.70 nM; Pb: 4.83 nM; Zn: 91.70 nM). Data are expressed as a % of the corresponding day 0 control value; *n=3*. Error bars represent the standard error of triplicate cultures.

5.4.2 Seaweed metal contents

Cd, Cu, Pb and Zn were present in all seaweeds and generally decreased in the order Zn > Cu > Cd > Pb (**Figure 5.2**). Multiple analyses of variance (MANOVA) for all 4 metals showed no significant effects of time or treatment on the mean vectors of the tissue metal concentrations, with the exception of Pb that increased significantly with time in the low metal mixture ($F=1214.53$, $p<0.05$). Several significant differences were found when the seaweed metal contents determined here were compared to those previously published for unary metal exposures: Zn was higher in seaweeds exposed to the low metal mixture on day 1 ($F=15.37$, $p<0.05$); Pb was higher in all seaweeds exposed to multiple metal mixtures on day 4 ($F=17.02$, $p<0.05$); and Cu was higher in seaweeds exposed to the low metal mixture on day 7 ($F=5.31$, $p<0.05$) (Murray et al. a, b).

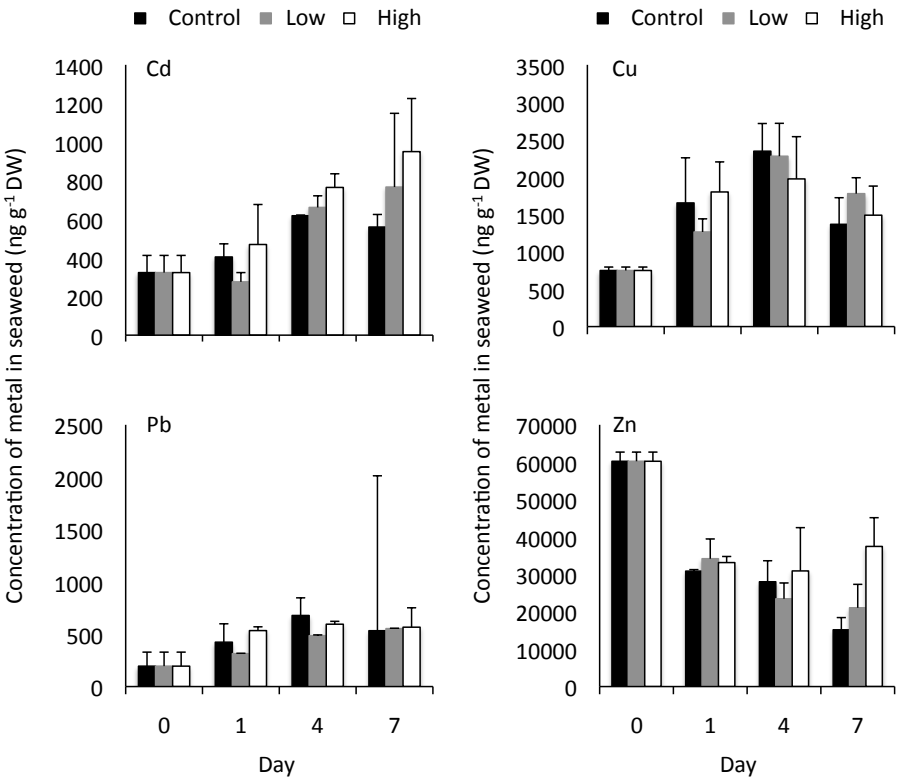

Figure 5.2. Concentration of the four metals in *F. vesiculosus* cultured for 7 d in different concentrations of a quaternary metal mixture: control (Cd: 0 nM; Cu: 0 nM; Pb: 0 nM; Zn: 0 nM), low (Cd: 0.89 nM; Cu: 7.86 nM; Pb: 1.40 nM; Zn: 15.20 nM), and high (Cd: 8.89 nM; Cu: 15.70 nM; Pb: 4.83 nM; Zn: 91.70 nM); *n=3*. For other culture conditions, see Fig. 5.1. Error bars represent the standard error of triplicate cultures. Note the Y-axes have different scales.

5.4.3 Metal speciation

The results of the metal speciation analyses over 7 days are presented in

Table 5.1. In all cultures, metal additions significantly increased the total dissolved metal concentration (F_{Cd}=2.64, $p<0.05$; F_{Cu}=79.64, $p<0.05$; F_{Pb}=5.70, $p<0.05$; F_{Zn}=7.41, $p<0.05$). Similarly, $[M]_L$ increased in the order control > low metal addition > high metal addition (F_{Cd}=336.18, $p<0.05$; F_{Pb}=11.74, $p<0.05$; F_{Zn}=93.71, $p<0.05$), with the exception of $[Cu]_L$ which did not vary with treatment ($p>0.05$).

Table 5.1. A summary of the DPASV and CLE-CSV speciation values measured for *F. vesiculosus* under simultaneous multiple metal exposure, mean ± standard error, *n=3*.

Day	$[M]_D$ (nM)	$[M]_L$ (nM)	[ML] (nM)	$[M]_D$ (nM)	$[M]_L$ (nM)	[ML] (nM)	$[M]_D$ (nM)	$[M]_L$ (nM)	[ML] (nM)
Cd									
	Control			Low			High		
0	-	-	-	0.81 ± 0.12	0.78 ± 0.06	-	7.69 ± 0.02	7.43 ± 0.23	-
1	-	-	-	0.41 ± 0.01	-	-	5.34 ± 0.18	5.27 ± 0.40	0.07
4	-	-	-	0.20 ± 0.26	-	-	4.52 ± 0.74	2.88 ± 0.26	1.64
7	-	-	-	0.29 ± 0.003	-	-	3.92 ± 0.19	3.30 ± 0.26	0.62
Cu									
	Control			Low			High		
0	-	-	-	7.31 ± 0.03	7.21 ± 0.69	-	16.34 ± 0.89	16.93 ± 0.30	-
1	0.36 ± 0.41	0.42 ± 0.55	0.00	4.40 ± 0.33	0.50 ± 0.03	3.90	7.36 ± 0.16	0.41 ± 0.06	6.95
4	0.62 ± 0.64	0.80 ± 0.85	0.00	5.07 ± 0.02	0.31 ± 0.04	4.76	6.81 ± 0.74	0.45 ± 0.06	6.36
7	1.02 ± 0.78	0.52 ± 0.02	0.50	6.31 ± 0.42	0.46 ± 0.05	5.85	4.93 ± 0.04	0.10 ± 0.03	4.83
Pb									
	Control			Low			High		
0	-	-	-	0.94 ± 0.04	1.21 ± 0.02	-	5.13 ± 0.04	5.54 ± 0.03	-
1	0.39 ± 0.22	0.04 ± 0.02	0.35	1.00 ± 0.05	1.13 ± 0.41	0.00	2.10 ± 0.02	1.73 ± 0.06	0.37
4	0.76 ± 0.80	0.81 ± 0.79	0.00	1.75± 0.002	0.85± 0.08	0.90	2.20 ± 0.05	1.66 ± 0.18	0.54
7	1.07 ± 0.07	0.35 ± 0.15	0.72	2.75 ± 0.06	1.06 ± 0.005	1.69	1.23 ± 0.003	0.30 ± 0.07	0.93
Zn									
	Control			Low			High		
0	-	-	-	15.31 ± 0.83	16.32 ± 0.09	-	90.43 ± 1.23	89.43 ± 2.34	-
1	5.33 ± 0.10	0.81 ± 0.43	4.52	8.67 ± 0.87	1.50 ± 0.19	7.17	69.90 ± 3.04	6.92 ± 3.31	62.98
4	1.45 ± 1.25	2.15 ± 1.81	0.00	9.03 ± 0.04	1.41 ± 0.30	7.62	58.59 ± 2.08	6.77 ± 1.23	51.82
7	1.28 ± 0.84	0.12 ± 0.11	1.16	8.71 ± 0.18	0.83 ± 0.11	7.88	32.91 ± 1.47	4.86 ± 0.47	28.05

- represents concentrations below the detection limits.

Beyond day 0, labile metal ranged from 2.88-5.27 nM for Cd, 0.10-0.50 nM for Cu, 0.04-1.73 nM for Pb, and 0.12-6.92 nM for Zn. However, there was no consistent trend amongst metals for maximum or minimum values. Instead, the maximum $[Cd]_L$ and $[Cu]_L$ were measured in the high metal-treated and low metal-treated cultures, respectively, on day 1, whereas the minimum $[Cd]_L$ and $[Cu]_L$ were measured in the high metal-treated cultures on day 4 and 7, respectively. By contrast, minima and maxima for $[Pb]_L$ and $[Zn]_L$ occurred in the same cultures where maximum concentrations were measured in the high metal-treated cultures on day 7 and minimum concentrations in the control cultures on day 1. A Pearson correlation analysis revealed that the concentration of bound metal covaried with $[M]_D$ and $[M]_L$ for Cd, Pb and Zn ($p>0.05$; $n=48$). For Cu, however, [CuL] varied only with $[Cu]_D$. The greatest proportion of bound metal on day 7 increased in the order Cd (16%) > Pb (39%) > Zn (90%) > Cu (98%). For each metal, the maximum [ML] did not correspond to the culture with the greatest proportion of bound metal.

Pair-wise comparisons of the metal speciation results obtained here with those determined previously for single metal cultures, by day and treatment, revealed significant differences for Cd and Pb but not Cu and Zn. On day 1, both $[Cd]_L$ and $[Pb]_L$ were significantly higher in the high metal mixture cultures than the relevant single metal cultures ($F_{Cd}=159.56$, $p<0.05$; $F_{Pb}=47.82$, $p<0.05$). Beyond day 1, there was no similar significant difference in $[Pb]_L$. By contrast, $[Cd]_L$ remained significantly higher in the low metal mixture on day 7 ($F_{Day\ 4}=39.86$, $p<0.05$; $F_{Day7}=65.12$, $p<0.05$). In both instances, [CdL] was significantly higher in cultures containing the highest concentration of mixed metals than the cultures containing the corresponding 8.89 nM of Cd ($F_{Day\ 4}=8.17$, $p<0.05$; $F_{Day7}=15.08$, $p<0.05$).

5.4.4 Organic ligands and conditional stability constants

The complexation parameters of Cd, Cu, Pb and Zn were measured on day 7 only and are presented in **Figure 5.3**. For each ligand, the addition of metal increased the $[L_M]$ in cultures in comparison to the relevant controls. For Cd and Pb, titrations were found to fit the presence of a single complexing ligand, whereas Cu and Zn titrations fit the presence of 2 ligands.

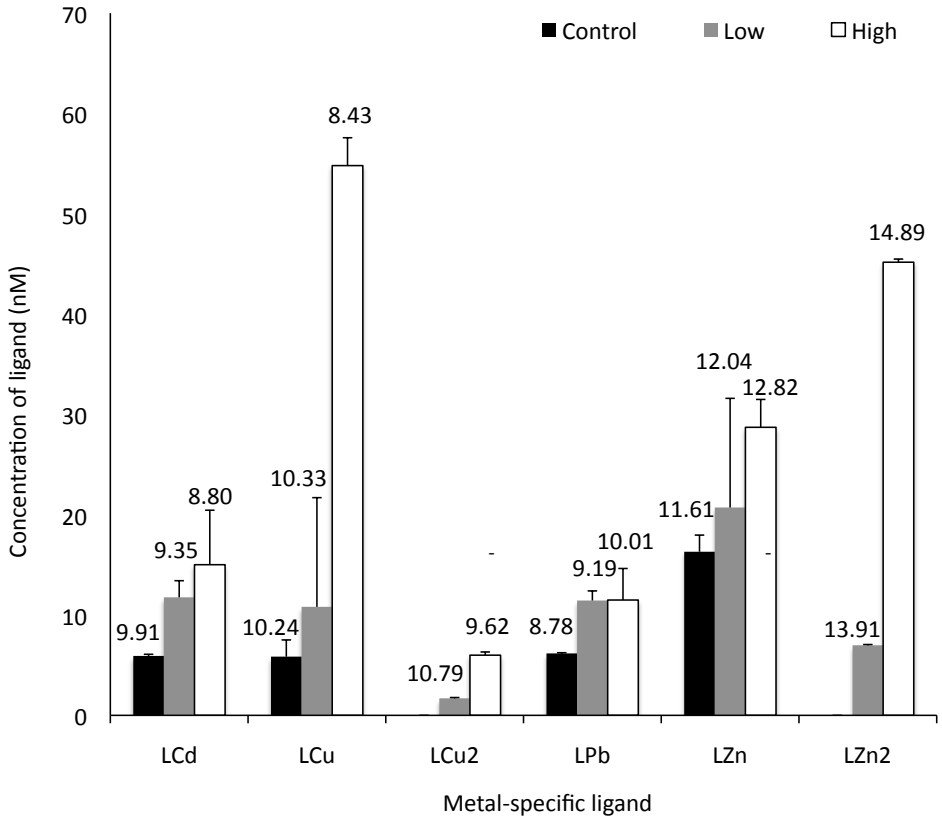

Figure 5.3. Ligand concentrations measured for F. vesiculosus cultures under different concentrations of a quaternary metal mixture: control (Cd: 0 nM; Cu: 0 nM; Pb: 0 nM; Zn: 0 nM), low (Cd: 0.89 nM; Cu: 7.86 nM; Pb: 1.40 nM; Zn: 15.20 nM), and high (Cd: 8.89 nM; Cu: 15.70 nM; Pb: 4.83 nM; Zn: 91.70 nM); *n=3*. Error bars represent the standard error of triplicate cultures. Dash represents concentrations not detected. Values above each bar are the corresponding conditional stability constant.

The concentration of L_{Cd} ranged from 5.92-15.02 nM and was within the range previously reported for the single metal (5.13-32.52 nM). However, at the highest metal treatment, $[L_{Cd}]$ was significantly lower ($F=9.61$, $p<0.05$) than that determined previously for *F. vesiculosus* cultured in 8.89 nM Cd. A Pearson correlation applied to the ligand concentration revealed only a strong positive correlation with [Cys], and a weaker but non-significant positive correlation with $[Cd]_D$, $[Cd]_L$ and [CdL]. Similar to L_{Cd}, the concentration range of L_{Pb} (6.17-11.49 nM) was within the range previously reported for the unary mixture (5.06-12.08 nM). In contrast to Cd, a Pearson correlation showed a significant positive correlation of $[L_{Pb}]$ with GSH, yet no similar significant correlation with $[Pb]_D$, $[Pb]_L$, [PbL] or [Cys].

The concentration of both Cu and Zn ligands increased significantly with increasing metal concentration ($F_{LCu}=31.26$, $p<0.05$; $F_{LCu2}=325.57$, $p<0.05$; $F_{LZn}=7.63$, $p<0.05$; $F_{LZn2}=132689.02$, $p<0.05$). In each case, $[L_M]$ was negatively correlated with final $[M]_L$ but

positively correlated with initial $[M]_D$. At the highest metal addition, $[L_{Zn}]$ and $[L_{Zn2}]$ were significantly lower and $[L_{Cu}]$ significantly higher in the quaternary metal cultures than the corresponding 91.70 nM Zn only and 15.70 nM Cu only cultures (F_{LZn}=15.10, $p<0.05$; F_{LZn2}=114325.89; $p<0.05$; F_{LCu}=65.86, $p<0.05$;); the same phenomenon observed for L_{Cd}. By contrast, L_{Cu2} was detected only in those cultures where multiple metals were added.

Despite differences in $[L_M]$ amongst treatments, the conditional stability constants of the detected ligands remained relatively constant. The logK's for L_{Zn} and L_{Zn2} increased significantly with increasing metal addition ($F_{LogK'LZn}$=1034.79, $p<0.05$; $F_{LogK'LZn2}$=157101.64, $p<0.05$), displaying the same trend as the unary mixture. Likewise, simultaneous exposure to multiple metals did not result in any significant changes ($p>0.05$) in the logK's for L_{Cd}, L_{Cu}, L_{Pb}, L_{Zn}, and L_{Zn2} when compared to the corresponding individual metal treatment.

5.4.5 Glutathione and Cysteine

As shown in **Figure 5.4**, GSH was present in all cultures and cysteine was detected only in cultures where metal was added. This is in agreement with previous single-metal experiments where cysteine was detected only in the 4.83 nM Pb cultures, and GSH was omnipresent. Similar to the findings using single metal additions, MANOVA revealed that glutathione decreased significantly with increasing metal treatment yet increased significantly with time ($F_{Treatment}$=16.97, $p<0.05$; F_{Time}=51.57, $p<0.05$). Cysteine was significantly lower in the high quaternary metal treated culture than in the 4.83 nM Pb only culture ($F=84.96$; $p<0.05$).

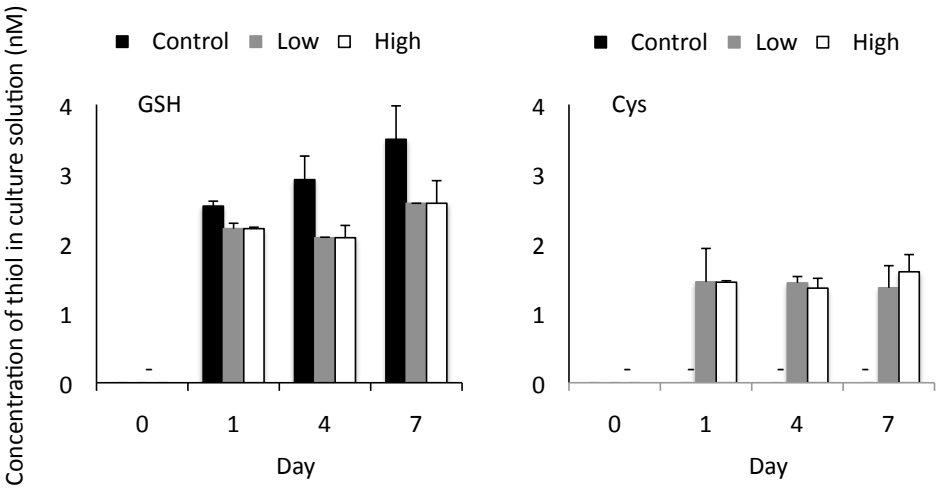

Figure 5.4. Glutathione and cysteine concentrations measured for F. vesiculosus cultures under different concentrations of a quaternary metal mixture: control (Cd: 0 nM; Cu: 0 nM; Pb: 0 nM; Zn: 0 nM), low (Cd: 0.89 nM; Cu: 7.86 nM; Pb: 1.40 nM; Zn: 15.20 nM), and high (Cd: 8.89 nM; Cu: 15.70 nM; Pb: 4.83 nM; Zn: 91.70 nM); *n=3*. Error bars represent the standard error of triplicate cultures. Dash represents concentrations not detected.

5.5 Discussion

5.5.1 Macroalgal responses and seaweed metal accumulation

Despite the fact that the photochemical quantum yield of *F. vesiculosus* exposed simultaneously to Cd, Cu, Pb and Zn differed from that of the control cultures, macroalgal response in multiple metal cultures did not show any deviation from single metal cultures. This indicates that, at least at the environmentally relevant metal concentrations employed, competitive metal interactions do not alter metal effects on the fraction of photosynthesis considered here.

By contrast, simultaneous additions of metals to *F. vesiculosus* cultures resulted in several variations in metal uptake when compared to the single metal systems. Increases in Cu, Pb and Zn uptake from the multiple metal cultures suggest that the degree of accumulation does not depend on competition between metals for binding or uptake sites but rather, in this instance, is synergized by the presence of Cd. This is in direct contrast to the antagonistic interactions of Pb and Cd on Cu uptake by microalgae previously reported by Vasconcelos et al. (2001) yet in agreement with the findings of Munda and Hudnik (1986) where the accumulation of Zn by *F. vesiculosus* increased in the presence of Cd.

The fact that increases in metal uptake are not reflected in the photosynthetic response indicates a divide between metal presence and toxicity. While Cu and Zn are likely used as nutrients, no similar explanation exists to clarify the decoupling of Pb uptake and toxicity. Baumann et al. (2009) also reported no obvious relationship between internal metal contents and effects on photosynthesis for Cd and Cu at internal concentrations far greater than those determined here (Cd < 22482 ng g^{-1} and Cu < 508320 ng g^{-1}). Ultimately, the observed metal tolerance combined with increased metal accumulation is indicative of an efficient internal sequestration mechanism capable of preventing photosynthetic inhibition or more specifically, substitution of the central Mg^{2+} atom of the chlorophyll molecule as well as binding to other important sites such as enzymes (Küpper et al. 2002).

5.5.2 Metal speciation

Differences in metal uptake between the quaternary and unary cultures may be the result of differences in metal speciation. Results obtained for the speciation of Cu, Pb, and Zn in the multiple metal system revealed marked decreases in the labile proportions by 55%, 88%, and 37%, respectively, in comparison with the single metal systems while $[Cd]_L$ showed a 57% increase. In general, the opposite differences are expected when considering the relative tissue metal contents alone. The contrasting behaviors of $[M]_L$ and tissue metal contents in the metal mixtures is particularly interesting as it suggests that the combinatorial effect of multiple metals can not be predicted by the simple addition of the individual metal results. However, predictions of speciation using models established from data on individual metals effectively ignore the interactive behavior of metals and may lead to incorrect predictions of bioavailability.

Unlike $[M]_L$, complexation of Cu, Pb and Zn showed no deviation from the single metal systems. This is not surprising given that the degree of competition in the multiple metal cultures was small; in general the amount of metal complexed was $\geq$ 0.6 % of the amount bound in individual metal cultures. On the contrary, [CdL] was higher in the metal mixture than the corresponding Cd only culture. Increased Cd complexation, in the presence of unchanged Cu, Pb and Zn complexation, indicates the existence of high affinity binding sites that are absent in the Cd-only cultures, and different from those already occupied by the other three metals.

5.5.3 Macroalgae-exuded organic ligands

The present study provides definitive evidence that brown seaweeds are a source of metal complexing ligands, as earlier reported by Andrade et al. (2010), Karavoltsos et al. (2013), and Murray et al. (a, b). As in the single metal system, it appears that the presence of multiple metals stimulated the release of ligands that increased in response to increasing $[M]_D$. For all metals except Pb, however, the concentration of ligands were substantially different in the multi-metallic cultures from the corresponding individual metal equivalents. The presence of all four metals suppressed the exudation of L_{Cd}, L_{Zn}, and L_{Zn2} by 45%, 39%, and 36%, respectively, yet nearly doubled the exudation of L_{Cu}. The increase in L_{Cu} and the appearance of a 2nd Cu ligand may be attributed to the unique production of new ligands or, more likely, the interaction of Cu with those ligands produced in response to Cd, Pb, and Zn. A low inhibition of Cu complexation, combined with an increase in detected ligand, can be explained by a greater affinity of Cu for L_{Cd}, L_{Pb}, L_{Zn}, and L_{Zn2} compared to Cd, Pb, and Zn. As shown in **Table 5.1**, both $[Cd]_L$ and $[Zn]_L$ increased in spite of an excess of metal ligands, and Cu complexation showed little inhibition in combination with increased L_{Cu}. This suggests that copper ligands do not bind Cd or Zn yet cadmium and zinc ligands bind Cu. Of course, inhibition of the remaining ions as a result of increased Cu competition would not be, and is not, evident due to the relatively large excess of L over M_D. Overall, this suggests that while macroalgae-exuded ligands may be produced in response to a certain metal, these ligands are not necessarily entirely specific to a single metal.

It is not surprising given the large excess of L_{Cu} in the culture solutions that the majority of Cu occurs organically complexed. Similarly, when taken in total, $[L]_M$ is more than one order of magnitude higher than the M_D suggesting a large fraction of other metals not included in this study could be bound by the exuded ligands. However, should that be the case one would expect more than 19% of Cd to be complexed. The fact that Cd remains dominantly labile, in spite of excess L and the absence of metal competition ($L_M > M_D$), indicates that at least some of the ligands exuded by macroalgae are metal-selective.

5.5.4 The nature of L

Based on the comparison of the logK'L_Ms determined here to those already present in the literature, it remains clear that the nature of L_{Cd} appears to be sulfide while L_{Pb} appears to be humic. Similar to the observation made during the single metal studies, the nature of L_{Cu} and L_{Zn} cannot be determined by simple comparison of the logK'L_Ms to known stability constants.

Still, the higher affinity of sulfide for Cu ($logK_{SCu}$=11.50) supports the hypothetical complexation of L_{Cd} to Cu even in the presence of Cd. Unfortunately, the slightly higher logK' of L_{Cu2} is nearer that of sulfide than the original ligand, yet not quite within its range meaning the logK' alone cannot verify this theory. Of course, any further discussion on the identity of L is complicated by the known presence of both multiple metals and multiple ligands, which are likely a mixture of discrete compounds and continuous binding sites.

In agreement with our previous studies (Murray et al. a, b), GSH and Cys do not appear to be good candidates for the metal complexing ligands measured here. Glutathione decreased with increasing L and although Cys increased with L, the concentrations found here compose only a minute fraction of $[L]_M$. Thus, even if Cys displayed metal complexing capacity, its contribution to the total ligand pool would be negligible.

5.6 Conclusions

This study is the first to report the exudation of organic ligands by macroalgae exposed simultaneously to environmentally relevant concentrations of Cd, Cu, Pb, and Zn. The results presented here confirm the conclusions of previous studies using individual metal treatments; seaweeds produce strong ligands in response to metal stimuli and similarities exist between L_{Cd} and sulfides, and L_{Pb} and humic substances. In addition, the present study has demonstrated that macroalgae-exuded ligands could be either metal- or non-metal- specific. Thus, based on the differences between ligand exudation in multiple metal and single metal studies highlighted here, we propose that ligands released in response to non-essential cations are non-metal specific, while those released in response to required nutrients are metal-specific. Of course, validation of this theory requires further investigation. Only once ligands are identified and binding mechanisms are elucidated can we make any concrete conclusions regarding the ion selectivity of macroalgae-exuded ligands.

5.7 Acknowledgements

This research was funded by the Science Foundation Ireland (SFI) and the Irish Research Council for Science Engineering and Technology (IRCSET). The authors gratefully acknowledge the Agri-Food and Biosciences Institute (Belfast) for ICP-OES analyses.

5.8 Supplementary Information

Table S1. Artificial seawater used for cultures, prepared according to Kester et al. (1967).

Salt	Concentration in solution
Sodium chloride	23.926 g kg^{-1}
Sodium sulfate	4.008 g kg^{-1}
Potassium chloride	0.677 g kg^{-1}
Sodium bicarbonate	0.196 g kg^{-1}
Potassium bromide	0.098 g kg^{-1}
Boric acid	0.026 g kg^{-1}
Sodium fluoride	0.003 g kg^{-1}
Magnesium chloride	0.0532 mol kg^{-1}
Calcium chloride	0.01033 mol kg^{-1}
Strontium chloride	0.00009mol kg^{-1}

Table S2. Accuracy of the analytical procedures employed for seaweed metal concentration and seawater total dissolved metal concentration (mean ± s.d., *n*=3). The algal CRM (NIES no. 9) certified by the National Institute for Environmental Studies, Japan Environment Agency and the near-shore seawater CRM (CASS-5) certified by the National Research Council of Canada.

Element	NIES no. 9			CASS-5		
	Measured (µg g^{-1} DW)	RSD (%)	Certified (µg g^{-1} DW)	Measured (nM)	RSD (%)	Certified (nM)
Cd	0.19 ± 0.034	17.89	0.15 ± 0.002	0.22 ± 0.032	14.54	0.19 ± 0.016
Cu	4.87 ± 0.10	2.05	4.9 ± 0.2	6.12 ± 0.15	2.45	5.97 ± 0.44
Pb	1.33 ± 0.04	3.01	1.35 ± 0.05	**0.055 ± 0.002**	3.64	0.053 ± 0.01
Zn	16.10 ± 0.34	2.11	15.6 ± 1.2	12.10 ± 0.20	1.66	10.92 ± 1.034

Bold represents measurements where deposition time was increased from 1 to 5 min.

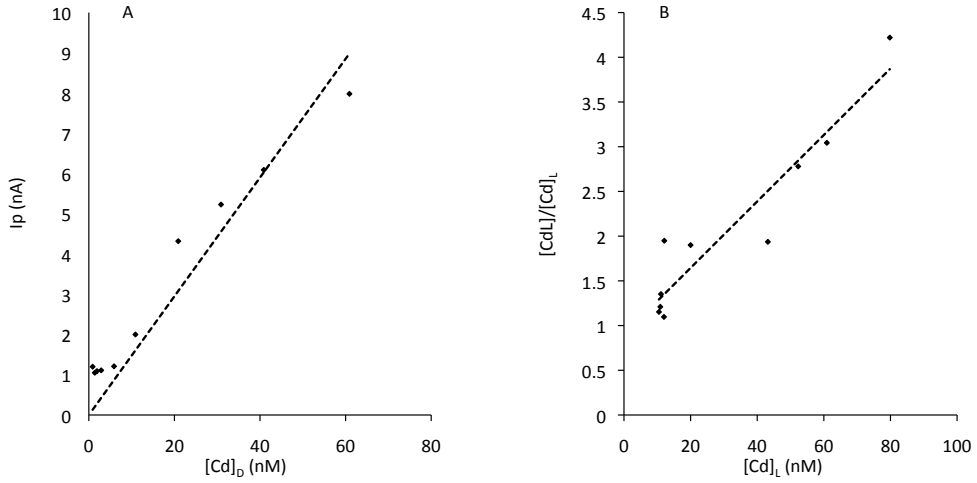

Fig. S1. Representative Cd titration curve for the day 7, high treated *F. vesiculosus* culture: (a) the current (nA) plotted versus the total amount of Cd (nM), and (b) the linear least squares fit using the van den Berg method (1982).

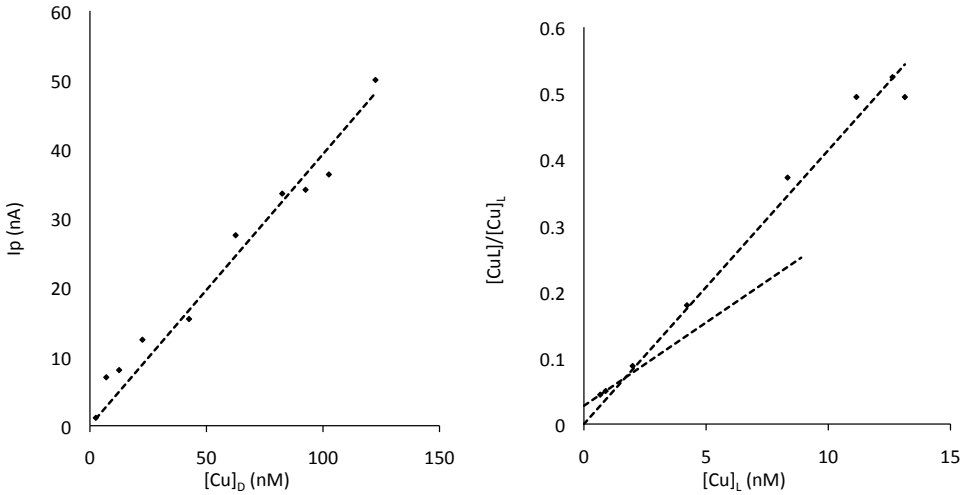

Fig. S2. Representative Cu titration curve for the day 7, high treated *F. vesiculosus* culture: (a) the current (nA) plotted versus the total amount of Cu (nM), and (b) the linear least squares fit using the van den Berg method (1982).

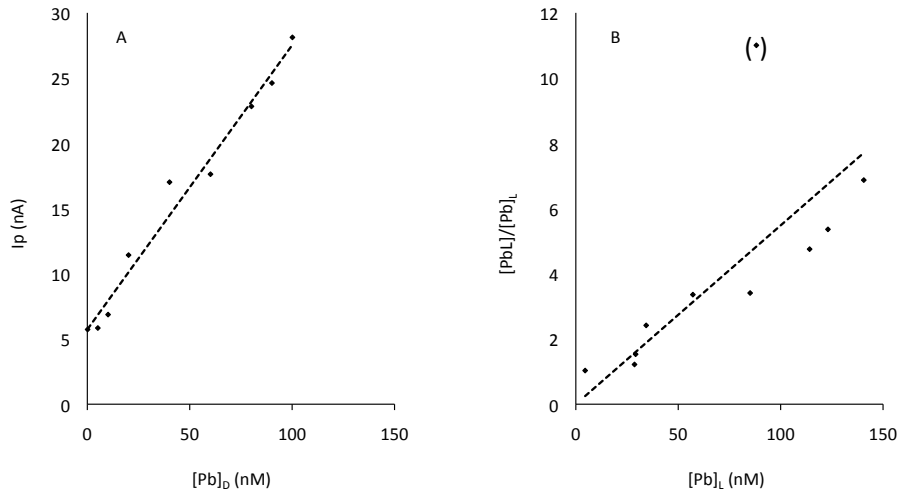

Fig. S3. Representative Pb titration curve for the day 7, high treated *F. vesiculosus* culture: (a) the current (nA) plotted versus the total amount of Pb (nM), and (b) the linear least squares fit using the van den Berg method (1982).

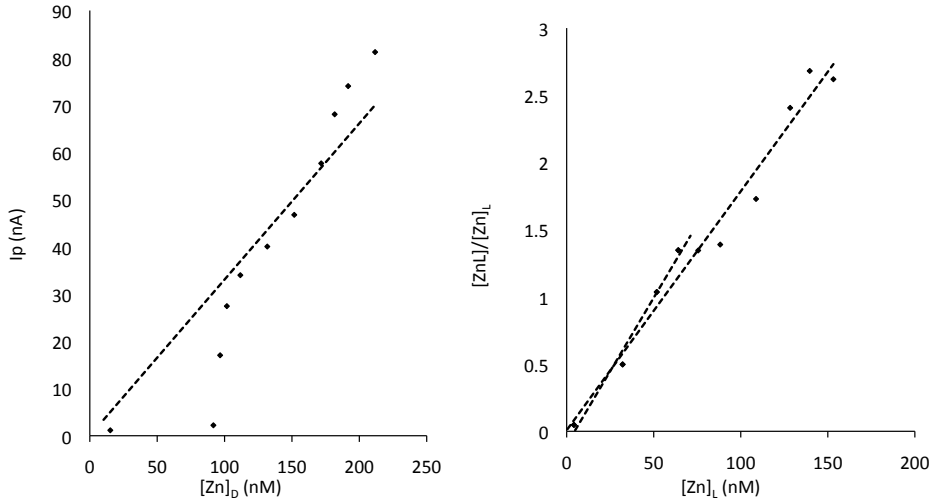

Fig. S4. Representative Zn titration curve for the day 7, high treated *F. vesiculosus* culture: (a) the current (nA) plotted versus the total amount of Zn (nM), and (b) the linear least squares fit using the van den Berg method (1982).

Chapter 6 Murray et al. (d)

Metal complexation by organic ligands (L) in near-pristine estuarine waters: evidence for the identity of L

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Hollydawn Murray[A,B*], Guillaume Meunier[A], Dagmar B. Stengel[B], and Rachel R. Cave[A]

[A]Earth and Ocean Sciences, School of Natural Sciences, Ryan Institute, National University of Ireland Galway, Galway, Ireland

[B]Botany and Plant Science, School of Natural Sciences, Ryan Institute, National University of Ireland Galway, Galway, Ireland

6.1 Abstract

Trace metal interactions with organic ligands largely control metal bioavailability in marine systems, although little is known about the identity of the ligands. To gain insight into the identity of metal complexing ligands (L), surface water and four species of brown seaweed were sampled from four sites in a region of the Lough Furnace Estuary, Ireland with varying salinity. We measured metal (Cd, Cu, Pb, Zn) speciation, organic complexing ligands, stability constants (logK'), glutathione (GSH), cysteine (Cys) and seaweed metal contents. Although prevalent in seaweed tissue, dissolved Cu and Pb concentrations in water were below the detection limits. Both Cd and Zn occurred in seaweed tissue. In water, both Cd and Zn occurred predominantly complexed to organic ligands. Levels of complexed Zn increased with decreasing salinity, increasing from 77% at high salinity to 100% at low salinity. Total dissolved Cd showed a mid-salinity decrease in complexation. Zinc ligand (L_{Zn}) ranged from 27.41 nM at high salinity to 95.81 nM at mid-salinity. Cd ligand (L_{Cd}) occurred in the highest concentration, 8.72 nM, at mid-salinity. Comparison of the logKs with known ligands provided clues to their identity: similarities were identified for L_{Cd} and fulvic acid (FA), and L_{Zn} and macroalgal exudates.

6.2 Introduction

In comparison to the open ocean, estuarine waters contain high concentrations of metals due to riverine inputs and cycling of historical anthropogenic inputs accumulated in sediments. Though few speciation studies of such estuarine water bodies exist, those that do show a wide range of metal concentrations. For instance, literature values for dissolved Cu vary by a factor of 3 from 10-150 nM (van den Berg et al. 1987; Baeyens et al. 1997; Paucot and Wollast 1997; Zwolsman and Van Eck 1997; Laglera and van den Berg 2003). These variations are likely to be due to the effects of characteristic geochemical gradients including salinity, O_2, and pH. This is particularly important because toxicity depends on metal speciation (Moffett and Brand 1996).

As in open ocean waters, the majority of metals in estuaries are organically bound with reported concentrations of ligands ranging from 5-300 nM (van den Berg et al. 1987; Gerringa et al. 1998; Laglera and van den Berg 2003; Santos-Echeandia et al. 2008; Gerringa et al. 1996). Variations in salinity are known to impact ligand concentration and complex stability (van den Berg and De Luca Rebello 1986; Gerringa et al. 1998; Tang et al. 2001; Shank et al. 2004), thus influencing metal bioavailability. Sources of estuarine ligands include sediment pore waters (Shank et al. 2004), sewage effluent (Bender et al. 1970), humic substances (Ytreberg et al. 2011), phytoplankton (Croot et al. 2000), and algal exudates (Andrade et al. 2010). Although evidence for the existence of metal specific marine ligands is prevalent in the literature, uncertainties remain regarding their chemical structure and source. There is a lack of progress on the identification of compounds controlling metal bioavailability, with the exception of Fe-siderophores (Vraspir and Butler 2009). Here, we focus on native seaweeds as a likely source of binding ligands. In recent laboratory studies, we identified both glutathione and cysteine in algal exudates (Murray et al. a, b, c). Both thiols are key constituents of phytochelatins and metallothioneins, which are involved in detoxification mechanisms (Ahner et al. 1995), and present in estuarine waters (Tang and Johannesson 2010).

Macroalgae are of particular interest in estuaries since they not only produce metal binding ligands, but also bioaccumulate metals. It is well known that tissue metal concentration is dependent on the concentration of dissolved metals in seawater (Braungardt et al. 2007). Brown seaweeds are the highest accumulators of metals and so, are often employed in marine monitoring programs (Sanchez-Rodriguez et al. 2001). However, accumulation is site-specific (Morrison et al. 2008) and is influenced by the complex water chemistry of estuaries including temperature (Munda 1979), light availability (Nielsen and

Nielsen 2010), nutrients (Parkhill et al. 2001), and salinity (Connan and Stengel 2011a). In particular, reduced salinities have been reported to enhance metal accumulation by algae (Munda 1984).

Despite evidence of contamination due to mining and other industrial activities, data on metal contents of Irish transitional waters is scarce (Boelens et al. 1999). Lough Furnace is one of few permanently stratified lagoon lakes in western Europe and as such is listed as an Annex I Priority Habitat (National Parks and Wildlife Service Special Areas of Conservation; NPWS SAC). The estuary is situated on the west coast of Ireland and has a semi-saline lake with an area of about 141 ha. Strong tidal currents are responsible for saline intrusions from Newport Bay as well as the re-suspension of coastal sediments. The resultant salinity gradient means that speciation and accumulation studies provide an insight into salinity effects as well as potential metal sources and sinks (where salinity serves as an indicator of freshwater and seawater mixing).

Generally, the metal speciation in estuarine waters is poorly described due to difficulties in analysis resulting from low metal concentrations, high reactivity, and few available *in-situ* techniques (Santschi et al. 1999). Advances in voltammetry, however, satisfy the requirements of speciation in estuaries and have been fundamental in the recent multi-metal characterization of such dynamic environments (Braungardt et al. 2007). Metal-specific complexing ligands can also be determined via voltammetry. The linearization of the titration data for a given sample with (competitive ligand exchange-cathodic stripping voltammetry; CLE-CSV) or without (differential pulse anodic stripping voltammetry; DPASV) a competing ligand yields both concentration and conditional stability constant (logK'). In the literature this technique is used to functionally classify marine ligands into groups of strong of weak ligands, although, a continuum of natural ligands is likely.

In this study, we determined the speciation of Cd, Cu, Pb and Zn at 4 sites characterized by different salinity regimes in the Loch Furnace estuary using both differential pulse anodic stripping voltammetry (DPASV) and cathodic stripping voltammetry with competitive ligand exchange (CLE-CSV). At the same time, we measured the concentrations of complexing ligands, conditional stability constants, glutathione, and cysteine and seaweed metal contents. Our objective was to establish a relationship between salinity, complexing ligands, and metal bioavailability in order to progress towards the identity of L.

6.3 Materials and Methods

6.3.1 Field site

The study area was the estuary of Lough Furnace (9° 34' 20" W, 53° 55' 22" N), which was sampled at 4 sites (**Figure 6.1**). The sites were selected for ease of sampling, variations in salinity, and potential influences of macroalgae.

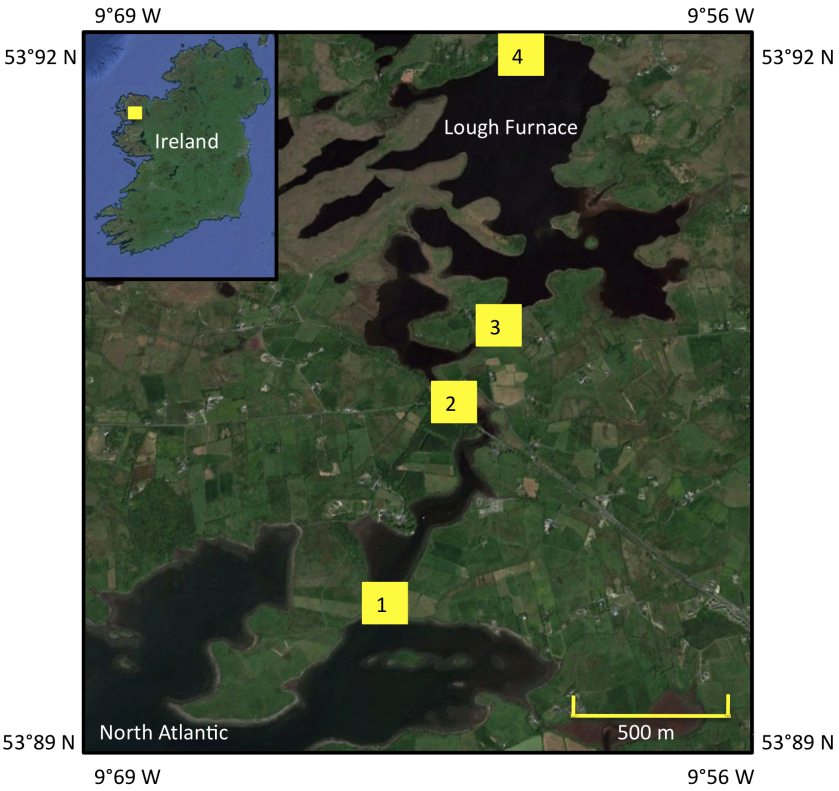

Figure 6.1. The sampling sites in the variable salinity region of the Loch Furnace Estuary, Mayo, Ireland (9° 34' 20" W, 53° 55' 22" N). The lower estuary site (1) is nearest the North Atlantic Ocean. The mid-estuary sites (2) and (3) are where predictable tidal mixing of fresh and seawater occurs. The upper estuary site (4) is nearest the freshwater input and is the same location as a station for long-term monitoring by the Marine Institute.(Irish Marine Institute 2013) Inset: a map of Ireland indicating the location of the Lough Furnace Estuary.

6.3.2 Seaweed collection

Tips of the intertidal macroalgae *Ascophyllum nodosum* (Linnaeus) Le Jolis, *Fucus ceranoides* (Linnaeus), *Fucus spiralis* (Linnaeus), and *Fucus vesiculosus* (Linnaeus) (Fucales, Phaeophyceae) were sampled haphazardly (*n=3*) at each site (where present), rinsed twice

with 1:9 ethanol/seawater (Gledhill et al. 1999), placed in acid cleaned bags, and frozen immediately.

6.3.3 Seaweed analyses

Tips were freeze-dried to a constant weight and then ground using an agate mortar and pestle. Approximately 1 g of the powdered biomass was dry-ashed at 450˚C and then brought up to 10 mL volume using 20% nitric acid. Cadmium, copper, lead and zinc contents were measured using inductively coupled optical emission spectrometry (ICP-OES; Varian Vista Pro), with the respective limits of detection as follows: Cd 0.1 ng g^{-1}, Cu 0. 2 ng g^{-1}, Pb 5 ng g^{-1}, Zn 0.2 ng g^{-1}.

6.3.4 Water collection

Parallel to seaweed collection, unfiltered seawater was collected in 500 mL acid cleaned HDPE bottles, at a depth of approximately 1 m. Samples were frozen immediately until the day prior to analysis. At this point, samples were thawed overnight at 4˚C and thoroughly mixed to ensure the dissolution of particulates formed due to freezing. Salinity (psu), and pH (NBS scale) were recorded *in-situ*, at the surface, using a hand held field meter (YSI 30) (**Table 6.1**).

Table 6.1. Ancillary data for water samples collected from Lough Furnace on July 26th 2011, and corresponding GSH and Cys concentrations measured by CSV, mean ± s.d., *n*=3.

Site	Salinity (psu)	pH	DOC (μM)	Nitrate (μM)	GSH (nM)	Cys (nM)
1	34	8.1	562.83 ± 45.54	16.59 ± 0.62	2.29 ± 0.03	3.11 ± 0.09
2	20	8.5	582.70 ± 65.84	20.29 ± 4.63	2.94 ± 0.13	3.04 ± 0.01
3	15	8.4	636.14 ± 23.87	20.85 ± 0.38	2.01 ± 0.10	2.83 ± 0.08
4	4	8.7	220.08 ± 40.42	14.94 ± 1.26	3.85 ± 0.07	2.90 ± 0.08

Most reported applications of voltammetry involve sample freezing, post-filtration. Therefore, due to the unconventional approach used here, procedures for sample storage and treatment were tested on spiked artificial seawater and seawater samples collected from the Lough Furnace estuary (site 1, June 2013) (**Table S1**). The methods tested were: filtration and immediate analysis; filtration, freezing, thawing, then analysis; and freezing, thawing, filtration, then analysis.

6.3.5 Water analyses

Instrumentation and reagents

The voltammeter used here was either a Metrohm 757 VA Computrace or a Metrohm 797 VA Computrace, controlled by a computer (PC). In both instances, the working electrode was a hanging mercury drop (HMDE) with a drop size of approximately 0.52 mm^2. The reference electrode was a double junction Ag/AgCl (3 M), saturated KCl. The counter electrode was a platinum wire. A polytetrafluoroethylene (PTFE) stirrer, rotating at 2 000 rpm, was employed during the adsorption step. Water used for dilutions was Milli-Q (MQ; $\geq$18.2 MΩ) and all reagents were analytical grade. Stock solutions of Cd, Cu, Pb and Zn were prepared in 0.01 M HCl, by dissolution of atomic absorption standard solutions (Fluka). Thiol working standards of glutathione, cysteine, and thioactemide (all Aldrich) were prepared prior to use via dissolution in MQ water. Stock solutions of 0.01 M salicylaldoxime (SA, Aldrich) and 0.025 M ammonium pyrrolidinedithiocarbamate (APDC, Aldrich) were prepared monthly in 0.1 M HCl and MQ water, respectively. One molar 3-[4-(2-Hydroxyethyl)-1-piperazinyl] propanesulfonic acid (HEPPS, Aldrich) and 0.55 M NaOH (Aldrich) were used in voltammetric determinations where the addition of 70 µL buffer to 10 mL sample yielded a pH of 8.1. While all experiments were conducted at room temperature, when not in use, all metal, ligand, and buffer solutions were stored at 4˚C. Where possible, sample manipulations were carried out in a laminar flow hood.

Metal speciation

The total dissolved metal concentrations ($[M]_D$) were determined simultaneously using differential pulse anodic stripping voltammetry (DPASV) (Leal et al. 1999). Thawed seawater samples were filtered through 0.45 µm glass-fiber filters and acidified to pH 2.2 using 6 M HCl, and 50 µL H_2O_2 was added prior to UV digestion (Metrohm 705 UV Digester) in PTFE-capped quartz silica tubes for 2 h. Ten mL aliquots of sample were pipetted into the voltammetric cell. Acetate buffer (70 µL, final concentration 0.07 M) was added and the solution was de-aerated for 5 min using nitrogen. The voltammetric parameters were: deposition 300 s at -1.15 V whilst stirring; 10 s quiescence; potential scan using the differential pulse modulation: pulse height 0.05 V, scan from -1.15 to 0.05 mV. Initial concentration was determined via two

standard metal additions, each replicated twice. Peaks for cadmium, copper, lead and zinc occurred at –0.6 V, -0.1 V, -0.4 V and –1 V with the respective limits of detection at 0.1 nM, 0.3 nM, 0.2 nM, and 0.6 nM.

The labile dissolved metal concentrations ($[M]_L$) of Cd was carried out as described for $[M]_D$, except for the acidification and UV digestion and at a more positive deposition potential of -0.8 V and using HEPPS (0.01M). For Zn, CSV with ligand competition was used to determine $[M]_L$, as previously described by Leal et al. (1999), Campos and van den Berg (1994), and van den Berg (1985). For these determinations, the pH was fixed at 8.1 using HEPPS and APDC ligand (100 μM)was added before measurements. In all cases, initial concentration was determined via two standard metal additions, each replicated twice.

Metal complexing-capacities

Titration of filtered seawater with individual metal ions was used to determine the concentration of metal-specific complexing ligand (L_M) and the associated conditional stability constant ($\log K'L_M$). As outlined for $[M]_L$, Cd measurements were made using ASV while Zn measurements were made using CSV with ligand competition. HEPPS (0.01 M) and appropriate ligand (if any) were added to 120 mL of filtered seawater. Ten mL aliquots were then pipetted into 10 or 12-20 mL polystyrene vials (Sterilin) containing metal additions in approximately equal increments. Titration schemes employed were sample-specific and their range pended on the total metal concentration of the sample. In general, titration points ranged from 0-60 nM for Cd and 0-120 nM for Zn. Each titration consisted of 10 (Cd) or 12 (Zn) individual aliquots equilibrated overnight (12-15 h). The $[M]_L$ of each Sterilin was then measured as previously outlined.

In an effort to minimize loss of metal due to adsorption, all containers (including culture jars, HDPE storage bottles, titration tubes, and voltammetric cells) were preconditioned twice by rinsing with artificial seawater containing the metal of interest at a concentration similar to that expected in the sample. Alternatively, where sample volume allowed, containers were rinsed twice with the sample itself. In both instances, the rinse solution was discarded and replaced by new sample. In order to minimize the constraints of the conditioning requirement, Sterilin tubes were rinsed with MQ between titrations and used again in the same order to maintain conditioning for the sample replicates. Preliminary experiments showed metal losses were insignificant using this method.

Identification and quantification of thiol compounds

Thiols were both identified and quantified using CSV at pH 8.1 (0.01 M HEPPS), according to Leal et al. (Leal et al. 1999), where cysteine, glutathione and thioacetamide peaks present at -0.47 V, -0.58 V and -0.68 V respectively. The voltammetric parameters were: deposition 60 s at -0.25 V whilst stirring; potential scan using the square wave modulation: scan rate 20mV/s, modulation amplitude 25 mV, and frequency 50 Hz. Initial concentration was determined via two standard thiol additions (cysteine, glutathione, or thioacetamide), each replicated twice.

6.3.6 Calculations

A modified van den Berg linearization (1982) was used to determine the complexing ligand concentration and corresponding conditional stability constant for each metal based on the following relationship:

$$[M]_L/[ML]=[M]_L/L_M + \alpha/(K'_{ML}L_M)$$

Where [ML] is calculated as the difference between $[M]_D$ and $[M]_L$ while the α-coefficient represents the overall side reactions producing labile complexes for each metal. Values of α were calculated, as a function of the salinity of the sample, as described previously by Vasconcelos et al. (2002) using the following values: $\alpha_{Cd}=35$ and $\alpha_{Zn}=2.1$ (Turner et al. 1981) at salinity 35; $\log K'_{ZnAPDC}$ was calculated for each salinity based on literature values established from α_{ZnAPDC} (van den Berg 1985; Donat and Bruland 1990).

Linearly transformed plots of $[M]_L/[ML]$ versus $[M]_L$ were used to elucidate the number of ligands present wherein a linear plot was indicative of a single ligand and a curved plot of more than one ligand. The concentrations of L_M and $\log K'L_M$ were determined from the slope and y-intercept of a least squares regression of this titration curve.

The calculation of sensitivity was modified as per Turoczy and Sherwood (1997) using multiple iteration of the final four titration points in order to avoid typical underestimation.

6.3.7 Quality assurance

The accuracy of the analytical procedures for seaweed metal concentrations and seawater total dissolved metal concentrations were checked by analyzing certified reference materials (CRM) for trace metals from the National Institute for Environmental Studies, Japan Environment Agency algal standard (NIES no. 9) and the National Research Council of Canada Reference standard seawater (CASS 5) for coastal seawater. The metal concentrations

determined were never significantly different than the expected values ('t' test, p>0.05), showing a good accuracy of measurement (**Table S2**).

6.3.8 Data analysis

Significant differences in seawater metal contents, complexing ligands, conditional stability constants, and thiol concentrations amongst the 4 sampling sites were tested using a one-way between subjects ANOVA. Where significant differences were identified, a post hoc Tukey's multiple comparison test was employed. Seaweed tissue metal concentrations were compared among species and between sites by means of multiple analyses of variance (MANOVA). Pearson product-moment correlation coefficients were used to measure the strength of relationship between $[M]_L$ and concentrations of complexing ligands. Correlations were declared significant at $P < 0.05$. Normality and correlation tests were undertaken prior to data testing. All statistics were performed using SPSS version 20.0.

6.4 Results

6.4.1 Seaweed metal contents

In all seaweeds analyzed, Zn displayed the highest concentrations whereas Cd concentrations were consistently the lowest (**Table 6.2**). A non-parametric Spearman correlation analysis applied to all samples showed significant positive correlations ($p < 0.05$, n=22) for the metal pairs Cd-Cu, Cd-Zn, and Cu-Pb. Multiple analyses of variance (MANOVA), including all 4 elements (Cd, Cu, Pb, Zn) measured in the estuary, revealed significant site effects on the mean vectors of the tissue metal concentrations (Wilks' l = 0.01; F= 29.751; $p < 0.001$). Seaweeds from the low salinity site 4 had higher concentrations of metals than those from the other three sites, with the exception of Cu, which did not differ significantly with site. Multiple analyses of variance also detected significant species effects on the mean vectors of the tissue metal data (Wilks' l = 0.001; F=34.04; $p > 0.001$). The total metal content increased in the order of *F. ceranoides* < *F. vesiculosus* < *A. nodosum* < *F. serratus*. Of the four species, only *F. serratus* was found at all sites. For this species, both Cd and Zn contents increased with decreasing salinity, displaying a significant ($p<0.05$) increase at the low salinity sites 3 and 4 in comparison to the other two sites.

Table 6.2. Concentration of metals (μg g^{-1} DW) in tips of several seaweed species collected from sites 1-4 of the Lough Furnace Estuary, mean ± standard error, *n*=3. AN: *Ascophyllum nodosum*, FC: *Fucus ceranoides*, FS: *Fucus spiralis*, and FV: *Fucus vesiculosus*.

Site	Species	Cd	Cu	Pb	Zn	Total
1	AN	0.072 ± 0.024	0.23 ± 0.18	0.24 ± 0.10	57.10 ± 22.64	57.63 ± 22.94
	FC	0.43 ± 0.07	2.33 ± 0.40	0.61 ± 0.07	36.26 ± 0.07	39.63 ± 0.54
	FS	0.44 ± 0.02	1.28 ± 1.28	0.51 ± 0.03	70.34 ± 2.92	72.57 ± 4.21
	FV	0.45 ± 0.05	1.17 ± 1.05	0.76 ± 0.05	39.08 ± 4.84	41.46 ± 3.89
2	AN	0.31 ± 0.02	0.38 ± 0.03	0.21 ± 0.02	68.91 ± 11.99	69.80 ± 11.92
	FC	0.34 ± 0.06	1.91 ± 0.17	0.58 ± 0.06	44.32 ± 0.68	47.15 ± 0.59
	FS	0.51 ± 0.02	1.84 ± 0.17	0.30 ± 0.00	87.04 ± 4.99	89.69 ± 5.21
3	AN	0.32 ± 0.02	0.89 ± 0.07	0.30 ± 0.13	108.65 ± 19.47	110.18 ± 19.65
	FS	0.76 ± 0.14	1.69 ± 0.06	0.63 ± 0.07	112.14 ± 16.31	115.22 ± 16.45
	FV	0.66 ± 0.020	1.13 ± 0.09	0.19 ± 0.02	72.67 ± 2.26	74.64 ± 2.30
4	FS	1.38 ± 0.01	1.80 ± 0.16	0.42 ± 0.07	156.76 ± 10.36	160.35 ± 10.28

6.4.2 Metal speciation in water as a function of salinity

Results of the speciation analyses are presented in **Figure 6.2**. Total dissolved Cd and Zn concentrations ranged from 0.12-0.38 nM and 2.74-5.14 nM respectively with the highest total measured metal concentration at site 1. Zinc showed conservative mixing behavior, decreasing with increasing salinity, implying a 2 end member relationship. Cd was characterized by a mid-salinity maximum at site 3. By contrast, total dissolved Cu and Pb concentrations were below the detection limits at all sites.

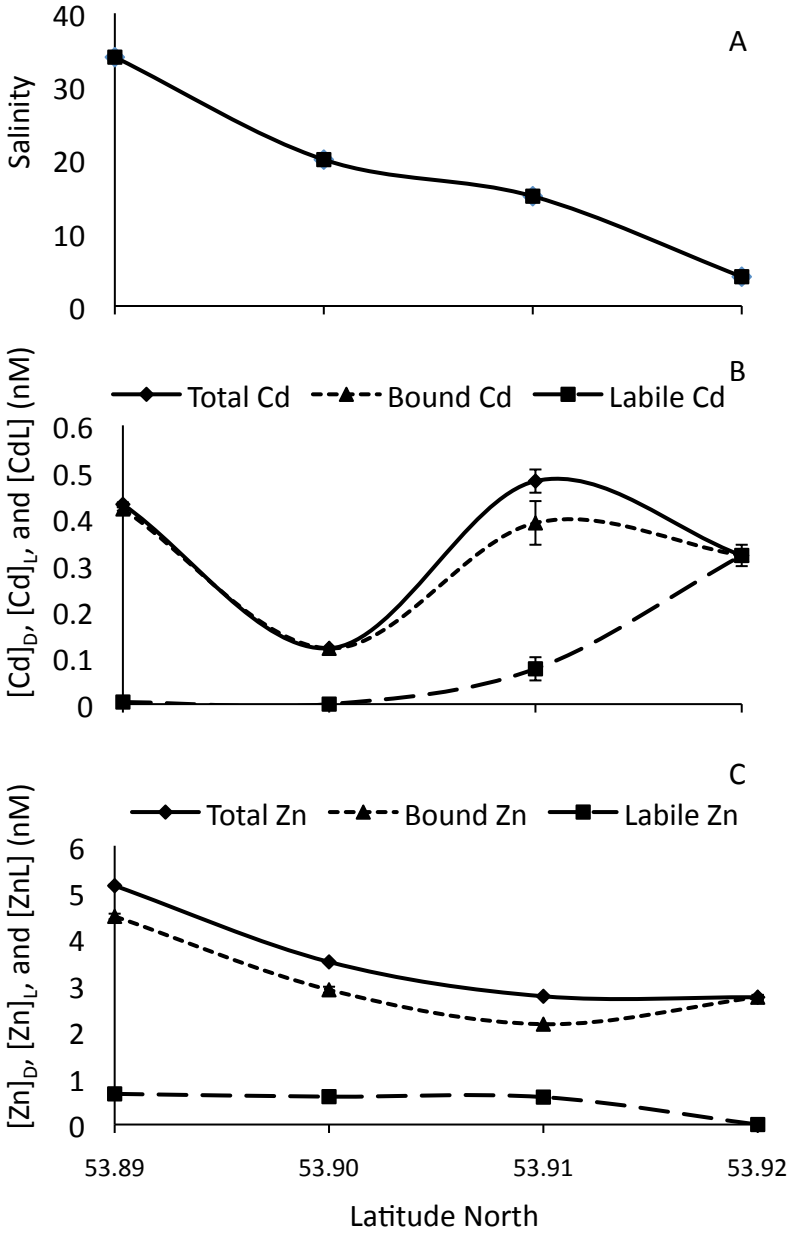

Figure 6.2. Salinity (A), DPASV Cd speciation (B), and CLE-CSV Zn speciation (C) measured in the dissolved surface water along the Lough Furnace Estuary, mean ± standard error, *n=3*.

Where labile species were detected, [ML] covaried with $[M]_D$ with a maximum [ZnL] at minimum salinity (i.e. site 1) and maximum [CdL] at mid salinity (i.e. site 3). Labile Zn constituted 22.74, 29.35, 35.55 and 0 % of M_D at sites 1, 2, 3, and 4 respectively. Speciation of Cd, for which data are only available for sites 1 and 3, was less variable with corresponding labile proportions of only 2.04 and 27.61%.

Table S1 reports the results obtained for $[Cd]_L$ and $[Zn]_L$ in testing the sample handling and treatment procedure. For Cd, results show substantial agreement for all 3 methods, including that employed here. Thus, the post-freeze filtration method adopted in this study maintains sample integrity with regards to Cd. By contrast, Zn results were significantly higher for post-freeze filtration samples than those obtained using the more conventional methods. In order to avoid erroneous interpretation, the possible overestimation of $[Zn]_L$ and its subsequent effect on Zn speciation must be considered when analyzing the discussion where the results obtained here are compared to those previously determined in other studies.

6.4.3 Organic compounds and metal complexation

Both Cd and Zn occurred predominantly complexed to organic ligands. Total percent complexed Zn increased with decreasing salinity, ranging from 77% at site 1 to 100% at site 4. As for total dissolved Cd, the concentration of complexed Cd showed no specific trend but rather was characterized by a mid-salinity decrease in complexation at site 3. For all samples, titration data yielded a straight line according to the equation from van den Berg (1982), indicating both metals were best modeled by a single complexing ligand. Results of the titration analyses are presented in **Table 6.3**. Zinc ligand estimates ranged from 27.41 nM at site 1 to 95.81 nM at site 3. For each site, $[L_{Zn}]$ was greater than $[Zn]_D$. Similarly, the single class of Cd ligand occurred in the highest concentration, 8.72 nM, at site 3 and the lowest concentration, 7.30 nM, at site 1. At site 3, the $[L_{Cd}]$ was 35x higher than $[Cd]_D$. In spite of concentration differences, the conditional stability constants showed very little variation across the 4 sites. For Zn, LogK' decreased slightly with increasing salinity. For Cd, LogK' appears to be independent of salinity. In any case, the stability constants for Zn were always higher than those for Cd indicating stronger binding of Zn ions.

Table 6.3. A summary of the DPASV and CLE-CSV metal titration for the dissolved surface water obtained from the 4 sampling sites, mean ± standard error, *n=3*.

Site	M	$[L_M]$(nM)	$LogK'L_M$
1	Cd	7.31 ± 0.57	9.92 ± 0.11
	Zn	27.41 ± 3.11	12.21 ± 0.18
2	Cd	8.05 ± 1.06	9.32 ± 0.92
	Zn	41.86 ± 1.10	11.50 ± 0.01

Site	M	$[L_M]$(nM)	$LogK'L_M$
3	Cd	8.72 ± 0.47	10.95 ± 1.58
	Zn	95.82 ± 4.19	11.71 ± 0.28
4	Cd	7.47 ± 0.99	9.73 ± 0.27
	Zn	63.99 ± 6.91	11.33 ± 0.04

- represents concentrations below the detection limits.

6.4.4 Nature and distribution of glutathione (GSH) and cysteine (Cys) in estuarine water

Both GSH and Cys were present at low nanomolar concentrations at all sites (**Table 6.1**). Glutathione increased with decreasing salinity, ranging from 2.29 nM-3.85 nM. Cysteine showed the opposite trend, with the highest concentration (3.11 nM) measured at the high salinity end of the estuary.

6.5 Discussion

6.5.1 Bioaccumulation by seaweeds

Despite some studies on the Irish coast (Morrison et al. 2008; Baumann et al. 2009), concentrations of metals have not been previously reported for macroalgae in Irish estuaries. A comparison of the results found here to those recently reported in estuaries of southwest England show few similarities. Our values for Zn accumulation by *A. nodosum* (57.1-108.65 μg g^{-1}) fall within the range reported for the Gannel and Looe estuaries of the UK (63.7-107 μg g^{-1}). However, values for both Pb and Cu are approximately 100 times lower in this study. Values reported here for Cu, Pb, and Zn accumulation by *F. vesiculosus* are also lower than those reported by Rainbow et al. (2011) for 5 estuaries. These concentration differences are not surprising given the history of Pb mining at the mouth of the Gannel estuary. Despite the presence of two smelting furnace ruins, observed concentrations of metals in seaweeds indicate Lough Furnace is relatively uncontaminated.

This study demonstrates that decreased salinity may cause increased metal accumulation in seaweeds. The total metal content of *F. serratus* was approximately 122% higher at 0 salinity than at full salinity. Decreased bioavailability at higher salinities may be due to the formation of metal complexes with the chloride ion or the result of Na, Ca, and Mg competition for uptake sites at the plasma membrane and in the free spaces of the cell walls. Competition for the biotic ligand between metal ions and other cations in solution is well-

described by the Biotic Ligand Model (BLM) (Di Toro et al. 2001). Higher bioaccumulation at the lake-end of the estuary may be the result of longer immersion time (Fuge and James 1973). Species differences in metal accumulation can also be attributed to differences in morphology. According to the form-function model developed by Littler and Littler (1980), thallus morphology can be used to predict productivity and survival of macroalgae. However, in this case the form-function model does not apply. Despite all 4 species being part of the 'thick leathery' group, there are systematic differences in accumulation. Similarly, differences in the surface area: volume (SA:V) ratio do not explain the species-specific differences in accumulation found here. In contrast to previous studies (Stengel et al. 2004; Baumann et al. 2009), whilst *F. vesiculosus* has a greater SA:V ratio than *A. nodosum*, accumulation is actually lower in the former. As proposed by Padilla and Allen (2000), groupings based on specific functions (here, bioaccumulation) may be more appropriate than those based on gross morphology. Connan et al. (2011a) attributed species-specific Cu accumulation in *A. nodosum* and *F. vesiculosus* to differences in binding sites and uptake mechanisms between the species. These data suggest differences in accumulation are not due to differences in metal availability or algal morphology but rather competitive interactions at the algal surface.

6.5.2 Metal speciation

Despite being measured in the seaweed tissue, concentrations of Cu and Pb in our water samples were below detection limits at each site. This suggests sampling of the surface layer may not yield a representative measure of metal concentration in the water column. It is possible that sampling season compounded this effect whereby concentrations of Cu and Pb were lower in summer and spring than winter (Hatje et al. 2003). Only filtered samples were analyzed and so the concentrations of some colloidal and particulate bound metals were discounted. Lower levels of Cu and Pb in comparison to Cd and Zn may also be due to differences in binding characteristics. Metal oxides are known to partially control metal speciation (Nelson et al. 1999). In previous studies, Cu and Pb were found to be preferentially bound to oxyhydroxide surfaces (Förstner 1987). Thus metal partitioning in the Fe/Mn oxyhydroxide phase of the sediments may be the cause of such extremely low concentrations of Cu and Pb in the surface layer. Similarly, increased Cd and Zn concentrations in comparison to Cu and Pb may be the result of greater prevalence in the geology of the catchment. In each case, detailed sediment analyses would be required.

For both metals, the labile concentrations in this study were similar to those reported for the pristine Bathurst Harbor (0.02-0.6 nM Cd; 6-45 nM Zn) (Mackey et al. 1996), yet slightly lower than those reported for the contaminated Narragansett Bay, USA (0.07-0.22 nM Cd; 8-26.6 nM Zn) (Kozelka and Bruland 1998). Results of our chemical speciation suggest more than 64 and 66% of Zn and Cd, respectively, were complexed by ligands with conditional stability constants ranging from 11.33–12.21 and 9.32–10.94, respectively. These results compare well with those reported for Cd in estuarine and coastal waters by Muller (1996a) who found 20-80% of Cd to occur complexed by a single ligand with a conditional stability constant between 9.81 and 10.49 (**Table 6.5**). In each case, the conditional stability constants decrease with increasing salinity, as would be predicted due to major cation competition. Similarly, the $[L_{Zn}]$ reported here is within the range of that determined by Skrabal et al. (1996) in an organic rich estuary. As in our study, the existence of only a single Zn complexing ligand has been previously reported for Narragansett Bay, USA, (Muller and Kester 1991; Kozelka and Bruland 1998) Chesapeake Bay, USA (Henry and Donat 1996), Delaware Bay, USA (Lewis et al. 1995), and the Scheldt River, NL (van den Berg et al. 1987) (**Table 6.4**). Despite the existence of only a single ligand in these studies, two ligands have been proposed by other authors (van den Berg 1985; Morelli et al. 1989). As outlined by Bruland and colleagues, error in the number of ligands detected can occur when the concentration of the competitive ligand is so high that it outcompetes any weaker ligands. As a result, even in our study, there may be unidentified ligands outside the analytical window employed. This window can be moved higher or lower simply by decreasing or increasing [APDC], in the specific case of Zn. While multiple titrations at varying competition strengths would reduce the likelihood of error, they would not necessarily eliminate it. The redeeming feature here is that our ligand concentrations were always greater than $[M]_D$ which, as indicated by Miller and Bruland (1997) improves the accuracy of the ligand approach.

Table 6.4. A comparison of current literature values for the speciation of dissolved Zn in various estuarine waters, including this study. Adapted from Kozelka and Bruland (1998).

Location	Salinity (psu)	$[M]_D$ (nM)	$[M]_L$ (nM)	ML (%)	$[L_M]$ (nM)	$LogK'L_M$	Method	References
Lough Furnace, Ireland	4-34	2.74-5.14	0.59-0.66	78-100	27.41-95.81	11.33-12.21	CLE-CSV	(Murray et al. d)
Adour Estuary, France	0, 33	44.35, 45.1	9.63, 27.1	78, 40	-	-	ICP-MS	(Point et al. 2007)
Narragansett Bay, USA	23-30	16-72	0.3-13	50-97	11-48	9.0	DPASV	(Kozelka and Bruland 1998)
Scheldt Estuary, UK	1-32.5	25-226	-	90-96	-	7.0-9.1	DPASV	(Baeyens et al. 1997)
Chesapeake Bay, USA	0-31	4-20	0.01-0.3	95-99	8-200	9.5-99	CLE-ASCV, DPASV	(Henry and Donat 1996)
Delaware Bay, USA	30	3.5	0.04	98	15	>10.9	SWASV TMF-RGCDE	(Lewis et al. 1995)
Narragansett Bay, USA	30	22-28	2.5-4.2	55-66	50-104	7.5-7.9	DPASV TMF-RGCDE	(Muller and Kester 1991)
Scheldt River, Netherlands	9-25	46-450	2-16	60-95	22-220	8.6-10.6	CLE-ASCV	(van den Berg et al. 1987)
River Dee and Ribble Estuaries, Wales	13.9-36.5	110-900	1.8-446	13-26	6-40	7.4-9.3	DPASV	(van den Berg and (Dharmvanij 1984)

- represents data not provided in the literature.

Murray et al. (d)

Table 6.5. A comparison of current literature values for the speciation of dissolved Cd in various estuarine waters, including this study.

Location	Salinity (psu)	$[M]_D$ (nM)	$[M]_L$ (nM)	Complexed (%)	$[L_M]$ (nM)	$logK'L_M$	Method	References
Lough Furnace, Ireland	4-34	0.12-0.47	0.004-0.07	83-100	7.30-8.72	9.32-10.94	DPASV	(Murray et al. d)
Adour Estuary, France	0, 33	0.1, 0.27	0.062, 0.25	38, 7.4	-	-	ICP-MS	(Point et al. 2007)
Narragansett Bay, USA	24-30	0.8-0.29	$< 2x\ 10^{-12}$	73-83	4	8.9	DPASV	(Kozelka and Bruland 1998)
Scheldt Estuary, Netherlands	1-32.5	0.38-1.8	-	10-35	-	6.9-8.9	DPASV	(Baeyens et al. 1997)

- represents data not provided in the literature.

The separation of site 3 was reflected in both the Cd speciation and DOC data. For Cd, a mid-salinity concentration increase indicated an internal metal source. This trend has been previously reported for Cd in the Galveston Bay estuary where concentrations reached a broad maximum at salinities between 12 and 15 psu (Tang et al. 2002). This feature is common to many estuaries (Kraepiel et al. 1997; Zwolsman and Van Eck 1997; Elbaz-Poulichet et al. 1999; Hatje et al. 2003) and is generally attributed to the release of particle-phase Cd resulting from chloride competition for complexation (Comans and van Dijk 1988). Similar sediment-water exchange processes have been reported previously for Galveston Bay (Warnken et al. 2001). Sundby et al. (1986) concluded that Mn, Fe, and Co fluxes out of sediments into the water column of the Gullmars Fjord were the direct result of low oxygen concentration. As a thick anoxic bottom layer characterizes Lough Furnace, the observed mid-salinity Cd increase may be attributed to a similar solubilisation of metals in the near-sediment anoxic layer or, alternatively, the oxidation of reduced sediments (solubilizing CdS). In either case, this suggests that sediments could be an important source of Cd to the water column in this estuary, although *in-situ* benthic flux studies are required to validate this hypothesis. The same studies may provide insight into why the same trend is not observed for Zn.

The DOC concentration of the sample with the lowest salinity (220.15 μM ± 28.61) is approximately half that of the full salinity sample (562.83 μM ± 31.19), a trend previously observed for fresh versus seawater (Cauwet 1991; Louis et al. 2009). However, the relationship between DOC and salinity is not entirely linear. Instead, DOC exhibited an increase at mid-salinity indicating an additional carbon source that may be attributed to biological ligand exudation. Further, the relatively high seawater DOC values are indicative of a local carbon source.

6.5.3 The nature of L

The relationship between salinity and ligand concentration is useful in assessing ligand source where salinity serves as proxy for seawater and freshwater mixing. The concentration of L_{Zn} decreased with increasing salinity indicating a low salinity origin of the ligands with subsequent dilution by seawater at higher salinities. Conservative behavior of the complex suggests there may be neither dissociation nor sorption across the estuary. This is likely to be

the result of a slow dissociation rate relative to the residence time. This theory is supported by the corresponding stability constants obtained in this study where a decrease along the salinity gradient is indicative of competition for binding by other ions. Constant values for L_{Cd} along the salinity gradient may reflect continuous lateral input or more likely, are the result of a balance between river flow and tidal effects such that no loss of strong ligand occurs within the estuary.

Current values in the literature for thiols in estuarine surface waters are presented in **Table 6.6**. Our estimates of GSH are within the range reported by Tang et al. (2001) for Galveston Bay. Like Loch Furnace, Galveston Bay is a shallow estuary with trace metal concentrations similar to pristine sites for sediments, water, and biota (Wen et al. 1999). In contrast, our results are higher and more variable than those determined for San Diego Bay, Cape Fear Estuary, and Norfolk Estuary (Tang et al. 2004). A negative linear correlation between GSH and $[Zn]_L$ suggests that GSH is a contributor to the natural ligand pool. This near stoichiometric relationship (Pearson correlation coefficient=-0.881; $p<0.01$) is in agreement with previously published literature (Krezel and Bal 1999). This is in contrast to cysteine, which shows a significant decrease (Pearson correlation coefficient=-0.886; $p<0.01$) with increasing ligand concentration, indicating it is possibly consumed in ligand production. This is not surprising, given Cys is a known precursor for GSH, and GSH is a known precursor for phytochelatin. Despite this, GSH shows greater correlation with metal concentrations than the ligands themselves. Glutathione complexes metals via sulphydryl coordination (Scott et al. 1993) albeit having a lower sequestration potential than phytochelatins (Kneer and Zenk 1992), however it may only be a minor fraction of the total ligand abundance. Nevertheless, sediment porewaters and humic substances cannot be discounted as additional ligand sources in Lough Furnace. However, it is difficult to explain estuarine variations in thiol concentrations as both compounds are involved in biochemical processes other than metal detoxification and may be rapidly consumed by bacteria and phytoplankton (Noctor and Foyer 1998). Further to this, estuary thiol concentrations are likely to change as a function of seaweed community structure. Also as demonstrated by Pawlik-Skowronska et al. (2007) for phytoplankton, the role of thiols in metal detoxification and resistance varies with species. The same may be true for macroalgae.

Table 6.6. A comparison of current literature values for thiols in various estuarine surface waters, including this study.

Location	Salinity (psu)	[GSH] (nM)	[Cys] (nM)	DOC (mg L^{-1})	Method	References
Lough Furnace, Ireland	4-34	2.29-3.85	2.83-3.11	3.10-9.08	CSV	(Murray et al. d)
Elizabeth River, USA	8.7-22.3	14.7-25.3	21.1-35	-	HPLC	(Dryden et al. 2007)
Norfolk Estuary, UK	18-22	0.37-0.45	-	2.4-3.5	HPLC	(Tang et al. 2004)
San Diego Bay (1), USA	18-22	0.17-0.52	-	0.86-1.6	HPLC	(Tang et al. 2004)
San Diego Bay (2), USA	18-22	0.23-0.79	-	0.80-1.7	HPLC	(Tang et al. 2004)
Cape Fear Estuary, USA	18-22	0.08-0.13	-	2.6-10.4	HPLC	(Tang et al. 2004)
Galveston Bay, USA		0.23-6.1	-	1.7-5.5	HPLC	(Tang et al. 2000)
Mersey Estuary, UK	23-33	20-300	-	-	CSV (with and without Cu addition)	(Le Gall and van den Berg 1993)

- represents data not provided in the literature.

Like any natural water body, the estuarine ligands found in this study may be of humic, fulvic, organic, or inorganic nature. In the absence of direct identification, their conditional stability constants serve as a means for characterization. For L_{Cd}, the measured conditional stability constants are similar to those determined previously for fulvic acid ($logK_{FACd}$=9.4; Xue and Sigg 1998). Thus it is likely that the fulvic acid fraction of the freshwater DOM is representative of the general class of cadmium ligands found here. The discrepancy between ZnL reported here and that for fulvic acid in previous studies ($logK_{FAZn}$=8.79; Yang and van den Berg 2009) rules out humic substances as the zinc ligand source. Fittingly, Cd-FA complexes are more stable than Zn-FA complexes (Ramalho et al. 2007). Previous work by Skrabal et al. (1996) drew similar conclusions: humic substances are not strong complexants of estuarine Zn. Organic ligands produced by living organisms constitute another possible identity of L_{Zn}. Marine organisms identified as ligand producers include: cyanobacteria (Moffett and Brand 1996), dinoflagellattes (Croot et al. 2000), coccolithophores (Leal et al. 1999), phytoplankton (Croot et al. 2000), microalgae (Vasconcelos et al. 2002), and macroalgae (Andrade et al. 2010). Copper conditional stability constants measured previously for *Chaetomorpha firma*, *Ulva lactuca*, *Porphyra columbina*, *Gelidium lingulatum* and *Lessonia nigrescens* ranged from 7.6-8.9 (Andrade et al. 2010). LogK's for such exudates have yet to be published for Zn. In recent laboratory studies we have observed that ligands exuded by several seaweed species under Zn exposure are within the range of the ambient ligands measured in Lough Furnace (Murray et al. a) The conditional stability constants reported here are similar to those determined for *A. nodosum*, *F. vesiculosus*, and *L. hyperborea* (from 11.82-12.15, 11.61-12.81, and 11.78-12.41 respectively). Such strong similarities in binding characteristics point to macroalgae as a source of L_{Zn} in Lough Furnace. Taking into account recent results obtained for macroalgae-exuded organic ligands (Murray et al. a), the average concentration of exuded L_{Zn} after 7 days (9.10 nM mg^{-1} C) would contribute significantly to the Lough Furnace ligand pool, where ligand density ranged from 3.47-20.60 nM mg^{-1} C.

The existence of unspecified ligands outside of the humic fraction has serious implications for environmental risk assessment. Commonly employed chemical-equilibrium models such as WHAM and SHM predict speciation using HA and FA as representative dissolved organic ligands. This assumption may be the cause of modeling inaccuracies. Even the highest binding constants employed by WHAM and SHM for HA (8.80 and 8.95) and FA

(9.40 and 8.75) are 33% lower than those determined under natural conditions in Loch Furnace (although the concentrations of HA and FA in these samples are unknown). The use of inappropriate stability constants may explain why WHAM-predicted $[Zn]_L$ is found to differ from natural concentrations by an entire order of magnitude (Stockdale et al. 2011). Accordingly, the inclusion of strong non-humic ligands, or at the very least higher conditional stability constants, in speciation models is necessary for the reliable prediction of speciation under natural conditions. Incorporating realistic ligand parameters into biogeochemical models is pivotal in predicting how ligands and subsequently, metal speciation will respond to large-scale environmental alterations such as climate change.

Differences in water chemistry between sites may be due to differences in algal communities. In this estuary, there was a strong influence of environmental factors on seaweed community structure as evidenced by decreasing seaweed abundance with decreasing salinity and increasing metal concentration. Assuming seaweeds were the primary source of complexing ligands, concentrations would be expected to decrease along the salinity gradient in parallel with seaweed abundance. However, data presented here do not follow this trend. Differences may be due to adaptation wherein those seaweeds dominating the high metal-low salinity waters may be adapted to such condition, as previously reported for phytoplankton (Gavis et al. 1981). As a result, they are likely to be capable of exuding higher amounts of metal binding ligands. Increased ligand exudation in response to metal stress has been previously reported for metal resistant microalgae (Croot et al. 2000). Connan et al. (2011a) also found that reductions in salinity and increases in Cu resulted in increased phenolic exudation by both *A. nodosum* and *F. vesiculosus.* Further to this, it is well established that resource availability affects algal secondary metabolism. For instance, negative correlations between nutrient availability and the production of phenolics have been frequently reported (Yates and Peckol 1993; Steinberg et al. 1995; Peckol et al. 1996; Pavia and Brock 2000). Thus, it is possible that increased ligand concentration in the upper estuary was a result of decreased nutrient availability, rather than salinity or labile metal concentration. However, the uniform distribution of nitrate across the sampling sites ($F=2.784$, $p=1.74$) makes this unlikely. Although there are current uncertainties whether seaweeds act to facilitate metal uptake or mitigate toxicity, they are emerging as strong candidates for the biological control of metal speciation, although further work is needed.

Although this dataset is limited, it highlights the importance of the metal complexing ligand pool. Still, it is crucial to note that any patterns inferred from such a limited dataset are

largely constrained. As such, the assumptions presented in this discussion are inherently open to question.

6.6 Conclusions

This study is the first to report Cd and Zn speciation for the Lough Furnace estuarine system. The results presented here include the influence of salinity on metal speciation, metal accumulation by macroalgae, and the concentration of dissolved organic ligands such as GSH and Cys. The voltammetric techniques employed were suitable for the speciation of Cd and Zn at low nanomolar concentrations, whereas an increased deposition time (lowering the detection limit) may benefit Cu and Pb measurements.

This study provides evidence on both the source and chemical characteristics of ligands in near-pristine estuarine conditions. The flux of ligands from sediments and exudation by macroalgae appear to be the major dissolved ligand sources in this study. Where direct identification is not possible, comparison of natural ligand complex stabilities with those of known complexes is useful in gaining insights on the nature of metal complexing ligands. In this case, similarities in magnitude were identified for the L_{Cd} and FA, and the L_{Zn} and algal exudates. These results are a direct response to the urgent call made by Stockdale et al. (2011) to measure DOM in conjunction with multiple metals to improve current models. While these findings reinforce ongoing efforts to characterize metal complexing ligands, they also indicate the need to include strong complexing ligands in speciation models.

Recent advances in LC-ESI-MS and NMR have led to the direct identification of hydroxamate siderophores in seawater (Mawji et al. 2008; Velasquez et al. 2011). As proposed by the SCOR working group 139, these techniques, if combined with voltammetry, could allow for both the qualification and quantification of metal complexing ligands. Thus, there is potential to significantly improve our understanding of marine ligands and to further advance the field. Only when ligands are fully characterized can we begin to ask more fundamental questions regarding the relationships between ligand stimuli, source, and function.

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6.8 Supplementary Information

Table S1. Comparison of sample handling and treatment methods for spiked artificial seawater and seawater samples collected from the Lough Furnace estuary, for total dissolved labile metal concentration (mean ± s.d., *n*=5). Methods tested include: (A) filtration and immediate analysis (conventional); (B) filtration, freezing, thawing, then analysis (conventional); and (C) freezing, thawing, filtration, then analysis (unconventional and employed in this study).

Element	Artificial seawater $[M]_L$ (nM)			Lough Furnace seawater $[M]_L$ (nM)		
	A	B	C	A	B	C
Cd	1.11 ± 0.19	1.03 ± 0.05	1.07 ± 0.05	0.69 ± 0.19	0.66 ± 0.04	0.66 ± 0.03
Zn	4.57 ± 0.20	4.40 ± 0.62	5.20 ± 0.16	2.42 ± 0.28	2.16 ± 0.25	3.34 ± 0.09*

* denotes significant difference in labile metal concentration between methods, within a water type.

Table S2. Accuracy of the analytical procedures employed for seaweed metal concentration and seawater total dissolved metal concentration (mean ± s.d., *n*=3). The algal CRM (NIES no. 9) certified by the National Institute for Environmental Studies, Japan Environment Agency and the near-shore seawater CRM (CASS-5) certified by the National Research Council of Canada.

Element	NIES no. 9			CASS-5		
	Measured (μg g^{-1} DW)	RSD (%)	Certified (μg g^{-1} DW)	Measured (nM)	RSD (%)	Certified (nM)
Cd	0.17 ± 0.026	15.3	0.15 ± 0.002	0.20 ± 0.021	10.5	0.19 ± 0.016
Cu	5.001 ± 0.58	11.59	4.9 ± 0.2	6.16 ± 0.21	3.41	5.97 ± 0.44
Pb	1.31 ± 0.15	11.5	1.35 ± 0.05	**0.052 ± 0.001**	1.92	0.053 ± 0.01
Zn	16.13 ± 0.21	1.31	15.6 ± 1.2	12.01 ± 0.25	2.1	10.92 ± 1.034

Bold represents measurements where deposition time was increased from 1 to 5 min.

Chapter 7 General Discussion and Conclusions

Chapter 7 General Discussion and Conclusions

7.1 Macroalgae as a source of L

Our studies measuring the exudation of organic ligands by macroalgae in response to increasing metal concentrations have demonstrated that macroalgae are a source of L. As reported in Chapters 3 and 4 (Murray et al. a, b), all algal species released metal complexing ligands yielding concentrations ranging from 2.22-72.45 nM. In each case, the concentration of exuded ligand on day 7 was significantly higher (up to 97%) in cultures with added metals than the relevant controls. Although algae-exuded organic ligands have been the focus of several studies, there are very few macroalgae-relevant data published. Instead, most data pertain only to microalgae and/or Cu. Therefore, this work affords novel insight into the production of Cd, Pb and Zn ligands by macroalgae and confirms those results already published for Cu.

Results clearly indicate that the exudation of L is seaweed-specific. For example, *L. hyperborea* produced significantly less L_{Cu} and L_{Zn} and significantly more L_{Cd} and L_{Pb} than the other two species. This indicates that the contribution of macroalgal species to L in seawater varies and subsequently, the ligand pool is likely a function of the species-specific biomass at a given location. Thus, the contribution of an algal species to the ligand pool is a balance between its biomass and production efficacy.

Despite species differences in production efficacy, it appears that similar response mechanisms may be responsible for ligand production as evidenced by the positive covariation between $[M_L]$ and ligand release for all combinations of metal treatment and macroalgal species. This response is most likely a general defence against metal toxicity or a sequestration strategy resulting from nutrient deficiency. The fact that $[L_M]$ is lowest in the control cultures described in Chapters 3 and 4 (Murray et al. a, b) points to ligand production as a defence mechanism rather than a means to acquire metals. Similarly, although exuded ligands maintain low metal availability in the culture solutions, these ligands cannot be considered specific indicators of metal toxicity, based on their omnipresence in the control cultures.

Despite these findings, our study on ligand production by *F. vesiculosus* in response to a multiple metal mixture presented in Chapter 5 (Murray et al. c) suggests that macroalgae-exuded L_{Cu} and L_{Zn} are metal-specific and, by contrast, macroalgae-exuded L_{Cd} and L_{Pb} are non metal-specific. Thus it appears that the ligand system in place produces cation-recruiters for the purposes of normal sequestration but toxin-only complexants when assuming a

detoxifying role. The same dual functionality has been previously speculated for phytochelatins produced by metallophytes (Schat 2002).

Additional information on the characteristics of L_M may be obtained by normalization of the ligand data for DOC. The density of metal ligands per unit of organic carbon (nM mg^{-1} C) determined for the highest metal treated macroalgae cultures on day 7 follow the order Cu (14.49) > Zn (9.10) > Cd (6.50) > Pb (3.61). The DOC exuded by macroalgae has more metal binding sites than that of natural aquatic DOM. For instance, at the seawater end of Lough Furnace the normalized concentrations of both L_{Cd} (1.09 nM mg^{-1} C) and L_{Zn} (3.47 nM mg^{-1} C) are substantially lower than those of the culture solutions. As outlined by Scoullos et al. (2004), low normalized field values are generally the result of several DOC sources, not all which attribute metal binding capacity. Still, Karavoltsos et al. (2013) found that seaweeds did not contribute significantly to the Cu ligand density per mass unit of C in the Aegean Sea. Of course, this may be due to differences in the chemical compositions of the waters as well as different environmental conditions.

7.2 Metals as stimuli for the exudation of L

In addition to identifying macroalgae as a source of L, our studies provide evidence that metals act as production stimuli of naturally occurring organic ligands. As demonstrated in Chapters 3, 4, and 5 (Murray et al. a, b, c), all seaweeds produced L and the $[L_M]$ was always significantly higher in cultures where metals were added than the relevant controls. The strong apparent coupling of $[M]_D$, $[M]_L$, and $[L_M]$ present in all cultures was also evident in the Lough Furnace Estuary (as outlined in Chapter 6; Murray et al. d). In practical terms, this means field stimulation of ligand production must be considered when evaluating the potential impact of a particular metal on a given system, and its subsequent speciation.

For all species, ligand production was greatest in response to Zn, followed by Cu, Pb, then Cd. As such, Zn is the most effective stimulus of ligand production by *A. nodosum*, *F. vesiculosus*, and *L. hyperborea* at environmentally relevant metal concentrations. The remaining metals also induce ligand exudation, though to a lesser extent. While other studies on the production of L are generally limited to Cu (Andrade et al. 2010; Karavoltsos et al. 2013), comparing the induced L production in response to metal additions typical of coastal metal concentrations allows for assessment of similar effects in natural systems. In each case, organic ligands were in excess of metal concentration. Thus, based on these findings, it is likely that Cd, Cu, Pb and Zn in near-pristine coastal waters are maintained at low

concentrations by organic ligands. Of course, metal-specific variability in the concentration of ligand exuded may be the result of differences in stoichiometry. The detected ligand concentrations for the given metal treatments suggest that 1:1 (Cu, Zn; Murray et al. a) and 3:1 (Cd, Pb; Murray et al. b) ligand: metal complexes are formed. Earlier studies on Cu speciation also found concentrations of L in a similar 1:1 relationship with dissolved metal (Croot 2003). The fact that L_{Cd} and L_{Pb} remain lower than L_{Zn} despite their higher ligand:metal ratios supports the assumption that Zn is the most effective inducer of ligand exudation.

7.3 On the identity of L

The electrochemical analysis of all algal culture solutions and field water samples confirmed the presence of excess amounts of metal-binding ligands. However, the voltammetric methods employed indicate only the presence and concentration of the ligands and do not distinguish between the metal-binding groups. Discerning ligand identity is crucial as metal bioavailability is influenced by the chemical nature of L. Comparison of the logK' values determined experimentally in Murray et al. 2013a-d with those reported in the literature provides the only information so far on possible ligand identity.

Compounds previously identified in seawater as trace metal ligands include humic substances (Laglera et al. 2007), sulfides (Han et al. 2006), thiols (Al-Farawati and van den Berg 2001), phlorotannins (Swanson and Druehl 2002), and phytochelatins (Ahner et al. 1998). As outlined in Chapter 3 (Murray et al. a), the chemical composition of macroalgae-exuded L_{Cu} and L_{Zn} is likely quite different than those ligands already identified in seawater. Dissimilarities between logK' values indicate functional differences between the macroalgae-exuded ligands and model compounds that could be related to the biogenic source of L. It is important to note that this lack of comparability with model ligands does not rule out the existence of macroalgae-exuded organic ligands in seawater. Rather, it suggests that these ligands constitute a portion of the "ligand soup" in seawater that remains poorly defined. Exudation of unknown ligands affecting Cu chemistry has already been reported for *Laurencia* spp. (Rhodophyta) (Karavoltsos et al. 2013).

By contrast, sulfides and humic substances are proposed as strong potential candidates for the Cd and Pb ligands determined in Chapter 4 (Murray et al. b). It is already well established that sulfide ions play an important role in Cd detoxification in plants and yeasts, where the incorporation of sulfides into PCs increases the intracellular Cd complexing capacity. Thus, it is conceivable that the same mechanism is employed by seaweeds for the

external control of Cd speciation. Still, the incorporation of sulfides in exogenous metal-PC complexes requires further confirmation. Similar to sulfides, metal-humic substance complexation has been extensively investigated in seawater (Town and Filella 2002). Correlation of L_{Pb} with humic substances suggests similar complexation sites, namely carboxylic and phenolic functional groups that bind Pb. However, any further insight would require a more complete structural characterization.

A striking result described in Chapter 6 (Murray et al. d) is that all points of the estuarine system exhibited Zn binding capacity similar to that reported for the isolated macroalgae-exuded organic ligands in Chapter 3 (Murray et al. a). The link between the $logK'L_{Zn}$ values of the experimental culture solutions and native seawater suggests macroalgae-exuded ligands dominate the Lough Furnace Zn ligand pool, and provides evidence that the exudation of L in the laboratory is in fact relevant to the marine environment. Yet, the same correlation does not exist for L_{Cd}. Instead, the L_{Cd} measured in the Lough Furnace estuary are most likely FAs whereas those determined in the culture solutions were thought to be sulfides. This suggests that, at least in the case of Cd, macroalgae exuded ligands do not dominate the complexing capacity of the estuary. This is not surprising given that ligands in natural waters may be derived from a vast array of terrestrial and aquatic sources resulting in a diverse range of metal complexers such as humic substances and thiols. As described by Town and Filella (2002) for Cu, it is more likely that metal binding curves of natural systems such as Lough Furnace lie somewhere between those for humic substances and biotic complexants (including, but not limited to, macroalgae-exuded organic ligands). Still, a 2nd hypothesis that the simple organic sulfide ligands exuded by macroalgae are subsequently incorporated into fulvic acids, where present, cannot be entirely dismissed.

Although some variability exists in the magnitude of the thiol response to $[M]_L$, the [GSH] on the whole was relatively low and stable in both culture solutions and field samples. In fact, the estimated GSH and Cys concentrations were always much lower than the measured L, indicating that overall thiols do not form a substantial portion of the ligands measured in Chapters 3, 4, 5, and 6 (Murray et al. a, b, c, d) Instead it is more likely that the production/presence of GSH is regulated by environmental factors other than metal concentration alone.

7.4 General Conclusions

As outlined in **Figure 7.1**, the studies presented here have clearly demonstrated that macroalgae-exuded organic ligands significantly reduce labile metal by forming strong complexes. In fact, those ligands produced by macroalgae were found to have concentrations and logK' values similar to those reported for natural waters, including the Lough Furnace Estuary. From these results, we can conclude that macroalgae produce metal-complexing organic ligands and trace metals in solution condition the concentration and nature of L exuded. These data represent a significant contribution to the current state of knowledge regarding the marine ligand pool.

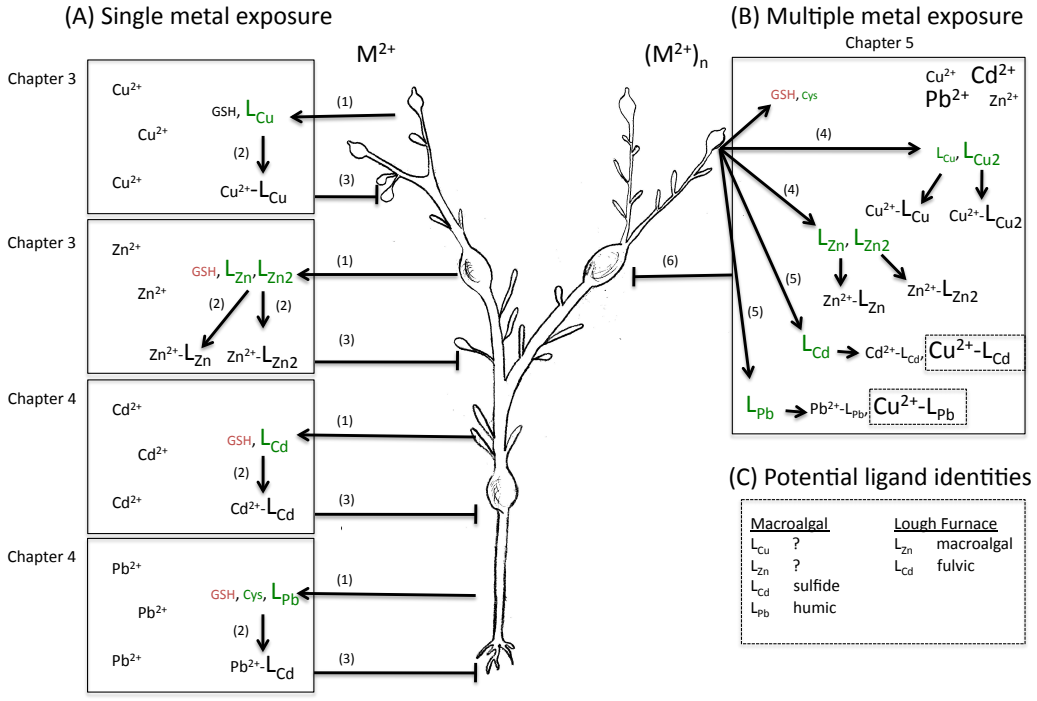

Figure 7.1. Schematic diagram summarizing our results on macroalgal ligand production in response to trace metals. (A) In response to single metal exposures macroalgae released ligands (1) which decreased the concentration of labile metal through complexation (2) but had no effect on the overall bioaccumulation (3). Within a panel, font size is proportional to the concentration of the metal species and color font indicates an increase (green) or decrease (red) in ligand concentration with increasing metal treatment. (B) In response to multiple metal exposure, macroalgae released ligands which were hypothesized to be (4) metal or (5) non-metal specific yet had no effect on the overall bioaccumlation (6). Within this panel, font size is proportional to the concentration of the metal species in comparison to the single metal cultures and color indicates an increase (green) or decrease (red) in ligand concentration with increasing metal treatment. (C) For laboratory and field experiments, a comparison of conditional stability constants provided clues on the identity of ligands. In all panels, dotted lines denote compounds that were not measured directly but whose presence is inferred.

Although we can say with certainty that macroalgae release ligands in response to metals, the relative importance of macroalgae-exuded ligands in natural systems will depend

on the nature the system itself. Recently, Muller and Batchelli (2013) established that *in-situ* ligands of biological origin are responsible for the buffering of Cu^{2+} in the river Thurso, where a stronger class of terrestrial ligands are saturated with Cu throughout the plume. This suggests that, at least in certain systems, macroalgae-exuded ligands may have the capacity to regulate M_L. Still, because both the speciation of metals and the physiology of macroalgae are affected by environmental conditions, the physico-chemical properties of seawater are likely to have as yet unidentified feedback effects on the ligand pool. The ability of seaweeds to produce ligands under low or very high salinity and the importance of these ligands in the presence of high humic substances are only a few of the considerations which must be thoroughly explored. Accordingly, many questions remain unanswered.

This thesis highlights several important points, each with significant implications for future work. Although the data reported here suggest that ligand exudation and subsequent metal complexation is a means of detoxifying trace metals by scavenging loss rather than acquiring them by ligand-aided dissolution, additional toxicological studies are required to quantify the role of both macroalgae-exuded organic ligands and other natural ligands in controlling the trace metal toxicity of natural systems. Similarly, a failure to develop broader conceptual models that employ a continuum of binding sites based on actual L measurements, such as those presented in Chapter 6 (Murray et al. d), could easily result in the inaccurate speciation of certain waters (particularly those with low humic substance content). In practical terms this means future models should place emphasis on ligand parameters determined from field studies and current models should be re-evaluated to account for differences in bioavailability in the presence of biogenic ligands. Likewise, single metal systems are not representative of metal-mixtures and as such, results from multiple metal mixture studies should be used where possible. The most warranted extension of this work is the complete structural characterization of L. Only once the chemical nature of the ligand pool is fully understood can we elucidate the binding mechanisms and decipher whether macroalgal response is by design (for the purposeful regulation of metal-uptake) or by chance (where ligands may be the by-product of an alternate metabolic process, stimulated by one or several other environmental factors, or intended for the regulation of structurally or chemically similar compounds).

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Appendices

Appendix 1

Optimization of Voltammetric Parameters

Prior to the analysis of real samples, voltammetric methods were optimized in the laboratory using bulk seawater. Conditions were chosen so as to fulfill the necessary requirement of sensitivity while maintaining manageability of both methodology and length of analysis.

Purge Time

Purge time had no effect on the peak height or slope of the voltammetric analyses (**Figure A1**). A purge time of 5 min was chosen for analyses, as it guaranteed the absence of the oxygen wave.

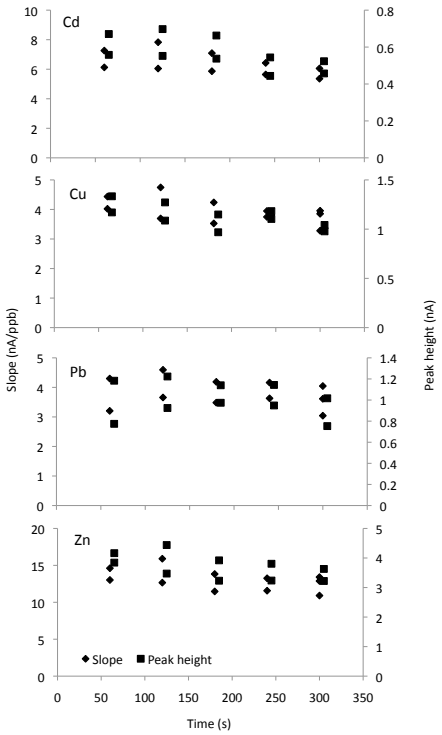

Figure A1. Effect of varying the purge time on the peak height and slope of the current response for Cd, Cu, Pb and Zn.

Stir Rate

Sensitivity increased linearly with stir rate (**Figure A2**). However, it was noted that stirring beyond 2000 rpm often resulted in drop loss during analyses. As a result, the stir rate was set to 2000 rpm for each method in order to optimize sensitivity and maintain the mercury drop throughout analysis.

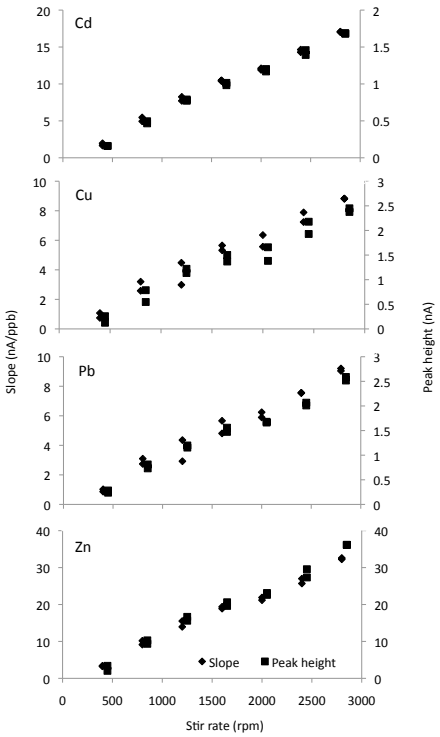

Figure A2. Effect of varying the stir rate on the peak height and slope of the current response for Cd, Cu, Pb and Zn.

Drop Size

As seen in **Figure A3**, sensitivity increased with drop size. However, beyond size 6 (0.52 mm^2) there was a tendency for the drop to fall approximately once in every three analyses. Accordingly, drop size 6 was employed in all analyses.

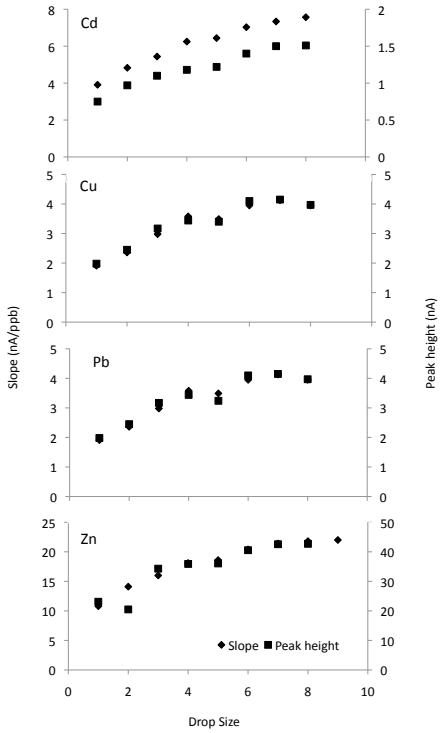

Figure A3. Effect of varying the HMDE drop size on the peak height and slope of the current response for Cd, Cu, Pb and Zn.

Deposition Time

As expected, the strength of the analyte signal increased with deposition time (**Figure A4**). From 10 s-480 s, the current response increased linearly with time. For the majority of the analyses, a deposition time of 60 s was employed in order to maintain sensitivity and manageability.

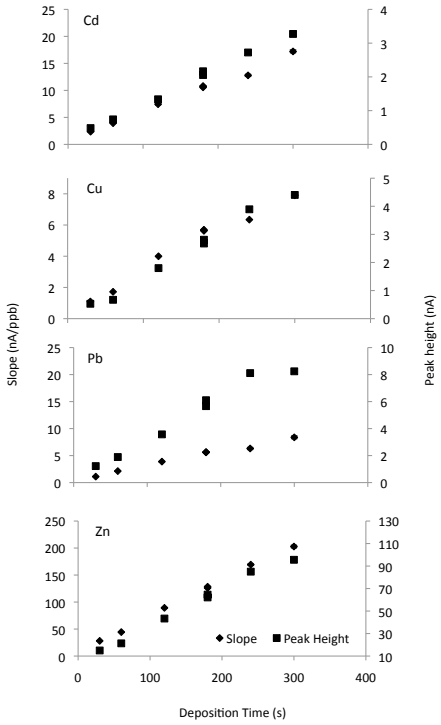

Figure A4. Effect of varying the deposition time on the peak height and slope of the current response for Cd, Cu, Pb and Zn.

Appendix 2

Generating metal complexation data by reverse titrations
(using Excel:MAC 2008; adapted from van den berg 1982, Turoczy and Sherwood 1997)

1. Make an independent measurement of total metal concentration: $[M]_D$

2. Enter the complexation capacity titration data in columns:
   1: added M
   2: i_p (measured peak height)

3. Make an initial estimate of sensitivity (S') by applying the Excel array function 'LINEST' to the final 4 titration points as follows:

   1st argument=the range of cells containing the [added metal] (nM)
   2nd argument=the range of cells containing the peak height (i_p)
   3rd argument=true (i.e.: force the y-intercept to 0)
   4th argument=true (i.e.: provide all statistical parameter rather than just m and b)

   Press Apple-Shift-Enter simultaneously

   S' is equal to the calculated slope (m).

4. For each row in Excel, calculate the following as separate columns:
   3: $[M]_{Total}$ = $[M]_D$ + added M
   4: $[M]_L = i_p * S$
   5: $[M]_L/[ML] = 4/(3-4)$

5. Make an initial estimate of C_L' and K' using S' and applying the Excel array function 'LINEST' as follows:

   1st argument=the range of cells containing the $[M]_L$ (nM)
   2nd argument=the range of cells containing $[M]_L/[ML]$
   3rd argument=true (i.e.: force the y-intercept to 0)
   4th argument=true (i.e.: provide all statistical parameters)

   Press Apple-Shift-Enter simultaneously

   Use these values to solve the following equation:
   $y=mx + b$ as $[M]_L/[ML]=[M]_L/L_M + (\sum\alpha_{AL})/(L_MK'L_M)$

   $[L_M]$ is equal to the inverse of the calculated slope (m).
   $LogK'L_M$ is equal to the log of the slope (m)/y intercept.

6. Generate model data for columns 2, 4, and 5 using the initial estimates of S', C_L', K'.

7. Make a 2nd estimate of sensitivity (S'') by applying the Excel array function 'LINEST' to the final 4 titration points of the model data (from step 6) as follows:

   1st argument=the range of cells containing the [added metal] (nM)
   2nd argument=the range of cells containing the model peak height (i_p)
   3rd argument=true (i.e.: force the y-intercept to 0)
   4th argument=true (i.e.: provide all statistical parameters rather than just m and b)

   Press Apple-Shift-Enter simultaneously

   S'' is equal to the calculated slope (m).

8. In a new cell, adjust S' as follows: $S_{adj}'=S'*(S'/S'')$

9. Recalculate columns 4 and 5 using the original data and the new S_{adj}'
   4: $[M]_L = i_p * S$
   5: $[M]_L/[ML] = 3/(1-3)$

10. Apply the Excel array function 'LINEST' to the new data (from step 8) as follows:

    1st argument=the range of cells containing the $[M]_L$ (nM)
    2nd argument=the range of cells containing $[M]_L/[ML]$
    3rd argument=true (i.e.: force the y-intercept to 0)
    4th argument=true (i.e.: provide all statistical parameters)

    Press Apple-Shift-Enter simultaneously

    Use these values to solve the following equation:
    $y=mx + b$ as $[M]_L/[ML]=[M]_L/L_M + (\sum\alpha_{AL})/(L_M K'L_M)$

    $[L_M]$ is equal to the inverse of the calculated slope (m).
    $LogK'L_M$ is equal to the log of the slope (m)/y intercept.

    *Note: for Cd and Pb, there is no α_{AL} as no added ligand (AL) is used.

11. Repeat this process until C_L' and L' have converged to constant values. Twenty iterations are typically sufficient. Recording and employing a Macro of this procedure in Excel is not necessary but will save a considerable amount of time.