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Trace metal chemical speciation and acute toxicity to Pacific oyster larvae

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TRACE METAL CHEMICAL SPECIATION AND ACUTE TOXICITY TO PACIFIC OYSTER LARVAE

by

Cathryn Money

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Controlled laboratory studies showed that the toxicity induced by biologically relevant trace metal species of Cu, Cd, Pb and Zn on embryo-larval development occurred at concentrations in excess of those found in the natural environment, except for Cu in metal perturbed areas. Average free ion concentrations inducing 50% abnormal development (EC_{50free}) were determined as 0.23 nM Cu^{2+}, 88.0 nM Cd^{2+}, 128 nM Zn^{2+} and 362 nM Pb^{2+}. However, the response to some binary metal combinations indicated enhanced (synergy) toxicity at concentrations relevant for estuarine waters (e.g. EC_{50free} for Cu^{2+} in the presence of Cd^{2+}, Zn^{2+} and Pb^{2+} was 0.004, 0.02 and 0.04 nM, respectively).

A comparison of voltammetric instrumentation (voltammetric *in situ* profiling (VIP) system versus Hanging Mercury Drop Electrode with potentiostat) highlighted the advantage of high resolution measurements (ca. 20-60 min intervals) for environmental studies and the minimisation of artefacts associated with discrete sampling methodologies.

Field-based studies were carried out in two contrasting estuaries in SW England, one heavily impacted with metal contaminants (Fal Estuary) and another subject to greater variety of anthropogenic influences (Tamar Estuary). High resolution *in situ* trace metal speciation measurements, carried out over tidal cycles, identified important information on the temporal and spatial distributions of biologically relevant dynamic (<4 nm) metal species of Cd, Pb and Cu. Variation in embryo-larval responses to discrete samples from these estuaries, effectively paralleled the metal speciation measurements showing enhanced toxicity when the marine water influence was at its lowest. In both systems, the results indicated that the combined effect of the metals studied was likely to have provided a significant contribution to the bioassay response. However, the difficulty in de-coupling the speciation measurements with biological responses was evident and supports the need for more comprehensive campaigns to study the impact of contaminants on ecosystem functioning. Bioassay and metal speciation analysis techniques were complementary, exhibiting high sensitivity and rapid responses, and would be considered effective screening tools for waters subject to intermittent inputs of metal contaminants and areas with recognised pressures. The integrated approach has extended our knowledge of trace metal speciation in estuarine environments and their effects on the developing embryos of the Pacific oyster. This approach has the potential for wider application.

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LIST OF ABBREVIATIONS

$[B]_{const}$	Constant buffer concentration
$[M]_{const}$	Constant metal concentration
AL	Added ligand
AdCSV	Adsorptive cathodic stripping voltammetry
ASW	Artificial sea water
BLM	Biotic ligand model
CE	Counter electrode
C_L	Concentration of natural ligand
C_M	Total metal concentration in the presence of added ligand
C_T	Total metal concentration
DGT	Diffusive gradient in thin-film
DW	Detection window
EA	Environment Agency
EC_{05}	Effective concentration causing 5% abnormal development
EC_{50}	Effective concentration causing 50% abnormal development
EDTA	Ethylenediamine tetraacetic acid
E_p	Peak potential
EPA	Environmental Protection Agency
EQS	Environmental Quality Standard
FIAM	Free ion activity model
FSW	Filtered sea water
GIME	Gel-integrated microelectrode
HEPES	*N*-hydroxyethylpiperazine-*N'*-*2'*-ethanesulphonic acid
HMDE	Hanging mercury drop electrode
i_b	background current
i_c	capacitive current
i_p	peak current
L′	Concentration of natural ligand not complexed by M

LDPE	Low density polyethylene
Log K′	Conditional stability constant
Log K'_{ML}	Conditional stability constant for the complex ML
M′	Inorganic metal concentration
MAL	Metal-added ligand
ML	Metal-natural ligand
M_{labile}	Labile metal concentration
M^{z+}	Free metal ion
OEL	Oyster-embryo larval bioassay
PLM	Permeation liquid membrane
PNR	Percent net response
r	Electrode radius
RE	Reference electrode
S	Sensitivity
SA	Salicylaldoxime
SWCSV	Square wave cathodic sweep voltammetry
SWASV	Square wave anodic stripping voltammetry
VIP	Voltammetric *in situ* profiler
WE	Working electrode
WFD	Water Framework Directive
α'	Overall side reaction coefficient
$\alpha_{M'}$	Inorganic side-reaction coefficient for complexation of M^{z+} by the major anions
α_{MAL}	Inorganic side reaction coefficient for the metal added ligand complex
α_{ML}	Inorganic side reaction coefficient for the metal natural ligand complex
β'_{MAL}	Conditional stability constant for the formation of MAL in seawater

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AUTHORS' DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate committee.

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Chapter 1:

Introduction

1.1. Introduction

Estuarine and coastal systems are subject to natural (e.g. from geochemical weathering) and anthropogenic inputs from agricultural, domestic and industrial sources, which can increase the concentrations of contaminants above baseline levels. Although metals are ubiquitous in aquatic environments, estuarine and coastal areas subjected to high levels of metal contaminants, for example from past and/or present mining activities and the marine industry (e.g. anti-fouling paints), present environmental concern with regard to human health risks (through seafood consumption) and ecosystem functioning.

A number of trace metals, such as Fe, Cu and Zn, can act as micronutrients or toxicants, depending on their concentration and physico-chemical speciation and therefore play an important role in regulating the structure of aquatic communities (Engel *et al.*, 1981). Traditionally, marine pollution was documented in terms of the concentrations of chemical contaminants with no measure of the deleterious effects on living organisms. While bioassays measure a direct response by specific organisms to all biologically available compounds within a medium, the bioassays do not identify the compounds inducing the effect. Another approach is to predict the biologically available metal species or group of metal species that induce a biological response using simplified theoretical models where certain assumptions are made (e.g. free ion activity model (FIAM) and the biotic ligand model (BLM) which assume that thermodynamic equilibrium is attained). Nonetheless, in order to properly evaluate natural water systems, and provide more meaningful data on their ecological status it is necessary to both quantify environmental contaminants and assess their biological effects. This is recognised in legislation, such as the EU Water Framework Directive (WFD, 2000/60/EC), which aims to improve and protect the quality of aquatic ecosystems with the monitoring of chemical, biological and physical parameters.

1.2. EU Water Framework Directive (WFD)

The WFD is based on a risk assessment exercise and requires that natural waters within the EU achieve 'good' ecological status by 2015. For successful implementation, information representative of water quality across EU member states is required.

It is accepted that a number of tools and techniques need to be identified or developed and fully validated in order to meet the diverse monitoring requirements of the directive. The monitoring aspects of the WFD was scheduled to commence in December 2006 (Allan *et al.*, 2006), and includes surveillance, operational and investigative monitoring. Waters achieving good quality status undergo surveillance monitoring, while operational monitoring will be required for waters that fail to achieve the necessary quality due to identified causes or where there are recognised pressures. This applies to all surface water bodies, including complex systems (e.g. tidal waters) or systems subject to temporal fluctuations in pollutant levels (e.g. seasonal use of pesticides or weather patterns), where more frequent or widespread monitoring will be necessary. If a water body fails to meet the quality standards and the cause is unknown then investigative monitoring will be needed (Roig *et al.*, 2007).

A wide range of mandatory and recommended quality elements (e.g. elements chosen to represent the quality of a water body) covering the biological, chemical and hydro-morphological status of water bodies have been defined. However, it is important that appropriate indicators of quality elements are selected for monitoring purposes, although the relevance of a parameter as an indicator can be established only when more detailed investigations of a water body have been undertaken. While there is no specific requirement in the WFD to link biological or chemical data to assess the health of an ecosystem, it is helpful in identifying valid indicators of water quality. Moreover, there are advantages to the use of systems that can detect early changes in water quality with affects on aquatic organism health, particularly where intermittent, accidental or deliberate release of a contaminant(s) is likely to occur (Allan *et al.*, 2006).

The importance of investigating the speciation and toxicological impacts of trace metals is recognised. In terms of the WFD and specific to the four metals chosen for this study, Cd and its compounds have been identified as priority hazardous substances (Annex V), Pb and its compounds as priority substances (Annex V), while Cu and Zn are recognised as specific pollutants (Annex VIII), the total concentrations of which are required to be below their respective Environmental Quality Standards (EQS), currently set at 5 $\mu g\ L^{-1}$ Cu and 40 $\mu g\ L^{-1}$ Zn.

1.3. Trace metal chemical speciation

The biological availability and toxicity of trace metals are determined by their chemical forms, which in the aquatic environment includes free hydrated ions, complexes with inorganic and organic ligands and associations with colloids and particulate matter (Cobelo-Garcia *et al.*, 2003; van den Berg *et al.*, 1987). It has been well documented that the biological availability of a number of trace elements is generally correlated with the inorganic or free metal ion concentration (Lorenzo *et al.*, 2002; Brand *et al.*, 1986). Notwithstanding this, the chemical speciation of many dissolved trace metals in seawater (e.g. Cu, Pb, Fe, Co, Ni) is dominated by organic complexation that lowers their free ionic concentrations and consequently their availability to biota (Buck and Bruland, 2005; Wells *et al.*, 1998). This is particularly important for trace metals, such as Cu, which has been widely studied because of its importance as a micronutrient and high toxicity to marine organisms, such as unicellular algae, metazoans, fish and bivalves (Rivera-Duarte *et al.*, 2005; Moffett *et al.*, 1997; Blust *et al.*, 1991, 1986; Zamuda and Sunda, 1982; Engel *et al.*, 1981; Anderson and Morel, 1978; Sunda and Guillard, 1976).

The reaction of a hydrated metal ion with the different constituents present in the aquatic environment is schematically represented in Figure 1.1.

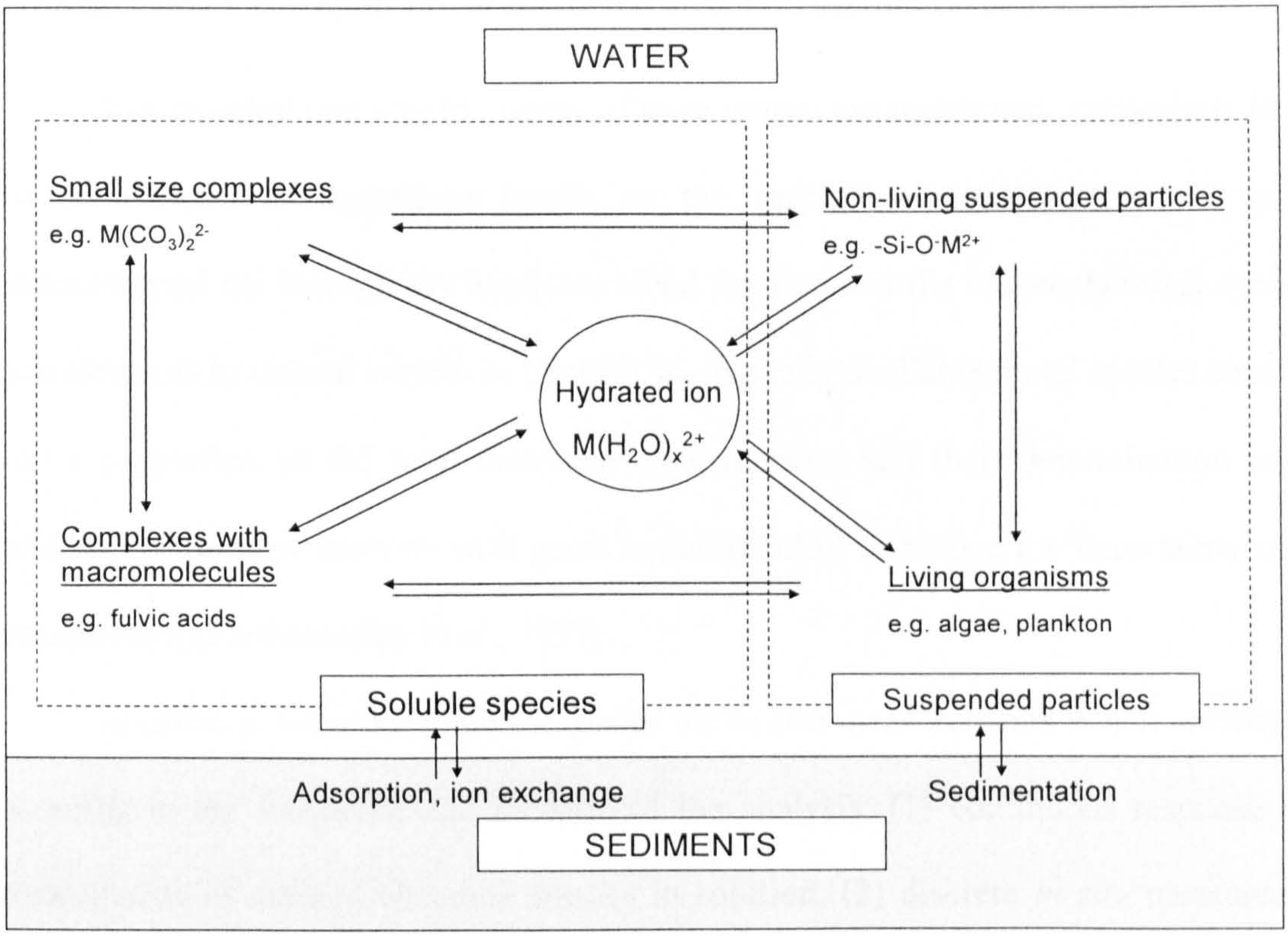


Figure 1.1: Schematic representation of the reactions of a metal ion, M, with the different types of aquatic system constituents (adapted from Buffle, J. *Complexation reactions in Aquatic systems*, Ellis Horwod, Chichester, 1988).

1.4. Monitoring approaches

To meet the WFD requirements for monitoring trace metals (and other species) three generic approaches can be used: (1) water sampling followed by sample storage, handling and analysis in the laboratory, (2) on-site analysis after manual or automatic sampling, and (3) *in situ* analysis at the exact location of interest (Buffle and Horvai, 2000). Near real-time measurements can identify important trends where discrete sampling methods can oversimplify or provide inaccurate data on environmental processes and may be poor indicators of the quality of a water body (Tercier-Waeber *et al.*, 2000). Moreover, the dynamic nature of natural waters, particularly estuarine systems, requires monitoring at high spatial and/or temporal resolution, which is not easily achieved using discrete sampling methods followed by laboratory analysis.

1.5. Analytical techniques for *in situ* trace metal speciation measurements

It is essential that specific forms of trace metals are monitored, particularly in areas that are subject to significant inputs, as the speciation measurements will provide information on the biologically available metal fraction and the biogeochemical cycling of trace elements in natural waters. In general, biologically available metal species constitutes only a proportion of the total dissolved concentration and their determination requires sensitive methods of analysis with good reproducibility to reduce the uncertainty in their measurement (Parthasarathy *et al.*, 1997).

In essence, there are three categories for *in situ* measurements which are classified according to the frequency and location of the analysis: (1) continuous response to the concentration of defined chemical species in solution, (2) discrete *in situ* measurements, either directly or after collection of discrete samples (e.g. flow injection analysis (FIA) and voltammetric techniques), and (3) fractionation of chemical species *in situ* followed by analysis in the laboratory (e.g. permeation liquid membrane (PLM) and diffusive gradients in thin-films (DGT) techniques) (Davison *et al.*, 2000).

There are few techniques which combine speciation capabilities with high sensitivity for *in situ* applications. The metal species measured with these techniques are defined by the nature of the technique itself and the operating conditions used, and therefore each technique measures a different fraction (e.g. is 'operationally defined'). The techniques available for speciation analysis include:

Flow Injection Analysis: Flow injection systems allow analyses to be performed in a highly reproducible manner. Methods have been developed that have suitable detection limits for the determination of total dissolved concentrations of a number of metals present in seawater, including Cu, Fe, Mn, Co, Al, and Zn (Yu *et al.*, 2007; Tyrell *et al.*, 2006; Coale *et al.*, 1992; Lui and Ingle, 1989). These methods most often use a column with an immobilised ligand to separate and pre-concentrate the analyte and use either chemiluminescence, kinetic spectrophotometric or fluorescence methods of detection. The

potential for speciation measurements was demonstrated by Zamzow *et al.* (1998) where flow injection-chemiluminescence (FI-CL) was used to examine copper speciation in discrete seawater samples using a titration approach. In general, these systems are compact, rugged and well suited to field deployment.

Permeation Liquid Membrane (PLM) techniques: Permeation liquid membrane (PLM) techniques are an emerging tool for separation and preconcentration of target elements or species (Slaveykova *et al.*, 2004; Parthasarathy *et al.*, 2004, 1997; Jonsson and Mathiasson, 1992) and have been shown to be a promising technique for *in situ* applications (Ndungu *et al.*, 2005; Parthasarathy *et al.*, 2004; Djane *et al.*, 1997; Papantoni *et al.*, 1995). The technique is selective for free ions and/or lipophilic forms of the test compounds and therefore provides information on biologically relevant species. Principally, PLMs pre-concentrate the analyte from the test solution into a so-called strip or receiver solution from which the analyte is measured with a suitable analytical instrument (e.g. AAS, voltammetry, ICP-MS, fluorimetry or coupled chromatographic techniques). Since the analyte is preconcentrated in a solution, the coupling of the PLM with various *in situ* detectors is also possible. Furthermore, PLMs have the potential to pre-concentrate both inorganic and organic species thereby widening its application (Buffle *et al.*, 2000).

Diffusive Gradients in Thin-films (DGT): Diffusive gradients in thin-films (DGT) is a kinetic rather then an equilibrium technique, whereby solutes freely diffuse through a layer of hydrogel (e.g. polyacrylimide) and are then immobilised in an underlying layer of binding agent (e.g. Chelex resin). The amount of analyte accumulated is proportional to the external concentration of labile species (e.g. where labile refers to metal-ligand complexes that have fast formation/dissociation reactions and generally include the free ion, inorganic metal complexes and small organic complexes), the deployment time, the surface area exposed to the solution, and the diffusion coefficient of the analyte, and is inversely proportional to the diffusive layer thickness. In essence, the chemical separation is made *in situ* but the analysis is performed under controlled laboratory conditions. DGT provides a

time-integrated response to biologically available trace metal species, and has been shown to be a more reliable indicator for the assessment of the impact of trace metals on organisms than total dissolved metal concentrations (Dunn *et al.*, 2007; Allan *et al.*, 2007, 2006; Forsberg *et al.*, 2006; Larner *et al.*, 2006; Stark *et al.*, 2006 Alfaro-de la Torre *et al.*, 2000; Davison and Zhang, 1994).

Voltammetric techniques: Voltammetry is an electrochemical technique where the current, *i*, produced from the oxidation or reduction of an analyte(s) is recorded as a function of the potential, *E*, imposed on an electrode system. The *in situ* voltammetric systems developed allow continuous near real-time monitoring of trace elements as well as the simultaneous determinations of several trace metals (e.g. Cu, Cd, Pb and Zn). The technique is based on size separation whereby an operationally defined metal fraction <4 nm is measured. This size fraction is considered to be biologically available. The voltammetric *in situ* profiler (VIP) system has shown its potential for *in situ* applications with the advantages of improved capability to detect rapid changes in speciation and minimisation of artefacts associated with sample collection, handling and analysis (Howell *et al.*, 2003a; Tercier-Waeber *et al.*, 2005). It has successfully been applied in lake water, river water, groundwater, fjord water and estuarine and coastal marine waters to measure the dynamic metal fractions of Cu(II), Cd(II), Pb(II), Zn(II) at pM levels and Mn(II) and Fe(II) at nM levels using either square-wave anodic stripping voltammetry (SWASV) or square wave cathodic sweep voltammetry (SWCSV) (Tercier-Waeber *et al.*, 2005; Howell *et al.*, 2003b; Pauwels *et al.*, 2002; Tercier-Waeber *et al.*, 2002, 1999, 1998a, 1998b; Tercier-Waeber and Buffle, 2000; Tercier *et al.*, 1998a; Tercier *et al.*, 1995).

Voltammetric techniques are particularly well suited to investigate the speciation of trace metals such as Cu, Cd and Pb, allowing the quantification of different forms present in the water column (Braungardt *et al.*, 2007). Moreover, the recent advances in *in situ* voltammetric instrumentation enable the speciation of trace metals to be examined in more detail, with the advantage of detecting short-term in-water variability (Howell *et al.*,

2003b; Tercier-Waeber *et al.*, 2005). This is a distinct advantage when compared with, for example, a time-integrated response that cannot fully characterise these changes.

1.6. Biological methods for the assessment of toxic impacts: Marine bioassays

In the EU WFD, ecological assessment of water quality is fundamental to the management of surface waters and the protection of aquatic ecosystems. The use of aquatic toxicity tests plays an important role in toxicity screening and assessment of the impact of contaminants on the natural environment (da Cruz *et al.*, 2007). Routine ecotoxicological monitoring requires simple, rapid and inexpensive methods (His *et al.*, 1999). Bioassays measure a direct response by specific organisms to all biologically available compounds within a medium. A variety of marine organisms at different life stages and from different trophic levels have been used for studying the effects of contaminants, and include echinoderms (Rosen *et al.*, 2005; Lorenzo *et al.*, 2000), bivalves (Beiras and Albentosa, 2004), crustaceans (Ferrer *et al.*, 2006) and ascidians (Bellas *et al.*, 2003). However, the parameter or species used for the measurement will influence the observed responses, and this can be likened to the operational nature of speciation techniques.

Bivalves are euryhaline species and adapt well to fluctuations in environmental conditions, and are particularly suitable for monitoring brackish and saline waters. Moreover, the Pacific oyster is an ideal subject for such studies because of its importance in pollution monitoring and aquaculture. The eggs, embryos and larvae of the Pacific oyster, have commonly been used in acute tests to assess the toxicity of environmental contaminants, such as heavy metals (Brooks *et al.*, 2007; McPherson and Chapman, 2000; His *et al.*, 1999; Beiras and His, 1994; Thain, 1991; Roberts, 1987; Robert and His, 1985; Martin *et al.*, 1981; Hrs-Brenko *et al.*, 1977; Calabrese and Nelson, 1974; Brereton *et al.*, 1973; Calabrese *et al.*, 1973), industrial and other organic pollutants (Geffard *et al.*, 2002, 2003; Nice *et al.*, 2000), and to evaluate the biological quality of waters and sediments

subjected to anthropogenic inputs (Geffard *et al.*, 2002; Beiras and His, 1995; Thain, 1992; McFadzen, 1992; Chapman and Morgan, 1983; Woelke, 1972).

1.7. Integration of chemical and biological measurements

The importance of integrating trace metal speciation and biological measurements in order to relate toxic impacts on biota is now recognised. Increasingly, studies are performed that specifically relate chemical measurements to biological effects. These have been carried out in freshwaters (Apte *et al.*, 2005; Devez *et al.*, 2005; Royset *et al.*, 2005; Tusseau-Vuillemin *et al.*, 2004; Meylan *et al.*, 2004; Meylan *et al.*, 2003), chemically defined waters (Sanchez-Marin *et al.*, 2007; Apte *et al.*, 2005; Lorenzo *et al.*, 2002; van Ginneken *et al.*, 2001; Lage *et al.*, 1996;) and marine waters (Brooks *et al.*, 2007; Stark *et al.*, 2006; Stauber *et al.*, 2005). A variety of different organisms have been tested from different trophic levels, such as amphipods (Stark *et al.*, 2006), algae (Devez *et al.*, 2005; Stauber *et al.*, 2005; Meylan *et al.*, 2004; Meylan *et al.*, 2003), dinoflagellates (Lage *et al.*, 1996) and invertebrates (Sanchez-Marin *et al.*, 2007; Lorenzo *et al.*, 2002). Largely, voltammetric techniques and DGT samplers have been used for the speciation measurements.

In general, these studies have shown the operationally defined labile fraction to be directly related to the toxicological response and these findings support the general concept that it is this fraction that is biologically available. For example, in a study by Brooks *et al.* (2007) the toxicity of Cu to the developing embryos of *C. gigas* diminished in the presence of humic acids. Humic acids form strong complexes with Cu that effectively prevents their assimilation by living organisms and these metal-organic complexes are generally considered not to be biologically available. In contrast to the study by Brooks *et al*, a study by Sanchez-Marin *et al.* (2007) showed that humic acids increased the biologically available fraction of Pb to the echinoderm *Paracentrotus lividus*.

1.8. Simplified models used for speciation studies

In general, analytical techniques are not available for the determination of all dissolved trace element species. Principally, the existing techniques are operational in nature thus defining the types of chemical species that can be distinguished. It follows that the operating conditions of a technique controls the fraction of metal species that can be determined and that for each technique a slightly different fraction is measured (Section 1.7).

The challenge in aquatic toxicology is to predict the biologically available fraction of dissolved metals as a function of their speciation in the natural environment. To address this challenge models such as the free ion activity model (FIAM) and the biotic ligand model (BLM), for freshwaters systems, were developed. The models assume that in a system at equilibrium the free metal ion activity reflects the chemical reactivity of the metal (e.g. the extent to which a metal reacts with binding sites on the surface of a biological membrane and hence its biological availability). These models have been widely applied and numerous studies have provided evidence in support of the FIAM (Brooks *et al.*, 2007; Voets *et al.*, 2004; Lorenzo *et al.*, 2002; Campbell, 1995 and references therein; Blust *et al.*, 1986, 1991; Zamuda and Sunda, 1982) and BLM (Arnold *et al.*, 2005; de Schamphelaere and Janssen, 2004; Paquin *et al.*, 2002 and references therein; Playle *et al.*, 1993). Indeed the US Environmental Protection Agency has included the BLM into its regulatory framework for Cu in freshwaters (Arnold *et al.*, 2005). Notwithstanding this, contradictory results have been reported questioning the general applicability of both models (Sanchez-Marin *et al.*, 2007; Erracalde *et al.*, 1998; Campbell *et al.*, 1995 and references therein; Phinney and Bruland, 1994).

1.9. Summary

In terms of the EU WFD, a range of monitoring practices may be adopted by different member states, and any strategy adopted should ideally take account of short-term

changes associated with, for example, seasonal, weekly, diurnal or tidal cycles. Therefore the strategic use of a range of tools is necessary to meet the varied needs of monitoring. The development of new analytical tools is progressing and as new tools emerge it is likely that they will be incorporated into monitoring programmes, although cost effectiveness and utility need to be demonstrated. It is anticipated that advances in the development of techniques for rapid on-site measurements will have a significant effect on the successful implementation of the WFD (Allan *et al.*, 2006). Indeed, the development of additional tools/techniques for monitoring purposes will provide an alternative to or complement classical methods and may help to reduce the risk associated with decisions made on the quality of a water body.

1.10. Project aims and objectives

The overall aim of this research was to integrate trace metal chemical speciation measurements with biological effects in order to (1) investigate the effect of specific metal species on embryo-larval development of the Pacific oyster, *Crassostrea gigas*, (2) test the applicability of an integrated approach (combining chemical and biological measurements) to the monitoring requirements outlined in the EU WFD, and (3) to improve our understanding of trace metal biogeochemical processes in estuarine environments.

The objectives of this project were to:

(1) Examine the effect of Cu, Cd, Pb and Zn and their chemical speciation on the developing embryos of the Pacific oyster, *Crassostrea gigas* in laboratory studies, by computing the concentrations of individual metal species in a buffered, chemically defined seawater, using a thermodynamic equilibrium model (MINEQL+). The work will specifically test the hypothesis that the free metal ion activity determines physiological effects. While a number of marine invertebrate species (e.g. grass shrimp, oysters, annelid, brine shrimp, crab and sea urchin

larvae) have been used at various developmental stages to test this hypothesis (Campbell, 1995 and references therein; Lorenzo *et al.*, 2002; Voets *et al.*, 2004), to the authors' knowledge no such studies have been conducted with the developing embryos of the Pacific oyster, *C. gigas* (Chapter 2).

(2) Discuss and compare *in situ* and laboratory-based voltammetric instrumentation applied to detect operationally defined metal fractions of Cu, Cd and Pb that in literature have been linked to the biologically available fraction (Chapter 3).

(3) Examine the relationship between trace metal chemical speciation and toxicity in an estuarine system heavily influenced through past mining activities, by undertaking high resolution *in situ* speciation measurements of Cu, Cd and Pb and comparing embryo-larval responses to discrete samples collected over tidal cycles (Chapter 4).

(4) Apply the same methods used in Chapter 4 in a contrasting estuarine environment, subject to greater anthropogenic influences, to investigate the benefits of combining chemical and biological measurements as a means to (1) assess the status of natural waters and (2) improve our understanding of biogeochemical processes (Chapter 5).

Chapter 2:

Model studies to investigate the toxicity of Cu, Cd, Pb and Zn to larvae of the Pacific oyster, *Crassostrea gigas*

2.1. Introduction

The continuing deterioration of estuarine and coastal systems by anthropogenic pollutants is a major concern, particularly with regard to human health and ecosystem functioning. Although metals are ubiquitous in aquatic environments, areas subjected to high inputs of metal contaminants present environmental concern. For some metals such as copper (Cu), cadmium (Cd), lead (Pb) and zinc (Zn), the free aqueous ion is considered to be the most toxic metal species.

The main aim of this study was to study the effect of biologically relevant trace metal species on the developing embryos and larvae of the Pacific oyster. It is not the intention to describe the mechanisms of uptake in detail but rather to use the end-point of abnormal larval development to illustrate the induction of toxic impacts.

The objectives of this work were to:

(1) Assess the suitability of using developing oyster embryos to study the biological availability of metal species (Cu, Cd, Pb and Zn) in buffered, chemically defined seawater.

(2) Determine the relationship between dissolved trace element species and larval development and deformities to *C. gigas* by computing individual metal species with the use of a thermodynamic equilibrium model, MINEQL+.

(3) Establish dose-response curves for the biologically relevant metal fractions, in particular the free metal ion and inorganic metal species, and determine EC_{50} and EC_{05} values for the investigated metal species.

(4) Examine the effect of chemical speciation on larval response in binary metal exposure experiments.

2.2. Background

2.2.1. The function of selected metals in biological systems

The concentrations of metals in natural waters are strongly influenced by anthropogenic inputs and geochemical weathering, and at enhanced concentrations can

cause toxic effects. It is well documented that the physico-chemical forms in which trace metals exist in natural waters determine their biological availability (Campbell, 1995; Blust *et al.*, 1991; Florence, 1982; Zamuda and Sunda, 1982). Copper is required for a number of biochemical processes, which include enzyme catalysis and oxygen transport (Rivera-Duarte and Zirino, 2004). Zinc is a cofactor of approximately 300 enzyme systems which are involved in nearly all aspects of metabolism (Lohan *et al.*, 2002; Anderson and Morel, 1978). Therefore, depending on their aqueous concentrations, both Cu and Zn could be either an essential element or a toxicant. Cadmium is generally considered toxic, but there is evidence to indicate that it can nutritionally substitute for Zn in some key Zn enzymes, such as carbonic anhydrase (Vasconcelos and Leal, 2001; Cullen *et al.*, 1999; Morel *et al.*, 1994; Price and Morel, 1990). Lead is a non-essential element, with no known biological role, and like Cd is known to produce toxic effects in marine organisms (Fernandez and Beiras, 2001; Hrs-Brenko *et al.*, 1977).

The importance of investigating the speciation and toxicological impacts of these metals has recently been recognised in legislation, such as the EU Water Framework Directive (WFD) which requires that all EU waters acquire good ecological status by 2015 (See Section 1.2).

2.2.2. Processes that control bio-uptake

Key processes that are widely accepted to control biological uptake and availability of metals in solution include (Figure 2.1): (1) mass transport of the solute to the biological membrane; (2) modification in the chemical speciation during transport (i.e. complexation/dissociation); (3) adsorption/desorption of the metal with a receptor site on the biological membrane; and (4) internalisation of the solute through the biological membrane (Kola and Wilkinson, 2005; Wilkinson and Buffle, 2004; Erracalde *et al.*, 1998; Campbell, 1995). The plasma membrane of organisms is typically composed of a bilayer of phospholipids that act as a barrier to simple diffusion of ions due to its hydrophobic

nature and it is assumed that internalisation is mediated by transmembrane proteins with complexing groups that bind the solute (Simkiss & Taylor, 1995) and/or through ion channels.

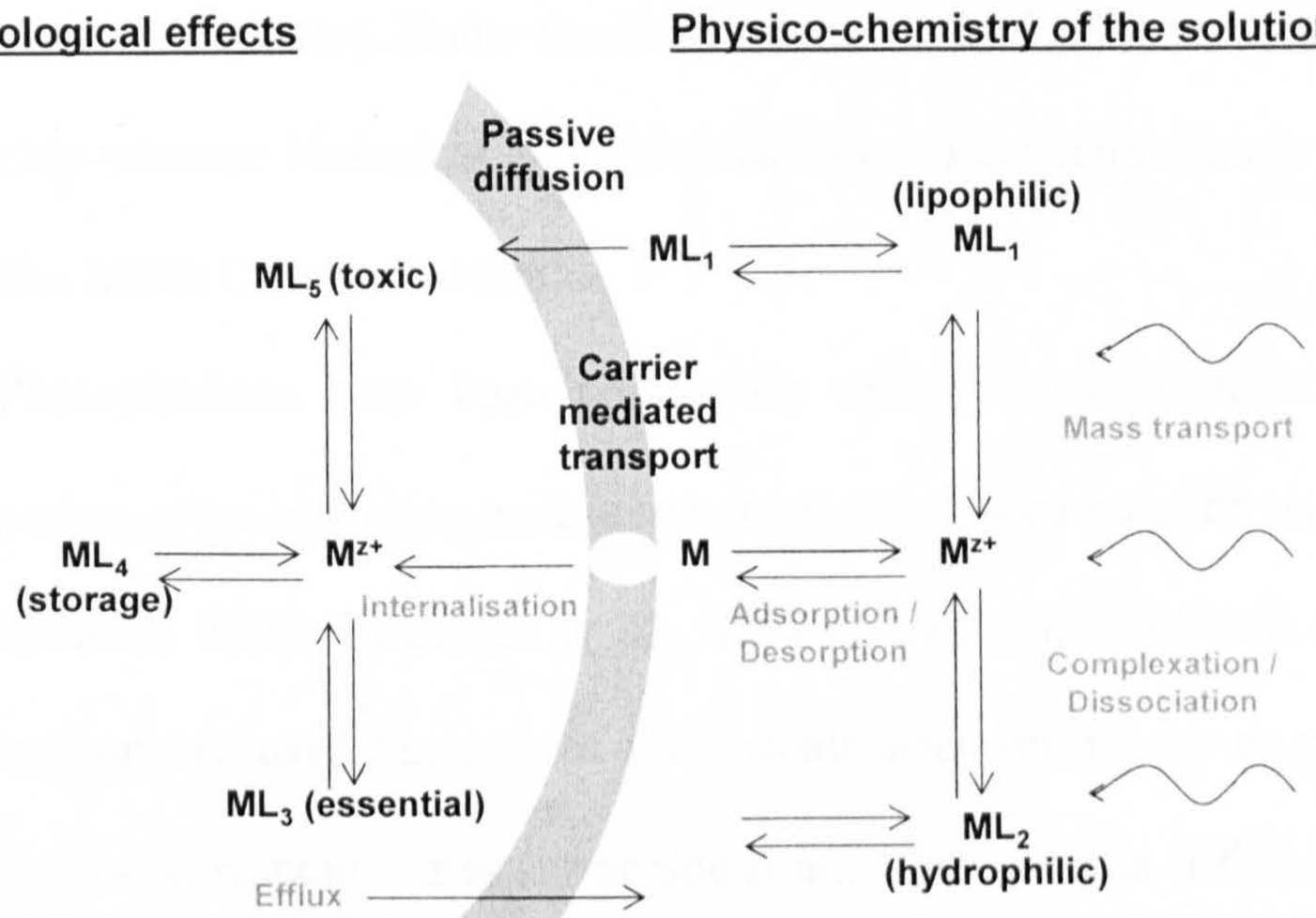


Figure 2.1: A conceptual model of the important physicochemical processes leading to the uptake of a trace element by an aquatic organism (taken from Wilkinson and Buffle, 2004 *Physicochemical Kinetics and Transport at Biointerfaces* Eds. van Leeuwen and Kosler, John Wiley & Sons, Ltd).

2.2.3. Free ion activity model (FIAM)

The free hydrated metal ion is considered to be the most toxic species (e.g. for Fe, Cu, Cd, Pb and Zn) to marine organisms, such as unicellular algae, metazoans, fish and bivalves (Zamuda and Sunda, 1982; Engel *et al.*, 1981; Anderson *et al.*, 1978; Sunda and Guillard, 1976). The importance of free metal ion activities in determining physiological effects was first proposed in 1993 by Morel in the 'free-ion activity model' (FIAM). The model indicates that the physiological effect is directly dependent on the free metal ion activity {M} (e.g. irrespective of whether it is the free hydrated metal ion or other complexes that react with cellular sites) and is not dependent on the mechanisms of the

reactions. Moreover, it is independent of the metal considered (e.g. whether it is a nutrient or toxicant), the nature of the complexing ligand or of the taxonomy of the affected organisms. In the FIAM the following assumptions are made: (1) the internalisation flux is rate-limiting; (2) the concentration of carriers or sensitive sites remains constant (i.e. they are not saturated); and (3) the membrane surface is chemically homogeneous (e.g. it only contains one kind of site). Under these assumptions, the FIAM predicts a first order, linear relationship between biological effect and the free ion concentration in solution (Wilkinson and Buffle, 2004; Campbell, 1995).

Phytoplankton have been universally used to investigate biologically available metal species, often including measurements of metal accumulation and uptake rates (Kola and Wilkinson, 2005; Erracalde *et al.*, 1998; Campbell, 1995 and references therein;). Phytoplankton are used because of their small size, sensitivity to trace metals, ease in culture and rapid response rates (Erracalde *et al.*, 1998; Luoma, 1995; Walsh, 1988). Fewer studies are reported for marine organisms at higher trophic levels.

Nonetheless, numerous reports have provided evidence in support of the FIAM and include studies using the diatom *Thalassiosira weissflogii* (Harrison and Morel, 1983), the oyster *Crassostrea virginica* (Zamuda and Sunda, 1982) and the brine shrimp *Atremia franciscana* (Blust *et al.*, 1991, 1986). Primarily these studies focused on the effect of divalent trace metals in experiments where metal-buffered (using known quantities of a well-defined synthetic ligand), artificial media was used. Notwithstanding this, some experiments have shown that lipophilic metal chelates (i.e. 8-hydroxyquinoline, ethyl xanthate and dithiocarbamate) and neutrally charged, non-polar complexes (i.e. $HgCl_2$ and CH_3HgCl) (Campbell, 1995; Phinney and Bruland, 1994) can diffuse directly across the cell membrane. Less polar, neutrally charged chloro-complexes such as AgCl and Cu(I)Cl may also be taken up by the same diffusion mechanism (Sunda and Huntsman, 1998). Small organic metal complexes (e.g. citrate, glycine) have also been linked to accidental transport. Rather than a generalised effect of citrate on algal membrane permeability,

membrane transport of a charged Cd-citrate complex was suggested to explain the enhanced biological availability of Cd (Erracalde *et al.*, 1998).

2.2.3.1. Buffered, chemically defined media

Determination of individual chemical species is currently beyond the scope of most analytical techniques. At best, operationally defined species such as labile, non-labile and inert can be measured (Twiss *et al.*, 2001). Chemically defined media, such as the artificial seawater AQUIL, have been developed to aid the prediction of the different chemical species present in solution, using thermodynamic principles (Price *et al.*, 1989). An important aspect in determining the biological effect of metals is to ensure the constancy of the metal concentrations throughout the exposure time, for which purpose metal buffers, such as ethylenediaminetetraacetic acid (EDTA) and nitrilo-triacetic acid (NTA) are often used. In addition, metal buffers (1) help to minimise speciation changes caused by metal uptake and any exudate release by the organisms, and (2) allow toxicity to be examined at the low concentrations of free metal ions representative of the natural environment. Moreover, biological membranes are largely impermeable to buffers such as EDTA, which form hydrophilic complexes with metals (Simkiss and Taylor, 1995).

2.2.3.2. Speciation calculations

In order to better understand the chemistry of aquatic systems, computational models have been developed that calculate the equilibrium behaviour of compounds (Schecher and McAvoy, 1992). Stability constants of metal complexes with inorganic ligands are generally well known (Martell and Smith, 1977; MINEQL+ database), and therefore inorganic speciation of metals can be evaluated by thermodynamic calculations when the composition of the solution is well-defined (e.g. buffered, artificial sea water) (Stumm and Morgan, 1996; Turner, 1995). Key chemical properties that control chemical speciation included in these models are ionic strength, salinity and pH.

Chemical equilibrium models, such as MINEQL+ (Schecher, 1994), calculate chemical speciation based on thermodynamic principles and hence rely on reliable thermodynamic data used in the calculation. Errors in thermodynamic databases (e.g. MINETAQ, MINQEL+ version 3.01b) have previously been reported primarily for reactions involving organic ligands (Twiss *et al.*, 2001; Serkiz *et al.*, 1996). In addition, errors occurred because the stability constants (log K) were not referenced to zero ionic strength (μ = 0) and 25^{0}C and/or the reaction was not correctly expressed in terms of the program components. For example, all reactions must be written as formation reactions from the MINEQL+ components (Twiss *et al.*, 2001; Serkiz *et al.*, 1996) so that the stability constant for the species $CuHEDTA^-$ is log K = 24.0, and is formulated from the reactions:

$$CuEDTA^{2-} + H^+ \leftrightarrows CuHEDTA^- \qquad \log K = 3.5$$

$$Cu^{2+} + EDTA^{4-} \leftrightarrows CuEDTA^{2-} \qquad \log K = 20.54$$

$$Cu^{2+} + H^+ + EDTA^{4-} \leftrightarrows CuHEDTA^- \qquad \log K = 24.0$$

Programs, such as MINEQL+, consider every reaction at chemical equilibrium (e.g. kinetic effects are ignored) when in fact some reactions, i.e. precipitation-dissolution and oxidation-reduction, can be very slow (Rand, 1995). It is important, therefore, that reagents introduced into an experimental system are given enough time for equilibrium to be reached (e.g. EDTA-complexes, Price *et al.*, 1989). Furthermore, it is necessary to prevent perturbation of this equilibrium through aeration processes (in redox-sensitive systems) and precipitate formation that could invalidate the equilibrium assumption.

2.2.4. Oyster-embryo larval bioassay

Invertebrates are an integral part of marine ecosystems and an important marine resource. The early life stages of bivalve mollusca have been shown to be sensitive to metal contaminants in a number of laboratory studies that exposed developing embryos to

increasing metal concentrations in both filtered seawater and chemically defined media (Table 2.1 and references therein). The oyster-embryo larval bioassay (OEL) is a rapid, relatively simple and low cost test that has been routinely used in marine monitoring programmes and to test areas subject to intermittent contaminant exposures by government agencies (Environment Agency of England and Wales (EA), Environmental Protection Agency (EPA), US). The embryos are simply incubated in the water samples at a constant temperature (20-24°C) for a given period of time (24-48 h). The number of larvae developing and surviving to the 'D' stage (first prodissoconch shelled larvae), characteristic for 'normal' or healthy development, is then counted. An advantage of the short exposure time is that the influence of the organisms on the exposure medium is minimised, as could occur, for example, through the depletion of metal concentrations in the medium from biological uptake and release of metal complexing exudates.

Table 2.1: Toxicity of heavy metals to oyster larva. EC_{50} values: Chlorides were used for Hg, Cu, Zn and Cd; nitrates for Ag, except where otherwise stated (adapted from His *et al.*, 2000).

Test species	Initial stage (time after fertilisation, where stated)	Exposure conditions (time, exposure, salinity, embryo density, water matrix)	EC50 (µg metal ion L^{-1})						References and notes
			Hg	Ag	Cu	Zn	Pb	Cd	
C. gigas	fertilized egg	27°C	10- 32		32-100	1000-3200			Okubo and Okubo (1962) Cu and Zn sulphates
C. gigas	fertilized egg	48 h, 20-22°C, 5 µm FSW UV sterilised				250			Brereton *et al.* (1973) ; abnormal larvae
C. gigas	fertilized egg	48 h, 20°C, 34 psu, 24-28 ml^{-1} 1µm FSW UV sterilised	5.7						Glickstein (1978); abnormal larvae excluded; Se decreases Hg toxicity
C. gigas	fertilized egg	48 h,20°C, >25 psu, 35 ml^{-1} FSW UV sterilised						>2500*	Cardwell *et al.* (1979); *nitrate
C. gigas	fertilized egg	48h, 20°C, 33 psu, 28-36 ml^{-1} 1 µm FSW		16-18	10				Coglianese and Martin (1981); Cu nitrate; abnormal larvae excluded
C. gigas	fertilized egg	48 h, 20°C, 30 psu, 30 ml^{-1} 1 µm FSW UV sterilised			12, 40* 100**				Knezovich *et al.* (1981), *humic matter 2 mg l-1, **EDTA 10E-6 M
C. gigas	fertilized egg (15 min)	48 h, 25°C, 34-35 psu 5 µm FSW	6.7	22	5.3	119	758	611	Martin *et al.* (1981); Cu and Zn nitrates abnormal larvae excluded
C. gigas	egg	48 h, 26°C, 28 psu, 30 ml^{-1} 0.8 µm FSW			130*	>100	>100	>100	His and Robert (1981); abnormal larvae excluded
C. gigas	egg	48 h, 20°C, 28-36 ml^{-1}, 33 psu 1 µm FSW UV sterilised 22 psu			10				Coglianese (1982); Ag nitrate; abnormal larvae excluded
C. gigas	fertilised egg	24 h, 24°C, 20, 25, 30 psu		>18	5-6.5				
		0.2 µm FSW		10-14	5-6.5				
C. gigas	fertilised egg (15 min)	24 to 72 h, methods not given, 0.2 µm FSW						>50	Roberts and His (1985); abnormal larvae excluded
C. gigas	fertilised egg	0.2 µm FSW, 33 psu, 30 ml^{-1}, 24 h	24°C 12.3		37				His *et al.* (1999)

Test species	Initial stage (time after fertilisation, where stated)	Exposure conditions (time, exposure, salinity, embryo density, water matrix)		EC50 (µg metal ion L^{-1})						References and notes
				Hg	Ag	Cu	Zn	Pb	Cd	
C. gigas	fertilised egg	0.22 µm FSW, 30 psu, 30 ml^{-1}, 24 h	23°C			5.8-12				Damiens *et al.* (2004)
C. gigas	fertilised egg	0.22 µm FSW, 30 psu, 30 ml^{-1}, 24 h	24°C			12				Quiniou *et al.* (2007)
C. gigas	fertilised egg	0.22 µm FSW, 30 psu, 30 ml^{-1}, 24 h	24°C			22.8 (41.1*)				Brooks *et al.* (2007) * in the presence of HA (1.02 mg/L)

2.3. Materials and methods

2.3.1. Reagents and equipment

Chemicals used in this study were of analytical reagent grade (AnalaR) or better, unless otherwise stated, and obtained from VWR International Ltd (Lutterworth, UK), unless otherwise stated. Low density polyethylene containers (LDPE, Nalgene, Hereford, UK), used for sampling and to store bulk reagents, were cleaned following trace metal clean techniques (Achterberg *et al.*, 2001) (Table 2.2). The protocols followed for other laboratory-ware are included in Table 2.3. All sample handling and preparation of reagents were carried out under a class-100 laminar flow hood. High purity, Milli-Q (MQ) water was used in the preparation of working solutions, and was purified by reverse osmosis (Milli-Q3, Millipore, Molsheim, France) followed by a de-ionization stage (Millipore, $R \geq 18\ M\Omega\ cm^{-1}$). Hydrochloric acid (HCl) and nitric acid (HNO_3) were purified by distillation in a sub-boiling quartz still (Q-HCl and Q-HNO_3) and used for the preparation of metal standards (0.1%) by serial dilution of atomic absorption spectrometry standards (1000 mg L^{-1}, Merck, SpectrosoL grade). Ammonia (NH_3) was purified by isothermal distillation (iso-NH_3) and used for neutralisation. Unless otherwise stated these purified reagents were used throughout. In order to minimize pH variation in the bioassay samples, metal standards were not stabilised with acid but were used within 4 h of preparation.

Table 2.2 Protocols for cleaning LDPE containers

Step	Procedure
In general laboratory	
1	Immerse in detergent bath (Decon 2% v/v) for 24 h
2	Rinse 3 times with MQ water
3	Immerse in HCl (6 M, VWR, Aristar grade) for 1 week
4	Rinse 3 times with MQ water
5	Immerse in HNO_3 (3 M, VWR, Aristar grade) for 1 week
6	Rinse 5 times with MQ water
For sample bottle storage (performed in clean air (class-100) laminar flow hood)	
7	Fill with MQ water and acidify to pH 2 with quartz distilled HCl (Q-HCl)
8	Double-bagged and stored in a re-sealable bag

Table 2.3: Protocols for cleaning laboratory equipment

Equipment	Step	Procedure
Polycarbonate filtration unit	1	Immerse in detergent bath (Decon 2% v/v) for 24 h
	2	Rinse 3 times with MQ water
	3	Immerse in HCl (6 M, VWR, Aristar grade) for 24 h
	4	Rinse 3 times with MQ water
	5	Dry in a class-100 laminar flow hood
	6	Store in a re-sealable bag
Filter papers	1	Immerse in HCl (1% v/v, VWR, Aristar grade) for 24 h
	2	Rinse and store in MQ water
O' rings	1	Immerse in detergent bath (Decon 2% v/v) for 24 h
	2	Thorough rinse with MQ water
	3	Immerse in HCl (1% v/v, VWR, Aristar grade) for 24 h
	4	Thorough rinse with MQ water
	5	Dry in a class-100 laminar flow hood
	6	Store in a re-sealable bag
Quartz ultra-violet (UV) digestion tubes	1	Rinse 3 times in MQ water
	2	First wash in 10% HCl
	3	Rinse 3 x with MQ water
	4	Second wash in 10% HCl
	5	Rinse 3 x with MQ water
	6	Dry in laminar-flow hood
Bioassay equipment PP beakers PP measuring cylinders Petri plates Mesh filters	1	Thorough rinse with MQ water
	2	Immerse in HCl (10% v/v, VWR, Aristar grade) for 1 week
	3	Rinse 5 times with MQ water
	4	Dry in a class-100 laminar flow hood
	5	Store in re-sealable bags

2.3.2. Preparation and treatment of an artificial seawater/culture (ASW) medium

An artificial seawater/culture media (ASW), based on the AQUIL recipe (Price *et al.*, 1989), was prepared according to the formulation shown in Table 2.4, using reagent grade salts. The mass of each salt was measured on a 4-figure balance (Oxford Ltd) and dissolved in MQ water separately before mixing to the final volume and allowed to equilibrate for >3 days. To remove trace metal impurities in the preparation of the ASW, Chelex-100 (50-100 mesh, Bio-Rad Laboratories, Richmond, California), a styrene-divinylbenzene polymer resin with iminodiacetate functional groups, was used to chelate transition metals such as Fe, Cu, Zn, Ni, Cd and Co, using a batch method. Before use, the

resin itself was treated to remove metal contamination and leachable metal-binding organic substances as detailed in Table 2.5.

Table 2.4: Composition of the artificial seawater salts (S=31)

Salt	Mass in g L^{-1}	Final concentration (mol L^{-1})
NaCl	21.37	3.66×10^{-1}
Na_2SO_4	3.565	2.51×10^{-2}
KCl	0.610	8.18×10^{-3}
$NaHCO_3$	0.174	2.07×10^{-3}
KBr	0.087	7.31×10^{-4}
H_3BO_3	0.026	4.22×10^{-4}
NaF	0.003	6.22×10^{-5}
$MgCl_2.6H_2O$	9.657	4.75×10^{-2}
$CaCl_2.2H_2O$	1.344	9.14×10^{-3}
$SrCl_2.6H_2O$	0.148	5.56×10^{-5}

Steps 2-5 (see Table 2.5) were repeated prior to further use in freshly prepared ASW. Cleaned Chelex-100 resin was added (~5 g L^{-1}) to the total volume of ASW, agitated periodically over a 48-72 h period, and filtered through a 0.2 μm cellulose acetate filter (Whatman Inc. Clifton, NJ, USA), to collect the resin. In most instances a decrease in pH was observed after the cleaning process and the pH was re-adjusted using NaOH (1.0 M, analytical reagent grade, Sigma) to pH ~ 8.1 (monitored using Jenway 3010 pH meter). The preparation of the ASW resulted in a theoretical salinity of 31 for the bioassay exposures, and this was confirmed with a portable refractometer (DIGIT-100 ATC).

In order to reduce bacterial growth during the incubation period the preparation equipment and the ASW were sterilised by microwave (Cookworks M8017P-F, 800 W) for 10 min in 2 min intervals. Complete removal of bacteria was not tested, since bacteria are present within the gonads of the oysters and therefore was introduced to the system with the sperm and eggs. Before and during each experiment, the pH, dissolved oxygen (DO) and salinity of the samples were monitored and remained in the range of 8.1 ± 0.2, 95 to >100% saturated (Jenway 9070, oxygen meter) and 31 ± 0.5, respectively.

Table 2.5: Protocol for cleaning Chelex-100 resin

Step	Procedure
1.	Slurry resin in 50 mL MQ water (for new Chelex-100)
2.	Rinse and decant x 3 then suspend in 50 mL MQ water for 1-2 h
3.	Rinse and decant x 3 in 50 mL 0.1 M HCl then suspend for 6-12 h
4.	Rinse and decant x 3 in 50 mL MQ water then suspend for 1-2 h
5.	Rinse and decant x 3 in 50 mL 1.0 M NaCl adjusted to pH 8.1 with 1.0 M NaOH (analytical reagent grade), then suspend for 12 h

Background metal content in ASW

The background concentrations of total dissolved Cu, Cd, Pb and Zn were measured using square wave anodic stripping voltammetry (SWASV). In preparation for analysis, samples were acidified to pH 2 with Q-HCl (0.1%), transferred into quartz glass UV digestion tubes (30 mL, 18 mm i.d.) fitted with PTFE caps and placed into an ultra-violet (UV) digestion unit (refer to section 3.3.1.3 for a detailed description of the UV digestion unit). The samples were irradiated in order to break down any organic metal complexing matter for 6 h in the presence of H_2O_2 (15 mM). Prior to voltammetric analysis the samples were neutralised to pH ~ 4 with the addition of iso-NH_3.

The voltammetric system consisted of a Metrohm (663 VA Stand), static mercury drop interfaced with a μAutolab voltammeter (Eco Chemie), controlled by a computer. The reference electrode was a double junction, Ag/AgCl, KCl (3 M) saturated AgCl electrode and the counter electrode was a glassy carbon rod (see section 3.3.1.1). The voltammetric conditions were: sample purge time: 240 s (nitrogen gas); stirrer speed: 2500 min^{-1}; Hg drop size: ~0.52 mm^2; quiescent period: 10 s; deposition time: 180 s; scan form: square wave modulation frequency: 50 Hz; modulation amplitude: 25 mV; step height: 2.44 mV; potential scan: -1.2 V to -0.05 V. The reduction peaks corresponding to the four metals

were in the region of: - 1.1 V Zn; -0.6 V Cd; -0.4 V Pb; -0.1 V Cu. The background metal concentrations in the ASW media were 0.05 ± 0.02 nM Cu, 0.18 ± 0.03 nM Cd, 0.19 ± 0.07 nM Pb and 0.50 ± 0.08 nM Zn (n=2). These concentrations were considered to form a negligible contribution in the metal exposure experiments.

2.3.3. Speciation calculations

The thermodynamic equilibrium model MINEQL+ (Schecher, Windows version 4.5) was used to calculate the chemical speciation for the exposure conditions used in the bioassays. The NIST (National Institute of Standards and Technology) database was used to review the MINEQL+ database, since it is considered a 'Standard Reference Database' because the given stability constants have been thoroughly examined and independently selected by the authors, with bibliographic references provided for each value. Good agreement was shown between the databases (MINEQL+ and NIST) when corrections for ionic strength and formation constants were taken into account. As a result, it was not considered necessary to correct any constants used in this MINEQL+ version.

For the speciation calculations with MINEQL+, all dissolved salts, metals and EDTA were entered at their respective concentrations and the temperature set to 24°C and the pH fixed at 8.1. The system was left open to the atmosphere which assumes equilibrium with atmospheric inputs of carbon dioxide, CO_2, and therefore the partial pressure of CO_2 was fixed at $pCO_2 = -\log 3.5$. The ionic strength adjustment was calculated within the program using the Davies equation. Dissolved solids were removed from the system as they were not expected to be present. A list containing the calculated speciation of Cu, Cd, Pb and Zn in the bioassay exposures is included in Appendix I. A sensitivity test confirmed that the variation of 0.2 pH units and a salinity change of 0.5 did not induce significant changes in metal speciation.

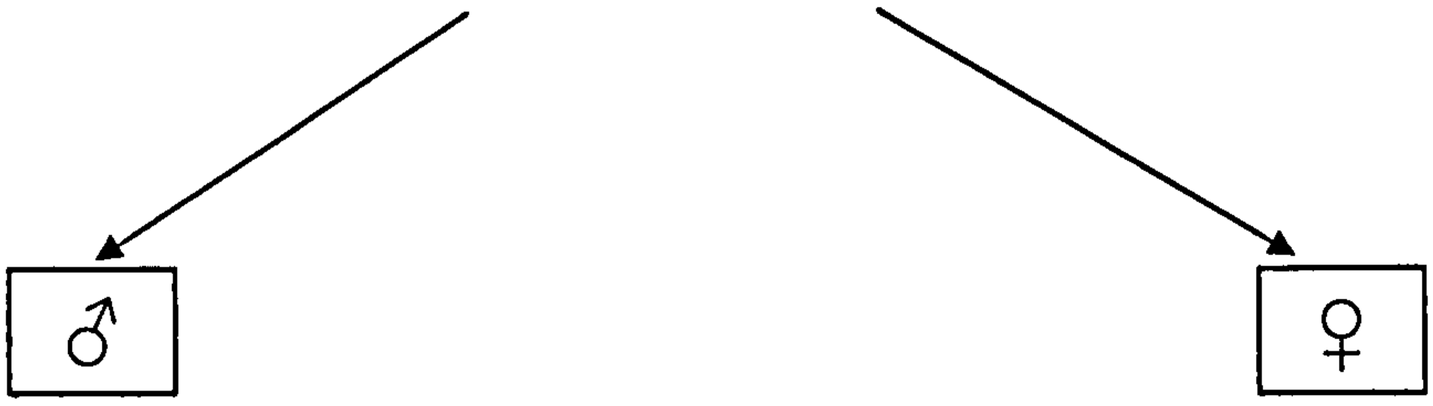
2.3.4. Bioassay methodology

2.3.4.1. Bioassay protocol

The protocol adopted for the bioassays was based on the Standard Operating Procedure used by the Environment Agency (EA Guidelines, 2001 and the revised issue, 2004). The term 'exposure' will be used to describe the solutions in which the oyster embryos were incubated for bioassay tests. Exposures may contain natural water samples (see Chapters 4 and 5) or, in this chapter, be made up of artificial seawater with added metals and metal buffer. An overview of the experimental protocol used in the bioassays is detailed in Figure 2.2. Pre-sexed pairs of conditioned adult oysters (e.g. oysters that have been reared on a high quality diet, are pre-sexed and ready to spawn) of the genus *Crassostrea gigas* (size 5 to 7 cm) were supplied by a recognised hatchery (Guernsey Sea Farms, Guernsey, Channel Islands) to ensure good quality gametes so that a viable control was obtained. Ideally, abnormalities should not exceed 20% in control experiments (His *et al.*, 1997), although as much as 40% is considered acceptable by the EA (EA Guidelines, 2004).

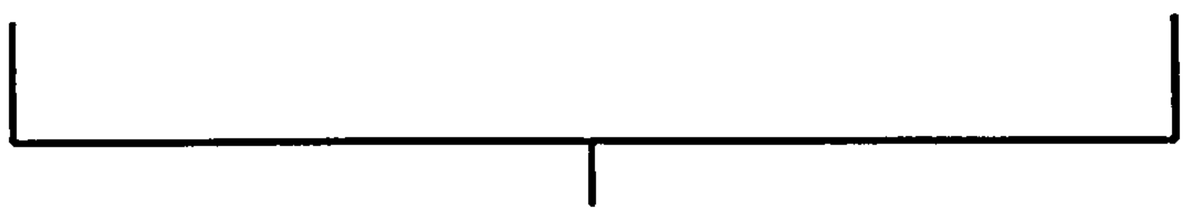
Following tests on different exposure volumes and egg densities (refer to section 2.3.4.3), 3 mL test volumes that contained nominally 50 embryos mL^{-1} were selected for experimental treatments. In the preparatory stages, an embryo density of ~ 5000 embryos mL^{-1} was prepared which allowed for an inoculum volume of 0.05 mL. The volume was kept low in order to avoid salinity and pH changes in the exposure vessels. The 24 h exposure time allowed complete development of the embryos to a characteristic D-shape (see Figure 2.3a) (His *et al.*, 1999, 1997). All larvae were counted by direct observation with an inverted microscope (Nikon Eclipse TS100, x100 magnification), and the number of abnormal larvae arising from the exposure conditions were determined, using the criteria proposed by His *et al.* (1999) and shown in Figure 2.3.

1. With a standard oyster knife open each oyster
2. Rinse body cavity to remove debris and body fluids
3. Strip the gonads with a sterile Pasteur pipette

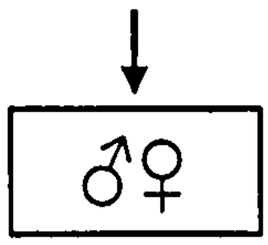


4. Filter sperm through a nylon mesh (60 µm) into a polypropylene (PP) beaker that contains 50 mL ASW
5. Leave for 10-15 min
6. Check sperm mobility*

4. Filter eggs through a nylon mesh (100 µm) into a PP beaker that contains 200 mL ASW
5. Leave for 15-20 min
6. Check for egg rounding*
7. Gently mix and record egg density (3 x 50 µL) and adjust to ~ 5000 eggs mL^{-1}



8. Add 600-1000 µL sperm suspension to the egg suspension



9. Verify ~ 5 -10 sperm attached to each egg membrane* and adjust if necessary (attachment of >24 sperm can result in polyspermy, whereby >1 sperm fertilises the egg and abnormal development can occur)
10. Gently mix and incubate# at 24^{0}C for 30 min
11. Inspect for successful fertilisation* (i.e. extrusion of polar bodies)
12. Return to incubator and assess progress hourly* (typically 1–3 h) with periodic mixing to optimise the fertilisation process
13. Pipette 10 µL aliquots to assess fertilisation rate (FR)
13. Provided FR ≥ 85%, inoculate prepared exposure volumes when the 16-32 stage cleavage stage is reached (50 µL contains nominally 150 embryos)
14. Leave for 24 h at 24±2^{0}C in the dark
15. Fix with formalin (40% v/v)
16. Count all larvae by direct observation with an inverted microscope** and determine the number which developed abnormally

Figure 2.2: Experimental protocol used in the bioassays
* inspection under a light microscope (Leitz Wetzlar, Orthoplan; 100x and 400x magnification); # Sanyo 20302264, Gallenkamp growth chamber; ** Nikon Eclipse TS100, x100 magnification

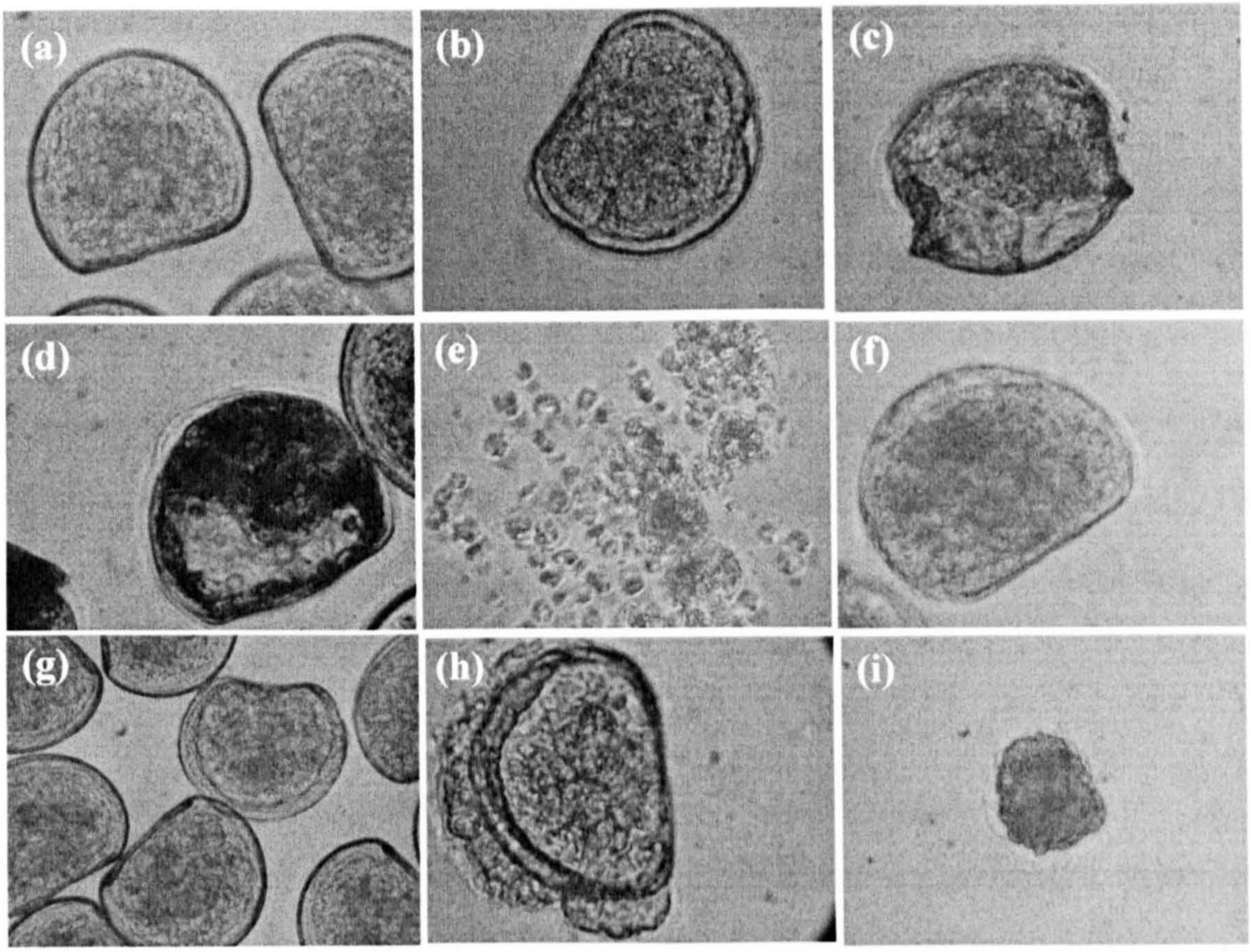


Figure 2.3: The different abnormalities observed in the development stages of the Pacific oyster, *Crassostrea gigas* after 24 h incubation period: (a) normal D-larva; (b) indented shell margin; (c) spherical shell shaping (d) transparency through the shell; (e) impairment of cell division (groupings of spherical bodies); (f) incomplete shell definition; (g) convex-hinge; (h) protruding mantle; (h) trochophore.

2.3.4.2. Preparation of metal exposure media

All preparations were carried out under a class-100 laminar flow hood. Fresh unacidified working metal standards of Cu (1.0×10^{-6} to 5.0×10^{-3} M), Cd (5.0×10^{-3} to 5.0×10^{-3} M), Pb (1.25×10^{-3} to 3.0×10^{-3} M) and Zn (2.5×10^{-3} to 1.0×10^{-3} M) and EDTA (1.0×10^{-1} to 5.0×10^{-3} M) standards were prepared in MQ water for each experiment and used within 4 h. The concentrations of EDTA concentrations used in the initial experiments were chosen to cover effective concentrations (i.e. EC_{50}) for total dissolved metal concentrations reported in previous studies (His *et al.*, 2000). A number of EDTA concentrations were required to ensure that dose-response curves were established. Chelex-cleaned and sterilised ASW (refer to section 2.3.2) was used in the preparation of

individual metal exposure media and made up to a final volume of 25 or 50 mL aliquots in polypropylene (PP) volumetric flasks, transferred into sterilised LDPE bottles, double-bagged and equilibrated overnight at 4°C.

On the day of the bioassay, the prepared exposure media were brought up to room temperature for ~1 h. Aliquots of 2.95 mL from each prepared medium were then pipetted, in triplicate, into sterile culture well-plates (Nunclon, 12x Polystyrene plates, Fisher Ltd), marked and stored at 24°C until required (e.g. fertilisation had been achieved). The exposure vessels and small exposure volume allowed assessment of all larvae under an inverted microscope.

2.3.4.3. Bioassay experiments

The effect of exposure volume (30 mL and 3 mL) and egg density (30, 50 and 100 embryos mL^{-1}) on embryo-larval responses of *C. gigas* was tested in a single experiment. No significant difference ($P>0.05$) in percent net response (PNR) was observed between an exposure volume of 30 mL (PP beaker with loose fitting polyethylene covering, PNR = 82 ± 3.9, n=3) and a volume of 3 mL (well-plate, PNR = 86 ± 1.3, n=3). Similarly, no significant difference ($P>0.05$) in responses to egg density was observed (30 embryos mL^{-1}: PNR = 84 ± 2.7%, 50 embryos mL^{-1}: PNR = 88 ± 4.2%, and 100 embryos mL^{-1}: 84 ± 0.8%, all n=3). An exposure volume of 3 mL and an egg density of 50 embryos mL^{-1} were selected for further experiments as this allowed (1) all larvae in each well to be counted under an inverted microscope, (2) allowed for some deviation from nominal egg density values and reduced the counting time, and (3) avoided further handling, sieving or sampling.

In order to study the relationship between chemical speciation and Cu, Cd, Pb and Zn toxicity to the larvae, preliminary experiments were carried out. Firstly, to assess the toxicity of the metal buffer ethylenediamine tetraacetic acid (EDTA) and, secondly, to establish dose-response curves for the four metals under study (Bioassay 1 in Tables 2.6-

Table 2.6: Experimental conditions used for Cu in bioassays conducted in this study. * represents the use of embryos from three individual sets of parents, # represents the use of embryos from three individual sets of parents and from one male coupled with eggs from three females (S=31, pH ~8.1).

Bioassay	Expt.	Cu (μM)	EDTA (μM)	Cu^{2+} (M)	Cu_{inorg} (M)	No. of aliquots used (n=3)
1	$[B]_{const}$	0.2-20	5	$2.7x10^{-12}$ to $1.7x10^{-6}$	$1.4x10^{-10}$ to $1.5x10^{-5}$	12
2 and 3*	$[B]_{const}$	3.5-12	5	$1.6x10^{-10}$ to $8.0x10^{-7}$	$1.4x10^{-9}$ to $6.9x10^{-6}$	16 and 12
2 and 3*	$[M]_{const}$	6	0.5-8.5	$1.6x10^{-10}$ to $6.4x10^{-7}$	$1.4x10^{-9}$ to $5.5x10^{-6}$	16 and 12
4#	$[B]_{const}$	3.3-3.91	5	$1.4x10^{-10}$ to $2.5x10^{-10}$	$1.2x10^{-9}$ to $2.1x10^{-9}$	8

Table 2.7: Experimental conditions used for Zn in bioassays conducted in this study. * represents the use of embryos from three individual sets of parents, # represents the use of embryos from three individual sets of parents and from one male coupled with eggs from three females (S=31, pH ~8.1).

Bioassay	Expt.	Zn (μM)	EDTA (μM)	Zn^{2+} (M)	Zn_{inorg} (M)	No. of aliquots used (n=3)
1	$[B]_{const}$	0.5-20	5	$2.4x10^{-9}$ to $7.0x10^{-6}$	$5.2x10^{-9}$ to $1.5x10^{-5}$	12
2	$[B]_{const}$	3.5-12	5	$4.7x10^{-8}$ to $3.3x10^{-6}$	$1.0x10^{-7}$ to $7.0x10^{-6}$	16
2	$[M]_{const}$	6	0.5-8.75	$4.6x10^{-8}$ to $2.6x10^{-6}$	$9.8x10^{-9}$ to $5.5x10^{-6}$	12
3*	$[B]_{const}$	4-12	5	$7.4x10^{-8}$ to $3.3x10^{-6}$	$1.6x10^{-7}$ to $7.0x10^{-6}$	12
3*	$[M]_{const}$	6	0.5-7.67	$7.1x10^{-8}$ to $2.6x10^{-6}$	$1.5x10^{-7}$ to $5.5x10^{-6}$	12
4#	$[B]_{const}$	3.47-6.04	5	$4.5x10^{-8}$ to $5.8x10^{-7}$	$9.8x10^{-8}$ to $1.2x10^{-6}$	8

Table 2.8: Experimental conditions used for Pb in bioassays conducted in this study. * represents the use of embryos from three individual sets of parents (S=31, pH ~8.1).

Bioassay	Expt.	Pb (μM)	EDTA (μM)	Pb^{2+} (M)	Pb_{inorg} (M)	No. of aliquots used (n=3)
1	$[B]_{const}$	0.5-30	5	$3.7x10^{-11}$ to $1.6x10^{-6}$	$5.9x10^{-10}$ to $2.5x10^{-5}$	12
2	$[B]_{const}$	6-16	5	$6.5x10^{-8}$ to $7.0x10^{-7}$	$1.0x10^{-6}$ to $1.1x10^{-5}$	16
2	$[M]_{const}$	12	3-11	$6.7x10^{-8}$ to $5.7x10^{-7}$	$1.0x10^{-6}$ to $9.0x10^{-6}$	12
3*	$[B]_{const}$	6-20	5	$6.5x10^{-8}$ to $9.5x10^{-7}$	$1.0x10^{-6}$ to $1.5x10^{-5}$	12
3*	$[M]_{const}$	12	0.5-12.5	$6.5x10^{-9}$ to $7.3x10^{-7}$	$1.0x10^{-6}$ to $1.2x10^{-5}$	12

Table 2.9: Experimental conditions used for Cd in bioassays conducted in this study. * represents the use of embryos from three individual sets of parents, # represents the use of embryos from three individual sets of parents and from one male coupled with eggs from three females (S=31, pH ~8.1).

Bioassay	Expt.	Cd (μM)	EDTA (μM)	Cd^{2+} (M)	Cd_{inorg} (M)	No. of aliquots used (n=3)
1	$[B]_{const}$	5-60	5	$4.2x10^{-8}$ to $1.9x10^{-6}$	$1.2x10^{-6}$ to $5.5x10^{-5}$	12
2	$[B]_{const}$	4-12	5	$2.6x10^{-8}$ to $2.5x10^{-7}$	$7.4x10^{-7}$ to $7.3x10^{-6}$	16
2	$[M]_{const}$	6.5	2.15-9.16	$2.4x10^{-8}$ to $1.6x10^{-7}$	$6.8x10^{-7}$ to $4.5x10^{-6}$	12
3*	$[B]_{const}$	4-12	5	$2.6x10^{-8}$ to $2.5x10^{-7}$	$7.4x10^{-7}$ to $7.3x10^{-6}$	12
3*	$[M]_{const}$	6.5	1.1-9.16	$2.4x10^{-8}$ to $1.9x10^{-7}$	$6.8x10^{-7}$ to $5.5x10^{-6}$	12
4#	$[B]_{const}$	5.17-9.38	5	$4.6x10^{-8}$ to $1.7x10^{-7}$	$1.3x10^{-6}$ to $4.8x10^{-6}$	8

2.9). EDTA was chosen for this study because it has well-established stability constants for reactions with all the cations in solution, forms hydrophilic complexes with the metals under study to which biological membranes are largely impermeable (Erracalde *et al.*, 1998; Simkiss and Taylor, 1995), and has sufficient buffering capacity toward the metals under study (Twiss *et al.*, 2001). EDTA was tested at concentrations ranging from 1 to 1000 μM (n=2) and found to be toxic at concentrations >400 μM.

Based on the toxicity results and speciation calculations of these preliminary experiments, two experiments were designed and run in parallel, identified as $[B]_{const}$ and $[M]_{const}$, for each of the four metals tested. In the first experiment, $[B]_{const}$, the buffer concentration was held constant (5 μM EDTA) while total metal concentrations were varied. A concentration of 5 μM EDTA was selected as it was shown not to be toxic and this concentration allowed for a lower limit of 0.5 μM EDTA to be used in the parallel experiment ($[M]_{const}$), where 0.5 μM EDTA was considered high enough to prevent speciation changes caused by the presence or production of other complexing ligands within the exposure period (24 h). In the second experiment, $[M]_{const}$, the total metal concentration of each of the four tested metals was held constant while EDTA concentrations were varied so as to maintain the free ion concentrations at the same levels as those in the corresponding aliquots of experiment $[B]_{const}$. Tables 2.6-2.9 detail the ranges of total metal and EDTA with the computed (MINEQL+) free ion concentrations.

The inorganic metal speciation is constant in constant ionic media. As a consequence, concentrations of free metal ion and inorganic complexes are related to one another by constant ratios (Sunda and Huntsman, 1998). Therefore, a similar outcome (e.g. dose-response curve in experiments ($[B]_{const}$) and ($[M]_{const}$)) should be found if either the free ion and/or inorganic metal species are the most bioavailable. Furthermore, for a given free-metal ion concentration the biological response should be the same regardless of the nature of L or the concentration of the ML_n species in the exposure medium (Erracalde and Campbell, 2000).

Experiments with living organisms present challenges due to natural variability in response. In all experiments six controls were used to account for this variability (see Section 2.4.2). Statistical assessment of differences in response was facilitated by conducting metal exposures in triplicate. The natural variability was also tested in experiments where three different pairings of oysters (from the same supply) and a 'mix' of their eggs and sperm were used to assess parental and/or metal specific responses in experiments $[B]_{const}$ and $[M]_{const}$. Initial experiments mixing eggs and sperm from three females and three males failed to produce a viable control and therefore the eggs from three females and sperm from a single male were used in subsequent experiments, as shown in Figure 2.4.

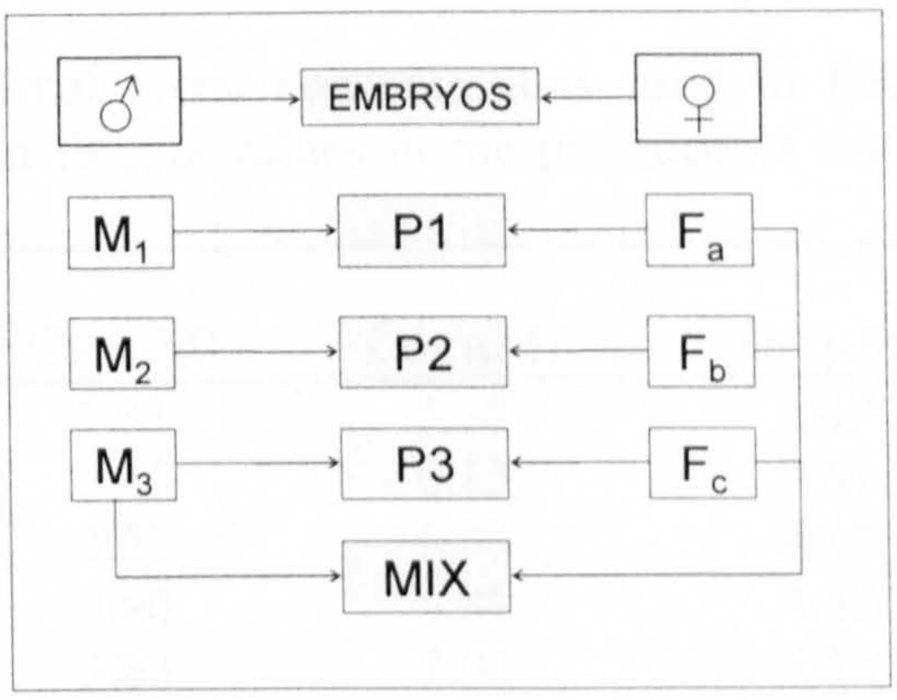


Figure 2.4: Experimental matrix used in the selection of different pairings (e.g. P1) and mix for bioassay exposures. The gametes from three adult male and female oysters (*C. gigas*) for different pairings were randomly selected. The most motile sperm was chosen for the mix.

2.3.4.4. Exposure to binary metal mixtures

Two experiments were carried out in which the developing embryos were exposed to binary metal mixtures. In the first approach, the same total metal concentrations used to establish dose-response curves in Bioassay 1 using 5 μM EDTA were combined (ranges shown in Tables 2.6-2.9), since the response to single metal exposures had not yet been established. For example, 0.2 μM Cu and 0.5 μM Zn were added to the first aliquot for the

Cu-Zn combination and 0.2 μM Cu and 0.5 μM Pb for the Cu-Pb combination. A cumulative approach was examined in the second method, mean EC_{xtotal} values were determined from previous dose-response curves from experiment $[B]_{const}$ (n=3). Binary combinations of Cu, Cd and Zn, at each EC_{xtotal} value for total metal concentrations (Table 2.10) were used for the exposures (e.g. EC01 Cu with EC01 Cd; EC05 Cu with EC05 Cd). Furthermore, the EC_{xtotal} values were combined in different proportions of EC01/EC20, EC20/EC01, EC50/EC05, EC05/EC50, EC25/EC10 and EC10/EC25 for each binary metal mixture. Total metal concentrations are needed for the preparation of the bioassays in the presence of a metal buffer, and accordingly total metal concentrations are specified for both approaches.

Table 2.10: Total metal concentrations used in binary metal exposures based on mean EC_{xtotal} values in the presence of 5 μM EDTA. S=31, pH ~8.1.

EC_{xtotal}	Cu (μM)	Cd (μM)	Zn (μM)
01	3.33	5.17	3.47
05	3.45	6.13	3.81
10	3.53	6.39	4.12
20	3.60	6.89	4.55
25	3.63	7.06	4.58
50	3.73	7.75	5.07
75	3.83	8.48	5.48
90	3.91	9.38	6.04

2.3.5. Data analysis

Results from the bioassays were normalised to the control values to take account of abnormal development of oyster larvae arising from the exposure conditions in the bioassays and to facilitate comparisons between sets of data. This was done according to Woelke (1972) by expressing experimental results as percent net response:

$$Percent\ net\ response = \frac{\%\ abnormality\ in\ exposures - \%\ abnormality\ in\ controls}{100 - \%\ abnormality\ in\ controls} \times 100$$

Dose-response curves were generated with Microsoft Excel and SigmaPlot® 8.0 (SYSTAT Software Inc, Richmond, CA 94804, 2004). A three or four parameter sigmoidal curve provided the best fit for the experimental data. These curves incorporate response values of 0 and 100% into plots and hence the manipulation of data is unnecessary. In addition, the calculation of theoretical concentrations that cause any given effect is possible, which allows comparison of data with literature values. For example, the EC_{05} has been reported by several authors to be a more reliable indicator of toxicity threshold than the No Observed Effect Concentration (NOEC) (Lorenzo *et al.*, 2002). From the function describing the fitted curve, EC_{50} and EC_{05} values were calculated for the metal exposures.

In general, the data did not follow a normal distribution (where measurements are distributed symmetrically about the mean and clustered towards the centre) and therefore the significance between different experiments was evaluated with non-parametric tests. The Friedman two-way analysis of variance by ranks test was used to test whether the larval response to the calculated free ion concentrations used in experiments $[B]_{const}$ and $[M]_{const}$ were significantly different. The Kruskal-Wallis non-parametric one-way analysis of variance test was used to test whether the response between different pairings to single metal treatments was significantly different.

2.4. Results and Discussion

2.4.1. Quality assurance

The levels of abnormality (PNR) in the controls ranged between 6 and 32% in all experiments (mean of 19.0 ± 7.8%, n=60), hence remaining below the acceptable level of 40% (da Cruz *et al.*, 2007; EA, 2004). The concentrations of EDTA used in the bioassays (i.e. 0.5 to 12.5 μM), were shown not to be toxic to the larvae in two experiments (refer to section 2.3.4.3). The experimental constraints during the bioassay incubations did not permit the measurement of labile metal concentrations with voltammetric methods.

Although larger exposure volumes could have been prepared and preserved for later analysis by freezing, the error introduced through differences in adsorption and evaporation between storage and exposure vessels, as well as speciation changes due to freezing and thawing was deemed too large. Therefore the metal speciation was calculated for each aliquot.

2.4.2. Results from larval exposure in parallel experiments ($[B]_{const}$ and $[M]_{const}$)

Whether the larval responses conform to the FIAM cannot be established unequivocally, although, statistically it was shown that there was no significant difference between the two experimental treatments, $[B]_{const}$ and $[M]_{const}$ (Freidman two-way analysis of variance by ranks test, $P>0.05$). Nonetheless, overall the data suggests that there is a difference between the experimental treatments for Pb and Zn (refer to Table 2.13 in Section 2.4.3).

Bioassay toxicity test results showed the sensitivity of *C. gigas* larvae, which responded with increasing abnormal development to progressively higher exposure to each of the metals (Cu, Cd, Pb, Zn) tested, as signified in increasing values of percent net response (PNR, abnormal development normalised to the controls). The results of parallel experiments, in which either the buffer concentration or the total metal concentration was held constant in order to maintain the same free ion concentration in corresponding aliquots ($[B]_{const}$ and $[M]_{const}$, respectively), are graphically presented in Figures 2.5a-h for Cu, Zn, Pb and Cd, using Bioassay 3 as an example (refer to Tables 2.6-2.9 for conditions). The figures show PNR as a function of the free ion concentration ($[M^{z+}]$ mol L^{-1}), where the $[M^{z+}]$ concentration was chosen as it represents the most toxic metal species for marine organisms (Zamuda and Sunda, 1982; Engel *et al.*, 1981; Anderson *et al.*, 1978; Sunda and Guillard, 1976). Differences in PNR between the three pairings were small for exposure to Cu and Zn, hence the mean of the three pairings are presented (P_{mean}) (Figures 2.5a and e, respectively). Larger differences in PNR in response to Cd and Pb exposure were

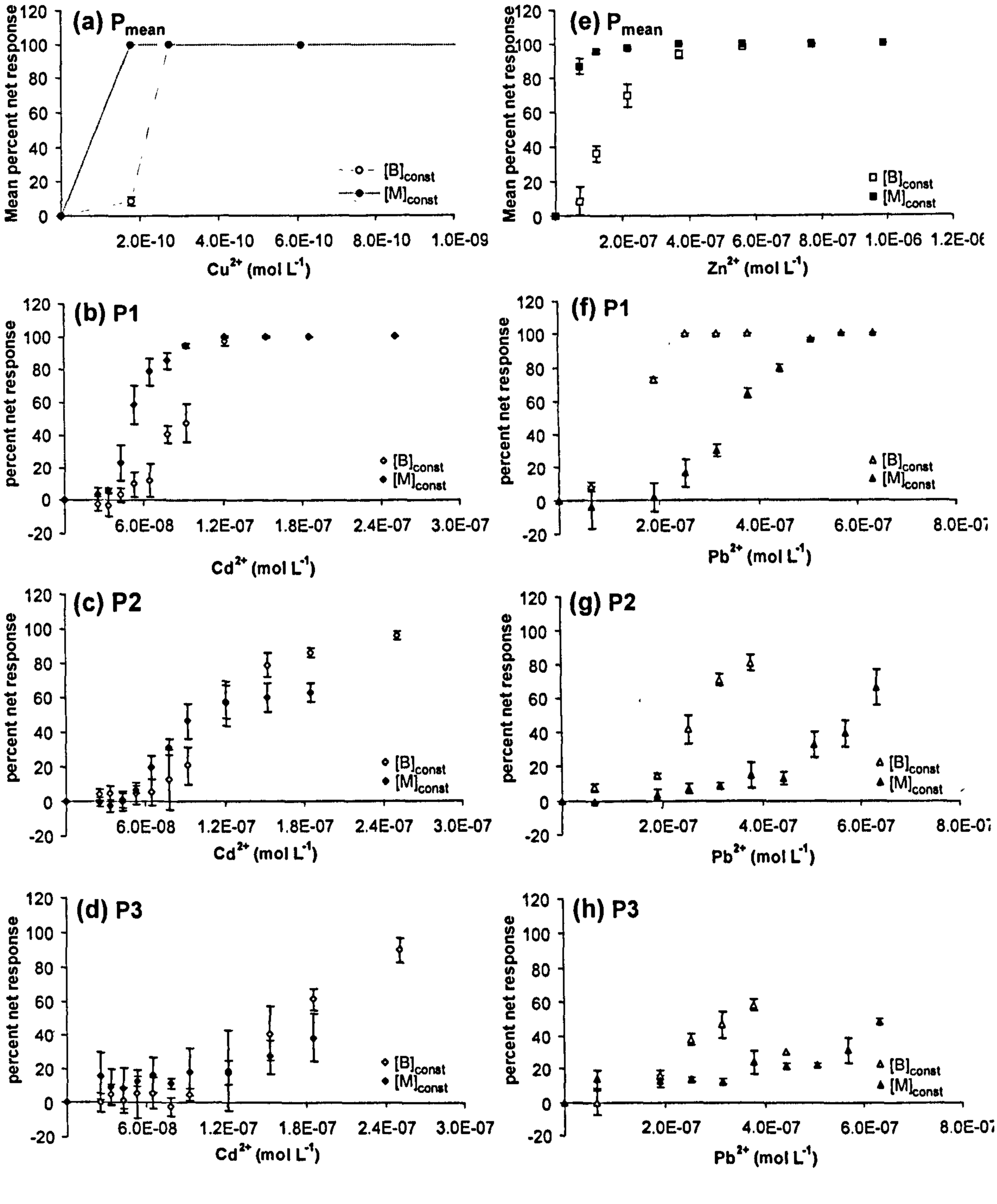


Figure 2.5: Percent net response (PNR) of oyster larvae exposed to Cu (a), Cd (b-d), Zn (e) and Pb (f-h) for experiments where the buffer concentration ($[B]_{const}$, 5 μM EDTA) and the total metal concentrations were held constant ($[M]_{const}$, 6 μM Cu, Zn, 6.5 μM Cd and 12 μM Pb) (n=3). The *x*-axis shows calculated free ion concentrations used in experiments. For Cu and Zn, differences between pairings were small (see error bars), and graphs show mean PNR for all pairings. For Cd and Pb, results from all pairings are shown. Error bars represent ± σ. Fertilised eggs were incubated for 24 h at 24 ± 1°C, pH 8.1. To aid visual representation, Cu plot contains trend lines and only part of the data since 100% abnormal larvae developed at concentrations >3E-10 M.

and therefore the results for each pairing are shown (P1, P2 and P3, Figures 2.5b-d and f-h, respectively). In Pb exposures a precipitate formed at 1 μM EDTA with a total Pb concentration of 12 μM for experiment $[M]_{const}$ and at 5 μM EDTA and 12 μM Pb for experiment $[B]_{const}$ and in all subsequent aliquots containing higher Pb concentrations. Due to the removal of Pb from solution, the percentage of D-larvae increased (e.g. PNR decreased), indicating that the bioavailable fraction of Pb had decreased and therefore, only data for exposures prior to precipitate formation are shown (see section 2.4.2.1 for further discussion).

The reproducibility between replicates (n=3) for this bioassay ranged from 16.7 to 33.1% for Pb, 13.3 to 26.1% for Cu, 26.0 to 55.3% for Cd and 2.5 to 24.9 % for Zn. Fernandez-Alba *et al.* (2002) report that the average reproducibility for bioassays using invertebrate and fish species is 38.8% based on a published summary of reproducibility by the US Environmental Protection Agency, 1991. For this set of experiments in the current project, the results for Cu, Zn and Pb were better or similar to this average, while some values for Cd were less reproducible. Statistically, there was a significant difference in inter-parental variability for the Cd exposures, based on a one-way analysis of variance (Kruskall-Wallis non-parametric test, $P<0.05$), whereas no significant difference was evident for Pb ($P>0.05$).

Although inter-pairing variability was most pronounced for Cd and Pb exposures, only P1 attained total abnormal development (PNR = 100%) in both experiments for all four metals. Indeed, the results indicated that for both Cd^{2+} and Pb^{2+} (n=3) the level of toxic response between pairings increased in the order P1 > P2 > P3 (e.g. P3 showed a relatively greater tolerance to the free ion concentrations of these two metals). Although the bioassays were prepared so that each exposure volume nominally contained 50 embryos mL^{-1}, there was some deviation from this figure as highlighted in Table 2.11. On average, P1 contained 30% less embryos per exposure volume and this probably had some influence on the results. A number of studies have established that micro-organisms, such

as bacteria and algae, exude metal-complexing ligands which is often attributed to metal stress (Lombardi *et al.*, 2005; Dryden *et al.*, 2004; Vasconcelos and Leal, 2001; Croot *et al.*, 2000; Gordon *et al.*, 2000; Pistocchi *et al.*, 2000; Capelo *et al.*, 1998; Santana-Casiano *et al.*, 1995), and it seems plausible that the higher number of developing embryos in the P2 and P3 exposures may have resulted in greater tolerance to metals due to the provision of additional complexing ligands from exudate release. Indeed, while best efforts were made to remove bacterial growth from the medium prior to bioassay exposures, their addition to the medium through the stripping process was unavoidable since bacteria are present within the gonads of the oysters. Bacteria and, on occasion, algae were observed in the exposure vessels under light microscopy. However, the concentration of EDTA used in the bioassays was chosen at a high enough level to render negligible any contribution from natural complexing ligands within the exposures. In addition, identical exposure conditions were used for all four metals and pairings, and therefore, the likely cause for the differences in PNR between pairings is genetic variability, although experimental errors (e.g. preparation and equilibration of exposure volumes, positioning in the growth chamber, bacterial growth etc.) will have contributed to the variability.

Table 2.11: Mean total larvae counted (±σ) in bioassay 3 in all exposure volumes for three pairings (n=3).

Metal	P1	P2	P3
Cd	112 ± 11.3	163 ± 9.44	159 ± 11.1
Zn	123 ± 10.6	179 ± 3.94	177 ± 16.9
Cu	118 ± 23.4	164 ± 4.17	160 ± 10.7
Pb	123 ± 5.30	177 ± 8.03	179 ± 12.5

The bioassay results show variability in the responses to the four metals, whereby the toxicity decreased in the following order: $Cu^{2+} >> Cd^{2+} > Zn^{2+} \approx Pb^{2+}$. Extreme sensitivity to Cu was evident frequently in bioassays that showed a complete disintegration

of the developing embryo following the addition of Cu to the 16 to 32-cell larval stage. Figure 2.3e shows an example of this phenomenon. Furthermore, in such cases unfertilised eggs, which occurred in low numbers in each bioassay and remained unaffected by addition of Cd, Pb and Zn, were not found after incubation with Cu, indicating damage to their integrity by the presence of Cu.

Overall, the toxic response between experiments (in Bioassay 3) showed $[M]_{const}$ > $[B]_{const}$ for Cu, Zn and for pairing P1 for Cd. However, in the case of Cd the variability in response between replicates (n=3) for pairings P2 and P3 was low, suggesting that there was little difference in the responses between experiments $[M]_{const}$ and $[B]_{const}$. In contrast, the toxic response to Pb exposures was higher for $[B]_{const}$ than for $[M]_{const}$, again at equal free ionic concentrations. Table 2.13 (see section 2.4.3) shows that the mean free ion EC_{50} values for experiments $[M]_{const}$ and $[B]_{const}$ were within the same order of magnitude for all metals, and a statistically significant difference was only observed for Zn and Pb (P=0.003 and P=0.020 for Zn and Pb, respectively). If the assumptions on which this study is based are valid, namely (1) the internalisation flux is rate limiting, (2) the concentration of carriers or sensitive sites remains constant and (3) the membrane surface is chemically homogeneous, and that inorganic metal speciation is constant in constant ionic media, then the results from $[M]_{const}$ should equal those from $[B]_{const}$. The difference in response to the two experiments therefore may reflect: (1) limitations of the thermodynamic equilibrium modelling approach; (2) EDTA-induced metal uptake by larva; and/or (3) a possible kinetic contribution in the uptake of the metal. These points are discussed in sections 2.4.2.1 to 2.4.2.3.

2.4.2.1. Speciation calculations for the bioassay exposures

Assuming that thermodynamic principles prevailed and equilibrium was reached, the speciation of metal ions is determined by their conditional stability constants for the formation of complexes with ligands present within the bioassay exposure media (e.g.

EDTA and inorganic species such as hydroxyl and carbonate species). In the preparatory stages of the bioassays, the buffer was added to the ASW before the metal in experiment $[B]_{const}$, while the metal was added to the ASW before the buffer in experiment $[M]_{const}$. This difference in procedure should not have influenced the metal speciation once equilibrium conditions have been reached. However, kinetic effects of the competition between major ions and metal ions for complexing sites on EDTA are not considered in MINEQL+. If kinetic effects resulting from the procedural difference in the addition of metals and buffer between the $[B]_{const}$ and $[M]_{const}$ prevented equilibrium conditions for Pb and Zn to be reached in the bioassay tests, then the free ionic metal concentration calculated using thermodynamic principles was erroneous, and could have resulted in the differences in PNR for Zn and Pb exposures.

Table 2.12 details a selection of the formation constants (e.g. log K values), from the MINEQL+ database, of primary species computed to be present within the exposures. The stability constants signify the relative binding strengths of these metal-ligand complexes and indicate the competition conditions. The affinity of EDTA for all the metals studied is higher than that of the carbonate ion, and therefore EDTA should theoretically out-compete the inorganic ions present for all four metals. It is unlikely therefore that formation of, for example, a Pb-carbonate species could explain the contrasting response between $[B]_{const}$ and $[M]_{const}$ experiments when compared with the other metals.

Equilibrium models, such as MINEQL+, calculate speciation based on thermodynamic principles. Systems that contain developing embryos are difficult to fully characterise and represent, limiting the models' validity.

In all speciation calculations dissolved solids were excluded as they were not expected to contribute. However, with dissolved solids included in the speciation calculations, no variation in dissolved species throughout the concentration range (detailed in Tables 2.6-2.9), including the free ion concentration, was predicted when compared to dissolved solids excluded from the calculation. In the case for Pb, speciation calculations

showed that the predominant species was likely to be the precipitated solid, hydrocerrusite ($Pb_3(CO_3)_2(OH)_2$). This suggests that after precipitation was observed in the exposure vessels that either: (1) hydrocerrusite does control the metal species distributions for Pb and determines the toxicological response, or (2) that calculations within the model that control the transformation between a dissolved solid (Type V) and a precipitated solid (Type IV) need refinement. This was a recognised limitation in MINEQL+ version 3.1a (Schecher, 1994), although improvements in the internal handling of Phase Rule violations have been incorporated into version 4.5.

Table 2.12: Formation constants from the MINEQL+ database of primary metal species predicted to be present in the defined culture medium (ASW). 'M' stands for the respective metal in the complex.

Metal	$MEDTA^{2-}$	$MHEDTA^{-}$	$MHCO_3^{+}$	$M(CO_3)_2^{2-}$
Cu	20.5	24.0	12.1	10.2
Pb	19.8	24.9	13.2	9.9
Cd	18.1	21.5	12.4	7.2
Zn	18.0	21.4	12.4	9.6
Ca	12.0	16.0	12.1	-
Mg	10.6	15.1	12.4	9.6

2.4.2.2. EDTA-induced uptake

While the preliminary tests showed that EDTA was not toxic to the developing embryos in the ASW medium at the concentrations used in this study (0.5 to 12.5 μM), these tests were not be able to resolve redox reactions that involve the reduction of M(II)EDTA to M(I) at the cell surface. This mechanism has been reported for causing higher uptake rates for Cu in phytoplankton (Jones *et al.*, 1987) and yeast (Hassett and Kosman, 1995). Also, utilisation of Cu(II)EDTA has been reported for *Thalassiosira*

weissflogii (Jones *et al.*, 1987). Hudson (1998) referred to a previous study by Sunda and Huntsman (1995), where Cu^{2+} uptake rates had exceeded the maximum supply of inorganic Cu species to the surface of three phytoplankton species. It was suggested that the high uptake rates could only be sustained if either the phytoplankton reduced Cu(II)EDTA to Cu(I) at their surfaces or some minor but labile Cu-EDTA species existed, although calculations indicated that dissociation would be too slow. Furthermore, it has been shown that mixed bacterial cultures can degrade metal-EDTA complexes and that Gram-negative bacteria can increase the permeability of the cell membrane resulting in greater metal uptake (Wilkinson and Buffle, 2004 and references therein). Tang *et al.* (2005) studied the uptake of Cu in the presence and absence of EDTA and it was suggested that EDTA-complexed Cu species could induce additional membrane damage and/or other toxic effects. In addition, Kohen and Nyska (2002) proposed that the formation of oxidative species from the reduction of Cu(II) could lead to harmful effects that include lipid oxidation, protein fragmentation, and DNA damage. Whether these phenomena are specific for exposure to Cu is not clear. However, one or more of these mechanisms could account for the somewhat greater toxic response toward Cu (and possibly Cd and Zn) in the exposures where the EDTA concentration was varied ($[M]_{const}$) since higher metal and EDTA concentrations were used at the lower calculated free ion concentrations compared with $[B]_{const}$ experiments. EDTA-mediated toxicity does not appear to play a role for Pb since, in general, a greater toxic response was observed in the $[B]_{const}$ experiments compared with $[M]_{const}$.

2.4.2.3. Kinetic aspects to bio-uptake

This study aimed to establish whether the free metal ion is the most biologically available metal species under the basic assumptions of the FIAM (i.e. thermodynamic control). If it is assumed that the toxicological response is the result of uptake by the organism, the different responses to experiments $[B]_{const}$ and $[M]_{const}$ could reflect a kinetic

contribution in the uptake process. The FIAM assumes that equilibrium, or more correctly pseudo-equilibrium, is reached, whereby the various metal species in the medium and bound to cellular ligands, achieve equilibrium, despite continuous depletion of the metal from the medium and its accumulation into the organisms. This, however, is dependent on the relative kinetics of the various processes at play and on the time scale of the observations (Morel, 1983). If transport of the metal to the cell surface is rate-limiting, then the FIAM no longer applies. The kinetics of the complexation reaction or the rate of metal transport in the reaction layer outside the cell will determine the biological response (Campbell, 1995). Therefore examination of transient uptake rates is necessary in order to discriminate between systems that are under thermodynamic or kinetic control (Hudson and Morel, 1993; Jackson and Morgan, 1978). Since uptake rates were not investigated in this study, it is impossible to evaluate whether the system was under thermodynamic or kinetic control.

2.4.3. Metal toxicity to *C. gigas* larva

Effective concentrations inducing an effect are often reported for comparative purposes. In the present study, the range and mean values for effective concentrations of free ionic species inducing a PNR of 5% (EC_{05free}) and of 50% (EC_{50free}), relative to the controls, from all experiments are shown in Table 2.13. For all four metals tested, increasing free ion concentrations produced increased abnormal development of oyster embryos above a certain threshold. This threshold was quantified by calculating EC_{05} values from dose-response curves as illustrated for Cd as an example in Figure 2.6.

The mean EC_{50free} values (referred to as mean* in table 2.13) include the data from both experimental treatments, $[M]_{const}$ and $[B]_{const}$, and suggest the order of toxicity $Cu^{2+} >> Cd^{2+} > Zn^{2+} > Pb^{2+}$. The mean EC_{05free} concentrations ($\pm\sigma$) determined were: 0.16 ± 0.05 nM Cu (n=9); 46.7 ± 19.7 nM Cd (n=12); 161 ± 84.4 nM Pb (n=8); 31.3 ± 12.8 nM Zn (n=10) (Table 2.13). The order of toxicity for total metal concentrations was Cu > Zn >>

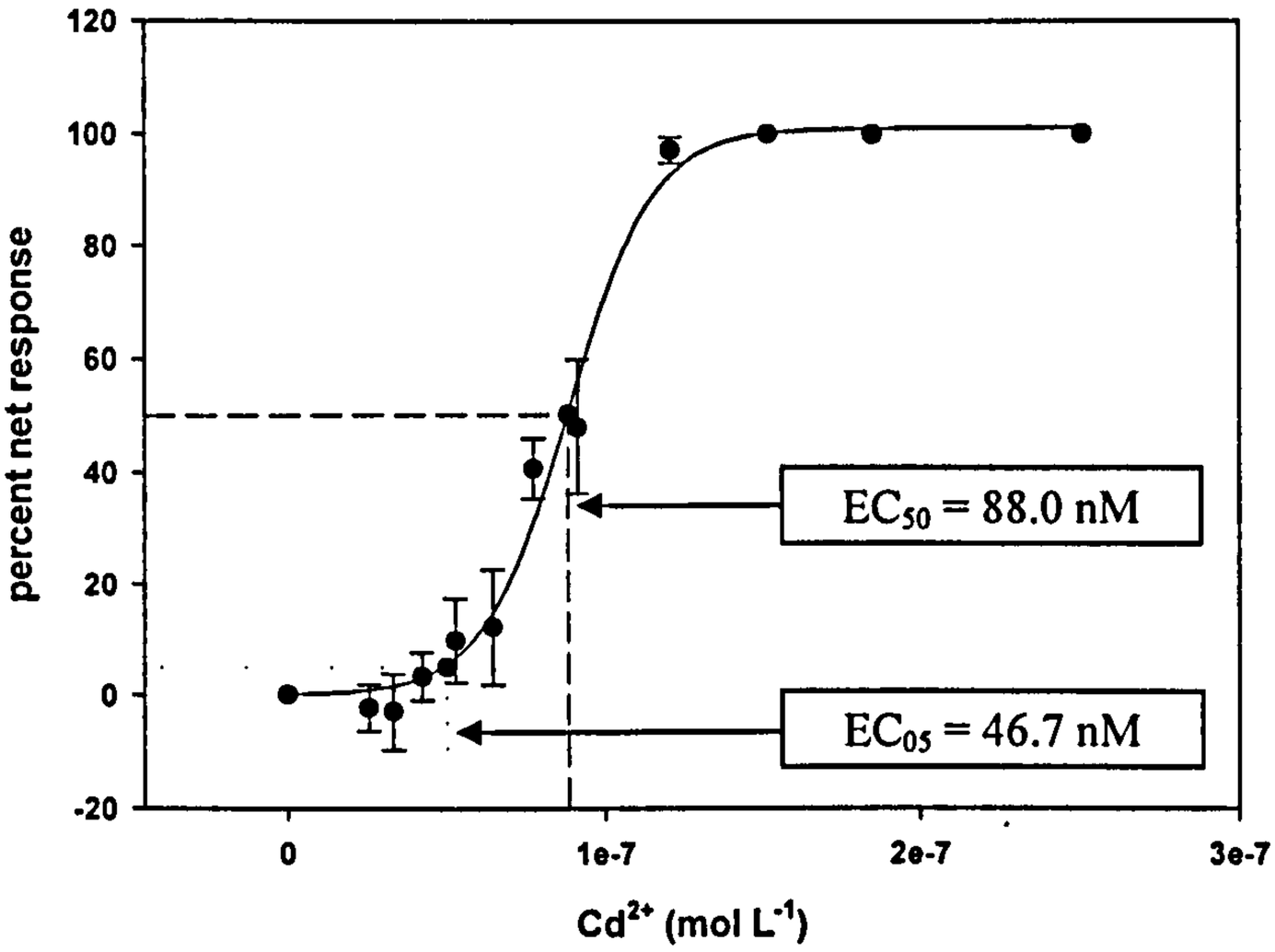


Figure 2.6: Percent net response (PNR) as a function of Cd^{2+} in the presence of 5 μM EDTA

Table 2.13: Effective free ion concentrations (±σ) inducing 5% (EC_{05}) and 50% (EC_{50}) abnormal development of larvae (relative to the controls). Mean* signifies the mean value of the data from all bioassays.

Metal	Treatment	EC_{50free} range (nM)	EC_{05free} range (nM)	Average EC_{50free} (nM)	Average EC_{05free} (nM)
Cu	$[M]_{const}$	0.12-0.29	0.09-0.16	0.20 ± 0.10 (n=4)	0.11 ± 0.04 (n=4)
	$[B]_{const}$	0.21-0.40	0.16-0.22	0.27 ± 0.06 (n=8)	0.20 ± 0.02 (n=5)
	Mean*			0.23 ± 0.08 (n=12)	0.16 ± 0.05 (n=9)
Cd	$[M]_{const}$	52.2-96	10-50	77.1 ± 22.5 (n=3)	31.3 ± 17.5 (n=4)
	$[B]_{const}$	57-168	35-90	88.1 ± 38.6 (n=8)	54.4 ± 16.5 (n=8)
	Mean*			88.0 ± 34.7 (n=11)	46.7 ± 19.7 (n=12)
Pb	$[M]_{const}$	356-560	50-300	522 ± 151 (n=3)	233 ± 57.7 (n=3)
	$[B]_{const}$	157-425	55-213	242 ± 69.4 (n=5)	95.0 ± 49.5 (n=5)
	Mean*			362 ± 178 (n=8)	161 ± 84.4 (n=8)
Zn	$[M]_{const}$	49.9-55.0	22.5-23.0	53.3 ± 2.94 (n=3)	22.7 ± 0.29 (n=3)
	$[B]_{const}$	92-341	10-108	173 ± 49.8 (n=8)	32.0 ± 16.0 (n=7)
	Mean*			128 ± 59.1 (n=11)	31.3 ± 12.8 (n=10)

Cd > Pb. The change in order for Zn and Cd suggests that Cd-inorganic complexes may contribute to the observed toxic response (e.g. are labile enough to be transported across

the biological membrane or that accidental transport may have occurred, although this has only been previously reported for Cd-citrate complexes by Erracalde and Campbell, (2000)). The range in EC_{05free} and EC_{50free} values, as well as the reported standard deviations, reaffirms the inherent variability in biological responses. The toxicological response toward Cu showed the extreme sensitivity of *C. gigas* to this metal, compared with Cd, Pb and Zn. Copper has been reported to be toxic to marine organisms, such as phytoplankton, at free cupric ion concentrations of 10^{-10} to 10^{-11} M. At these concentrations complete inhibition of cyanobacterial growth has been observed (Brand *et al.*, 1986), and the survival of naupliar larvae of the marine copepod *Acartia tonsa* reduced (Sunda *et al.*, 1990), and the grazing activity of *A. hudsonica* significantly diminished (Sharp and Stearns, 1997). Furthermore, at concentrations $>10^{-12}$ M the viability of many phytoplankton species, such as coccolithophores, dinoflagellates and diatoms declines (Sunda *et al.*, 1987). However, a study by Rivera-Duarte *et al.* (2004) with sea urchin (*Strongylocentrotus purpuratus*) and mussel (*Mytilus galloprovincialis*) larvae suggests that these marine invertebrates exhibit a greater tolerance to Cu^{2+} with EC_{50} values of <900 pM and <60 pM Cu^{2+}, respectively. The sensitivity of bivalve larvae towards trace metals has been shown previously (Brooks *et al.*, 2007; McPherson and Chapman, 2000; His *et al.*, 1999), and the EC_{50free} value of 230 ± 80 pM for Cu^{2+} obtained in the present study indicates that *C. gigas* larvae are less sensitive to Cu^{2+} than *M. galloprovincialis* larvae.

The effective concentrations determined in this study cannot be directly compared with the values reported by other workers since the buffer concentrations used in this work, to control the free ionic metal concentrations, governed the total metal concentrations used and were largely not biologically available. As a result, the total metal concentrations exceeded those used in other studies. However, the ranges of total metal concentrations reported in previous studies serve to illustrate the large variability that is found with this type of bioassay. For example, Arnold *et al.* (2005) reported a range of EC_{50} values for the mussel (*Mytilus* sp.) of 74-305 nM Cu (n=92) and His *et al.* (2000) in a comprehensive

review on bivalve embryo and larval bioassays include average EC_{50} data (±σ) of: 614 ± 50 nM Cu (n=27); 1974 ± 3312 nM Cd (n=18); 4672 ± 847 nM Pb (n=5); 4894 ± 497 nM Zn (n=16). The pooling of large datasets from different workers is likely to contribute to the large variability in the data presented by His *et al.* (2000). In the present study, the mean EC_{50free} values (±σ) were much lower since the free ion is a minor proportion of the total concentration and were determined as: 0.23 ± 0.08 nM Cu (n=12); 88.0 ± 34.7 nM Cd (n=11); 362 ± 178 nM Pb (n=8); 128 ± 59.1 nM Zn (n=11).

Overall, the toxicological impact of the free metal ions obtained in this study indicate that the concentrations of free metal ion that determined the toxicological impact on *C. gigas* larva may not be environmentally relevant, except perhaps for Cu^{2+}, although interactions between metals and other contaminants have been reported to enhance and/or decrease the toxic responses (Fleeger *et al.*, 2007; Gallego *et al.*, 2007; Xie *et al.*, 2006; Jonker *et al.*, 2004; Norwood *et al.*, 2003; His *et al.*, 2000 and references therein, Herkovits *et al.*, 1998). Since metals do not occur in isolation in the natural environment it is a valid consideration.

2.4.4. Toxicity of metal mixtures to *C. gigas* larva

The toxicity of the metals was measured singly and in binary mixtures of varying complexities. The combined toxic effect of multiple chemicals has been recognised as an important consideration in ecotoxicology because mixtures of chemicals can have a greater negative impact than the individual constituents of the mixture (Fernandez-Alba *et al.*, 2002), and natural waters contain mixtures of chemicals in varying proportions. In order to examine the effect of binary metal mixtures two different approaches were used.

Binary metal mixtures (1): In the first approach the metals were combined at concentrations (Tables 2.6 to 2.9) used to establish dose-response curves for single metal bioassays, in bioassay 1, and the results are shown in Figures 2.7 to 2.12. This approach

was defined by the need to establish individual dose-response curves and therefore the intention is to provide a qualitative assessment of the interactivity between metals, although EC_{50} values have been calculated for comparative purposes. The figures represent the response to individual metals as well as the response to binary metal mixtures, shown as PNR over the free ion concentration of one of the metals in the mix. In this way, the PNR resulting from metal combinations can be directly compared with that resulting from single metal exposures. The concentration of the free metal ion of each metal combination was calculated with both metals included in the modelling program (MINEQL+).

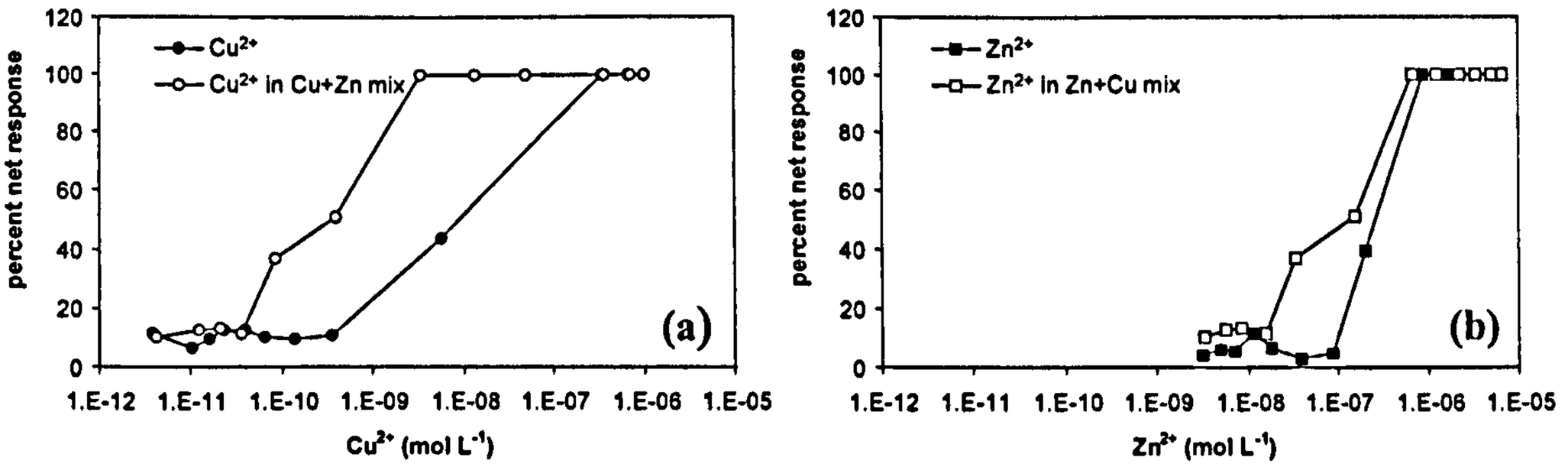


Figure 2.7: Acute toxicity of Cu^{2+} (a) and Zn^{2+} (b) in single metal exposures (filled symbols) in the presence of 5μM EDTA, pH 8.1; S=31. The open symbols show the percent net response when larva are exposed to binary metal combinations as a function of Cu^{2+} (a) and Zn^{2+} (b) concentrations in the binary mix. Note the logarithmic scale. Open symbols in (a) and (b) refer to the same binary mix. Therefore, Zn concentrations in the Cu+Zn mix (a) can be read from the open symbols in (b) and vice versa for Cu.

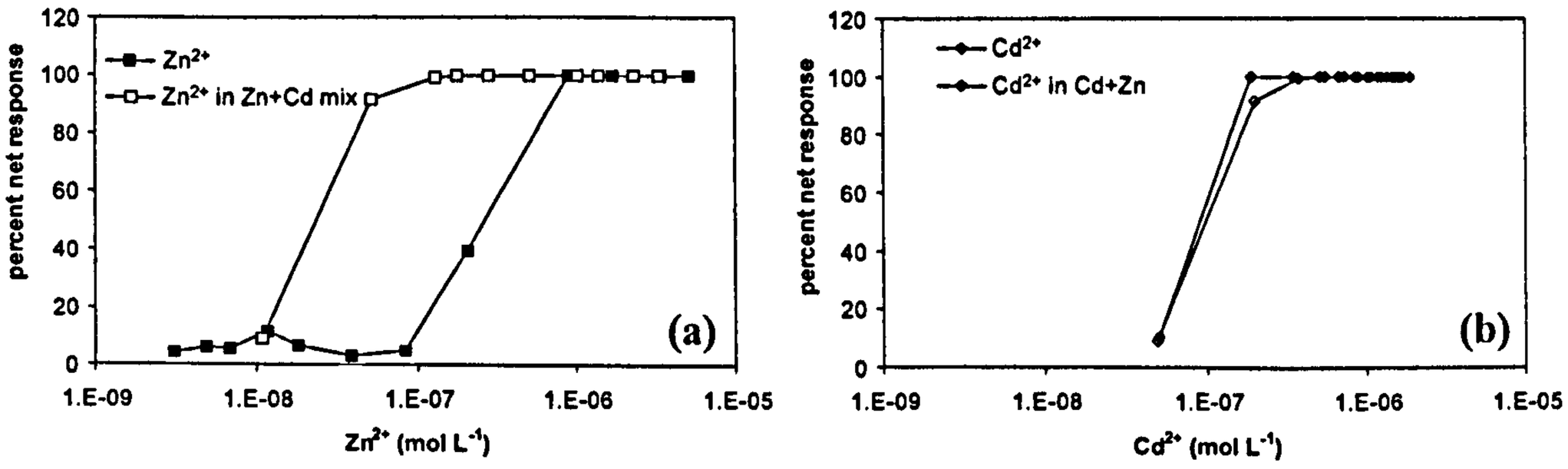


Figure 2.8: Acute toxicity of Zn^{2+} (a) and Cd^{2+} (b) in single metal exposures (filled symbols) in the presence of 5μM EDTA, pH 8.1; S=31. The open symbols show the percent net response when larva are exposed to binary metal combinations as a function of Zn^{2+} (a) and Cd^{2+} (b) concentrations in the binary mix. Note the logarithmic scale. Open symbols in (a) and (b) refer to the same binary mix. Therefore, Cd concentrations in the Zn+Cd mix (a) can be read from the open symbols in (b) and vice versa for Zn.

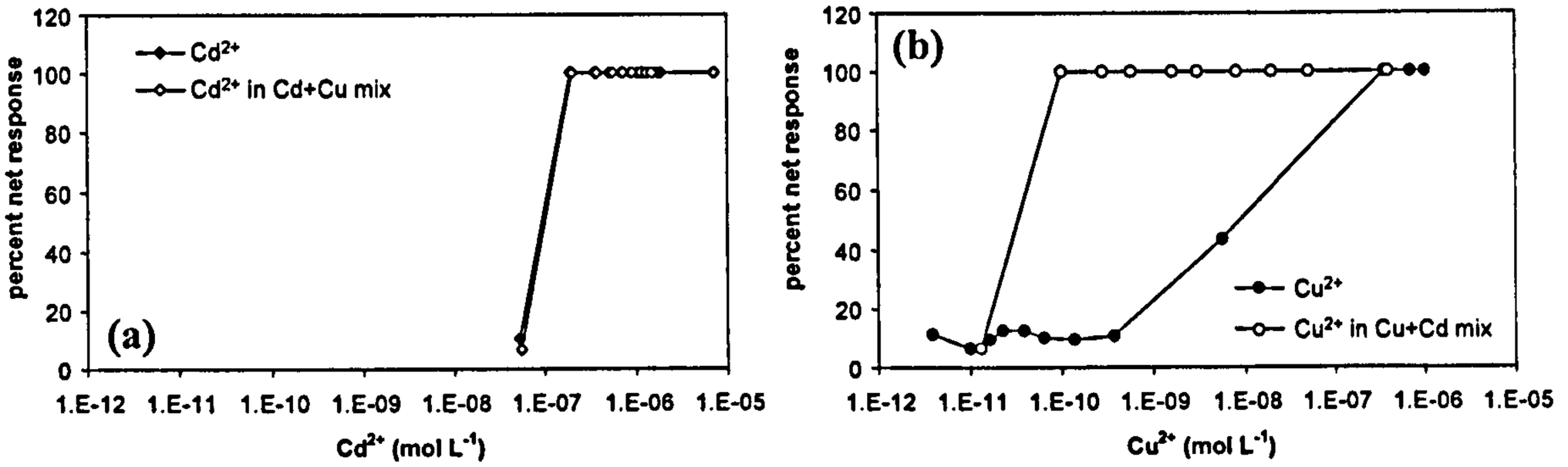


Figure 2.9: Acute toxicity of Cd^{2+} (a) and Cu^{2+} (b) in single metal exposures (filled symbols) in the presence of 5μM EDTA, pH 8.1; S=31. The open symbols show the percent net response when larva are exposed to binary metal combinations as a function of Cd^{2+} (a) and Cu^{2+} (b) concentrations in the binary mix. Note the logarithmic scale. Open symbols in (a) and (b) refer to the same binary mix. Therefore, Cu concentrations in the Cd+Cu mix (a) can be read from the open symbols in (b) and vice versa for Cd.

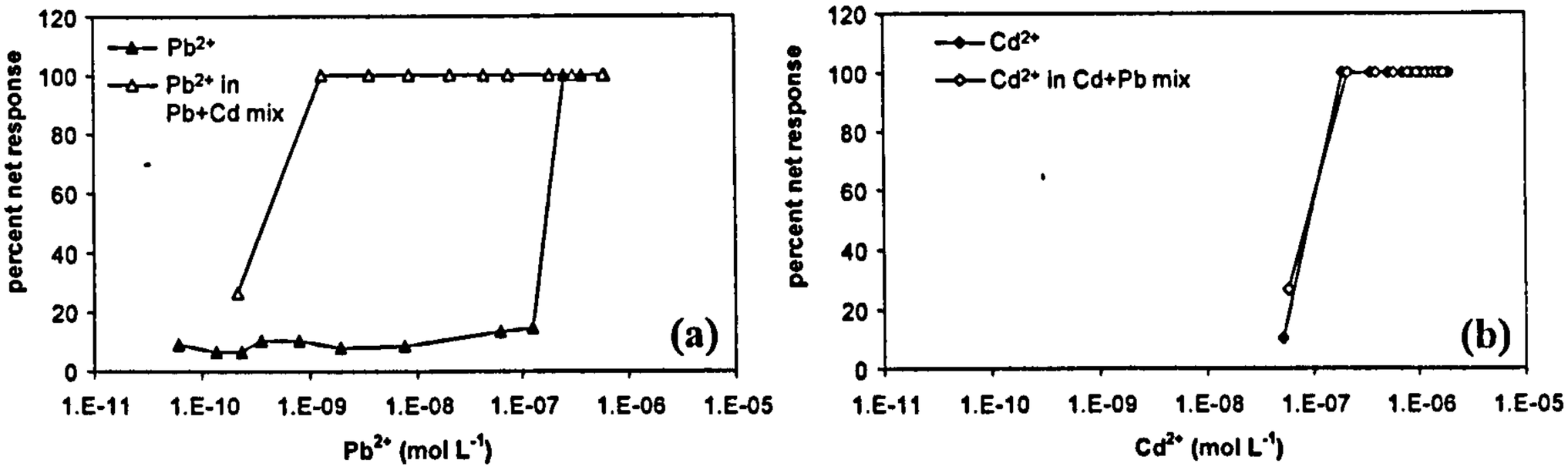


Figure 2.10: Acute toxicity of Pb^{2+} (a) and Cd^{2+} (b) in single metal exposures (filled symbols) in the presence of 5μM EDTA, pH 8.1; S=31. The open symbols show the percent net response when larva are exposed to binary metal combinations as a function of Pb^{2+} (a) and Cd^{2+} (b) concentrations in the binary mix. Note the logarithmic scale. Open symbols in (a) and (b) refer to the same binary mix. Therefore, Cd concentrations in the Pb+Cd mix (a) can be read from the open symbols in (b) and vice versa for Pb.

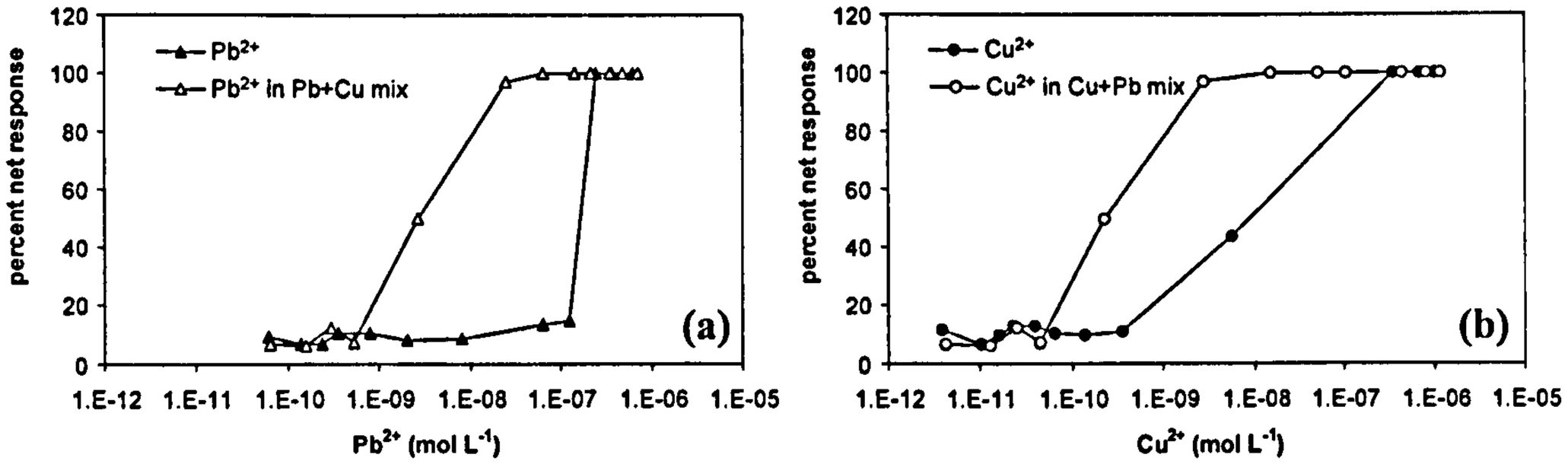


Figure 2.11: Acute toxicity of Pb^{2+} (a) and Cu^{2+} (b) in single metal exposures (filled symbols) in the presence of 5μM EDTA, pH 8.1; S=31. The open symbols show the percent net response when larva are exposed to binary metal combinations as a function of Pb^{2+} (a) and Cu^{2+} (b) concentrations in the binary mix. Note the logarithmic scale. Open symbols in (a) and (b) refer to the same binary mix. Therefore, Cu concentrations in the Pb+Cu mix (a) can be read from the open symbols in (b) and vice versa for Pb.

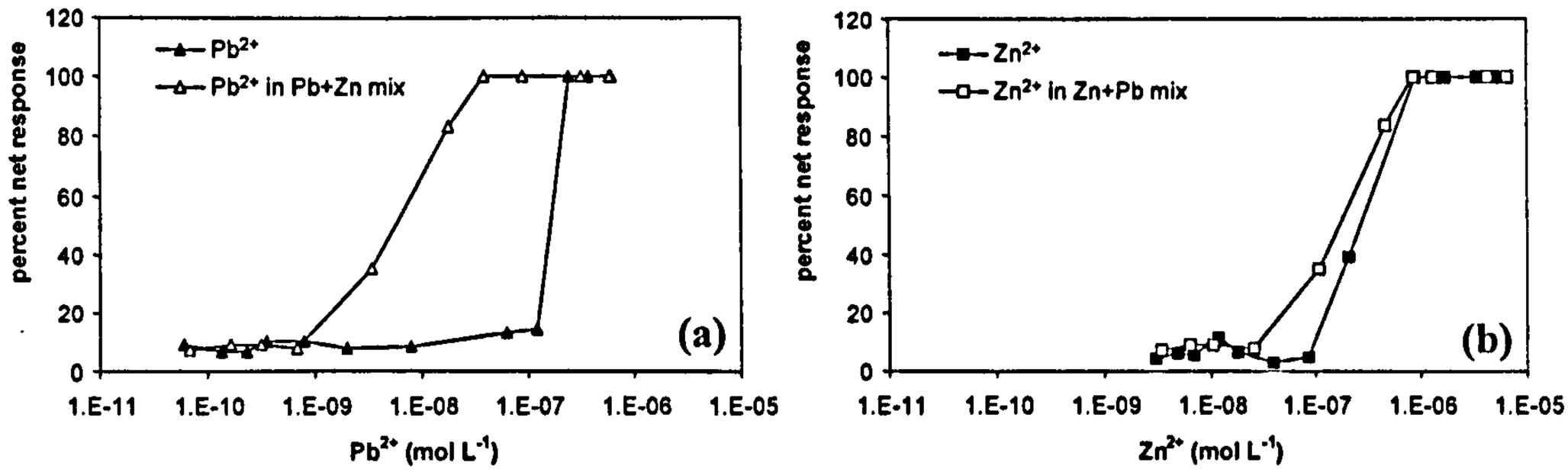


Figure 2.12: Acute toxicity of Pb^{2+} (a) and Zn^{2+} (b) in single metal exposures (filled symbols) in the presence of 5μM EDTA, pH 8.1; S=31. The open symbols show the percent net response when larva are exposed to binary metal combinations as a function of Pb^{2+} (a) and Zn^{2+} (b) concentrations in the binary mix. Note the logarithmic scale. Open symbols in (a) and (b) refer to the same binary mix. Therefore, Zn concentrations in the Pb+Zn mix (a) can be read from the open symbols in (b) and vice versa for Pb.

Interactions between compounds can affect their toxicity. If two or more toxicants act together with the same intensity as they do singly, their interaction is termed additive; and if the presence of one chemical enhances the toxicity of another, the interaction is considered to be synergistic (His *et al.*, 2000). Antagonistic effects occur when the presence of one chemical reduces the toxicity of another.

Enhanced levels of toxicity were observed for Cu, Zn and Pb when combined with one other metal, indicating synergism. However for Zn, in the presence of Pb and Cu, the

increase in toxicity is less marked and is probably within the experimental error. The toxic impact of Cd, however, appears to remain unchanged in the presence of either Cu, Pb or Zn (Figures 2.8 to 2.10). Table 2.14 details the EC_{50} values determined for the single and binary metal combinations and shows that significant changes in the free ion concentrations that induce a toxic response can occur in mixtures.

Table 2.14: EC_{50} values for free metal ion concentrations from single and binary metal combinations. The EC_{50free} concentration of each metal is reported in the columns and the rows refer to the interacting metal for each particular metal combination. Single metal exposure EC_{50free} in bold.

Interacting metal	Cu^{2+} (nM)	Cd^{2+} (nM)	Pb^{2+} (nM)	Zn^{2+} (nM)
Cu	**0.42**	74.6	0.28	23.5
Cd	0.004	**57.0**	0.04	3.12
Pb	0.02	50.3	**425**	21.0
Zn	0.04	105	0.71	**265**

According to His *et al.* (2000) mostly quasi-additive effects have been reported from studies on interactions between some heavy metals using embryo-larval tests. Additive interactions were reported by MacInnes (1981) for Cu/Zn and Hg/Ag combinations, and by Coglianese and Martin (1981) for Cu and Ag. Synergistic trends were shown by MacInnes (1981) for copper/zinc mixtures who inferred that the type of interaction may depend on the toxicant concentrations, a possible result of saturation of the complexing capacity of the seawater. Antagonistic interactions have been reported by various authors (His *et al.*, 2000 and references therein) using zinc/cadmium, mercury/silver, mercury/copper and mercury/zinc combinations with both oyster and mussel embryos.

In a review on metal mixture interactions, Norwood *et al.* (2003) reported that the responses to metal mixtures were extremely variable, even to the same binary metal combinations, and attributed to the different methodologies and species specific responses.

Gallego *et al.* (2007) found that for the ciliated protozoan, *Tetrahymena thermophila*, not only was the concentration of individual metals important but also the concentration ratio between them. For example, antagonism was found for Cd/Zn ratios of 1:1, 2:1 and 1:2 but at ratios of 3:1 and 4:1 the interactions changed to additive and synergistic, respectively. Other workers have found similar changes in responses based on concentration ratios with other organisms such as the marine microalgae, *Phaeosactylum trinutum* (Wang *et al.*, 1995), the shrimp *Callinassa australensis* (Negliski *et al.*, 1981) and two plant species (Otitoloju, 2002; Sharma *et al.*, 1999) and for different metal combinations (e.g. Cu/Zn and Cd/Cu).

The experimental design used in this approach was limited by the fact that total abnormal development occurred for many of the concentration combinations. However, the results still serve to illustrate that the toxicological impact can be modified in the presence of more than one metal and that the scale of these effects can vary considerably between the metal combinations as highlighted in Table 2.14. Indeed the increase in free aqueous metal ion concentrations upon addition of another metal may well result in the observation of synergistic effects.

Binary metal mixtures (2): In the second approach, the larvae were exposed to single and binary metal combinations based on mean EC_{xtotal} values determined from previous dose-response curves from experiment $[B]_{const}$ (n=3) (refer to section 2.3.4.4 and Table 2.10 for details). For the three metals tested, $EC_{50total}$ values from this experiment for single metal exposures were similar to the predicted values for Cu and Zn, whilst Cd showed a greater toxic response (Table 2.15).

Table 2.15: Mean $EC_{50total}$ values for single metal exposures for P1, P2, P3 and mix.

Metal	$EC_{50total}$ measured	Mean $EC_{50total}$ predicted
Cu	4.00 ± 0.01 (n=4)	3.73
Cd	5.87 ± 0.06 (n=4)	7.75
Zn	4.64 ± 0.30 (n=4)	5.07

The results of binary mixtures between Cu/Cd and Cu/Zn showed total abnormal development in all treatments, suggesting that additive or synergistic effects occurred. In contrast, Zn/Cd combinations resulted in a more varied response (Table 2.16). Single metal exposures indicated a greater toxic response than the average determined in previous experiments, for example, exposure to the mean $EC_{50total}$ value resulted in PNR = 100% for Cd and PNR= 89.7% for Zn. While this may account for the high PNR observed in binary exposures in this experiment, the less than total abnormal development in some combinations suggests that antagonistic effects may have occurred. This has been shown previously by Pavicic *et al.* (1994a,b) for Cd/Zn combinations using the mussel *Mytilus galloprovincialis*.

Table 2.16: Percent net responses for Cd and Zn from single and mixed exposures based on predetermined EC_x values.

	PNR %			PNR %			PNR %	
EC value tested	Cd	Zn	EC value tested	Cd/Zn	EC proportion	Zn/Cd	Cd/Zn
5	98.4	17.0	1	77.5	25:10	97.9	99.5
20	100	30.2	5	87.7	20:1	97.1	93.3
50	100	89.7	10	98.3	50:5	99.9	97.2
75	100	99.1	20	100			
>100	100	100	25	100			
			50	100			
			75	100			
			90	100			

2.5. Conclusions

The oyster-embryo larval bioassay has been shown to be sensitive to Cu, Cd, Pb and Zn, although the total concentrations used in this study were not environmentally relevant since the buffer concentrations used were in excess of the concentrations of natural ligands present in environmental systems. However, the free metal ion concentrations computed with an equilibrium modeling program, MINEQL+, indicated that the cupric ion concentrations inducing an extreme toxicological response were

relevant to coastal waters perturbed by metal contamination, such as harbours and industrialised estuaries. The results of this study support the use of this organism in studying the toxicity of environmental samples.

The main objective of this study was to use this bioassay to establish whether the free ion concentrations of Cu, Cd, Pb and Zn were the most toxic metal species (e.g. conformed to the FIAM). Based on parallel experiments using a buffered, chemically defined medium, the results were not fully conclusive. The inherent variability of biological systems was an important consideration in the observed responses. Whilst a synthetic ligand can control the speciation of metals it is not certain that its presence can induce abnormal larvae development and this requires further study.

The toxicities of the tested metals were measured singly and in binary mixtures. This is of particular interest since natural water systems are complex mixtures of interacting substances. Overall, the results indicated that additive and/or synergistic effects occurred between metal combinations. For Cu, more than additive effects were observed which further demonstrates the importance of investigating the speciation of this metal in environmental systems. In addition, there is clearly a need to investigate the effects of multiple metal exposures and the interactions with organic contaminants that are present in the natural environment. As different metals act differently and not all life forms are equally susceptible, it would be advantageous to use several different organisms to assess toxicity.

The large number of experiments carried out in the same laboratory gave rise to variable toxic responses, resulting in relatively large standard deviations for indicator values, such as EC_{05} and EC_{50}. Therefore it would be appropriate to use a worst case EC_{50} value for statutory purposes, rather than a mean value. Furthermore, it would be of benefit to consider using the free ion concentration in legislation since the free ion is considered to be the most biologically available metal species and therefore of more relevance in determining toxicological impacts. Although further work is needed, the effects of toxicant

mixtures, that can enhance the toxicity of single chemicals, should be considered in legislation, as well as the effects of other toxicant mixtures.

Chapter 3:

Voltammetric determination of biologically relevant metal fractions

3.1. Introduction

Stripping voltammetry has become an established analytical tool to investigate trace metal speciation in natural waters and has been widely applied (Buck and Bruland, 2005Cobelo-Garcia and Prego, 2004; Muller *et al.*, 2001; Kozelka and Bruland, 1998; Kozelka *et al.*, 1997; Collado-Sanchez *et al.*, 1996; Campos and van den Berg, 1994; Capadaglio *et al.*, 1990; Bruland, 1989; van den Berg, 1989, 1984). The combination of a preconcentration step with a stripping step provides the analytical sensitivity and selectivity necessary for the determination of trace metals at concentrations commonly found in natural waters (Achterberg and Braungardt, 1999).

Biogeochemical cycling of trace metals results in constant changes in their chemical speciation, and this in turn affects their mobility, bioavailability and toxicity to aquatic organisms. A major limitation of conventional sampling and analytical protocols is that perturbation of metal speciation occurs through sample collection, storage and handling procedures. The last decade has seen significant advances in on-site and *in situ* voltammetric instrumentation suitable for real time monitoring of trace metals in natural waters. This approach allows data collection at high temporal and spatial resolution, which aids the elucidation of biogeochemical processes in dynamic systems.

The main objectives of this chapter are to:

(1) Compare the analytical performance of a voltammetric *in situ* profiling (VIP) system and conventional laboratory voltammetric instrumentation using stripping voltammetric techniques (ASV and AdCSV) for speciation studies of Cu, Cd and Pb.

(2) Compare and contrast speciation measurements made with conventional laboratory voltammetric instrumentation (Metrohm 663 VA voltammeter) and the VIP system.

(3) Conduct copper-ligand titrations and illustrate the effect of data transformation on the estimated parameters.

3. 2. Background and Theory

3.2.1. Basic concepts of voltammetry

In its simplest form, voltammetric instrumentation consists of a voltammetric cell, a potentiostat and a current meter. The cell (Figure 3.1) has three electrodes that are immersed in the sample solution (i.e. solvent, electrolyte and analyte): (i) a working electrode (WE, generally a Hg electrode), (ii) a reference electrode (RE) and (iii) a counter electrode (CE). Stripping voltammetry (SV) involves a deposition (preconcentration) step followed by a stripping step. In the preconcentration step, a constant potential is applied for the duration of the preconcentration time between RE and WE, and the analyte is amalgamated with or adsorbed onto the WE. In the stripping step, a potential scan applied between the WE and RE leads to a redox reaction involving the analyte and results in a flux of electrons that is measured in the form of a current, i_p, between the WE and the CE. The change in oxidation state of the analyte produces the faradaic current, i_f, (Wang, 1985), and this is proportional to the concentration of the analyte in solution. In addition, a capacitive current, i_c, is generated as a result of the physical characteristics (e.g. resistance) of the cell. The sensitivity of voltammetric techniques is governed by the background current, of which the capacitive current is a significant component.

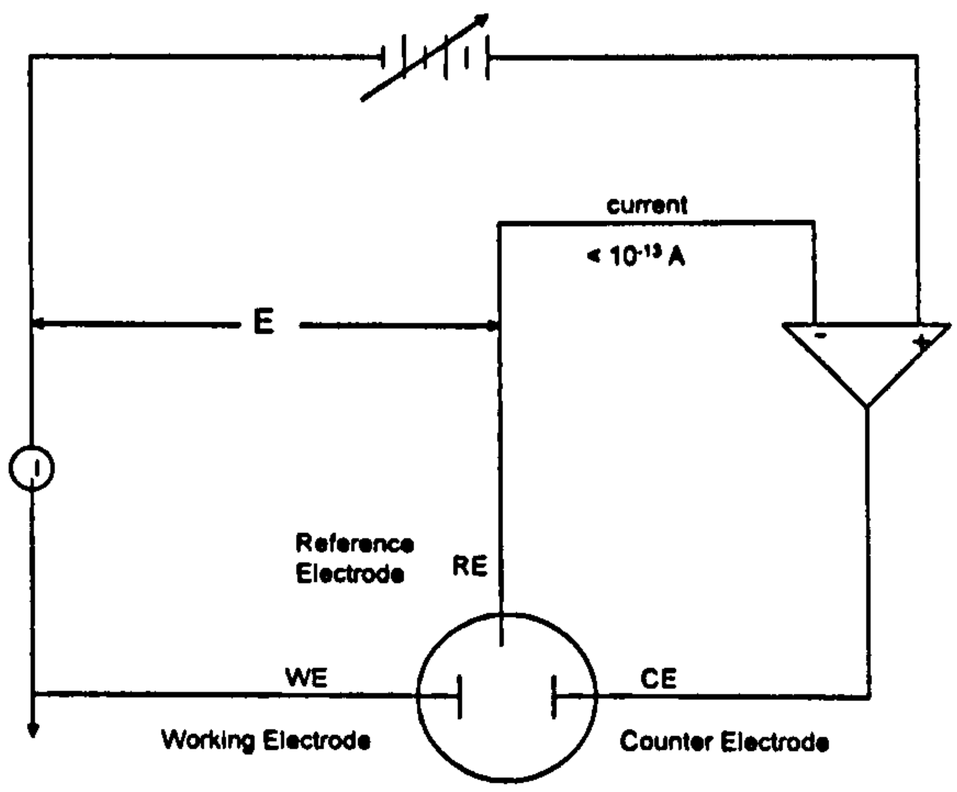


Figure 3.1: Schematic drawing of the electronic circuit of a potentiostat. E = imposed potential; i = measured current. WE, RE, CE = working electrode, reference electrode, counter electrode respectively (adapted from Buffle and Tercier-Waeber, 2000).

Most commercial instrumentation incorporates a variety of different potential-time (modulated) waveforms that improve the sensitivity of voltammetry, i.e. lower the limit of detection. Widely used waveforms include differential pulse (DP) and square wave (SW) forms, as they affect a reduction of the relative contribution of i_c to the measured signal, thus improving sensitivity (Buffle and Tercier-Waeber, 2000). Additional advantages include improved peak separation between analytes, increased speed of analysis and a reduction in interferences from surface active compounds in solution. More detailed theoretical and practical consideration of voltammetric techniques are available elsewhere (Buffle and Tercier-Waeber, 2000, 2005 and references therein; Mota and Correia dos Santos, 1995; van den Berg, 1989; Wang, 1985).

3.2.2. Working electrode

The WE is the electrode at which the reaction of interest takes place. Preferably, the WE should have a reproducible surface and area and a low capacitive current (Buffle and Tercier-Waeber, 2000; Wang, 1985). Mercury (Hg) electrodes are most often used for the determination of trace metals in natural waters because they fulfil both requirements and are more reliable and uniformly controlled than any solid substrate-water interface.

3.2.2.1. Mercury macroelectrodes

Mercury macroelectrodes (typical Hg radius >100 μm) include the dropping mercury electrode (DME), the hanging mercury drop electrode (HMDE) and the mercury film electrode (MFE). The MFE offers greater resolution and has a higher sensitivity when compared with the HMDE (Mota and Correia dos Santos, 1995; Wang, 1985) but is more strongly affected by interferences (i.e. intermetallic compound formation and adsorption of surface active compounds). The HMDE has the advantages of good reliability and reproducibility, as a new electrode surface is produced for each measurement. However, macroelectrodes present challenges for on-site and *in situ* applications due to their

configuration (i.e. amount of Hg required, drop stability etc.) (Buffle and Tercier-Waeber, 2000).

For macroelectrodes, the electrode radius, r, is larger than the diffusion layer thickness, δ (δ « r), so that linear diffusion is considered to dominate analyte transport. As a consequence, macroelectrodes are dependent on hydrodynamic transport, which is an important limitation for *in situ* applications where convection is difficult to control.

3.2.2.2. Mercury microelectrodes

Advances in microtechnology-based techniques have resulted in the development of rugged and reliable mercury microelectrodes (Hg radius typically 5-10 μm). The main advantages of microelectrodes relate to their small size and consequent low current flows, which reduces the capacitive current contribution. Spherical diffusion occurs as the electrode radius, r, is much smaller than the diffusion-layer thickness, δ (r « δ), which ensures that steady-state conditions and a constant flux at the electrode surface are maintained in unstirred solutions. As a consequence, mercury microelectrodes have a good signal-to-noise ratio and allow the determination of sub-nanomolar concentrations with short preconcentration times (Buffle and Tercier-Waeber, 2005).

3.2.3 Stripping voltammetric techniques

Figure 3.2 summarises the analytical sequence in a series of steps for ASV and AdCSV. The requirement for some of these steps is referred to in more detail in Sections 3.2.3.1 and 3.2.3.2.

3.2.3.1. Anodic stripping voltammetry (ASV)

For most applications of ASV, macroelectrodes (e.g. hanging mercury drop electrode (HMDE) or mercury film electrode (MFE)) have been utilised, but more recently, an increased number of reports show the use of microelectrodes (e.g. gel-integrated

microelectrode (GIME)) (Tercier-Waeber *et al.*, 2005; Howell *et al.*, 2003a,b; Pei *et al.*, 2000; Tercier-Waeber *et al.*, 2000a; Tercier *et al.*, 1998; Belmont *et al.*, 1996). The range of the electrical potential applied in ASV is confined by the oxidation current of Hg (at ca. 0 V) and by the reduction current of H_2O (at ca. -1.5 V), which limits the number of metals that can be measured with this technique. In natural waters, the limited sensitivity of ASV for other metals (van den Berg, 1991, 1988) has restricted studies primarily to copper (Cu), cadmium (Cd), lead (Pb) and zinc (Zn). Applications of ASV for the determination of these metals in natural waters have been carried out by Cobelo-Garcia *et al.* (2003) ; Huang and Wang, (2003); Capodaglio *et al.* (2002); Muller *et al.* (2001); Kozelka and Bruland, (1998); Kozelka *et al.* (1997); Collado-Sanchez *et al.* (1996); Capodaglio *et al.* (1990); Bruland, (1989).

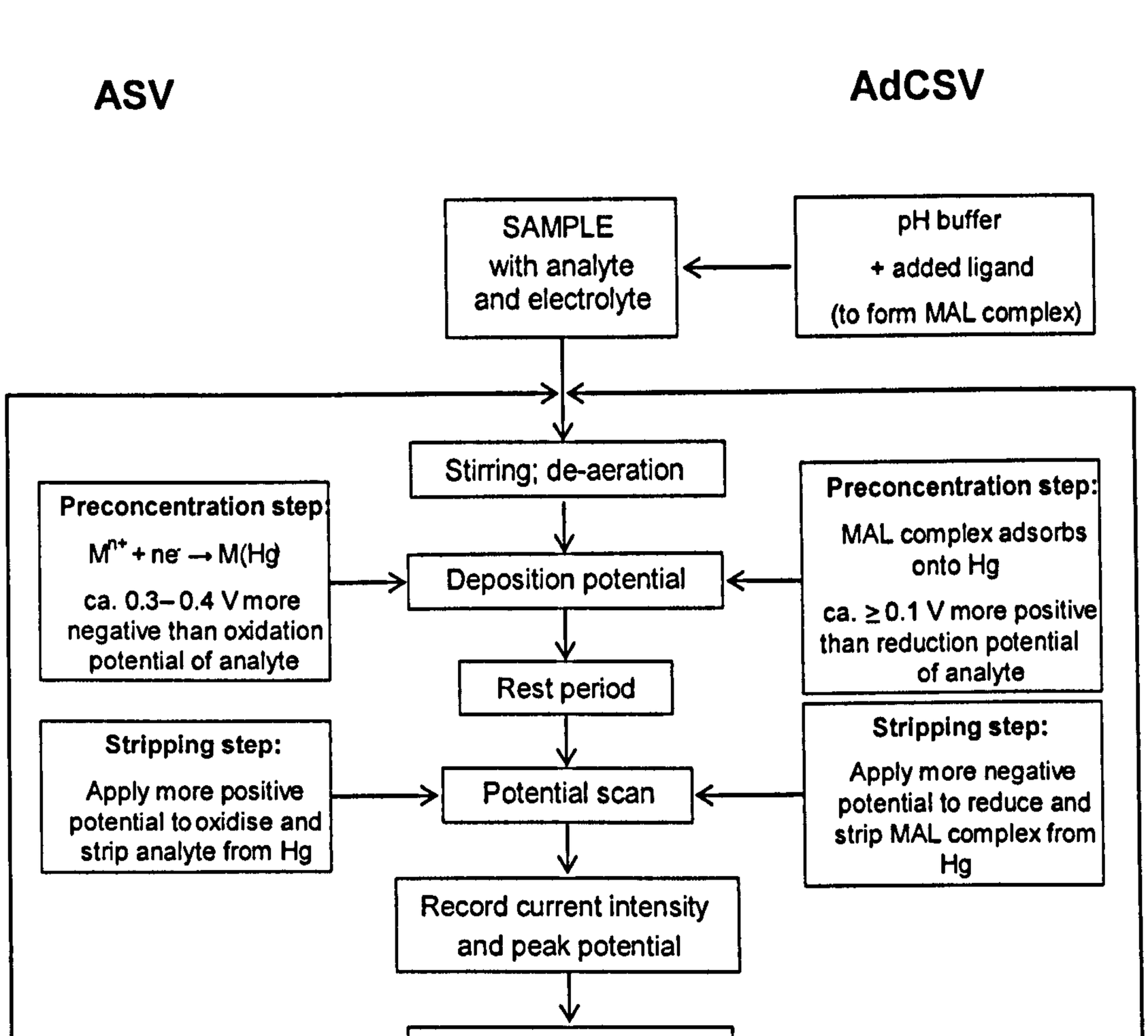


Figure 3.2: The series of steps common to stripping voltammetric techniques. NB. the potential scan continues towards a more positive or more negative final potential for ASV and AdCSV, respectively; MAL refers to metal-added ligand.

During the deposition or preconcentration step in ASV the metal ions are collected in the mercury (Hg) by reduction to the elemental form (M^0) and an amalgam is formed with the Hg (forward direction in Equation 3.1).

$$M^{n+} + ne^- + Hg \leftrightarrow M(Hg) \tag{3.1}$$

After a resting period, a potential scan is initiated at a potential more negative (by 0.3 - 0.4 V) than the oxidation potential of the metal(s) and continues towards a more positive final potential. At a potential specific to each analyte the metal is oxidised and stripped from the Hg (reverse direction in Equation 3.1). The resulting current is measured and plotted as a current-potential curve (voltammogram), from which the current intensity, i_p, and peak potential are obtained. The peak height above the baseline (i_p) is proportional to the metal concentration in the test solution. The analyte is quantified by standard additions to the test solution. Usually, two standard additions are performed using concentrations that increase i_p by 100%. The increase in i_p is calculated from:

$$\Delta i_p = i_{p1} - i_{p0} \tag{3.2}$$

where i_{p0} and i_{p1} are the peak currents measured before and after the standard additions, respectively. The peak current is directly related to the metal concentration by the sensitivity, S:

$$S = \frac{\Delta i_p}{\Delta M} \tag{3.3}$$

where ΔM is the increase in metal concentration from the standard addition. Thus the initial metal concentration, C_M, can be calculated from:

$$C_M = \frac{i_{p0}}{S} \tag{3.4}$$

3.2.3.2. Adsorptive cathodic stripping voltammetry (AdCSV)

In adsorptive cathodic stripping voltammetry (AdCSV), a well-characterised added ligand (AL) forms a complex with the metal(s) of interest (MAL) (Equation 3.5). Specific ligands, with known competition strength, are used for individual and/or groups of metals, for example tropolone, catechol, salicyldioxime and 8-hydroxyquinoline (oxine) have been used for Cu speciation studies (Buck and Bruland, 2005; Jin and Gogan, 2000; Campos and van den Berg, 1994; van den Berg and Donat, 1992; Donat and van den Berg, 1992; van den Berg, 1989, 1986, 1984) and ammonium pyrrolidine dithiocarbamate (APDC) and oxine have been utilised for Zn (Ellwood and van den Berg, 2000; van den Berg, 1985).

$$yM^{n+} + zAL^{m-} \leftrightarrow M_y(AL)_z^{(yn-zm)} \tag{3.5}$$

$$M_y(AL)_z{}^{(yn-zm)} \leftrightarrow M_y(AL)_z{}^{(yn-zm)}{}_{adsorbed} \tag{3.6}$$

$$M_y(AL)_z{}^{(yn-zm)}{}_{adsorbed} + e^- \leftrightarrow yM^{(n-1)+} + zAL^{m-} \tag{3.7}$$

The formation of MAL is pH-dependent and therefore pH buffers (van den Berg, 1991) such as HEPES (*N*-hydroxyethylpiperazine-*N'*-*2'*-ethanesulphonic acid, pH 7.8), PIPES (piperazine-*N,N'*-bis-*2*-ethanesulphonic acid, pH 6.8) and borate (boric acid, pH 8.5) are commonly used to control the pH of the sample solution. During the preconcentration (deposition) step a potential more positive (≥ 0.1 V) than the reduction potential of the analyte is applied and a fraction of MAL adsorbs on the electrode surface, forming a mono-molecular layer (van den Berg, 1989) (Equation 3.6). The potential scan to more negative values (stripping step) reduces and strips the adsorbed complex from the

electrode surface (at a specific potential for the MAL complex) (Equation 3.7). As for ASV, the peak current is measured and recorded and standard additions are used to quantify the analyte(s) (see Section 3.2.3.1).

3.2.4. Electrochemical trace metal speciation measurements

The chemical speciation of trace metals has important implications for their biogeochemical cycling in environmental systems. However, few techniques have the sensitivity and simplicity of electrochemical methods. Stripping voltammetry (ASV and AdCSV) is used to differentiate between labile and non-labile (see below for definitions) dissolved metal complexes, M_{labile} and ML, respectively. By definition, these metal fractions are operationally defined by the experimental conditions used during analysis. Labile metal complexes are the electrochemically reactive metal species that include the free hydrated metal ions and inorganic metal complexes (e.g. OH^-, CO_3^{2-}, Cl^-, SO_4^{2-}), and will include a fraction of relatively labile organic complexes that dissociate under the conditions used in the applied method. The non-labile fraction corresponds to the non-electroactive metal fraction that is primarily complexed by a range of strong organic ligands of different characteristics in terms of their molecular size and complexing strength. The voltammetric peak currents measured in natural water samples is proportional to $[M_{labile}]$ in solution. The total metal concentration, C_T, can be determined after sample treatments that convert non-labile into labile metal species (e.g. acidification and UV-irradiation). The non-labile metal fraction, ML, is then calculated by difference:

$$[ML] = C_T - [M_{labile}] \quad (3.8)$$

where $[ML]$, $[M_{labile}]$ and C_T are the non-labile, labile and total metal concentrations, respectively.

AdCSV speciation studies rely on competitive equilibrium between the added

ligand (AL) and metal complexing ligands (L) present in natural water samples. After equilibration, the measured labile metal concentration, $[M_{labile}]$, represents the concentration of metal complexed with AL (e.g. $[MAL] = [M_{labile}]$).

The detection window (DW) can be controlled by the choice and concentration of added ligand, and this fixes the competition conditions and defines which complexing sites or ligands are detected. The DW is framed either by: (1) the power to determine accurately a small decrease in the labile metal concentration (upper limit in AdCSV) or (2) the potential of the organic complexing material to compete with the inorganic complexation of the metal (upper limit in ASV), and by the limit of detection for labile metal (lower limit in AdCSV and ASV) (van den Berg and Donat, 1992). The centre of the DW is expressed as the α-coefficient for complexation of the metal, M, by AL (α_{MAL}), where the α-coefficient is a comparative measure of the abundance of the free metal ion concentration, M^{n+}, defined as: α = [metal-ligand complex]/$[M^{n+}]$ (see Section 3.2.4.2). It has been suggested that the DW spans one decade either side of the centre (Donat and van den Berg, 1992).

3.2.4.1. Metal-ligand titrations with AdCSV

Natural waters contain a range of ligands with different complexing sites and binding strengths. The range of ligands detected is controlled by the DW used (see Section 3.2.4). Therefore, in order to identify groups of natural ligands across a range of complexation strengths, different analytical competition strengths need to be applied (e.g. vary the DW).

With AdCSV, multiple analytical windows can be achieved by adjusting the analytical competition strength through use of added ligands of different complexation strength with the analyte, or by the use of different concentrations of the same added ligand (Buck and Bruland, 2005; Bruland *et al.*, 2000). Although the binding strength of natural ligands is likely to be a continuum, they are often categorised into different classes, e.g.

strong, intermediate and weak ligand classes, or as L_1, L_2 and L_3 ligands (Buck and Bruland, 2005; Cobelo-Garcia and Prego, 2004; Cobelo-Garcia *et al.*, 2003; Muller *et al.*, 2001; Bruland *et al.*, 2000). A linear (indicative of ligand saturation) or a curved response from the titration data reflects either the presence of only one group or more than one class of ligand/complexing sites, respectively (Buck and Bruland, 2005; Bruland *et al.*, 2000; Zhang *et al.*, 1990; van den Berg, 1984).

Metal-ligand titrations are employed to determine the free metal ion concentration and to estimate the capacity of a system to buffer additional metal inputs. This is achieved by calculating the concentration and binding strengths of the organic ligands present after suitable transformation of the titration data.

In this study, the data were transformed using the van den Berg/Ruzic linearisation method since this method is well established and data is available for comparative purposes (Ruzic, 1982; van den Berg, 1982). The mathematical transformation and calculations used are detailed below.

The uncertainties associated with the estimates of ligand concentrations and their binding strengths have been investigated (Miller and Bruland, 1997; Gerringa *et al.*, 1995; Apte *et al.*, 1988). The magnitude of the relative errors in the linearization processes shift between competition strengths and/or when characterising a single or two ligand system.

For a single ligand system, where the natural ligand dominates, $\alpha_{MAL} \ll \alpha_{ML}$ (low competition strength), there are large relative uncertainties in the early titration points. In this case, the stability constant cannot be determined with confidence as it is dependent on the slope and the y-intercept. However, the metal binding ligand concentration (C_L) is relatively well constrained by the later titration points. In contrast, for a system where $\alpha_{MAL} \gg \alpha_{ML}$ (high competition strength) the uncertainties are greater in the later titration points so that neither C_L nor log K′ can be determined with confidence, with C_L being particularly poorly defined. Therefore, in order to reduce the uncertainty in the estimation of both C_L and log K′, the competition strength should approximate the binding capacity of

the natural ligands (e.g. $\alpha_{MAL} \approx \alpha_{ML}$).

At weak and intermediate competition strengths, a curvature in the linearised data may indicate a two ligand system. However, the dependence on a few early titration points that are subject to large relative errors magnifies the uncertainties in relation to the slopes and intercepts. Accordingly, it is more appropriate to conduct at least two titrations at different DWs in order to characterise more than one ligand with greater confidence.

3.2.4.2 Theoretical aspects of metal-ligand titrations

Metal ligand titrations are often based on the assumption that a linear relationship exists between the metal and the organic ligand, L (for a 1:1 metal to ligand ratio):

$$C_L = [L'] + [ML] \tag{3.9}$$

where C_L is the total ligand concentration and [L'] is the concentration of L not complexed by M. The conditional stability constant for the complex ML, K'_{ML}, is given by:

$$K'_{ML} = \frac{[ML]}{[M^{n+}][L']} \tag{3.10}$$

Substitution of [L'] in Equations 3.9 with 3.10 and rearrangement provides the linear relationship:

$$\frac{[M^{n+}]}{[ML]} = \frac{[M^{n+}]}{C_L} + \frac{1}{K'_{ML} C_L} \tag{3.11}$$

The labile metal concentration, $[M_{labile}]$, is determined in each sample aliquot in a titration, so that [ML] can be calculated from the mass balance:

$$[ML] = C_M - [M_{labile}] \tag{3.12}$$

where for each aliquot, C_M equals the total dissolved metal concentration in the original sample plus the metal concentration added during the titration. The total metal concentration, C_M, in the presence of an added ligand, AL, is calculated from:

$$C_M = [M'] + [MAL] + [ML] \tag{3.13}$$

where [M′] is the inorganic metal concentration, and [MAL] and [ML] are the metal complexed by AL and the natural ligands L_x, respectively. The measured labile metal concentration, $[M_{labile}]$, includes [M′] (since a small constant fraction of added M remains uncomplexed) as well as the metal complexed with AL:

$$[M_{labile}] = [M'] + [MAL] \tag{3.14}$$

The labile metal concentration is related to the sensitivity, S, by:

$$i_p = S[M_{labile}] \tag{3.15}$$

where S is the slope of a plot of peak height, i_p, as a function of C_M provided the natural organic ligands have been saturated (e.g. $[M] > C_L$). This is established by standard additions to samples in the linear portion of the titration slope.

The free metal ion, M^{n+}, is related to $[M_{labile}]$ by:

$$[M^{n+}] = \frac{[M_{labile}]}{\alpha'} \tag{3.16}$$

where α' is the overall side reaction coefficient, not including complexation by L, and is expressed as:

$$\alpha' = \alpha_{M'} + \alpha_{MAL} \tag{3.17}$$

where $\alpha_{M'}$ is the inorganic side-reaction coefficient for complexation of M^{n+} by the major anions and α_{MAL} is the α-coefficient for the added ligand, so that Equation 3.16, after rearrangement, becomes:

$$[M_{labile}] = [M^{n+}](\alpha_{M'} + \alpha_{MAL}) \tag{3.18}$$

and:

$$\alpha_{MAL} = \beta'_{MAL}[AL'] \tag{3.19}$$

where [AL′] is the concentration of added ligand not complexed by M. In general, the added ligand is present in excess of C_M such that [AL′] approximates to [AL]. β'_{MAL} is the conditional stability constant for the formation of MAL in seawater:

$$\beta'_{MAL} = \frac{[MAL]}{([M^{n+}][AL'])} \tag{3.20}$$

It is more appropriate to use $[M_{labile}]$ in place of $[M^{z+}]$ as the former is directly measured in AdCSV. Substitution of $[M^{n+}]$ in Equation 3.11 with Equation 3.14 gives the linear equation (van den Berg/Ruzic plot):

$$\frac{[M_{labile}]}{[ML]} = \frac{[M_{labile}]}{C_L} + \frac{\alpha_{M'} + \alpha_{MAL}}{C_L K'_{ML}} \qquad (3.21)$$

For a 1:1 metal to ligand ratio, a plot of $[M_{labile}]/[ML]$ as a function of $[M_{labile}]$ will be linear. On this basis, the conditional stability constant, K'_{ML}, and the total ligand concentration, C_L can be obtained by linear least squares regression from the slope and intercept, respectively, where C_L = 1/slope and $K'_{ML} = \alpha'/(\text{intercept} \times C_L)$.

The free metal ion concentration can be calculated from the ligand concentration, conditional stability constant and the inorganic α-oefficient.

A curvi-linear response after linearisation indicates the presence of more than one class of natural ligands. In this case, examining the early titration points can be used to distinguish two classes of ligand for which K'_{ML} and C_L can be estimated for each ligand class. However, there are some uncertainties involved in the estimation of C_L and K'_{ML} as discussed in Section 3.2.4.1.

3.2.5. Voltammetric in situ profiling (VIP) system

Analytical systems that can be operated *in situ*, such as the VIP, can provide detailed spatial and temporal data on natural waters as well as the rapid detection of pollutant release so that prompt remedial action can be implemented. However, there are a number of requirements for such systems, including: (1) miniaturised flow-through cells that perform well-controlled speciation measurements, eliminate O_2 interference and minimise memory effects (e.g. prevent accumulation of residual metals between measurements); (2) rugged and reliable microsensors (that provide rigorous signals of speciation); (3) robust, submersible instrumentation; (4) high sensitivity and reliability; and (5) low energy consumption (Buffle and Tercier-Waeber, 2005). The VIP system was developed specifically to address these issues. An advantage of the VIP system is that in well-buffered marine waters, oxygen interference is minimised by the combination of

square wave anodic stripping voltammetry (SWASV) with a fast frequency (200 Hz; to reduce the sensitivity to oxygen reduction, an irreversible process) and by subtraction of the background current from the stripping current (Buffle and Tercier-Waeber, 2000a).

The heart of the VIP system is the gel-integrated microelectrode (GIME), which is an integrated microanalytical system that is used for water analysis and comprises individually addressable Hg-plated-Ir microelectrodes covered by an agarose gel (Buffle and Tercier-Waeber, 2005). The GIME sensor measures current intensities (by a direct or an anodic stripping voltammetric technique) as a function of the diffusion coefficient(s) of the analyte(s) inside the gel after an equilibration period. The main advantages of the gel layer include: (1) exclusion of fouling components (e.g. macromolecules and colloids); (2) stabilisation and protection of the Hg semi-drops, which are deposited through the gel, so that the GIME can be used for up to 3-4 weeks without renewal of the Hg drops; and (3) transport of the analyte(s) is controlled by molecular diffusion (e.g. the current is independent of the convection in solution) and therefore suitable equations can be applied to relate measured currents to concentrations. These characteristics improve the reliability and robustness of the GIME and enable continuous, long-term, *in situ* measurements to be performed in environmental systems.

The unique feature of the GIME is that it achieves speciation by size separation. When the GIME is used with ASV, the metal flux during the preconcentration step corresponds to the so-called dynamic metal complexes (e.g. those that are sufficiently labile and mobile with large dissociation and diffusion rates, respectively). Not included in the measurement are: (1) species that are unable to penetrate the gel either for steric reasons (radius >35 nm) or electric charge (applies to most colloids), (2) species with small diffusion rates (i.e. any complex, MX, with a radius >4 nm), and (3) organic ligands that form non-dissociable complexes on microelectrodes (Buffle and Tercier-Waeber, 2005). Thus, in natural waters, it is the free metal ion, small inorganic complexes (e.g. OH^-, CO_3^{2-}, Cl^-) and a few small organic ligands (e.g. malonate, citrate and fulvic acids) that contribute

to the reduction current during the preconcentration step. Given that i_p in the stripping step is proportional to the metal accumulated during the preconcentration step, it follows that this signal is equivalent to the flux of the dynamic species. Studies that employ the GIME are greatly simplified and the contamination is minimised compared to conventional methodologies involving discrete samples, as separation of colloids by filtration is not required.

The significance of the VIP system, in terms of environmental studies, is highlighted in Figure 3.3, which shows the analogy between size separation at the GIME/solution interface and the processes involved in bio-uptake at the microorganism/solution interface. The hydrophilic (agarose) gel mimics the cell wall that protects the hydrophobic plasma membrane. The cell/gel wall allows diffusion of hydrophilic species (M, ML, L) and very small colloids (X, MX), while most colloids (Y, MY) are excluded by size. Transport of M through the plasma membrane occurs by specific carriers or protein channels *via* adsorption to the transport site R. Enzymes present in the cell wall may also transform ML and MX so that M may adsorb to R. Whilst for the GIME this is analogous to the amalgamation into the Hg film step.

The main functional differences in the measurement cycle when compared with conventional voltammetric systems include: (1) a cleaning step that ensures the Hg layer is uniformly spread onto the Ir substrate of the GIME and any analytes accumulated during previous measurements are removed, and (2) after the first potential scan, a second cleaning step is carried out, followed by a second potential scan that gives the background current. The latter is subtracted from the former to calculate the current signal which is proportional to the analyte in solution.

The VIP system has been successfully applied *in situ* in lake water (Tercier-Waeber *et al.*, 2002, 1998; Tercier-Waeber and Buffle, 2000), river water (Tercier *et al.*, 1995), groundwater (Pauwels *et al.*, 2002), fjord water (Tercier-Waeber and Buffle, 2000a;

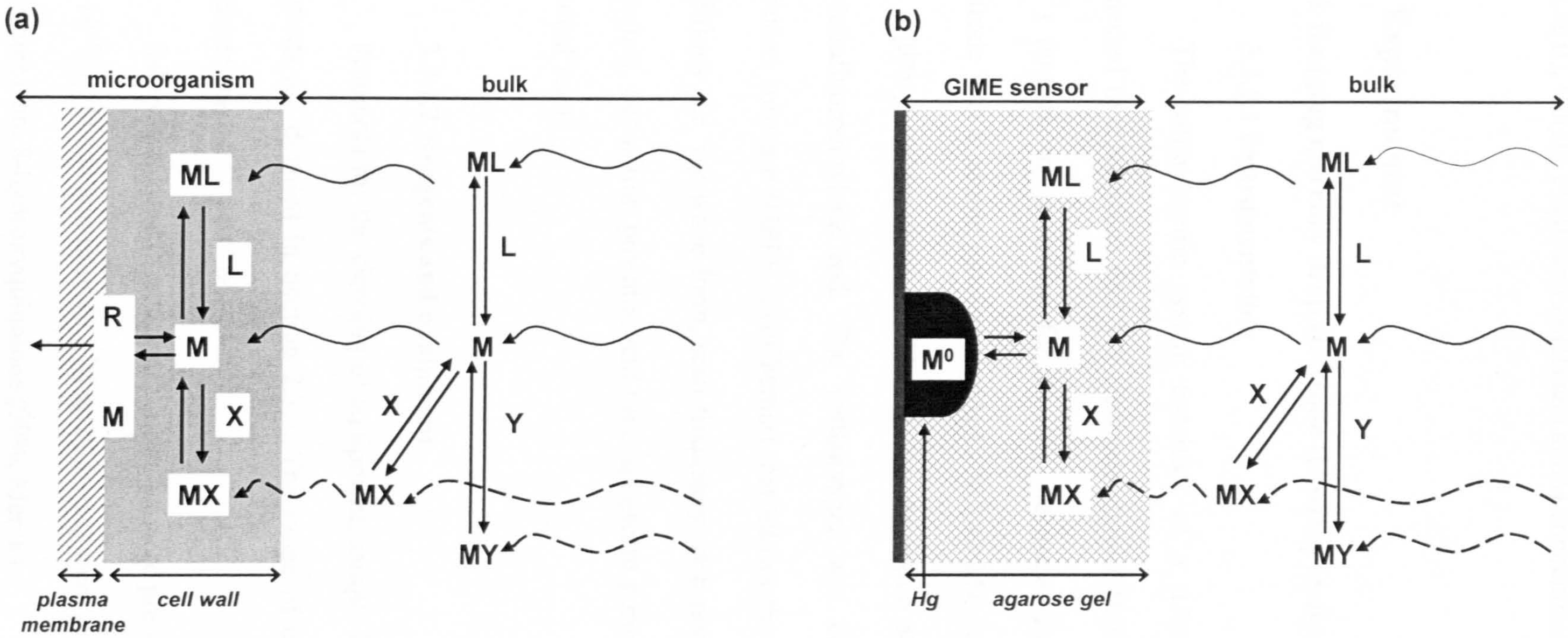


Figure 3.3: Schematic representation of (a) a microorganism/solution interface, (b) a GIME/solution interface (note: thicknesses of the various layers are not to scale). The test solution includes free metal ion (M), small ligands (L), small complexes (ML), colloidal complexants (X,Y) and colloidal complexes (MX, MY). Only one species of each type is shown for simplicity. X and MX are small enough to penetrate the gel, unlike Y and MY (adapted from Buffle and Tercier-Waeber, 2005)

Tercier *et al.*, 1998) and estuarine and coastal marine waters (Tercier-Waeber *et al.*, 2005; Howell *et al.*, 2003; Tercier *et al.*, 1998) to measure the dynamic metal fractions of Cu(II), Cd(II), Pb(II), Zn(II) and Mn(II). Detection limits at pM concentrations have been reported for Cu(II), Cd(II), Pb(II) and Zn(II) and at nM concentrations for Mn(II).

3.3 Experimental

3.3.1 Hanging mercury drop electrode (HMDE) analyses

3.3.1.1 Instrumentation

The voltammetric system consisted of a μAutolab voltammeter (EcoChemie) connected to a Metrohm 663-VA electrode stand (HMDE; drop size approximately 0.52 mm^2) through the interface for the Hg electrode (IME, EcoChemie). The reference electrode was a double junction Ag/AgCl/KCl (3 M) electrode, and the counter electrode was a glassy carbon electrode. During the adsorption step, solutions were stirred with a polytetrafluoroethylene rod. The voltammeter was controlled by a computer using dedicated software (GPES, EcoChemie) that allows the user to control the voltammetric conditions (i.e. the wave form, scan frequency, step potential and amplitude, modulation frequency, deposition potential and time, quiescent period, initial and final potentials in a potential scan).

3.3.1.2 Reagents and equipment

Protocols for the cleaning of equipment, sample handling and preparation of metal standards are detailed in Section 2.3.1. The range of concentrations used for the metal standards was 10^{-6} to 10^{-4} M.

Salicylaldoxime (SA; Sigma) solutions were prepared in MQ water (0.5 M, 0.01 M and 10^{-3} M). Oxine solutions (0.1 M and 0.01 M) were prepared in acidified (Q-HCl, pH 2) MQ water from 8-hydroxyquinolone (99%, Merck).

A stock borate solution (1.0 M) was prepared from ortho boric acid (Aristar, Merck). Organic contaminants were removed from the borate buffer by UV-irradiation (4 h, 400 W medium pressure Hg lamp, Photochemical Reactors). The pH value was adjusted with iso-NH_3 to pH 8.2 in seawater (final concentration 0.01 M).

A HEPES stock solution (1.0 M) was prepared from *N*-hydroxyethylpiperazine-*N'*-*2'*-ethanesulphonic acid (Biochemical grade, Merck) in MQ water. The pH was adjusted with iso-NH_3 to pH 7.8 in seawater (final concentration 0.01 M). To reduce metal contamination, oxine was added (~ 10 μM) to the HEPES stock solution to chelate any metals present. The solution was subsequently passed through two conditioned Sep-Pak® C_{18} columns (pre-packed octadecyl carbon units bonded to a silica gel support) which retained the oxine and oxine-metal chelates. This cleaning procedure resulted in background concentrations of ≤0.20 nM Cu.

A sodium nitrate ($NaNO_3$) solution (1.0 M) (Trace select, Sigma Aldrich, Dorset, UK) was prepared in MQ water to act as supporting electrolyte.

3.3.1.3 Laboratory measurements of trace metal speciation

Discrete samples for laboratory based speciation analysis were collected into LDPE bottles (after rinsing 3x with the sample). Immediately after collection samples were either: (1) vacuum filtered on-site using a polycarbonate filtration unit (Nalgene) fitted with a hand pump (Nalgene), using 0.45 μm acid washed cellulose acetate membranes (Whatman Inc. Clifton, NJ, USA), frozen on dry ice and stored at -20°C. Analysis was carried out after slow thawing at 4°C (18-24 h), or (2) samples were transported to the laboratory for filtration and analysis within 2 h of collection.

Measurements in the laboratory were performed in stirred, deaerated (oxygen-free N_2 (BOC)) samples using SWASV and/or square wave adsorptive cathodic stripping voltammetry (SWAdCSV) under the conditions detailed in Table 3.1. Before sample analysis, MQ blanks (10 mL) were analysed and the samples subsequently corrected if

required.

Table 3.1: Representative conditions used for voltammetric measurements using AdCSV and ASV with a HMDE.

Parameter	AdCSV* Cu	ASV Cu	ASV Cd and Pb
Modulation	SW	SW	SW
Deposition potential (V)	-0.10 to -0.15	-0.6	-0.8
Final potential (V)	-0.55 to -0.6	-0.05	-0.3
Scan frequency (Hz)	50	50	50
Deposition time (s)	10-120	60-120	180-360
Stirrer setting (#)	1 to 6	1 to 6	6
Equilibration time (s)	5 to 8	8	10
Step potential (mV)	2.44	2.44	2.44
Step amplitude (mV)	18	18	18
Hg drop size setting (#)	1 to 3	3	3
Purge time (s)	180 to 240	180 to 240	180 to 240

* using salicylaldoxime as competing ligand at 3 μM and 25 μM concentrations for labile and total Cu concentrations, respectively.

3.3.1.4 Total dissolved trace metal determinations

Prior to total dissolved metal determinations, filtered samples were acidified to pH<2 with Q-HCl for analysis using HMDE or with Q-HNO_3 for analysis with the VIP system. To breakdown organic metal-complexing matter the samples were UV-irradiated (see Section 2.3.2) in batches of 30 mL in quartz glass UV digestion tubes (18 mm i.d.) fitted (loosely) with PTFE caps. To compensate for evaporation during irradiation, each quartz tube was marked and any loss replaced with MQ water. For each sample set, a blank that contained MQ water and Q-HCl or Q-HNO_3 was analysed to establish the level of contamination. The digestion unit comprised a 400 W medium pressure Hg lamp (Photochemical Reactors), positioned in a purpose-built light tight aluminium lamp housing, which was cooled by a fan to maintain a temperature of 70^0C (RS, air flow 40 L min^{-1}) and vented into a fume cupboard. Irradiation of samples was carried out for 6 h in the presence of H_2O_2 (15 mM). Prior to voltammetric analysis the sample pH was adjusted (by the addition of iso-NH_3) to pH 8.2 and pH 4-5 for HMDE and VIP measurements,

respectively.

The accuracy of the analytical procedure was verified using certified reference waters for coastal (CASS-3) and estuarine waters (SLEW-4) by the method described above.

3.3.1.5 Copper ligand titrations

Non-expanded polystyrene portion cups with lids (60 mL, Sweetheart Holdings Inc/DE, USA) were used for the equilibration of samples with added copper. To reduce adsorption to the walls of the containers, the cups were first conditioned by overnight equilibration with seawater samples prepared in the same way as the planned titrations. The labelled cups were then rinsed and filled with MQ water, left for ca. 2 h, rinsed again and dried in a laminar flow unit. Only this last step was repeated for further titrations. The titrations were carried out in sequence of increasing total Cu concentrations in order to minimize possible carry-over between titrations. Possible carry-over was assessed by adding MQ water, with borate buffer (0.01 M) and ligand (25 μM SA), to the portion cups that had previously been used for high Cu additions, and equilibrated overnight before testing. An increase of 0.7 ±0.1 nM (n=3) was recorded, which was deemed a negligible contribution at the high concentrations used (80 to 500 μM Cu) for the titrations.

All equipment used to measure and transfer sample aliquots was pre-conditioned by rinsing three times with the sample seawater. For the Cu titrations, a volume of 300 mL of seawater was transferred into an acid-washed LDPE bottle using a 100 mL polypropylene volumetric flask. To this volume, borate buffer (0.01 M) and salicylaldoxime was added (2.5 to 25 μM SA, depending on the desired detection window). The prepared sample was pipetted into 11 pre-conditioned portion cups (cups 1, 4, 8, 11: 40 mL volume, allowing for three replicate measurements; remaining cups: 20 mL). Copper was added incrementally at concentrations that ranged from 0 to 500 nM, mixed carefully, covered with a lid and left to equilibrate overnight (12-18 h) in a laminar flow unit. The maximum Cu concentration

added was 3 to 5 times the total dissolved concentration determined previously on acidified, UV-irradiated sub-samples (see Section 3.3.1.4). The voltammetric conditions used for individual Cu titrations are detailed in Sections 4.3.4.2 and 5.2.4.2.

After equilibration, aliquots (10 mL) were pipetted into the voltammetric cell in order of increasing concentrations and the reduction current recorded. To verify that the linear range was not exceeded, once the last sample had been analysed a standard addition of the equivalent Cu concentration was added, thereby doubling the Cu concentration.

3.3.2. Voltammetric in situ profiling (VIP) system analyses

3.3.2.1 Instrumentation

The VIP submersible probe, shown schematically in Figure 3.4a, has been described in detail elsewhere (Tercier-Waeber *et al.*, 1999; Tercier *et al.*, 1998). Briefly, the VIP (Idronaut, Milan) system is composed of an upper case (Delrin) that contains electronic hardware, firmware and data storage, and a lower case that houses the preamplifier and peristaltic pump. The hardware and firmware manages the simultaneous control of the fluidic system, the SWASV measurements and data acquisition, storage and transfer. The analytical centre, a fully pressure compensated flow-through voltammetric cell (Plexiglas) (Figure 3.4b), is mounted between these cases and its flow system is connected to the peristaltic pump. The voltammetric cell is based on a three electrode system and consists of an internal cell, which houses the WE (gel-integrated micro array, μ-AMMIA) and a counter electrode (CE, Pt ring), and an outer compartment that contains the RE (Ag/AgCl/KCl saturated gel). An electrolyte gel (1.5% LGL agarose in 1 M $NaNO_3$) fills the compartment between the cells that acts as a double bridge, via two zirconium oxide junctions. Furthermore, it acts as a pressure equaliser and protects the inner electrodes.

The microelectrode array consists of 5 x 20 interconnected iridium (Ir) electrodes (which act independently) of 5 μm diameter with a centre to centre spacing of 150 μm. The

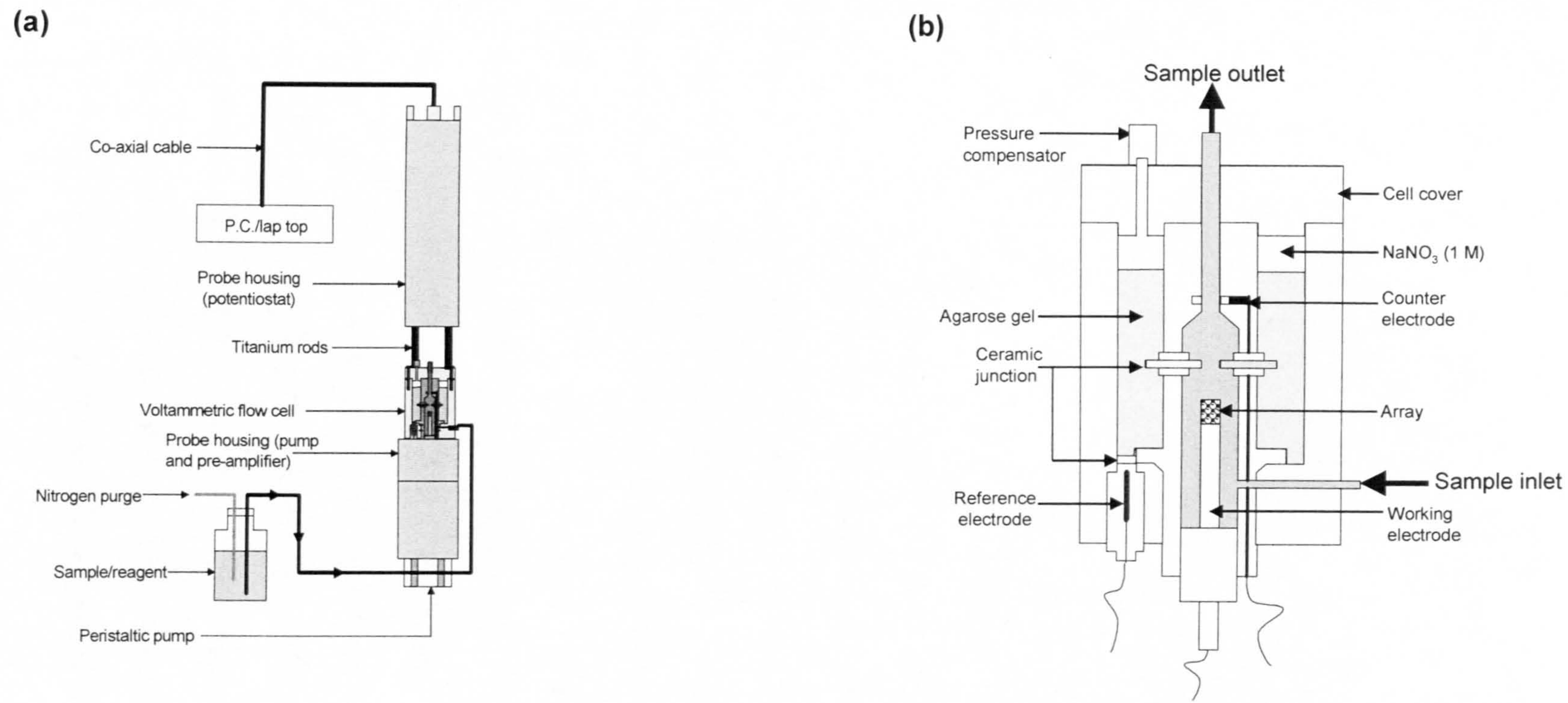


Figure 3.4: Schematic representations of (a) the VIP system and (b) the flow-through voltammetric cell (adapted from Howell *et al*., 2003)

GIME is coated with agarose gel (LGL 1.5% w/w, Biofinex, Switzerland) and contained by a 300 μm thick ring (Epon SU8) which produces a reproducible gel layer of this depth.

3.3.2.2 Reagents and equipment

In addition to the cleaning procedures detailed in Section 2.3.1 an extra cleaning step for equipment (i.e. bottles, volumetric flasks, filtration units etc.) and tubing was required for VIP measurements to reduce organic contamination, a recognised interference for VIP analyses (Idronaut, 1999). This involved immersion in NaOH (pH ~ 10) for at least 24 h between the detergent cleaning step and the first acid bath.

A solution of mercury acetate (5 mM $Hg(CH_3COO)_2$) was prepared in 10^{-2} M perchloric acid ($HClO_4$) and used for the electrochemical deposition of the Hg on the Ir microdiscs in the GIME. For the re-oxidation and removal of this Hg film from the electrodes, a potassium thiocyanate (1 M, KSCN) solution was prepared. Rinsing fluids for the fluidic systems were MQ water and a Q-HNO_3 solution (10^{-2} M in MQ). Stock solutions of $NaNO_3$ (1.0 M and 0.1 M) (Trace select, Sigma Aldrich, Dorset, UK) were prepared for baseline voltammograms and calibration purposes. Individual metal standard solutions of Cu(II), Pb(II) and Cd(II) (10^{-3} M Cu and 10^{-4} M Pb and Cd) (SpectrosoL) were prepared in 0.1 M Q-HNO_3, as well as 10^{-5} M Cu and a mixed Pb and Cd standard (10^{-6} M) prepared in 0.01 M Q-HNO_3. The metal standards were added to $NaNO_3$ (0.1 M) at concentrations that ranged from 5 to 20 nM Cu(II) and 0.75 to 2.5 nM Cd(II)/Pb(II) for calibrations. The metal concentrations in this electrolyte were below the limit of detection of the VIP (LOD: 47 pM Cd, 32 pM Pb and 680 pM Cu, refer to Section 3.4.1.2).

3.3.2.3 Preparation and covering of the gel-integrated microelectrode (μ-AMMIA)

Prior to covering the surface of the array with the agarose layer, the microarray surface was inspected under a microscope (Swift & Son, London) for the presence of impurities. Any dust particles were removed using a fine jet of MQ water, followed by

drying. The preparation of 1.5% (w/w) LGL agarose gel involved heating MQ water to ca. 70°C in a test tube within a water bath. The heated water (2 mL) was added to 30 mg agarose in a further test tube. The mixture was agitated under continued heating (to ca. 98°C) until the gel was fully dissolved. The heat source was removed and the test tube left in the water bath until all air bubbles in the gel dissipated and the temperature had decreased to ca. 70°C. The tip of the array (~1 cm) was briefly placed in the agarose gel solution and checked for air bubbles within the containment ring. It was then removed and gel wiped away from outside the containment ring. The array was allowed to cool for 60 s, and conditioned by immersing in MQ water for 30 min and then transferred to a solution of $NaNO_3$ (0.1 M) for 3-5 h.

3.3.2.4 Electrochemical deposition and re-oxidation of Hg

Hg hemispheres were plated onto the Ir substrate (i.e. microdiscs) by electrochemical reduction of Hg^{2+} using deaerated mercury acetate (5 mM) at a potential of -400 mV for 6-7 min. Following Hg deposition, three short (5 min deposition) measurement cycles were carried out in 0.1 M $NaNO_3$ immediately in order to spread the Hg layer evenly over the Ir microarray surface (see Table 3.2 for conditions). To remove the Hg layer, the Hg electrode surface was re-oxidised with potassium thiocyanate (1.0 M) using a linear potential scan from -300 to +300 mV (Idronaut, 1999). During electroplating, recorded voltammetric signals were proportional to the amount of Hg electroplated (i.e. the combined radii of the individual microdiscs/electrodes). The reproducibility of the Hg layer radius can be obtained from the charge consumed during the reduction, Q_{red}, (by integration of the deposition curve, Figure 3.5a, typical range of Hg radius: 5.2-5.8 μm), and is used to assess the deterioration of the electrode's quality (life-span ca. 4-5 weeks). In the same way, the efficiency of the Hg removal is quantified by integration of the re-oxidation curve (Figure 3.5b).

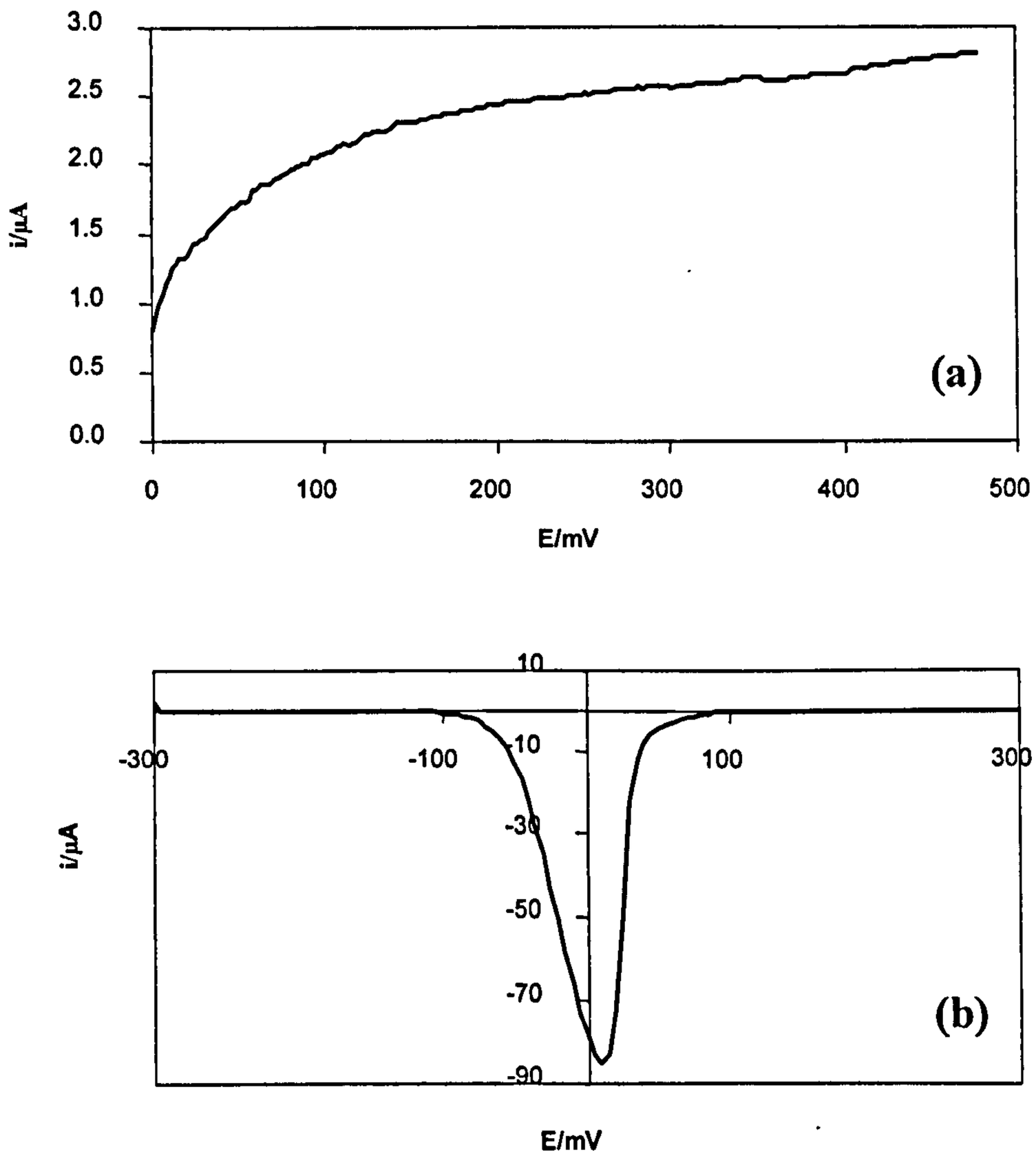


Figure 3.5: Typical mercury deposition curve obtained in deoxygenated 5 mM $Hg(COOH)_2$ in 10^{-2} M $HClO_4$, deposition potential -400 mV, deposition time 360 s (a); and re-oxidation curve in deoxygenated 1 M KSCN linearly scanned from -300 mV to +300 mV (b).

3.3.2.5 Trace element determinations with the VIP

Systematic studies of the influence of deposition potential, ionic strength, pH, temperature, pulse and step amplitudes, dissolved oxygen, pumped volume and calibration solutions ($NaNO_3$ electrolyte and seawater) on the analytical performance of the VIP system have been carried out previously (Howell *et al.*, 2003; Tercier-Waeber *et al.*, 1999). The parameters and conditions used in this study were based on the published literature.

Laboratory measurements were performed in deoxygenated (humidified N_2 gas, BOC) solutions which were either in electrolyte (0.1 M $NaNO_3$) adjusted to pH 3-6,

depending on application, or in saline water samples after filtration (0.4 μm polycarbonate membrane filter, Cyclopore, 47 mm diameter or 0.45 μm cellulose acetate membrane filters, Whatman, 47 mm). At the beginning of each measurement cycle, carry-over between samples was minimised by flushing the fluidic system with ca. seven times its own volume of sample (10 mL, or 3 min with the peristaltic tubing used) prior to equilibration of the sample with the micro-array gel membrane for 6.5 min (Figure 3.6). A second pumping period was followed by the pre-cleaning step aimed at stripping analyte metals from the electrode. The duration of the subsequent deposition step (5-30 min) depended on the analyte concentration in solution and served to pre-concentrate analytes into the Hg film. After a 10 s equilibration/rest period a SWASV scan was carried out and the stripping current measured and recorded. After a second pre-cleaning step, the background current was recorded from a second SWASV scan. The peak heights of analytes were identified at specific potentials (e.g. for $NaNO_3$ solution: ~ -500 mV for Cd, -350 mV for Pb and +50 mV for Cu) (Figure 3.7).

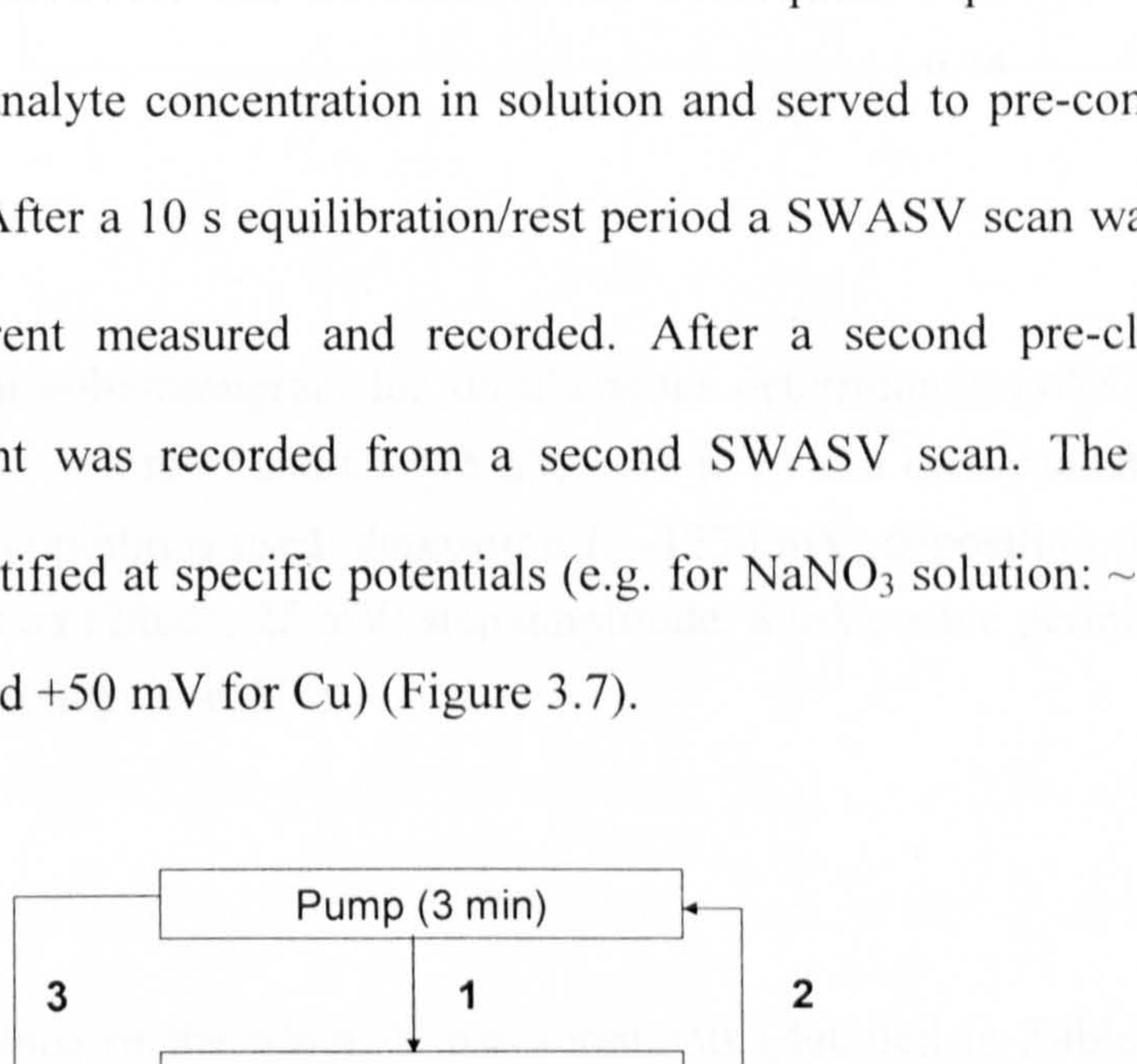


Figure 3.6: Series of steps involved in VIP measurements (adapted from Howell *et al.*, 2003b). Numbers represent the sequence of steps. The dashed lines correspond to background analysis.

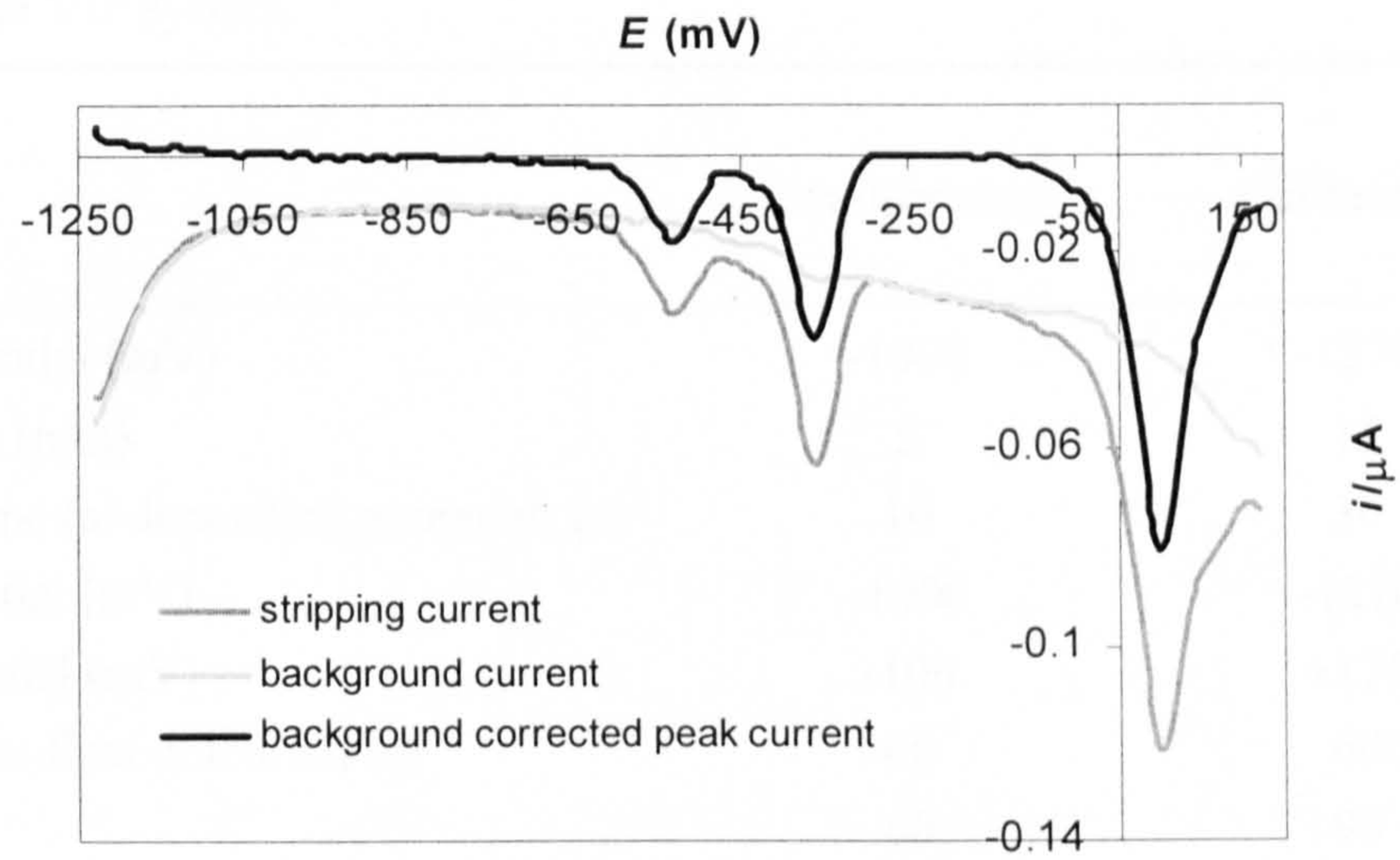


Figure 3.7: Typical voltammogram for simultaneous determination of Cd (1.75 nM, -526 mV), Pb (1.75 nM, -358 mV) and Cu (15 nM, +58 mV) in a deoxygenated 0.1 M $NaNO_3$ solution. SWASV conditions used: deposition *E*, -1230 mV; deposition time, 15 min; final *E*, +170 mV; pulse amplitude, 25 mV; step amplitude, 8 mV; wave period, 20 ms; cleaning *E*, -80 mV; and cleaning time, 60 s.

The conditions of the SWASV measurements (detailed in Table 3.2) allowed the simultaneous determination of Cu(II), Pb(II) and Cd(II). All potentials given are *versus* the Ag/AgCl/3 M KCl/1 M $NaNO_3$ reference electrode. Calibrations were performed by standard additions of each metal in 0.1 M $NaNO_3$ (pH 3-6). Both deposition time and analyte concentrations were recorded in order to calculate the sensitivity (nA nM^{-1} min^{-1}) of the instrument (Equation 3.22).

$$Sensitivity\ (nA\ nM^{-1}\ \min^{-1}) = \frac{response\ (nA)}{Concentration\ (nM) \times deposition\ time\ (\min)} \qquad (3.22)$$

Table 3.2: Conditions for the simultaneous determination of Cd(II), Pb(II) and Cu(II) using SWASV with the VIP system.

Step	Equilibration	Calibration
Deposition potential (mV)	-1000	-1270
Deposition time (min)	5	15
Equilibration time (at deposition potential) (s)	10	30
Scan start potential (mV)	-1000	-1270
Scan final potential (mV)	-100	+175
Cleaning time (at final potential) (s)	60	60
Pump time (s)	60	90
Pause (mV; s)	-80 ; 0	-80 ; 0
Pulse amplitude (mV)	25	25
Pulse step (mV)	8	8
Frequency (Hz)	200	200

Typically, a deposition time of 15 min was used for calibration purposes. Good linearity ($R^2 > 0.98$ for all) between peak current and deposition time was observed. After calibration, the deposition time can be extended or reduced within the linear dynamic range (typically 5-40 min).

Diffusion of trace metals through the agarose gel is temperature dependent. Typically water column temperatures vary between 4 and 25°C in mid-latitudinal regions (Howell *et al.*, 2003). As a result, temperature changes will influence the peak current response. It has been shown that a quantitative relationship exits between peak current response, *i*, and temperature that follows the Arrhenius equation (Idronaut, 1999):

$$i = i_0 \exp\left[\frac{-\Delta G^*}{RT}\right] \tag{3.23}$$

where ΔG^* is the activation Gibbs energy, R is the gas constant and T is

temperature (K). This behaviour allows temperature corrections to be made to data obtained from *in-situ* deployments. The parameters for the temperature corrections were determined empirically by Howell *et al.* (2003a).

Sample preparation for total dissolved metal determinations was as detailed in Section 3.3.1.4.

3.3.3 Sampling protocol for comparison of speciation studies with HMDE and GIME

The VIP system was deployed *in situ* over a tidal cycle at Percuil Creek and Restronguet Point in the Fal estuary, SW England (see Section 4.3.1 for map, sampling locations and description), and measurements of dynamic Cd, Pb and Cu were carried out with a frequency of between 30 and 45 minutes. In parallel, discrete samples were collected at regular intervals (1 – 1.5 h) for laboratory determination of dynamic/labile and total metals with the VIP system (GIME) and the voltammetric system with an HMDE. Details of this sampling campaign are described in Chapter 4. In brief, *in situ* deployment of the VIP was carried out for 9½ h at PC and 10 h at RP on 3rd August 2005 and 4th August 2005, respectively. These monitoring periods were chosen to cover low and high water conditions at each sampling site. The probe was deployed from an anchored vessel, with the sample inlet at about 2 m depth. Discrete samples were collected in close proximity to the sample inlet for the VIP, with the aid of a purpose-built sampler (Balls and Laslett, 1991) loaded with acid-washed LDPE bottles and equipped with an operator-controlled release mechanism. The samples were immediately vacuum filtered (0.45 μm) into LDPE containers to aid their preservation, as unfiltered samples change rapidly due to coagulation, sedimentation, and microbial activity (Pei *et al.*, 2000; Chen and Buffle, 1996). The filtered samples were placed in the dark on dry ice prior to transport to the laboratory.

The conditions used for the analysis of samples from the Fal estuary using the VIP system are detailed in Table 3.3. All other conditions used for the SWASV measurements are as specified in Table 3.2 for the VIP system and Table 3.1 for the HMDE.

Table 3.3: Conditions used for the Fal sample analysis for Percuil Creek* and Restronguet Point#

Sample	Size fraction (μm)	Sample treatment	pH of analysis	Initial potential (mV)	Final potential (mV)	Deposition time (s)
Dynamic (laboratory)	< 0.45	Frozen	7.5-8.3* 8.2-8.6#	-1280*#	-80*#	900-1200* 300-600#
Dynamic (*in situ*)	< 0.004	None	8.21-8.26* 8.85-8.91#	-1120* -1150#	-80* -80#	1200-1500* 600-900#

3. 4 Results and Discussion

3.4.1 Analytical figures of merit

This section compares the analytical performance of a Metrohm 663-VA voltammeter and a voltammetric *in situ* profiling (VIP) system using stripping voltammetric techniques for speciation studies of Cu, Cd and Pb.

3.4.1.1 HMDE

The limit of detection (LOD) was determined as blank + 3σ from repeated analysis of the Cu concentration in MQ water and coastal seawater that had been acidified and UV-irradiated (refer to Sections 3.3.1.3 and 3.3.1.4 for details). For MQ water, the LOD was 0.16 ± 0.06 nM Cu (n=4, deposition time 30s), 0.11 ± 0.05 nM Cd and 0.10 ± 0.03 nM Pb (n=3, deposition times 300s). In seawater the LOD was 0.20 ± 0.04 nM Cu (n=4, deposition time 30s), 0.13 ± 0.03 nM Cd and 0.10 ± 0.04 nM Pb (n=3, deposition times 300s). Typically an RSD <5%, n ≥3, was achieved between replicates. The accuracy of the analytical methods was verified by the analysis of certified reference materials (CRMs) for total dissolved metal concentrations. Estuarine water (SLEW-2) and coastal water (CASS-4) CRMs were UV-irradiated and neutralised with iso-NH_3 before analysis. Good agreement was obtained for both CRMs using ASV for Cd and Pb and AdCSV for Cu

(Table 3.4).

Table 3.4: Results from the analysis of certified reference materials CASS-4 and SLEW-2 using the HMDE. Confidence intervals refer to ± 2σ of the sample mean. The P value (two tail) for a t-test carried out on the sample means indicates no significant difference between the experimental and certified values at P=0.05.

Element	Experimental value (nM)	Certified value (nM)	n	P value
CASS-4				
Cu	9.40 ± 1.17	9.32 ± 0.86	5	0.985
Cd	0.21 ± 0.09	0.23 ± 0.03	3	0.422
Pb	<LOD	0.05 ± 0.02	3	
SLEW-2				
Cu	24.9 ± 2.56	25.5 ± 1.7	5	0.231
Cd	0.19 ± 0.12	0.17 ± 0.02	3	0.482
Pb	0.16 ± 0.08	0.13 ± 0.02	3	0.067

3.4.1.2 GIME

The limit of detection (LOD) for Cd, Pb and Cu in 0.1 M $NaNO_3$ (pH ~2, spiked with 0.5 nM Cd and Pb, 5.0 nM Cu) and in untreated coastal seawater are given in Table 3.5. These figures compare well with those previously reported (Howell *et al.*, 2003a; Idronaut, 1999). Good agreement with the CRMs was obtained for Cu (Table 3.6). A t-test was carried out on the sample means and showed no significant difference (P>0.05).

Table 3.5: Limit of detection (LOD) for analysis (n=3) in deoxygenated, pH ~2, 0.1 M $NaNO_3$ and in seawater (pH 7.9), using 15 and 20 min deposition times, respectively. LOD is defined as blank + 3σ.

Matrix	Metal ion	LOD (pM)	RSD (%)
$NaNO_3$	Cd(II)	47	4.4
$NaNO_3$	Pb(II)	32	5.8
$NaNO_3$	Cu(II)	680	6.7
Seawater	Cd(II)	38	4.8
Seawater	Pb(II)	43	3.8
Seawater	Cu(II)	673	5.9

Table 3.6: Results from the analysis of certified reference materials CASS-4 and SLEW-2 for copper using the GIME. Confidence intervals refer to ± 2σ of the sample mean. The P value (two tail) for a t-test carried out on the sample means indicates no significant difference between the experimental and certified values at P=0.05.

Element	Experimental value (nM)	Certified value (nM)	n	P value
CASS-4	8.72 ± 0.17	9.32 ± 0.86	1	0.363
SLEW-2	22.8 ± 2.14	25.5 ± 1.7	1	0.145

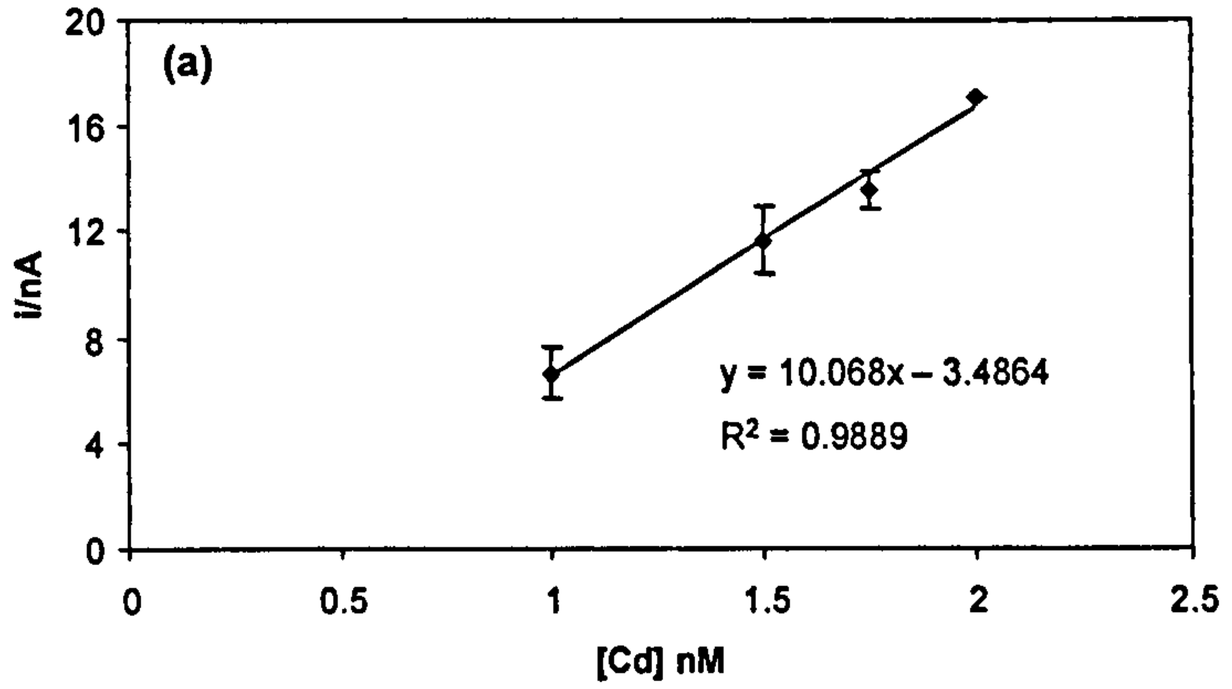
The concentrations of dynamic metal species measured *in situ* with the VIP were calculated using the sensitivity determined from the calibration slope for each metal. Typical calibration graphs for Cd, Pb and Cu are shown in Figure 3.8a-c.

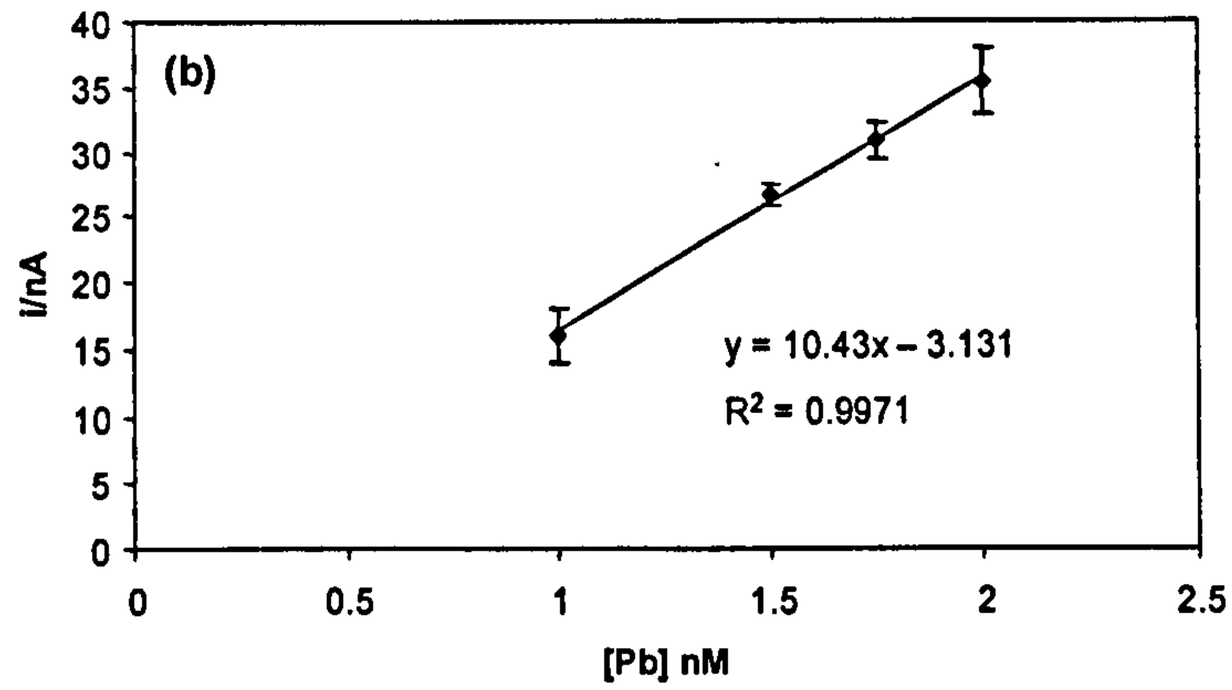
3.4.2 Comparison of voltammetric measurements

In this section, the results from *in situ* deployment of the VIP system in the Fal estuary (Percuil Creek and Restronguet point) are compared with metal concentrations measured in the laboratory with the HMDE in filtered discrete samples taken concurrently with the *in situ* deployment. The significance of the results in terms of environmental processes is discussed in more detail in Chapter 4.

3.4.2.1 VIP measurements

The results from the VIP system for Restronguet Point, both from *in situ* deployment and laboratory analysis, are shown in Figure 3.9a-c for Cd(II), Pb(II) and Cu(II), respectively. The results indicate that the VIP system can measure picomolar concentrations of Cd and Pb and nanomolar concentrations of Cu in estuarine waters (S = 30-34). The higher resolution of the *in situ* results shows the variability in the dynamic fraction over relatively short time periods, indicating that metal concentrations change





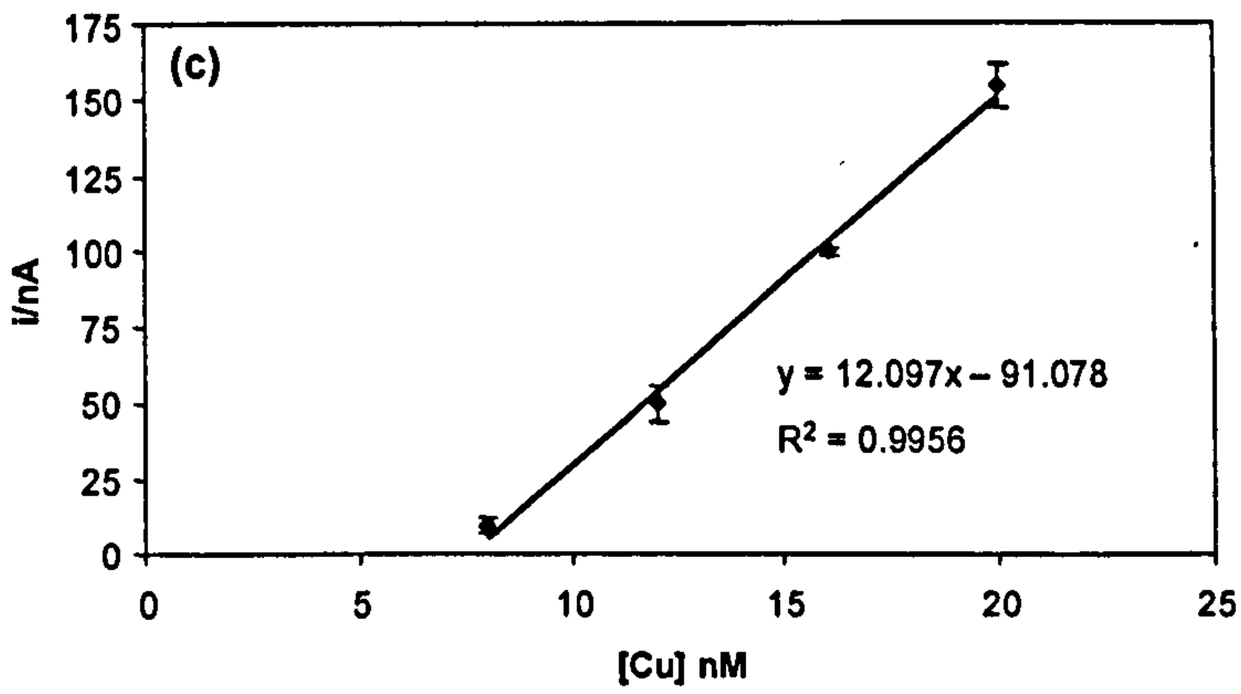


Figure 3.8: Typical calibration graphs for Cd **(a)**, Pb **(b)** and Cu **(c)** in deoxygenated 0.1 M $NaNO_3$. Error bars represent 2σ, $n \geq 3$. SWASV conditions used: deposition *E*, -1250 mV; deposition time, 15 min; final *E*, +150 mV, pulse amplitude, 25 mV; step amplitude, 8 mV; wave period, 20 ms; cleaning *E*, -80 mV; and cleaning time, 60 s. Concentration ranges: 1-2 nM Cd and Pb, 8-20 nM Cu.

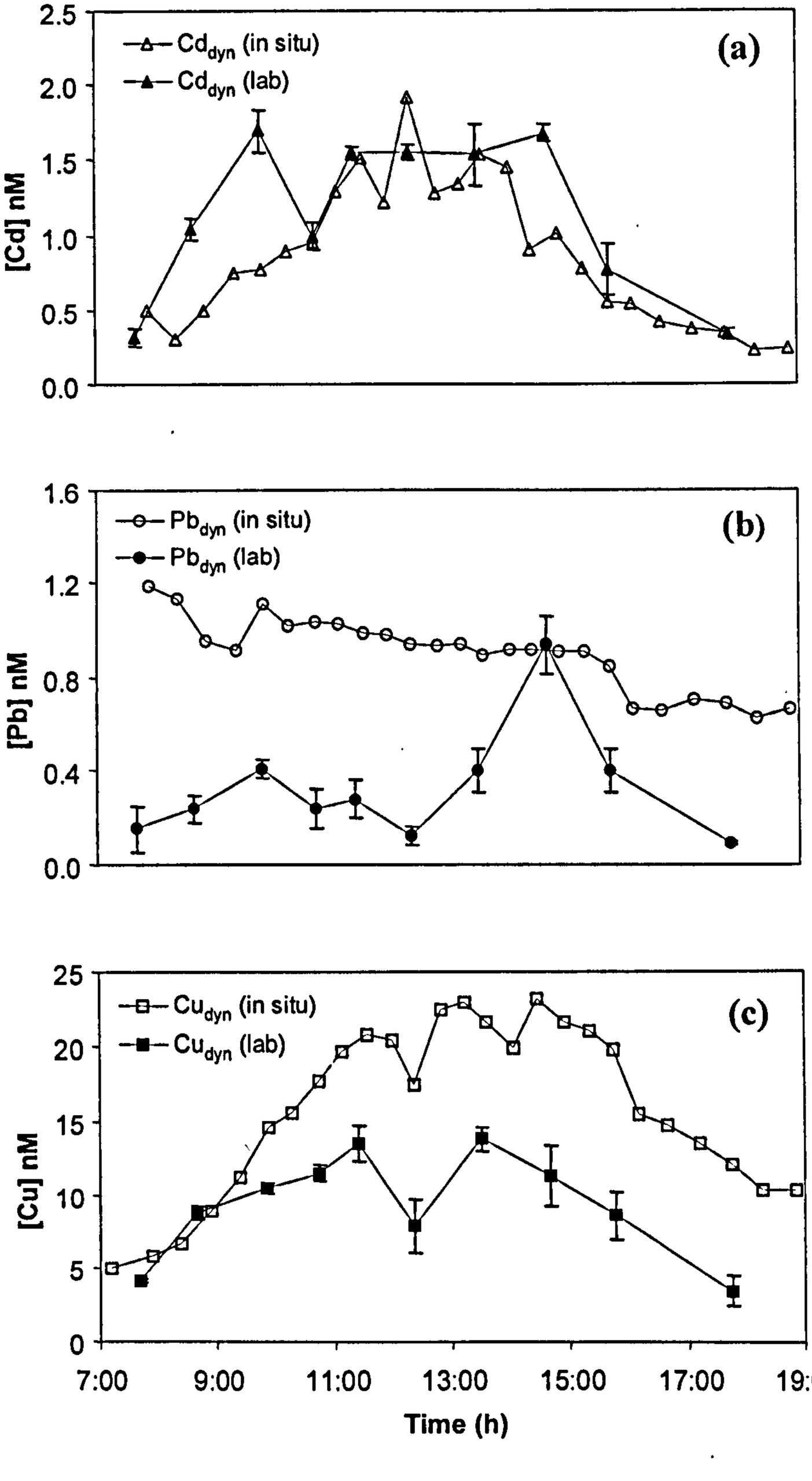


Figure 3.9: VIP time series measurements of Cd(II) **(a)**, Pb(II) **(b)** and Cu(II) **(c)** at Restronguet Point over a 10 h period on 04/08/05 for *in situ* (open symbols) and dynamic concentrations (<0.45 μm) (filled symbols) determined in the laboratory on discrete samples. Error bars for the laboratory determinations represent 2σ. Low water was at 12:22 h, 1.4 m and high water was at 17:49 h, 5.0 m.

rapidly in time and space in this system. It is evident that important spatial and temporal changes can go unnoticed with the discrete sampling method due to the limited temporal resolution from samples that can be achieved when manually collecting and filtering on-board.

Comparison of VIP dynamic measurements: In situ and laboratory determination

When the dynamic fractions measured *in situ* were compared with the laboratory measurements with the VIP (Figure 3.9) Cd showed the best agreement between the two datasets, while in general, the dynamic concentrations of Pb and Cu determined in discrete samples were lower than those measured *in situ*. The differences may lie in the different sampling methodologies and/or artefacts introduced by the methodology.

Discrete samples were collected as close to the sample inlet of the VIP system as possible, but slightly different water masses will have been collected and analysed by discrete and *in situ* methods, and this may account for some of the difference (e.g. through stratification in the water column). The concentrations measured in discrete samples were on average 72% and 38% lower than the values recorded *in situ* for Pb and Cu, respectively.

Among the three metals studied, Cd is least affected by organic complexation and sorption processes (e.g. to particles and the walls of containers) in saline conditions. Also, because of its high solubility it is likely to be least dependent on mobilisation from the sediment and influences related to stratification in the water column that may affect organic matter distribution (Comans and van Dijk, 1998; Millward, 1995). In contrast, Pb is a particle reactive element and therefore likely to be affected by surface sorption processes, and more strongly influenced by stratification in the system from mobilised sediments (Elbaz-Poulichet *et al.*, 1984). Transfer from the dissolved to the colloidal form and precipitation are also likely to influence the speciation of Pb and therefore the filtration process (<0.45 μm) may have affected the laboratory measurements (Howell *et al.*, 2006), which is discussed in more detail below. The difference between metal concentrations

measured *in situ* and in the laboratory appears to be most systematic for Cu, with an average 44 ± 0.32 % between the sets of data (see slope of Figure 3.10), and at P<0.02 the two methods of analysis were shown to be significantly correlated. Cu is most affected by organic complexation in estuarine waters (Buck and Bruland, 2005; Rivera-Duarte *et al.*, 2005; Wells *et al.*, 1998), and the main cause of the difference may be a result of complexation and/or the adsorption of complexes to the wall of the container.

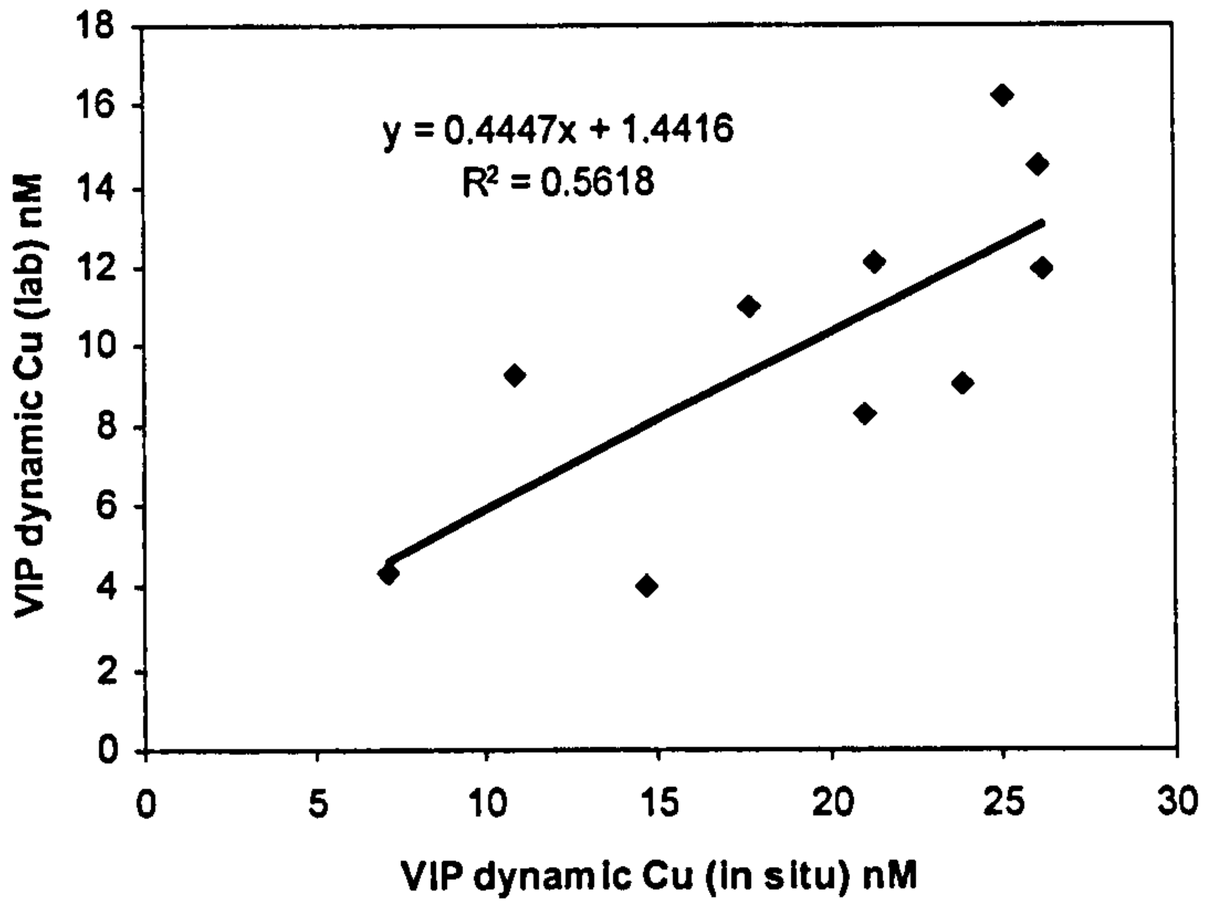


Figure 3.10: Regression analysis for *in situ* (<4 nm) and laboratory determinations (<0.45 μm) of Cu performed with the VIP system on samples collected at Restronguet Point over a 10 h period on 04/08/05.

Notwithstanding this, the differences in laboratory and *in situ* measurements could also be a result of (1) metal loss resulting from the filtration process (e.g. filter clogging, Howell *et al.*, 2006), (2) metal speciation changes, (3) loss due to sample storage and freezing/thawing processes, and/or (4) loss of sensitivity with the GIME. These points are discussed below in sequence.

***Effects of sample filtration*:** Studies on the effects of filtration on sample integrity (Howell *et al.*, 2006, 2003; Morrison and Benoit, 2001; Horowitz *et al.*, 1996; Hall *et al.*, 1996; Laxen and Chandler, 1982) have shown that concentrations of trace metals in the

filtrate decrease as filtration proceeds (e.g. with increasing filter volume). A rapid reduction in the nominal pore size of the filter membrane, caused by retention of colloids and fine particles (e.g. filter clogging), was cited as a possible cause. Furthermore, as particulate matter accumulates on the filter surface, contact with binding sites may increase metal retention. In this study, the filtration rate decreased with increasing filtrate volume, indicating that particulate matter had accumulated on the filter membrane, probably causing increased retention of colloidal material and associated metals. A limited number of acid-leached filter membranes were available so the membranes could only be changed after the filtration process had slowed appreciably. Copper, and in particular Pb, are particle reactive elements (Elbaz-Poulichet *et al.*, 1984) and therefore loss of these metals may have occurred through the filtration process.

Effects of sample storage and freezing/thawing processes: The effect of sample storage by freezing on trace metal speciation using laboratory-based electrochemical methods has been investigated. Sander *et al.* (2003) and Capodaglio *et al.* (1995) found no major effects for lake waters and open ocean, respectively. However, loss (as much as 20-30%) of analyte through freezing in Cu speciation measurements with high total filterable Cu was reported by Braungardt (2000) and Nelson (1985) for more complex estuarine waters.

Although the following discussion refers to measurements carried out with an instrument fitted with an HMDE, it is included here to illustrate the effects of the freezing/thawing processes, which should in principle be also valid for analysis using the VIP system. It should be noted that the repeatability and indeed reproducibility of these results was not tested due to time constraints and therefore these results may not be truly representative. Notwithstanding this, Table 3.7 details the results of Cu-ligand titrations carried out using AdCSV at three competition strengths on freshly filtered (<0.45 μm pore size) water collected in the Tamar estuary and on samples that were frozen (-4°C) and

subsequently thawed. At the lowest competition strength, 2.5 μM SA, duplicate analysis on the thawed sample was also performed which shows good repeatability. The results show that Cu_{labile} increased by factors of 1.2 to 1.5 over the three competition strengths with larger differences evident at 25 μM SA. In the duplicated titrations, the concentration of Cu^{2+} increased in the thawed samples by a factor of 1.7 compared with the fresh sample. Since Cu^{2+} is considered to be the most environmentally relevant Cu species (see Section 2.2.3. and references therein) and is known to produce biological effects at pM concentrations (Section 2.4.3), these results suggest that data generated from samples that have been frozen should be interpreted with caution as they may lead to an over-estimation of the most toxic metal species. The difference in the calculated concentration of natural ligand, C_L, and conditional stability constant, $\log K'_{CuL}$, between the fresh and thawed samples was small (at all competition strengths) and was within the analytical error of the method, in which the uncertainties can be large, as discussed previously in Section 3.2.4.1.

Table 3.7: Comparison of AdCSV competitive copper ligand titrations carried out at three competition strengths on fresh (F) and thawed (T) (where subscripts refer to duplicate analysis) filtered samples (<0.45 μm pore size) that were stored frozen for several months and collected in the Tamar Estuary. pH = 7.62; S = 10; Cu_T = 43.8 nM; $\log K'_{CuL}$= conditional stability constant for copper-natural ligand complex; C_L = concentration of natural ligand, n=1.

Method	SA (μM)	C_L (nM)	$\log K'_{CuL}$	Cu_{labile} (nM)	Cu^{2+} (pM)
F	2.5	52.5 ± 0.91	12.2 ± 0.03	6.28 ± 0.21	0.53
T_1		50.2 ± 2.49	12.2 ± 0.11	7.74 ± 0.84	0.89
T_2		51.1 ± 0.95	12.0 ± 0.03	8.38 ± 0.37	0.92
F	10	49.4 ± 0.87	13.4 ± 0.04	17.6 ± 1.08	
T		51.1 ± 1.47	13.1 ± 0.03	22.3 ± 1.21	
F	25	26.7 ± 1.29	14.6 ± 0.23	11.3 ± 1.03	
T		26.0 ± 0.89	14.7 ± 0.18	17.3 ± 0.63	

***Loss of sensitivity*:** The concentrations of individual metals within a sample are determined from their respective sensitivities determined before and after *in situ* deployment or analysis of a set of samples in the laboratory. The sensitivity is established from the slope of the calibration curve as a function of the deposition time (in pA nM^{-1} min^{-1}). Table 3.8 lists the sensitivities for Cd, Pb and Cu obtained from calibrations before and after sample analysis, and shows some variability from the average sensitivity reported by the VIP manufacturer for various analytical media (Idronaut, 2003). Good agreement between pre- and post calibration was obtained for the *in situ* deployment which gives a good indication of the stability of the electrode surface. For the dynamic laboratory measurements, loss of the Hg electrode surface precluded post-analysis calibration. This occurred during calibration and was caused by air trapped within the system, resulting in Hg oxidation. Changes in sensitivity during laboratory analysis may also occur by scanning at too positive or negative a potential causing degradation of the Hg film through Hg oxidation and/or reduction of H^+, respectively.

Table 3.8: Sensitivities for the VIP system analyses of Cd(II), Pb(II) and Cu(II) in pA/nM min^{-1} calculated before and after *in situ* and laboratory determinations. *Specified reported sensitivities obtained from VIP operators manual (Idronaut, 2003).

	Cd		Pb		Cu	
	before	after	before	after	before	after
Dynamic (*in situ*)	771	717	1207	1287	900	893
Dynamic (laboratory)	619	-	1231	-	913	-
Specified reported sensitivity*	680 ± 50 (n=5)		1070 ± 25 (n=8)		820 ± 25 (n=4)	

Most analytical techniques available for trace metal speciation measurements require the collection and preservation of samples prior to analysis, even though trace metal speciation changes are likely to occur when samples are taken out of their natural context (Buffle and Tercier-Waeber, 2000). This study shows that the integrity of samples was affected by filtration processes, sample storage and treatments. Above all, this demonstrates that reliable, robust *in situ* monitoring systems for speciation analysis are required.

3.4.2.2. Comparison of GIME with HMDE

This section compares laboratory measurements of the dynamic Cu fraction, in filtered (<0.45 μm pore size), frozen (-4^{0}C) and thawed samples, analysed by ASV with the VIP and GIME (gel-integrated microelectrode), with measurements of the labile Cu fraction analysed by AdCSV using a conventional voltammeter (Metrohm 663-VA Stand) and HMDE (hanging mercury drop electrode).

Results for Cu measured in discrete samples obtained during a tidal cycle time series in Percuil Creek and Restronguet Point in the Fal estuary (see Figure 4.1 in Chapter 4 for locations) are shown in Figures 3.11a and b, respectively. The labile fraction measured by HMDE was generally higher than the dynamic fraction measured with the GIME (VIP system). This result is consistent with the difference in analytical characteristics of the two methods. The dynamic fraction (GIME) includes metal species <4 nm in size, such as inorganic species and metals associated with small organic molecules. The labile fraction (HMDE) is defined by the size cut-off of the filter membrane (here 0.45 μm) and by the analytical conditions of the ASV or AdCSV method applied, which allows the free hydrated metal ion and varying fractions of inorganic complexes and weakly complexed organic species to be detected. A large number of different ligands with binding sites that form complexes of differing stabilities are present in seawater. Consequently it is the detection window of the technique used that determines

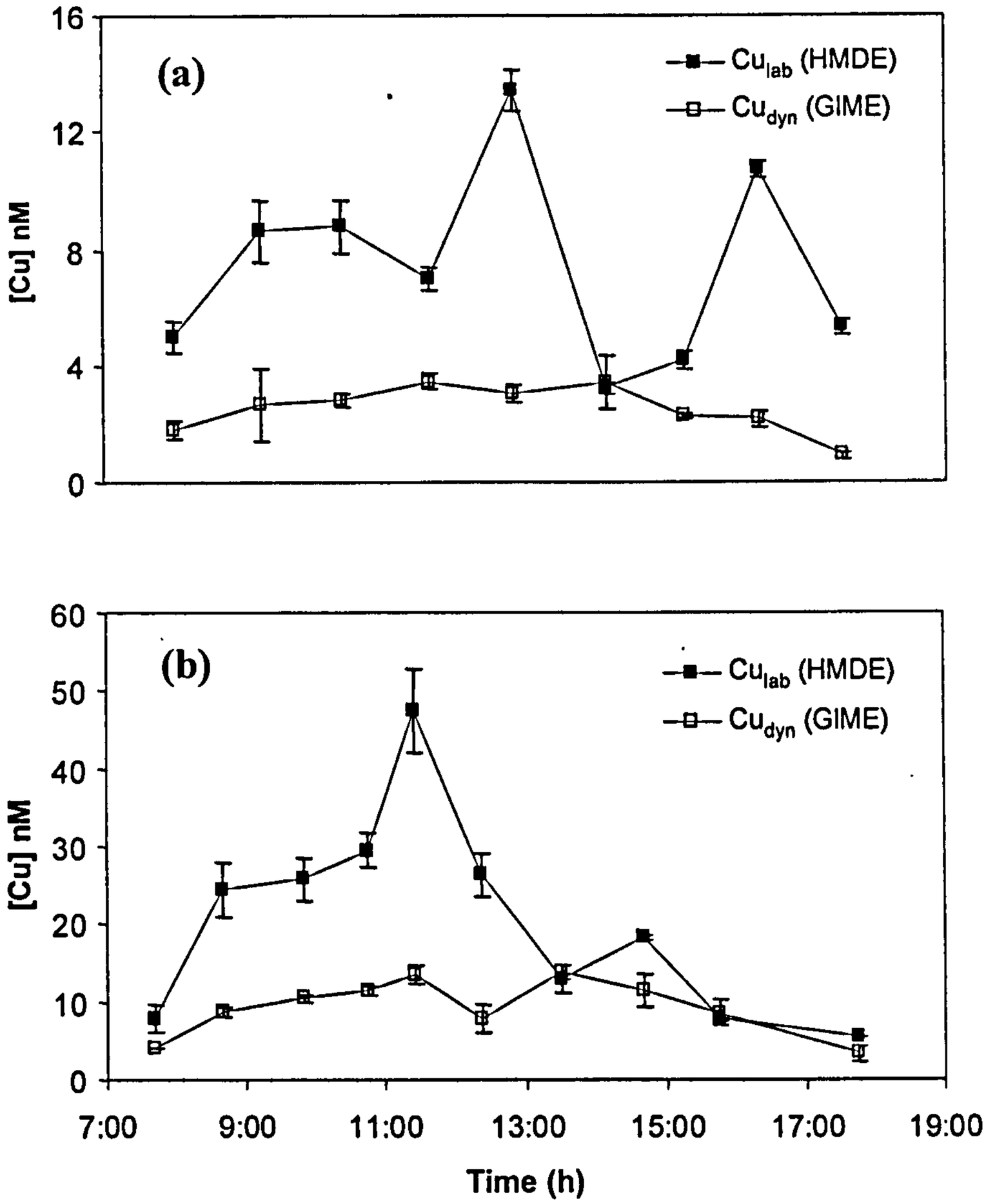


Figure 3.11: Laboratory time series measurements for dynamic (< 4 nm) and labile (< 0.45 μm) Cu using the GIME (ASV) and HMDE (AdCSV) for Percuil Creek (**a**) and Restronguet Point (**b**).

the ligands or binding sites detected. For the HMDE, the centre of the detection window (DW) approximates to the side reaction coefficient ($\alpha_{M'}$) for ASV, and lies in the range of $\log\alpha_{Cu'} = 1.1 - 1.3$ (Bruland *et al.*, 2000; Kozelka and Bruland, 1998; Coale and Bruland, 1988; Byrne *et al.*, 1988). For AdCSV the DW is defined by the α-coefficient of the metal-added ligand complex (α_{MAL}). In this study, the analytical competition strength using

salicylaldoxime ([SA] = 3 μM, pH=8.2-8.5, S=33.6-35.2) was approximately $\log\alpha_{CuSA}$ = 3.9, almost three orders of magnitude above that of the ASV method. Consequently, the AdCSV method introduces a higher analytical competition strength into the sample, therefore competing more effectively with the more strongly complexed natural ligands as well as detecting weaker ligand classes, and this accounts for the higher metal concentration determined, compared with the VIP dynamic measurements using ASV. In addition to the difference in α-coefficient between ASV and AdCSV methods, the diffusion dynamics and geometry of the GIME of the VIP system, which affects the size separation at 4 nm, may also influence $\alpha_{M'}$.

Overall, the <4 nm metal fraction measured with the GIME provides more environmentally relevant information, as this size fraction more closely overlaps with the metal species likely to be transported across biological cell membranes (Buffle and Tercier-Waeber, 2005).

3.4.3 Cu-ligand titrations

The toxicity of Cu has been shown to be related to its free ionic concentration (Cu^{2+}) and not to its total concentration (Gledhill *et al.*, 1997; Moffett and Brand, 1996; Simkiss and Taylor, 1995). Natural waters contain a range of ligands with different complexing sites and binding strengths that act to control the free cupric ion concentration. It is of interest therefore to determine the free cupric ion concentration and the capacity of a system to buffer additional Cu inputs by determining the concentration of natural complexing ligands and their binding strengths (Buck and Bruland, 2005; Sunda and Huntsman, 1991).

In the Cu-ligand titrations carried out in this study, different detection windows were considered by using three concentrations of the added ligand, salicylaldoxime (SA) (2.5, 10 and 25 μM), resulting in analytical competition strengths of $\log\alpha_{CuSA}$ = 3.77, 4.97 and 5.77, respectively. The detection windows, spanning a decade on either side of the

centre ($\log\alpha_{CuSA}$), overlapped (ca. 2.8-4.8, 4-6 and 4.8-6.8) and were chosen to cover a range of Cu-binding ligands. By applying more than one analytical window the influence of different ligand classes on the Cu speciation in the samples was investigated, as illustrated below with an example.

The total dissolved Cu concentration ($[Cu_T]$) was determined as described in Section 3.3.1.4, using AdCSV with SA (25 μM). The titration curves obtained for Percuil Creek (Figure 3.12) showed progressively higher labile concentrations ([Cu']) for titration series using 2.5, 10 and 25 μM SA, respectively. As the applied analytical competition strength increased, a larger proportion of the dissolved Cu concentration was determined within the labile fraction.

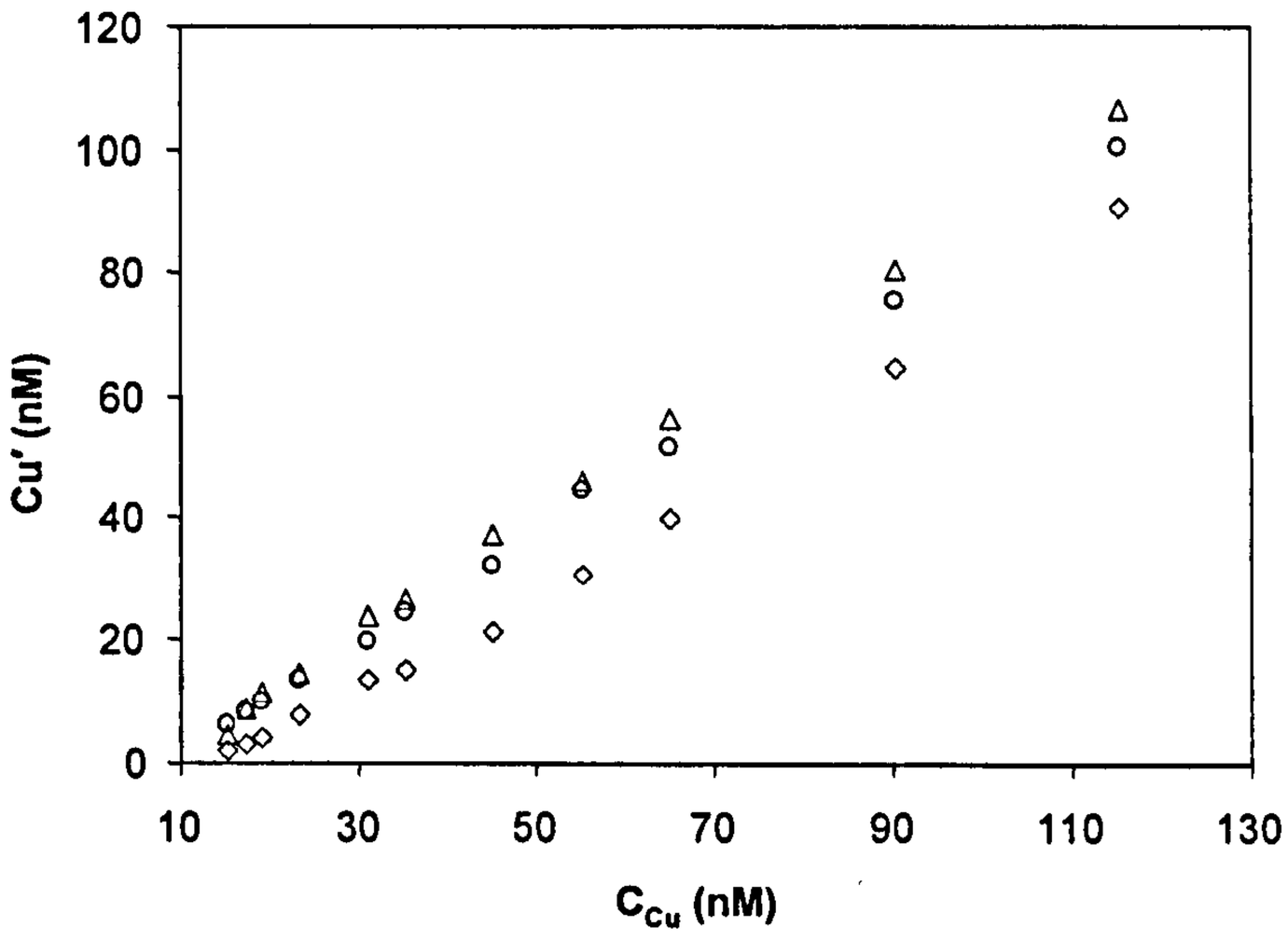


Figure 3.12: Labile Cu (Cu') as a function of increasing total dissolved Cu (sum of total dissolved plus added Cu, C_{Cu}) with three different concentrations of SA. Δ represents 25 μM SA; ○ represents 10 μM SA; ◊ represents 2.5 μM SA.

For each competition strength, the concentration of the Cu-binding ligand concentration (C_L) and its conditional stability constant ($\log K'_{CuL}$) was calculated using

the van den Berg/Ruzic linearization method (Ruzic, 1982; van den Berg, 1982) (Table 3.9). Graphical representations for the lowest and highest competition strengths are given in Figure 3.13.

Table 3.9: Copper speciation results at different detection windows calculated from the van den Berg/Ruzic linearisation method ($[C_T]$ =15.2 nM)

[SA] μM	Log α_{CuSA}	C_{L1} nM	Log K_1	C_{L2} nM	Log K_2
2.5	3.77	23.0	12.6	ND	ND
10	4.97	15.8	13.8	ND	ND
25	5.77	9.7	14.3	ND	ND
2.5*	3.77	17.3	12.8	36.7	11.6

ND: not detected; * represents data selected from low titration points.

The non-linear titration curve (Figure 3.13a) and the curve after linearization (Figure 3.13b) obtained at the lower SA concentration (2.5 μM) indicate that Cu was complexed by more than one group of naturally occurring metal-complexing ligands with different stability constants $\log K'_{CuL}$. In order to resolve this multi-ligand system, the non-linear part of the curve was investigated in more detail by focussing the van den Berg/Ruzic linearization on the low titration points (non-linear zone of titration curve) (Figure 3.14 and Table 3.9).

The calculated stability constants for the natural ligand system ($\log K_1$) from the van den Berg/Ruzic approach increased with the competition strength (log α_{CuSA}), while their concentrations ($[C_{L1}]$) decreased. The trend of weak Cu complexing natural ligands being present at higher concentrations than those of the stronger ligands has also been documented for other coastal systems (Buck and Bruland, 2005; Bruland *et al.*, 2000; Moffett *et al.*, 1997; Coale and Bruland, 1988).

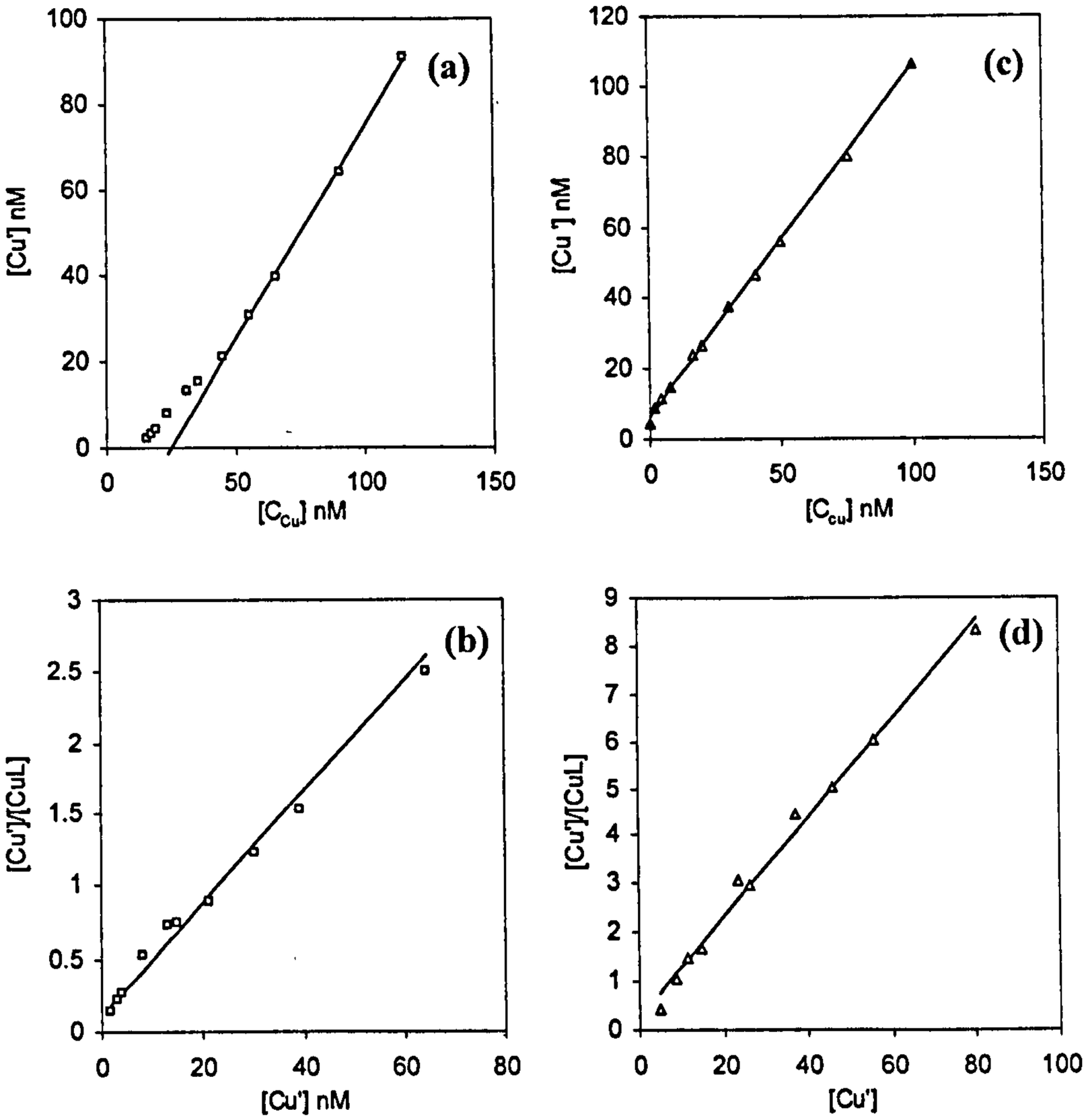


Figure 3.13: Copper titration results. □ represents 2.5 μM SA; Δ represents 25 μM SA. (a) titration curve using 2.5 μM SA, (b) van den Berg/Ruzic linearisation of data shown in (a), (c) titration curve using 25 μM SA, (d) van den Berg/Ruzic linearization of data shown in (c).

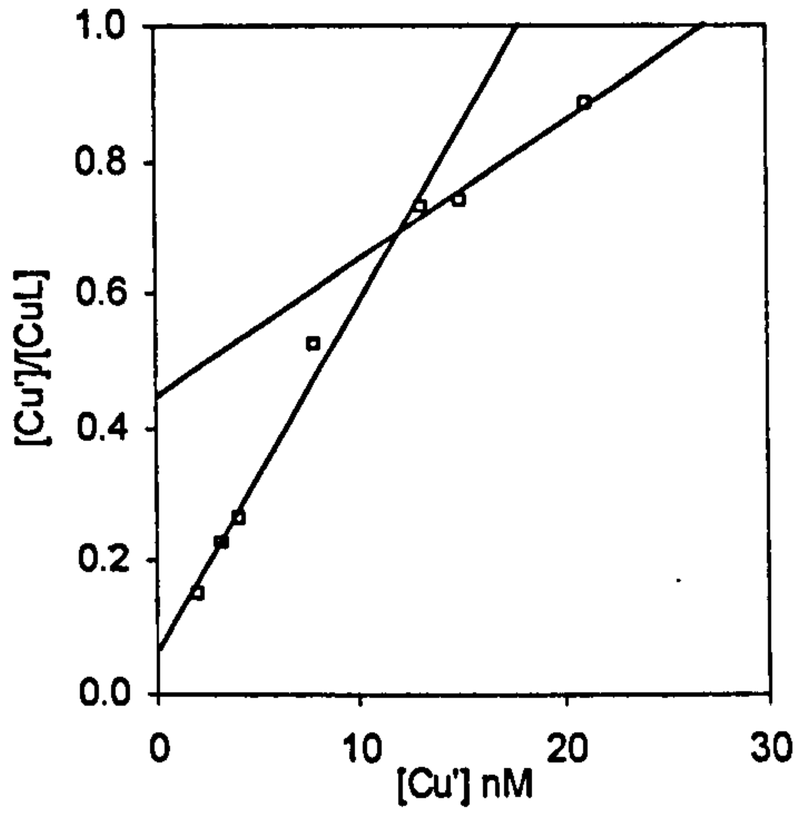


Figure 3.14: Data transformation for copper titration data, 2.5 μM SA; van den Berg/Ruzic linearization at low titration points. The lines represent the resolved contributions for the stronger and weaker ligand classes.

A linear response in the titration curve, at the higher detection window (25 μM SA) suggests that all natural Cu binding ligands in this sample were saturated with Cu (Figure 3.13c) and hence the weaker ligand classes were not detected. The greater competition from the strongest binding sites for Cu resulted in their (thermodynamically) preferred saturation, followed by the progressive occupation of weaker sites. It follows that at lower analytical competition strength the weaker natural ligands can compete more effectively with the added ligand for the available Cu. However, for systems in which C_{L1} is lower than $[Cu_T]$, the values of log K′ cannot be determined with confidence since all ligands are saturated prior to titration and the magnitude of errors on the slope is consequently large. Therefore, estimates of these parameters will be reported for comparative purposes only in Chapters 4 and 5.

By concentrating on the non-linear part of the titration curve, the ligand concentrations were determined as 17.3 nM C_{L1} and 36.7 nM C_{L2} at log α_{CuSA} = 3.77, with $\log K'_{CuL1}$ = 12.8 and $\log K'_{CuL2}$ = 11.6 (Table 3.7). For this system, the C_{L1} concentration was lower by a factor of 1.3 when compared to the data obtained by using the entire titration curve. The $\log K'_{CuL1}$ values differed by 0.2 log units and were within the estimated error of the slope and intercept (between 0.22 to 0.32 log units, see Section 3.4.3.1). It has been argued that adding metal at concentrations much greater than those that occur naturally introduces artefacts into the system that are difficult to interpret (Pei *et al.*, 2000). For example, metal complexes formed with the added ligand may be quite different from those formed with natural ligands, intermetallic compounds may form, and/or precipitation of metal ions may occur. Furthermore, it has been suggested that only data at the beginning of the curve should be considered in calculations, since the added metal is likely to be complexed by the same ligands and complexing sites as those that are active under natural conditions (Buffle and Tercier-Waeber, 2000; Pei *et al.*, 2000). On this basis, and where appropriate (e.g. non-linear curve), the van den Berg/Ruzic linearisation method using low titration points seems to be the most suitable for data

transformation, particularly when a two ligand system can be resolved.

The stability constant is dependent on the slope and *y*-intercept, and therefore strongly dependent on the early titration points, which suffer from large relative uncertainties. The relative importance of the high and low titration points shifts with the competition strength employed. For low competition strengths, the magnitude of the errors at low titration points makes the estimates for C_{L1} uncertain, and is therefore better represented at stronger competition strengths. In contrast, the estimates for C_{L2} are more certain at a low detection window.

Comparison of duplicate copper-ligand titrations

This section discusses the data from duplicate titrations, the environmental implications of which are considered in Chapters 4 and 5. Duplicate Cu-ligand titrations were carried out at low competition strength (2.5 μM SA) on discrete samples collected at different sites in SW England (Fal and Tamar estuaries) that had been frozen and subsequently thawed. For each site, titrations were prepared from bulk samples. The results after data transformation are detailed in Table 3.10 and show some variability in the parameters determined. Principally, the $\log K'_{CuL1}$ and C_{L1} values were within the estimated analytical precision of the method (for all titration data points), except for C_{L1} at Cremyll. This location also showed the most significant variation in the Cu_{labile} concentration (7.91 and 16.5 nM), followed by Neal Point (5.58 and 7.89 nM), while the other sites showed close agreement between duplicates. $\log K'_{CuL2}$ values determined from the low titration points showed reasonable agreement, with Neal Point showing the greatest variation of 0.6 log units. The C_{L2} concentrations were more variable between duplicates, particularly at Wilcove and Neal Point, which deviated by factors of 1.2 and 1.4, respectively. Of most concern were the variations in Cu^{2+} concentrations (varied by factors of 1.2 to 2.4) where only the duplicates for Wilcove agreed. This adds to the uncertainty associated with the estimation of Cu^{2+} by means of titration as determined in Section 3.4.2.1. However,

Table 3.10: Overview of the results of Cu-ligand titrations (after data transformation), performed in duplicate, on thawed samples collected in SW England. All sites other than Percuil Creek (Fal Estuary) were located in the Tamar Estuary. Physical parameters and analytical conditions are detailed in relevant Sections in Chapters 4 and 5.

Site	C_{L1} (nM)		$\log K'_{CuL1}$		Cu^{2+} (pM)		Cu_{labile} (nM)		C_{L2} (nM)		$\log K'_{CuL2}$	
	1	2	1	2	1	2	1	2	1	2	1	2
Percuil Creek	22.4 ± 0.77	23.6 ± 0.81	12.6 ± 0.32	12.5 ± 0.22	0.35	0.45	3.30	3.31	-	-	-	-
Percuil Creek*	17.2	17.4	12.9	12.7	-	-	-	-	35.3	38.0	11.6	11.6
Cremyll	39.1 ± 0.52	29.1 ± 0.64	12.7 ± 0.18	12.7 ± 0.31	0.31	0.74	7.91	16.5	-	-	-	-
Cremyll*	34.7	27.8	12.7	12.5	-	-	-	-	42.4	38.3	12.4	12.0
Wilcove	48.1 ± 1.90	46.2 ± 0.90	12.9 ± 0.58	12.5 ± 0.20	0.09	0.09	2.65	2.64	-	-		
Wilcove*	35.8	31.4	13.6	13.5	-	-	-	-	63.3	52.4	11.8	11.8
Neal Point	50.4 ± 1.09	50.3 ± 1.64	12.6 ± 0.23	13.4 ± 1.0	1.05	0.86	7.89	5.58	-	-	-	-
Neal Point*	47.6	43.7	12.7	13.1	-	-	-	-	72.5	53.5	11.8	12.4
Whitsand	9.39 ± 0.10	9.49 ± 0.23	12.7 ± 0.03	12.8 ± 0.09	0.24	0.12	1.37	1.38	-	-	-	-
Whitsand*	8.4	7.1	12.7	13.0	-	-	-	-	11.4	12.0	12.3	12.4

although competitive ligand titrations rely on data transformation and are subject to large uncertainties (refer to Section 3.2.4.1) few established analytical techniques are available that combine both speciation capabilities with high sensitivity (e.g. concentrations of free metal ions are typically <100 pM in natural marine systems) and therefore, the titration approach remains one of the preferred methodologies. Notwithstanding this, there are techniques emerging that selectively measure free ion concentrations that show some potential for the estimation of free ion concentrations in natural waters. These include permeation liquid membrane (PLM) techniques coupled to a sensitive analytical technique (e.g. flame atomic absorption spectroscopy, inductively coupled plasma-mass spectrometry or voltammetry), the Donnan Membrane technique, ion selective electrodes with improved sensitivities and the Complexing Gel Integrated Microelectrode (CGIME) (Noel *et al.*, 2006).

3.5 Conclusions

High resolution *in situ* speciation measurements enable the detection of short-term variability in water quality parameters, and this facilitates a more detailed examination of environmental processes. In this study, this advantage of *in situ* measurements with the VIP over laboratory measurements carried out on discrete samples taken with limited temporal resolution was apparent in the detection of short-term changes in metal concentrations and the absence of concerns related to maintaining sample integrity.

Few analytical techniques combine speciation capabilities with high sensitivity and the VIP system used has been shown to achieve both in *in situ* and laboratory applications. The high resolution *in situ* speciation measurements of Cd, Pb and Cu, determining the operationally defined metal species <4 nm in size, provided biologically relevant information at picomolar concentrations of Cd and Pb and nanomolar concentrations of Cu in dynamic coastal waters. Furthermore, the analytical procedure requires minimal sample handling, therefore minimising the risk of contamination.

The main limitations with respect to the VIP system are the requirement for experienced operators and the reliance on pre-and post-deployment calibrations. The former, at present, hampers wide use for routine applications. Degradation of the Hg surface over time can result in a change or loss of sensitivity during deployment, only detected after post-deployment calibrations. In addition, to characterise the distribution of trace metals in natural water in more detail, total dissolved metal concentrations are required. This necessitates the collection of discrete samples, and this study has shown that collection of slightly different water masses for *in situ* and total analysis can introduce uncertainty into data interpretation.

The advantage of analysis of discrete samples using a hanging mercury drop electrode (Metrohm) is the capability to estimate detailed speciation on individual samples. This includes labile, non-labile and total dissolved concentrations, in addition to parameters determined from metal-ligand titrations (ASV and AdCSV). Competitive ligand titrations with Cu were utilised using different detection windows to determine different ligand classes of different binding strengths and concentrations within a sample with confidence. This provided a more complete assessment of the buffering capacity of the system towards potential inputs of total dissolved Cu. Differences between titrations conducted on freshly filtered samples and samples that had been frozen, as well as duplicate titrations, indicated that labile and free cupric ion concentrations may be overestimated. Notwithstanding this, few established and sensitive analytical techniques are available to determine metal species at environmentally relevant concentrations and as such metal ligand titrations remain indispensable.

The fraction of metals detected using voltammetric speciation measurements is operationally defined by the sample treatment and analytical approach taken. Moreover, the different sampling methodologies and/or artefacts introduced by the methodology are important considerations that can impact on the speciation measurements, and this has to considered when comparing or evaluating data sets from different studies. In this work,

data generated using different voltammetric approaches have been used to complement each other (Chapters 4 and 5). On the whole, it was recognised that the VIP system measured a smaller size fraction and that this fraction would be more environmentally relevant for speciation studies, as it more closely overlaps with the metal species likely to be transported across biological membranes.

Chapter 4:

Fal Field Study:

Chemical and biological investigation in an estuary heavily contaminated with metals

4.1 Introduction

The work presented in this chapter integrates biological and chemical investigations in an effort to establish the ecotoxicological impact of trace metals on biological systems in marine waters, as well as to provide an insight into the biogeochemical cycling of trace metals. The study was undertaken at two contrasting sites within the Fal Estuary, southwest England, one heavily impacted by historical and chronic metal contamination and one more pristine location, influenced by waters from the English Channel.

The aims and objectives of this study were to:

(1) Assess the toxicity of waters in the Fal Estuary at contrasting sites by applying the oyster-embryo larval bioassay (using the Pacific oyster, *Crassostrea gigas*) to discrete samples collected over full tidal cycles.

(2) Investigate the relationship between metal speciation and overall toxicity of coastal waters by comparing high-resolution *in-situ* measurements of Cd, Pb and Cu determined with the VIP system with the embryo-larval responses.

(3) Use competitive ligand titrations in order to examine dissolved Cu speciation and relate these measurements to biological effect.

4.2. Estuarine processes

Estuarine environments are subjected to a complex interplay of chemical, biological and physical processes that act collectively to control the biogeochemical cycling and transport of trace metals (Millward, 1995; Morris *et al.*, 1981).

Estuaries are at the interface between rivers and oceans and as a result undergo continuous variations in supply of both matter and energy. The physical mixing of fresh and saline waters of markedly different composition results in strong physico-chemical gradients. Key processes that act to control the speciation of trace elements include salinity, pH, redox potential, and the concentrations of organic material, nutrients, organic constituents and particulate matter (Chester, 2003). Major variations in these parameters,

particularly in the mixing zone, result in a variety of dissolved-particulate interactions that modify the behaviour of the dissolved constituents. These modifications are driven by physical, chemical and biological factors, and include sorption at the surfaces of suspended particles, flocculation-aggregation, complexation, precipitation and uptake via biological processes (Figure 4.1). In general, the extent to which modifications occur depends on the nature and concentrations of both the particulate and the dissolved components. As a result of these interactions a large part of particulate discharge (with associated metals) from rivers becomes trapped in estuarine regions.

Particulate phases, of marine and terrestrial origin, act as binding sites for trace metals and are important in controlling the chemical behaviour of trace elements in estuaries, providing a fundamental link between the water column, bed sediment and food chain (Turner and Millward, 2002). These particles include various mineral phases and organics that are present either as flocculants or as coatings on the surfaces of mineral components (e.g. clays, quartz, feldspars and carbonates). In estuaries, the role of suspended particles is particularly important because of variations in tidal and wind-driven currents or river flow that give rise to changes in particle concentrations and character. The density of suspended particles is greater than that of water and particles are subject to successive cycles of deposition-resuspension. Consequently, they provide an important and recurrent link for chemicals between the aqueous phase, suspension and the bed sediment (Turner and Millward, 2002). The role of suspended sediments in biogeochemical processes is schematically represented in Figure 4.1.

The freshwater/seawater interface (FSI) and the Turbidity Maximum Zone (TMZ) are regions within an estuary where elevated concentrations of suspended particulate matter (SPM) occur and where particle-water interactions play an important role in geochemical cycling (Millward, 1995). Most simply the behaviour of dissolved constituents can be described as conservative (e.g. non-reactive) or non-conservative (e.g. reactive). The major dissolved components (e.g. the major ions Na^+ and Cl^-) appear to

behave conservatively, which indicates that their distribution is controlled only by physical mixing. Where dissolved-particulate reactions occur non-conservative behaviour results in either removal or addition of the dissolved component (e.g. Mn in the Tamar Estuary showed removal and addition, Knox *et al.*, (1981)). In essence, adsorption of dissolved metals onto particles leads to removal from the water column if the particle settles, and desorption of dissolved metals from particles leads to addition to the water column. Further inputs result from porewater infusions, industrial sources and from fluctuations in riverine sources. Additional losses occur through precipitation and/or flocculation of colloidal material (Millward, 1995).

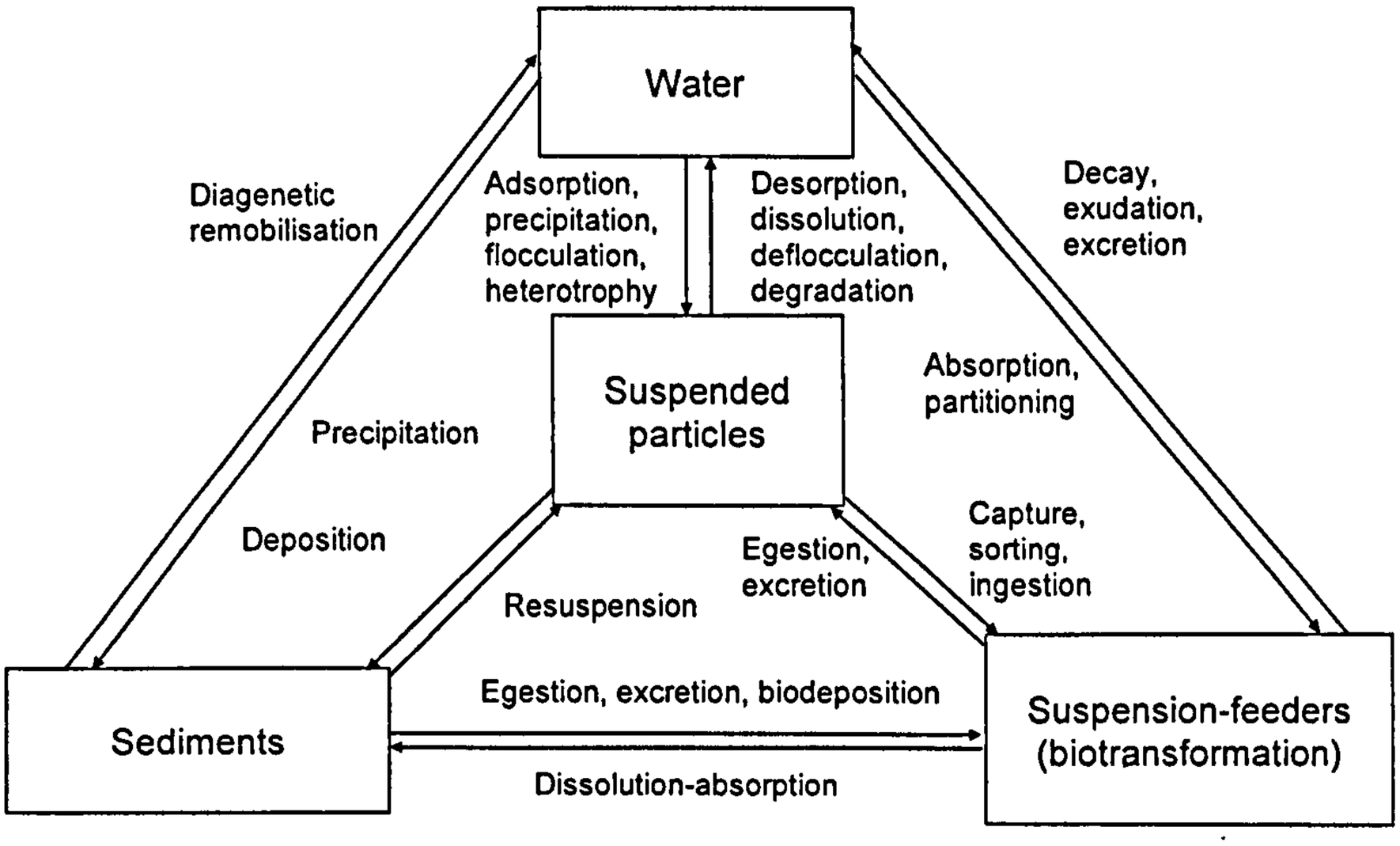


Figure 4.1: Schematic representation of the role of suspended sediments in estuarine biogeochemical processes. Boxes represent compartments hosting materials and chemical constituents, and arrows represent physical and biogeochemical processes responsible for transfer of such between compartments (adapted from Turner and Millward, 2002).

Aquatic colloids (defined as solid phase material with one dimension between 1 nm and 1 μm (Figure 4.2)) play a key role in the distribution and transport of trace compounds (Buffle *et al.*, 1998). Figure 4.2 illustrates the principal colloidal phases, iron

hydroxyhydroxides, manganese oxyhydroxides, aluminosilicates and organic carbon (and humics). Large fractions of trace pollutants, nutrients and pathogens are physically or chemically bound to colloidal material and these interactions strongly modify the biological availability of trace metals (Lead and Wilkinson, 2006).

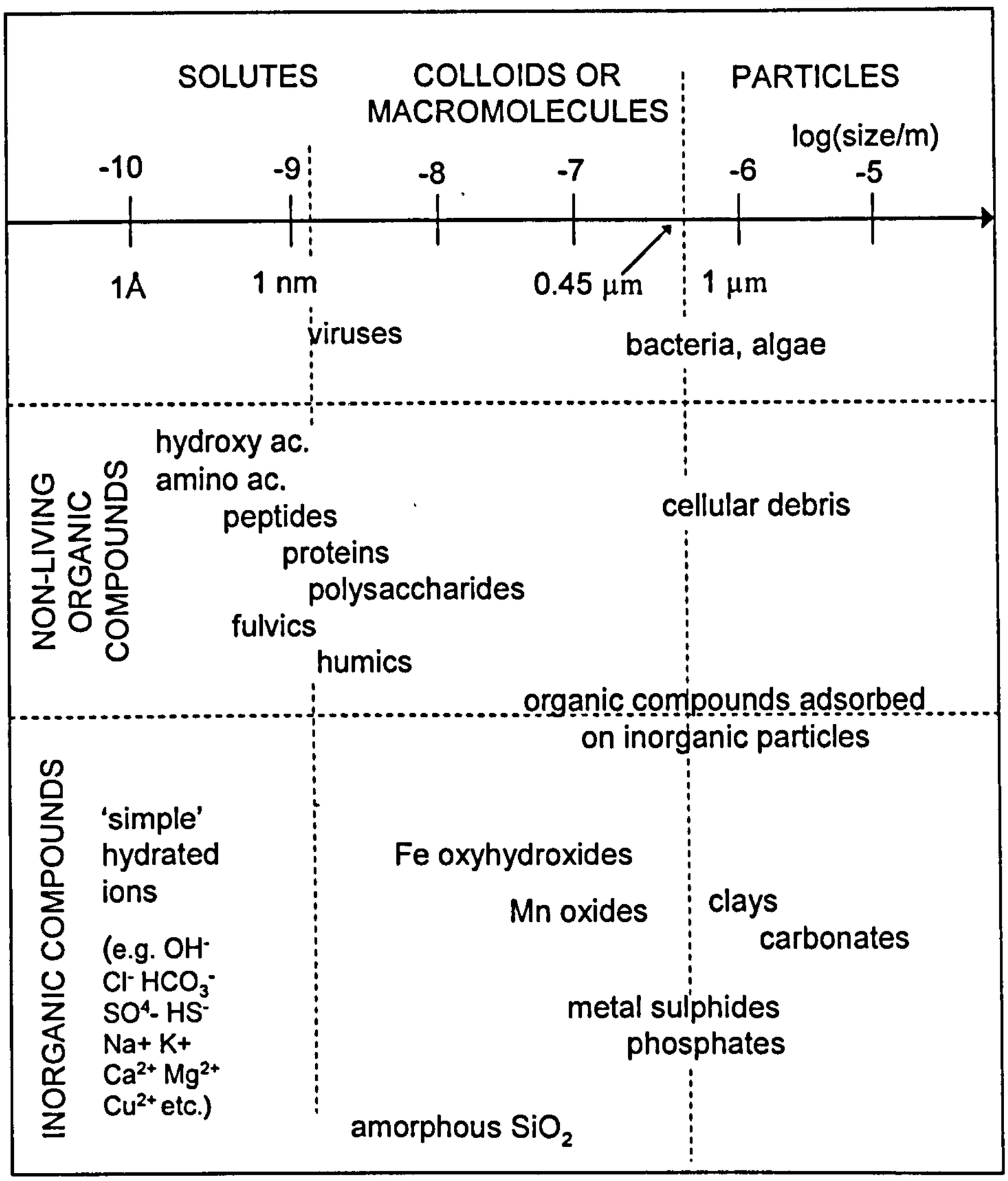


Figure 4.2: Size distributions of various types of environmental colloids and particles (adapted from Lead and Wilkinson, 2006).

Dissolved trace element species vary mainly as a function of salinity and to a lesser degree pH, and can be preferentially complexed by inorganic or organic ligands. As a result trace metals are transported in a complex manner in estuarine environments (Millward, 1995).

4. 3 Materials and methods

4.3.1 Site description and field sampling locations

The Fal Estuary is located in SW England (Figure 4.3) and its catchment is predominantly rural, supporting intensive mixed arable and dairy farming, and has relatively little heavy industry. However, it is profoundly influenced by the underlying geology of Carnmellis granite and surrounding metamorphic aureole to the west of the estuary (Langston *et al.*, 2003a). For centuries, mining and ore processing was a major feature of this area and this has strongly impacted the catchment and sediments of the Fal system (Pirrie *et al.*, 2003). In addition to the Fal river, a number of other rivers drain into creeks within the Fal Estuary system, including the Carnon (flowing into Restronguet Creek), Percuil and Penryn. The drainage from abandoned mines, as well as mobilisation of metals from sediments results in elevated metal concentrations in the waters and sediments of the Fal estuary. Particularly affected is Restronguet Creek (Langston *et al.*, 2006) through inputs from the Carnon river that drains parts of what was formerly the most productive of the south-west mining districts (St Day, Cambourne and Redruth) (Dines, 1969). Indeed, the sediments of Restronguet Creek are some of the most metal polluted in the UK (Pirrie *et al.*, 2003; Bryan and Langston, 1992), with mean observed sediment concentrations of 3.46 $\mu g\ g^{-1}$ Ag, 1732 $\mu g\ g^{-1}$ As, 1.1 $\mu g\ g^{-1}$ Cd, 2148 $\mu g\ g^{-1}$ Cu, 297 $\mu g\ g^{-1}$ Pb and 2700 $\mu g\ g^{-1}$ Zn (Bryan and Gibbs, 1983). Dissolved metal concentrations are reported in the range of 0.49 to 1.5 μM As, 2 to 4 nM Cd, 0.16 to 0.4 μM Cu and 0.18 to 6.5 μM Zn. In general, aqueous metal concentrations in other parts of the estuary (outside the immediate influence of Restronguet Creek) are two to three orders of magnitude lower (Langston *et al.*, 2003a).

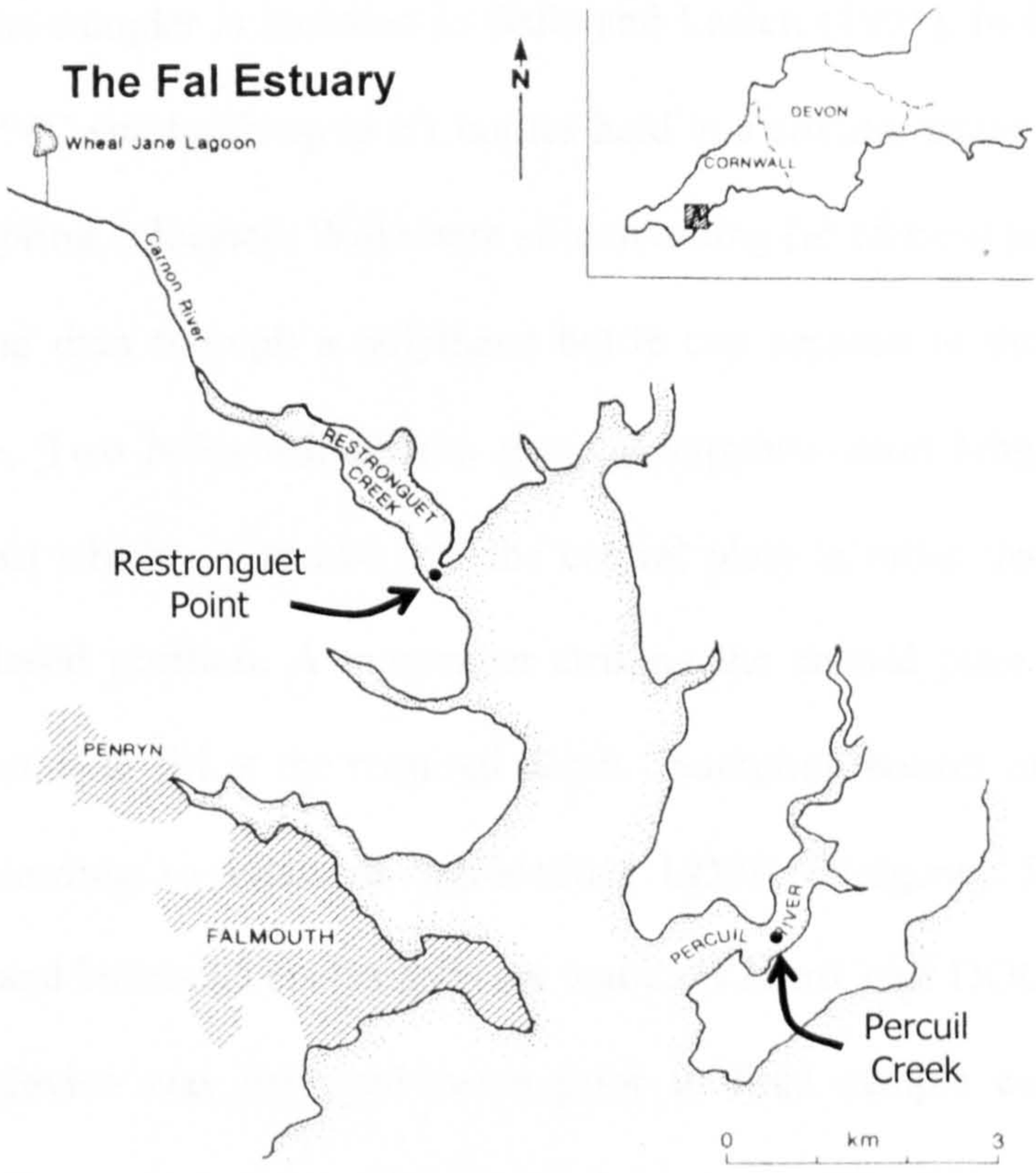


Figure 4.3: Survey locations in the Fal Estuary, southwest England. The Restronguet Point is affected by chronic metal inputs from abandoned mines in the catchment of the Carnon River. The catchment of Percuil Creek is predominantly agricultural and the sampling station is strongly influenced by coastal marine waters.

4.3.2 Sampling strategy and protocols

The surveys were carried out for periods of 9½ hours at Percuil Creek and 10 hours at Restronguet Point (see Figure 4.3 for locations) on 3rd and 4th August 2005, respectively. The monitoring periods commenced before low water (11:39 h GMT, 1.5 m on 3rd August and 12:22 h, 1.4 m on 4th August) and finished after high water (17:10 h GMT, 4.8 m on 3rd August and 17:48 h, 5.0 m on 4th August) conditions at each site. The VIP system was deployed from an anchored vessel (34 ft glass fibre leisure yacht) at ca. 2 m depth, and *in-situ* measurements were carried out with a temporal resolution of ca. 20 to 45 min. Discrete samples for complementary laboratory analysis of total dissolved metal concentrations, bioassay exposures, salinity, dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) measurements were collected from the vessel at hourly intervals with the aid of a purpose-built sampler equipped with an operator-controlled release mechanism. A detailed

description of the sampler is included in Balls and Laslett (1991). In brief, the sampler is constructed of PVC and holds up to six bottles held in a circular frame with a central plate under which a spring is located. Wide-bore silicon tubing (id 18 mm) passes through a hole in the frame and then through a polythene bottle cap secured to the frame to hold the bottles in place. Two holes drilled into the cap supports short lengths of smaller-bore tubing (id 6 mm) which are tucked into the central plate in order that the bottles can be lowered in a closed position. A messenger striking the central plate releases the tubing allowing the bottles to fill at the required depth. Sampling bottles of different materials were fitted according to intended application: LDPE (Nalgene, 500 mL) for metal determinations and bioassays analysis, Pyrex bottles (125 mL) for DOC and TDN analysis. The sampling device was deployed twice prior to final sample collection for rinsing purposes. Samples were vacuum filtered on-site using a filtration unit (polysulphonate, Nalgene, with 0.45 μm cellulose acetate filter membranes, Whatman, UK for metal analysis and bioassay exposures, or glass unit with 0.7μm GF/F filter membranes, Whatman, for DOC and TDN analysis) fitted with a hand pump (Nalgene), and the filtrate was collected in separate receptacles. Samples collected for total dissolved metal analysis (LDPE, 250 mL) were immediately acidified with Q-HNO_3 (0.1%). Samples for DOC and TDN analysis were transferred into Pyrex vials (I-chem, Techmate Ltd, Milton Keynes) and acidified with Q-HCl (0.1%). Samples collected for bioassay exposures (LDPE, 125 mL) and metal titrations (LDPE, 1 L) were stored on dry ice in the dark (maximum period 36 h) until return to the laboratory where they were stored frozen (-20°C). Sample bottles were rinsed three times with the sample before final sample collection. Trace metal clean techniques were used for all sample processing and analytical procedures.

Master variables were recorded on site using portable instrumentation. Temperature and pH was measured using pH meter (Hanna model HI9025; Hanna Instruments Ltd, Herts, UK) fitted with a GELPAS probe (BDH). The pH meter was calibrated before each tidal cycle using buffer solutions of pH 7 and pH 4 (NIST). Conductivity was measured

using a conductivity meter (Hanna model HI9635). Discrete samples were collected for salinity measurements using the sampling device. Salinity analysis was undertaken using an Autosal (Guildline) salinometer (National Oceanography Centre, Southampton).

DOC and TDN concentrations were determined simultaneously using high temperature catalytic combustion (Badr *et al.*, 2003) by X. Pan (National Oceanography Centre, Southampton).

4.3.3 Reagents and equipment

The quality, preparation and purification of reagents (Q-HCl, Q-HNO_3, iso-NH_3) have been given in Chapter 2, section 2.2.1. Reagents used for the AdCSV method (SA, oxine, borate, HEPES) and for the VIP system ($NaNO_3$, $Hg(CH_3COO)_2$, KSCN) have been described in Chapter 3, sections 3.3.1.2 and 3.3.2.2, respectively. Artificial seawater (ASW) salts (pH 8.1, S=31), used for the controls in bioassay exposures, were prepared and treated according to the method described in Chapter 2, Section 2.2. Sample handling and preparation of reagents was carried out under a class-100 laminar flow hood. Low density polyethylene (LDPE, Nalgene, UK) containers were used for sample collection and to store bulk reagents and were cleaned using the trace metal cleaning protocol of Achterberg *et al.* (2001) as described in Chapter 2, section 2.2.1. Filter apparatus (glass) and sampling bottles for DOC and TDN analysis were acid washed in 10% HCl for 24 h, dried, wrapped in aluminium foil and ashed (450°C, 4 h). The procedure was repeated for filter membranes (0.7μm GF/F, Whatman) without the acid leach step. The equipment was stored and transported in the ashed aluminium foil.

4.3.4 Trace metal analysis

4.3.4.1 Voltammetric *In situ* Probe

The VIP system and preparation of the VIP voltammetric cell and gel-integrated microelectrode (GIME) have been described in detail in Chapter 3. The instrument was

calibrated before and after field-deployment. The concentrations of dynamic metal species measured with the VIP were calculated using the sensitivity determined from the calibration slope for each metal (for details refer to Table 3.8, Section 3.4.2.1). Simultaneous *in situ* measurements of Cd, Pb and Cu were undertaken using the VIP operating in the square wave anodic stripping voltammetry (SWASV) mode with the analytical parameters detailed in Table 4.1. Temperature corrections for *in situ* deployment were carried out as described in Section 3.3.2.5. Total dissolved concentrations of Cd and Pb (<0.45 μm pore size) were determined after UV-irradiation following the method detailed in Section 3.3.1.4.

Table 4.1: Conditions for the simultaneous determination of Cd(II), Pb(II) and Cu(II) using SWASV with the VIP system.

Step	Calibration conditions	Field conditions
Deposition potential (mV)	-1200 to -1250	-1150
Deposition time (min)	15	10 to 25
Equilibration time (deposition potential) (s)	30	30
Final potential (mV)	+150	-90 to -80
Cleaning time (final potential) (s)	60	60
Pump time (s)	90	90
Pause (mV ; s)	-80 ; 0	-80 ; 0
Pulse amplitude (mV)	25	25
Pulse step (mV)	8	8
Frequency (Hz)	200	200

4.3.4.2 Hanging mercury drop electrode (HMDE)

Adsorptive cathodic stripping voltammetry (AdCSV) was used to determine total dissolved Cu (see Section 3.3.1.3 for methods), with salicylaldoxime (SA, 25 μM) as the competing ligand and borate as pH buffer (10 mM, pH 8.3). Calibration was carried out by standard addition to each sample. The working conditions for the Cu-ligand titrations are detailed in Table 4.2.

Copper titrations were carried out on samples collected at high water at Percuil Creek (16:40 h) and Restronguet Point (17:49 h), as well as at low water at Restronguet Point (12:22 h). AdCSV was used at three competition strengths (2.5, 10 and 25 μM SA) applying the method described in Section 3.3.1.5. For each competition strength ($\log\alpha_{CuSA}$ = 3.77, 4.97 and 5.77) or detection window (ca. 2.8-4.8, 4-6, 4.8-6.8 where the detection window spans a decade either side of the centre), the concentration of the Cu-binding ligand concentration (C_L) and its conditional stability constant ($\log K'_{CuL}$) was calculated using the van den Berg/Ruzic linearization method which assumes that Cu complexation can be described by a single ligand forming a 1:1 complex with Cu (Ruzic, 1982; van den Berg, 1982). Focussing on the low titration points allowed two ligand classes to be determined at low competition strength.

Table 4.2: Analytical parameters used for Cu titrations. Modulation was square wave, deposition potential was -0.12 V, scan frequency 50 Hz, scan was from -0.12 to -0.55 V, step amplitude 25 mV and step potential 2.44 mV. Salicylaldoxime (SA) 25 μM was used for total Cu determinations.

Parameter	Percuil Creek (16:40 h)			Restronguet Point (17:49 h)			Restronguet Point (12:22 h)		
SA (μM)	2.5	10	25	2.5	10	25	2.5	10	25
Cu_T (nM)	15.2	15.2	15.2	17.8	17.8	17.8	88	88	88
Upper limit of Cu added (nM)	100	100	100	120	120	120	500	500	500
Deposition time (s)	10	10	2	20	10	5	5	5	2
Stirrer setting (#)	6	6	3	5	3	3	3	2	1
Equilibration time (s)	8	8	5	8	5	5	5	5	5
Hg drop size setting (#)	3	3	1	3	3	3	3	2	1
Purge time (s)	240	240	240	180	180	180	180	180	180

4.3.5 Toxicity test

The toxicity of the water collected from the sample sites was assessed by means of the oyster-embryo larval bioassay and based on the method adopted by the Environment

Agency (EA, 2004) (see Section 2.3.4.1). Conditioned male and female oysters (*Crassostrea gigas*) were obtained from Guernsey Sea Farms Ltd and allowed to acclimatise in aerated ASW (1.5 L) for 3-4 h prior to commencement of the bioassay. The experimental set-up is shown in Figure 4.4, whereby the 'pairings' (i.e. denoted P1-P3 and Mix) of eggs and sperm were chosen at random from male and female adults. By keeping the 'pairings' separated the larval response to the sample waters could be assessed between sites as well as between 'pairings'.

Data was normalized to the control values using the procedure according to Woelke (1972) to take account of abnormal development of oyster larvae arising from the exposure conditions in the bioassays by expressing experimental results as percent net response:

$$Percent\ net\ response = \frac{\%\ abnormality\ in\ exposures - \%\ abnormality\ in\ controls}{100 - \%\ abnormality\ in\ controls} \times 100$$

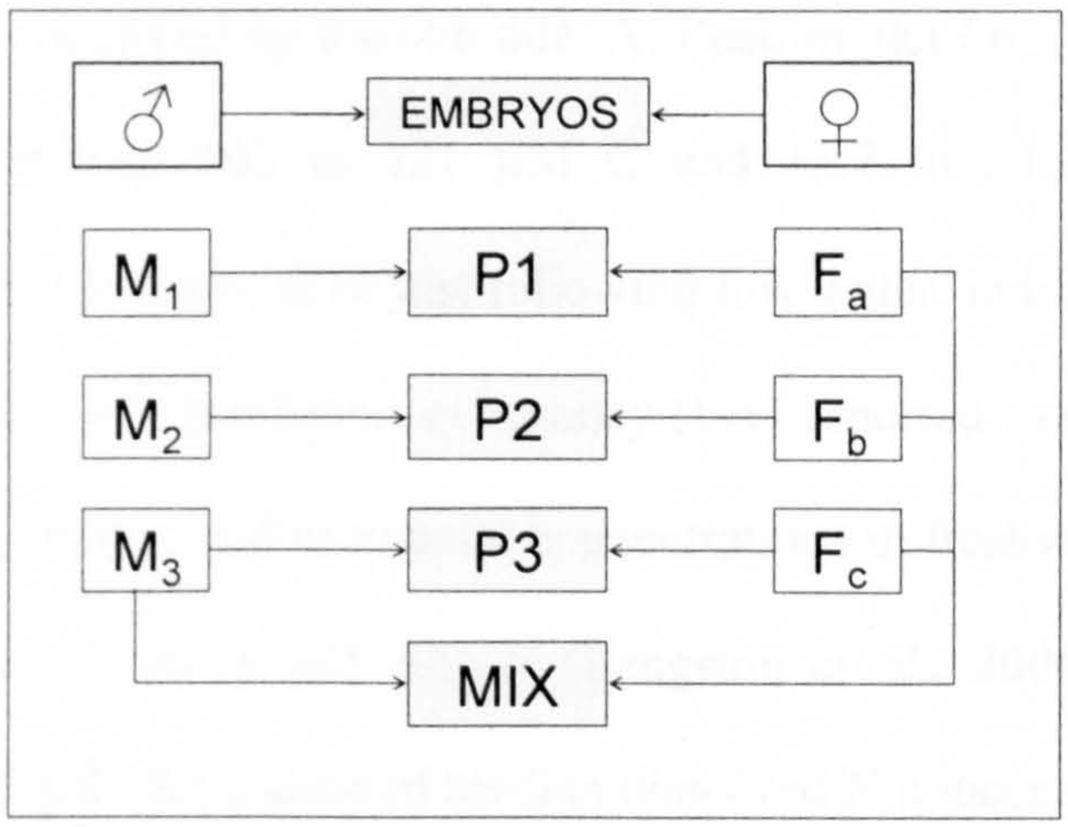


Figure 4.4: Experimental matrix used in the selection of different pairings (e.g. P1) and mix for bioassay exposures to the discrete water samples. The gametes from three adult male and female oysters (*C. gigas*) for different pairings were randomly selected. The most motile sperm was chosen for the mix.

4.4 Results and Discussion

4.4.1 Master variables

The physical parameters determined during the survey indicated that the estuarine waters were predominantly influenced by marine waters. The salinity measured at Percuil Creek remained constant throughout the tidal cycle at 35.0-35.2. At Restronguet Point the salinity ranged between 33.6 and 34.9, with the lowest salinity coinciding with low water. The water temperature ranged from 17.2 to 18.2°C at Percuil Creek and 14.2 to 17.5°C at Restronguet Point where the influence of freshwater inputs raised the temperature up to low water and then remained relatively stable through the remainder of the tidal cycle. The pH remained reasonably constant and high with ranges of pH 8.21-8.26 and pH 8.45-8.51 at Percuil Creek and Restronguet Point, respectively.

DOC and TDN largely co-varied (Figures 4.5e and 4.6e) throughout the tidal cycles. At Percuil Creek, DOC ranged between 98.9 to 138 μM C, with the highest concentration occurring at low water. TDN ranged between 8.5 and 15.5 μM, with higher concentrations recorded on the ebb tide. At Restronguet Point, concentrations of DOC and TDN were between 102 to 121 μM C and 13.7 to 24.7 μM N, respectively, with concentrations increasing at or just following low water, indicating a riverine TDN source. From 1999-2001 the Environment Agency (EA) reported median total inorganic nitrogen (sum of nitrate, nitrite and ammonia) concentrations in freshwater inputs to the Fal Estuary that ranged from 423 to 541 μM N (Langston *et al.*, 2006). A survey of tidal waters conducted in 2000 (EA), showed median dissolved N concentrations (calculated as nitrate) at Percuil of between 0.031-0.054 μM and approximately 0.071 μM at the mouth of Restronguet Creek (Langston *et al.*, 2006). Nutrient concentrations vary with freshwater flow and with salinity, and as a result can show considerable differences throughout the year and at different states of the tidal cycle, and these are likely causes for the differences in the concentrations observed in this study.

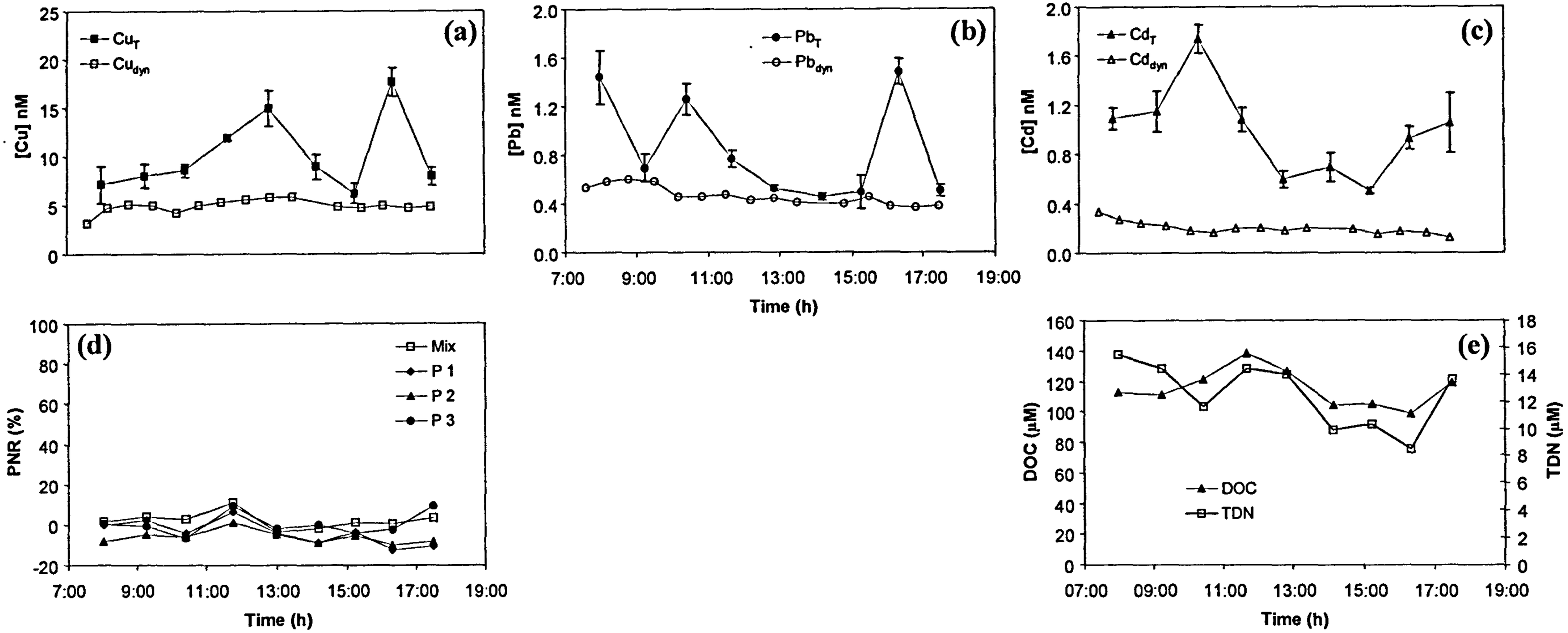


Figure 4.5: Percuil Creek: Time series of dynamic and total dissolved Cu (a), Cd (b) and Pb (c) concentrations (M_{dyn} and M_T, respectively), percent net response (PNR) of oyster larvae to discrete water samples (d) for four pairings (P1, P2, P3 and mixed), dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (e). Dynamic metal concentrations were measured *in situ*, the remaining parameters were determined in discrete samples. Low water: 11:40 h, high water: 17:10 h. Note: the trendline for total metal concentrations is added for clarity. Error bars represent ± 2σ.

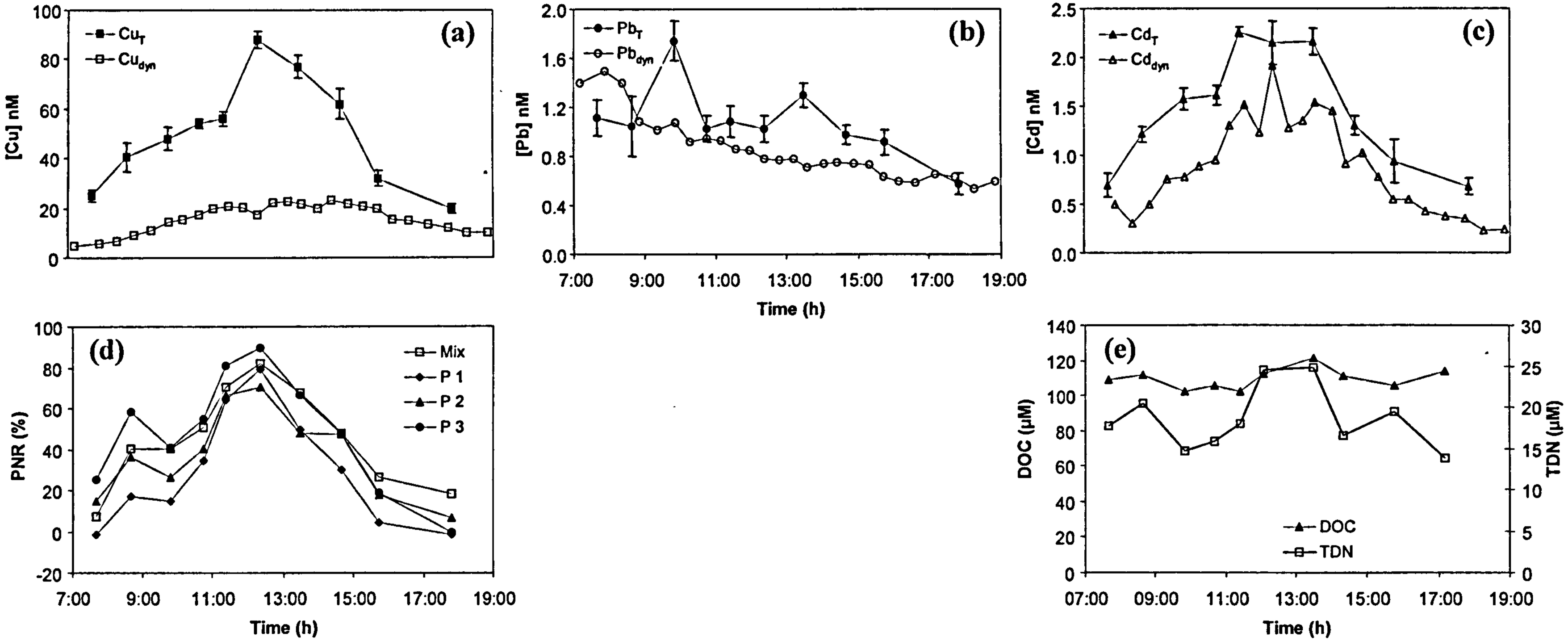


Figure 4.6: Restronguet Point: Time series of dynamic and total dissolved Cu (a), Cd (b) and Pb (c) concentrations (M_{dyn} and M_T, respectively), percent net response (PNR) of oyster larvae to discrete water samples (d) for four pairings (P1, P2, P3 and mixed), dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (e). Dynamic metal concentrations were measured *in situ*, the remaining parameters were determined in discrete samples. Low water: 12:22 h, high water: 17:48 h. Error bars represent ± 2σ.

4.4.2 Bioassays

The results from the bioassay exposures to water collected at Percuil Creek are shown in Figure 4.5d. The larval response varied little throughout the tidal cycle, with the maximum PNR of 1-11% occurring at low water for all pairings. This low PNR indicated a relatively low toxicity of the water and confirmed that Percuil Creek represents a reasonably pristine coastal site. The mean PNR (for replicates) ranged from -13 to 11% over the tidal cycle and the coefficient of variation between replicates was < 40% which is considered acceptable by the US Environmental Protection Agency (da Cruz *et al.*, 2007) and the UK Environment Agency (EA, 2004). The mean for the controls of all pairings in this study was 13 ± 6%. Some responses to Percuil Creek water (61%) were better than the controls, as indicated by negative PNR values, suggesting that the developing embryos and larvae were exposed to more favourable conditions. The reason for this is unclear and since the ASW was treated with Chelex-100 to remove metal contaminants it is unlikely that contamination of the controls was an issue, although total metal concentrations were not investigated in this instance.

In contrast, the maximum PNR at Restronguet Point was higher (PNR = 71-90%, Figure 4.6d) and also occurred around low water, indicating the main source of the observed embryotoxicity was upstream. Moderate responses of PNR up to 19%, comparable to the maximum PNR observed at Percuil Creek, were observed during the remainder of the tidal cycle.

The distinctly larger toxic response of the bioassay to waters collected at Restronguet Point, compared with Percuil Creek, can be mostly attributed to the higher level of contamination from past mining activity at the former site. This was reflected in the higher dynamic (potentially biologically available) concentrations of Cu, Cd and Pb determined at Restronguet Point and it is likely that other mining-related contamination (e.g. Zn, As) not included in this survey contributed to the toxicity at this site. Langston *et*

al. (2003) report UK minimum sediment values of 1.68 μg g^{-1} As and 26 μg g^{-1} Zn (data source: Marine Biological Association) and elevated surface sediment (<100 μm) concentrations of As and Zn at both Restronguet and Percuil with values of 1467 and 35 μg g^{-1} (dry wt) As and 2332 and 315 μg g^{-1} (dry wt) Zn, respectively. Interim marine sediment quality guidelines (Cole *et al.*, 1999) and probable effect levels for As are 7.24 and 41.6 μg g^{-1}, respectively and for Zn are 124 and 271 μg g^{-1}, which indicates that the sediments of both sites are potentially a source of trace metals to the water column and are likely to impact on biota. Dissolved metal concentrations entering Restronguet Creek have decreased since mining operations have ceased, however enhanced concentrations are still observed at 0.35 μM As and 35.5 μM Zn, where environmental quality standards (EQS) are set at 0.33 μM As and 0.61 μM Zn (Langston *et al.*, 2006). Previous studies on benthic communities that inhabit Restronguet Creek report a sparse assemblage that most often exhibit metal tolerance (Warwick, 2001; Bryan and Gibbs, 1983). Largely, bivalve larvae and juveniles are unable to withstand the toxic conditions and only one bivalve *Scrobicularia plana* reportedly survives within the Creek with the population confined to the margins (Bryan and Gibbs, 1983). Morillo *et al.* (2005) reported that bivalves are not found in large parts of the Huelva Estuary as a result of high levels of metal contaminants that drain from the Iberian Pyrite Belt (metalliferous mining area) through the Odiel and Tinto rivers.

The difference in response between pairings were not statistically significant to waters collected at Restronguet Point (P=0.35, Fisher's PSLD, StatView), however, there was a significant difference in the larval responses between P1 and the mix (P=0.012), P2 and the mix (P=0.01) and P2 and P3 (P=0.08) pairings to Percuil Creek waters. A significant difference was also indicated between Percuil Creek and Restronguet Point (P=0.0001).

4.4.3 Trace metals in the Fal Estuary

Percuil Creek

Dynamic and total concentrations of Cu, Pb and Cd measured in Percuil Creek are presented in Figure 4.5a-c. In general, the dynamic fraction of each metal was subject to small changes throughout the tidal cycle, while total dissolved concentrations showed marked variations. It follows that the variations in total concentrations resulted from short-term changes in the colloidal metal fraction (M_T-M_{dyn}), although it should be noted that the water masses sampled by the *in situ* and discrete sampling methods were not identical and therefore the calculated colloidal concentrations have a large uncertainty. Notwithstanding this, for clarity, trendlines have been inserted into the figures.

Since English Channel waters influence Percuil Creek, the range of concentrations of Cd, Pb and Cu determined in this study were compared with total dissolved (<0.4 μm) concentrations reported for waters sampled in the English Channel by Laslett (1995) (Table 4.3). By comparison, Cu concentrations were within the range reported whilst both dynamic and total dissolved Cd and Pb concentrations are enhanced indicating some level of contamination within this estuarine system.

Table 4.3: Metal concentrations determined at Percuil Creek and total dissolved concentrations (<0.4 μm) in English Channel waters reported by Laslett (1995).

Location	Cd (nM)		Pb (nM)		Cu (nM)	
	Cd_{dyn}	Cd_T	Pb_{dyn}	Pb_T	Cu_{dyn}	Cu_T
Percuil Creek	0.13-0.33	0.50-1.74	0.37-0.59	0.46-1.48	4.76-5.80	6.25-17.7
English Channel		0.09-0.20		0.10-0.20		2.0-24.0

Figure 4.5a-c show sharp increases in total dissolved concentrations for all the metals studied that reflect intermittent inputs of colloidal material of varying organic content, since the DOC concentrations did not follow the same profile. However, it may

also be a consequence of manually sampling waters at slightly different depths compared with the VIP system and/or sampling between different boundary layers caused by the tidal dynamics of the flood and ebb tides. To avoid this problem and to ensure the same water masses were analysed, a sample line was secured in close proximity to the sample inlet of the VIP system and water pumped to the surface and collected in a sealed carboy in subsequent sampling campaigns.

Cadmium chloride complexation dominates Cd speciation in marine waters as this element has a high affinity for chloride in marine systems (Millward, 1999). It was observed that the colloidal Cd fraction was high (69-89%) in the Percuil system, particularly when compared to the 15-22% reported for the Tamar Estuary (SW England) by Howell (2003). The colloidal Pb fraction was highly variable (8-75%) in the Percuil system. In agreement, a high proportion of Pb associated with colloids has previously been reported, based on discrete samples taken at a low temporal resolution (65-99%) (Kozelka and Bruland, 1998; Elbaz-Poulichet *et al.*, 1984). Also, in comparison to other workers who report Cu to be highly complexed by organic ligands in estuarine systems (>85-99.9%) (Buck and Bruland, 2005; Bruland *et al.*, 2000), the colloidal Cu fraction determined in this work showed a relatively wide range (24-73%).

No influence of riverine inputs on salinity and total metal concentrations was discernable in Percuil Creek, and the high-resolution measurements of dynamic metal species showed little variability and no link to the tidal dynamics typically found in estuaries. Therefore, waters at this sampling location can be regarded as strongly marine influenced. The marked short-term variation in total metal concentrations indicated anthropogenic inputs of metals which mix with the marine waters. The water depth at the mooring site varied from approximately 4.5 m at low water to 8.5 m at high water. It is likely, therefore, that intermittent inputs of metal-enriched colloidal material may have occurred through mobilisation of sediments caused by physical disturbance as a result of tidal currents or from passing vessels. Whilst sediment concentrations of heavy metals in

the Fal Estuary have been reported to be lowest in Percuil Creek (Somerfield *et al.*, 1994), metal concentrations in the surface sediments (<100 μm) are in general one to three orders of magnitude higher than UK minimum values of 0.003 μg g^{-1} Cd and 1.0 μg g^{-1} Cu (Bryan and Gibbs, 1983). Sediment concentrations determined in previous studies range from 0.24-2.2 μg g^{-1} Cd, 50-75 μg g^{-1} Pb and 179-<400 μg g^{-1} Cu (Braungardt, 1996 unpublished data; Somerfield *et al.*, 1994; Bryan and Gibbs, 1983). Thus the sediments can potentially provide a significant source of metals to the water column within this system.

The relatively constant dynamic concentrations in Percuil Creek at the time of sampling suggest that organisms experience little variation in exposure to biologically available Cd, Pb or Cu over the course of a tidal cycle which was also emphasised in the bioassay results (Figure 4.5d). Moreover, the rapid changes in the colloidal fraction were unlikely to adversely affect biota because of the low potential biological availability of this fraction.

Restronguet Point

The dynamic and total metal concentrations determined at Restronguet Point exceeded the concentrations determined at Percuil Creek. At the start and end of the monitoring period minimum concentrations (0.69 and 0.68 nM Cd_T, 0.31 and 0.23 nM Cd_{dyn}, 25.0 and 19.6 Cu_T, 5.03 and 10.4 nM Cu_{dyn}, respectively, Figure 4.6c,a) were observed. Metal concentrations gradually increased towards the time of low water to maxima of total (2.26 nM Cd_T, 88.0 nM Cu_T) and dynamic concentrations (1.91 nM Cd_{dyn}, 23.2 nM Cu_{dyn}), and subsequently declined again. This pattern, in combination with the observed salinity, indicated that the main source of dynamic and colloidal Cd and Cu was located upstream of the sampling point. In contrast, dynamic Pb declined gradually from 1.36 to 0.49 nM Pb_{dyn} (Figure 4.6b) throughout the tidal cycle, while the total dissolved concentrations showed more random variability (0.58 – 1.74 nM Pb_T) and was reflected in a relatively low and variable colloidal fraction (0-45%). At the sampling position, the wide

estuary narrows abruptly and water depths become very shallow. During our sampling campaign, depths upstream ranged from approximately 1.8-4.1 m at low water to 5.1-7.4 m at high water and at the sampling location 8.2-15 m at low and high water, respectively. Increased suspended matter density was observed at low water and after leisure boat activity in the vicinity of the sampling position. It is likely therefore that the increase in colloidal Pb was a result of mobilisation from the sediments during intermittent disturbance. As for Percuil Creek, the colloidal Pb fraction was lower (0-45%) than typically found for this particle-reactive element (Kozelka and Bruland, 1998; Elbaz-Poulichet *et al.*, 1984), although variable colloidal Pb fractions (6-92%) have previously been observed for this system (Braungardt pers. comm) and similarly for Cd and Cu. As presented in Figures 4.6a and c, Cd was found principally in the dynamic form (41 to 89%) while Cu was primarily in the colloidal fraction (38-80%). The colloidal Cu fraction was highest around low water indicating a riverine source that may include Fe-oxyhydroxides from the acid mine drainage, where scavenging of metals may be implicated.

Bryan and Gibbs (1983) have reported total dissolved concentrations of 2-44 nM Cd and 0.16-1.57 μM Cu in the waters of Restronguet Creek. A more recent study carried out at Restronguet Point determined Cd_T concentrations of between 0.13-8.5 nM, Pb_T from 0.35-3.7 nM and Cu_T between 22.0-315 nM (Braungardt pers. comm). In the same study, dynamic concentrations measured with the VIP system ranged from 0.20-5.1 nM Cd, 0.04-1.3 nM Pb and 2.8-190 nM Cu. By comparison, the total dissolved metal and Pb_{dyn}, concentrations observed in this study were at the lower limit of these ranges, whilst the dynamic concentrations of Cd and Cu were in the middle of the range. The wide range of metal concentrations observed in these studies indicates the complexity of estuarine systems which are subject to variable inputs influenced by tidal dynamics and seasonal variations in freshwater flow.

Overall, lower total dissolved metal concentrations have been determined in this study when compared with other studies, which may reflect on-going efforts to treat waste-

waters from abandoned mines (Neal *et al.*, 2005). However, dissolved metal concentrations in waters entering Restronguet Creek have only showed a limited decrease from 0.77 to 0.35 μM As, 59 to 41 nM Cd, 4.28 to 3.79 μM Cu and 47.5 to 35.5 μM Zn as reported by Langston *et al.* (2003a). Nonetheless, the high levels of Cu reflect the inputs from past mining activities and the high concentrations that remain in the sediments of Restronguet Creek which continue to be influenced by residual drainage from numerous old mines, spoil heaps and groundwater (Williams *et al.*, 1998; Bryan and Gibbs, 1983). For the metals studied, reported concentrations in the sediments of Restronguet Creek varied from 0.8-2.7 μg g^{-1} Cd, 100-330 μg g^{-1} Pb and 800-2800 0.8-2.7 μg g^{-1} Cu (Braungardt, 1996 unpublished data; Somerfield *et al.*, 1994; Bryan and Gibbs, 1983). In addition, concentrations of As and Zn were also elevated as discussed previously in Section 4.3.1. Based on probable effect levels, sediment concentrations of Cu in all the major rivers and creeks within the Fal system are at levels predicted to cause effects to sensitive biota (Langston *et al.*, 2006).

Based on the observation that the larval response effectively paralleled the dynamic metal concentrations determined *in situ* with the VIP system, it is probable that the higher dynamic concentrations measured at Restronguet Point (compared with Percuil Creek) impact on the toxicological response.

4.4.4 Copper speciation in discrete samples

Total dissolved Cu concentrations in discrete samples taken at high water were similar at the two sampling locations (Percuil Creek, PC: 15.2 nM, Restronguet Point, RP: 17.8 nM), while Cu_T at low water at RP was five times higher (88.0 nM, Table 4.4). The concentration of natural copper complexing ligands at low water RP (C_L = 98.2 nM, using 2.5 μM SA) was higher by a factor of 4-5 than that in the high water samples (C_L = 19.5 nM at RP and 26.8 nM at PC). Nevertheless, the labile Cu concentration was highest at low

water at RP (27.6 nM Cu_{lab}), compared with the high water samples (3.31 and 5.93 nM Cu_{lab}, for PC and RP, respectively).

With increasing competition strength higher labile concentrations were determined due to the increased competition for Cu by the added ligand SA and the natural ligands. At the lowest competition strength (2.5 μM SA), between 67% (high water at RP) and 78% (high water at PC) of the total dissolved Cu was complexed by SA. The non-labile Cu was complexed by a relatively weak class (mean log K'_{CuL}=12.1±0.13) of natural ligands, and it is typically the weaker ligands that have been found at highest concentrations in coastal marine waters. While in this study the concentrations of the stronger (C_{L1}) and weaker ligand (C_{L2}) classes differed only by factors of 1.6 at PC, 1.3 at RP (17:49 h) and 1.1 RP (12:22 h), order of magnitude differences in the concentrations of C_{L1} and C_{L2} have been reported previously. For example, Muller (1996) reported C_{L1}=12 nM and C_{L2}=101 nM and Cobelo-Garcia and Prego (2004) C_{L1}=20 nM and C_{L2}=172 nM, for Cu_T of 4.5 nM in both cases. In Percuil Creek, a stronger natural ligand class (log K'_{CuL}=13.1±0.08, with 10 μM SA) was determined that was close to saturation (Cu_L = 15.9 nM) and represented 52% of the total dissolved copper complexed by SA. Titrations using the higher competition strength (25 μM SA) resulted in a linear response of signal to increased Cu additions, indicating either the absence of ligands within this detection window or their full saturation. As discussed in section 3.2.4.1, to reduce the uncertainty in the estimation of C_L and $\log K'_{CuL}$ the competition strength should approximate the binding capacity of the natural ligands ($\alpha_{MAL} \approx \alpha_{ML}$), where α_{MAL} is the centre of the detection window and in this study was 3.77, 4.97 and 5.77 from the lowest to highest competition strengths employed. The results at the lowest competition strength estimated $\alpha_{MAL} < \alpha_{ML}$, consequently there is some uncertainty in the estimated values, although $\log\alpha_{CuL}$ was within the detection window applied (considered to span a decade either side of the centre). Saturation of the ligands by Cu was indicated at the highest competition strength (marked in bold in Table 4.4) with the estimated $\log\alpha_{CuL}$ outside the detection window and therefore the values for

log K'_{CuL} and C_L cannot be determined with confidence (see Section 3.4.3). Despite this, the values have been included for comparative purposes. There is a trend of increasing log K'_{CuL} values and decreasing ligand concentrations (Table 4.4) which has been noted in a number of studies (Buck and Bruland, 2005; Bruland *et al.*, 2000; Campos and van den Berg, 1994; van den Berg and Donat, 1992; van den Berg *et al.*, 1990) and attributed to systems that comprise a mixture of Cu-binding ligands that have a range of complexing strengths. Essentially, at higher competition strengths, the increased concentration of added ligand competes more effectively for the Cu with the natural ligand classes, and out-competes the weaker ligands, so that the natural Cu-binding ligands show an apparent decrease. Overall, these results demonstrate the importance of varying the analytical competition strength in determining the Cu-binding characteristics of natural ligands.

The free cupric ion [Cu^{2+}] concentration, the most toxic Cu species to aquatic organisms (Buck and Bruland, 2005; Sunda *et al.*, 1990), was lowest at Percuil Creek (0.45 pM) due to the presence of non-saturated ligands with stability constants between log K'_{CuL}=12.2 and 13.1. At low water RP, [Cu^{2+}] was an order of magnitude higher (3.60 pM; log K'_{CuL}=11.5) than at PC as a consequence of the higher metal concentrations and lack of buffer capacity from stronger ligand classes. A strong positive linear relationship between [Cu^{2+}] and [Cu_T] was observed across the two locations ($y = 4.10^{-5} x + 7.10^{-5}$; $R^2 = 0.99$) with the slope indicating that on average 0.004% of Cu_T was present as Cu^{2+}. This type of relationship has been found previously in the Gulf of Cádiz (Braungardt *et al.*, 2007a).

The cupric ion concentrations determined in this study were several-fold higher than lower estimates for sites in San Francisco Bay (0.005-0.1 pM) reported by Buck and Bruland (2005) and were similar to estimates of other perturbed estuaries, such as the Scheldt, Severn and Solent (Muller, 1996; Apte *et al.*, 1990; van den Berg *et al.*, 1987) where [Cu^{2+}] has been reported to range between 0.1-3 pM, 0.16-8 pM and 0.03-16 pM, respectively. Furthermore, the results from this study compare well with a study previously

Table 4.4: Total and labile Cu concentrations determined by AdCSV in the discrete samples used for the oyster larvae bioassays. Ligand titrations were carried out in all samples using three different concentrations of SA (2.5, 10 and 25 μM). Stability constants for Cu-ligand complexes (log K'_{CuL}), concentrations of natural ligands (C_L) and cupric ion [Cu^{2+}] are reported for all titrations. Numbers highlighted in bold refer to titrations in which the natural ligand L was saturated with Cu. Logα_{CuLx} is defined as [C_{Lx}]/[Cu^{2+}].

Site	[SA] μM	logα_{CuL1}	logα_{CuL2}	[Cu_{labile}] nM	[Cu_T] nM	Log K'_{CuL1}	C_{L1} (nM)	Log K'_{CuL2}	C_{L2} (nM)	[Cu^{2+}] pM
PC (16:40 h)	2.5	4.7	4.9	3.30	15.2	12.6 ± 0.32	23.6 ± 0.81	11.6	38.0	0.45
	10	4.6	ND	7.32	15.2	13.1 ± 0.08	15.9 ± 0.65	ND	ND	
	25	4.3	ND	9.28	15.2	**14.4 ± 0.16**	**9.64 ± 0.30**	ND	ND	
RP (17:49 h)	2.5	4.4	4.5	5.93	17.8	12.5 ± 0.20	19.5 ± 0.46	11.7	25.1	0.79
	10	4.3	ND	9.85	17.8	**13.1 ± 0.19**	**15.2 ± 1.94**	ND	ND	
	25	4.3	ND	10.7	17.8	**15.7 ± 0.65**	**14.1 ± 0.26**	ND	ND	
RP (12:22 h)	2.5	4.4	4.5	27.6	88.0	11.5 ± 0.09	98.2 ± 1.96	11.3	109	3.60

conducted in the Fal Estaury by Kawakami (2004) where [Cu^{2+}] ranged between 0.02-0.2 pM at Percuil Creek and 1-6 pM at Restronguet Point.

4.4.5 Embryotoxicity of Fal Estuary waters

Free hydrated Cu^{2+} ion concentrations of 10^{-10} to 10^{-11} M have been reported in estuarine environments such as the Elizabeth River Estuary, a polluted tributary of Chesapeake Bay (Sunda *et al.*, 1990) the Itchen River Estuary, north-west of the Solent, an area strongly influenced by sewage disposal, industrial and domestic effluents (Muller, 1996) and harbours on the south coast of Cape Cod, Massachusetts, that are subject to varying degrees of anthropogenic inputs (Moffett *et al.*, 1997). At these enhanced concentrations complete inhibition of cyanobacterial growth has been observed (Brand *et al.*, 1986), survival of naupliar larvae of the marine copepod, *Acartia tonsa* (Sunda *et al.*, 1990) significantly diminished, as did the grazing activity of *A. hudsonica* (Sharp and Stearns, 1997). Furthermore, the viability of many phytoplankton species, such as coccolithophores, dinoflagellates and diatoms declines at concentrations $>10^{-12}$ M (Sunda *et al.*, 1987), and on this basis, the concentration of Cu^{2+} observed at low water at Restronguet Point was biologically relevant.

Greater tolerance to Cu^{2+} has been exhibited by marine invertebrates. For example toxicity testing using bioassays of sea urchin (*Strongylocentrotus purpuratus*) and mussel (*Mytilus galloprovincialis*) larvae resulted in EC_{50} values of EC_{50} <900 pM Cu^{2+} and EC_{50} <60 pM Cu^{2+}, respectively (Rivera-Duarte *et al.*, 2004). Exposure of the oyster larval bioassay with single metals resulted in EC_{50} = 230 ± 80 pM Cu^{2+} and the threshold for toxic response, EC_{05}, has been determined as EC_{05} = 160 ± 50 pM Cu^{2+} (see Section 2.3.4). These values are one to two orders of magnitude higher than those determined at Restronguet Point (low water) and two to three orders of magnitude higher than at Percuil Creek. Therefore it is unlikely that dissolved Cu was the sole agent triggering the toxic response in the bioassay. Nevertheless, at Restronguet Point, the maximum toxic response

coincided with the highest total dissolved and dynamic Cd and Cu concentrations (determined *in situ*), indicating a likely contribution of metals to the overall toxicity of these estuarine waters. Indeed, the toxicity of Cu increased by an order of magnitude in bioassays exposed to a mixture of two metals under controlled laboratory conditions (e.g. for Cu/Cd mix: EC_{50} = 4 pM for Cu^{2+} and for Cu/Pb mix: EC_{50} = 20 pM for Cu^{2+}, see Section 2.3.5), indicating synergistic effects. Furthermore, dynamic Cd showed a positive linear relationship with the larval response ($y=0.02x +0.1531$; $R^2=0.89$) which may be an indication of its potential influence on the toxicological impact in these waters.

Metal contaminants are the principle source of pollution in the lower Fal system (Somerfield *et al.*, 1994). The significant inputs of total dissolved concentrations of Cu, Cd and Pb, as well as the presence of metals other than those studied here, are therefore likely to contribute to the toxicity observed, possibly enhanced through synergy. Furthermore, the heavily impacted sediments provide an additional source to the water column. Although a recent review of water quality in the Fal estuary (Langston *et al.*, 2003a) showed no evidence of regular breaches of water quality standards for the protection of saltwater life, maximum Cu, Pb, As and Zn concentrations near or above the environmental quality standards, EQS (5 $\mu g\ L^{-1}$ Cu, 25 $\mu g\ L^{-1}$ Pb, 25 $\mu g\ L^{-1}$ As, 40 $\mu g\ L^{-1}$ Zn) have been observed.

4.5 Conclusions

Metal contamination is the primary source of pollution in the mid and lower reaches of the Fal Estuary with some areas heavily impacted as observed in this study, notably Restronguet Creek. As such it was a prime location to test the suitability and value of the developed approach (e.g. combining chemical, biological and physical parameters) to characterise natural waters. Moreover, this type of approach is required for successful implementation of the EU Water Framework Directive. Although this study used parent

oysters from Guernsey that were not acclimatised to the Fal Estuary environment this approach allowed the most sensitive response to toxicity to be measured.

The toxicological response to waters collected at two contrasting sites, Percuil Creek and Restronguet Point, was pronounced. Restronguet Point was shown to be highly toxic to the developing larvae of *C. gigas* under low water conditions where the influence of riverine inputs and mobilisation of contaminated sediments was greatest. The concentrations of metals determined in this study were at the lower limit determined for this system in previous studies (Kawakami, 2004; Braungardt pers. comm), which were also shown to vary through time and seasons. Thus an even greater toxic response could be envisaged. Furthermore, when the total dissolved metal concentrations and free ion concentrations determined in previous studies are considered it is likely that other perturbed estuarine systems would elicit similar responses.

The contaminated sediments of Restronguet Creek impact on other areas of the Fal Estuary. Indeed maintenance dredging is commonly carried out in Falmouth harbour where contaminated sediments are problematic (Langston *et al.*, 2003a). Accordingly screening these waters to highlight specific areas of concern would be of benefit, particularly since shellfish farming takes place in some parts of the Fal (Langston *et al.*, 2003a).

Overall, the benefit of high resolution *in situ* measurements that detect small-scale changes in biologically relevant metal species was evident and comparison with bioassay results showed the levels of toxic response effectively paralleled the metal speciation measurements. Thus ecologically relevant information was provided. Moreover, this study showed that the embryos of *Crassostrea gigas* were sensitive to dissolved metal species and the bioassay to be an effective screening tool for metal perturbed areas.

Chapter 5:

Tamar Field Study:

Chemical and biological investigation in an estuary affected by urban and industrial inputs

5. 1. Introduction

The study presented in this Chapter took the methods that were successfully applied in the Fal Estuary to a contrasting coastal environment, the Tamar Estuary. The Tamar is affected by past mining activities, albeit not on the same degree as some areas within the Fal district, but is subject to other anthropogenic influences that are largely related to the anthropogenic activities conducted in the vicinity of the city of Plymouth. In order to provide a more comprehensive overview of the estuarine biogeochemistry and other processes that may affect biological responses, additional parameters were incorporated into the study of the Tamar.

The main aims and objectives of this study were to:

(1) Assess the toxic response of the early developmental stages of the Pacific oyster, *Crassostrea gigas*, to waters of the lower Tamar Estuary, in comparison to waters collected at coastal reference sites.

(2) Determine temporal and spatial trends in trace metal speciation, through high-resolution *in-situ* measurements with the VIP system over periods of full tidal cycles at three locations within the lower Tamar estuary.

(3) Study the dissolved Cu speciation in detail using competitive ligand titrations.

(4) Investigate the importance of the concentration and dissolved speciation of Cd, Pb and Cu in determining the toxic response observed in bioassays.

(5) Determine the nutrient status in the water column.

(6) Compare the results with a previous study, conducted in a similar manner, in the Fal Estuary.

5. 2. Materials and methods

5.2.1. Site description and field sampling locations

The Tamar Estuary (Figure 5.1) is located in SW England and stretches from Gunnislake weir (upper tidal limit) to the entrance to Plymouth Sound (lower tidal limit), a distance of about 31 km. As well as the Tamar, the estuary includes two tidal sub-estuaries, the Tavy and the Lynher (Miller, 1999). The Tamar has the following physico-chemical characteristics; a mean river flow of 19 $m^3 s^{-1}$, tidal range for neaps to springs of 2.2-4.7 m, tidal excursion 30 km, dissolved oxygen 5-10 mg L^{-1}, suspended particulate matter (SPM) <5-1000 mg L^{-1}, pH 6.8-8.5, and flushing time 7-12 d (Millward, 1995). The Tamar catchment covers an area of ca. 1700 km^2 (Langston *et al.*, 2003b) with the upper estuary dominated by rural land use and old mines and the lower reaches subjected to significant urban/industrial development. The eastern shore of the lower estuary is influenced by the City of Plymouth and the naval dockyard with its associated ships and boat-building yards, and more recently nuclear submarine maintenance. Past metal mining activities (principally Cu and As and to a lesser extent Sn, Zn, Pb, Ag and W) in the Tamar, Lynher and Tavy catchments continue to influence the waters and sediments of the Tamar Estuary. Elevated dissolved As, Cu and Zn concentrations have been observed in the upper reaches of the Tamar (and in the Tavy and Lynher), although in general do not exceed environmental quality standards (EQS, 0.232 μM As, 0.079 μM Cu and 0.612 μM Zn Langston *et al.*, 2003b). Notably, porewaters may contain Cu and Zn concentrations of 0.21-3.15 μM and 0.20-13.9 μM, respectively, which are one to two orders of magnitude higher than in the overlying water and therefore of potential toxicological significance (Ackroyd *et al.*, 1986). Metals in the sediments of the Tamar Estuary, Lynher, Restronguet and Tees Bay (baseline values) are detailed in Table 5.1 for comparison. Whilst the concentrations of these elements are enriched in the sediments of the Tamar catchment, compared with baseline values, they are in general an order of magnitude lower than those observed in parts of the Fal system (Langston *et al.*, 2003b).

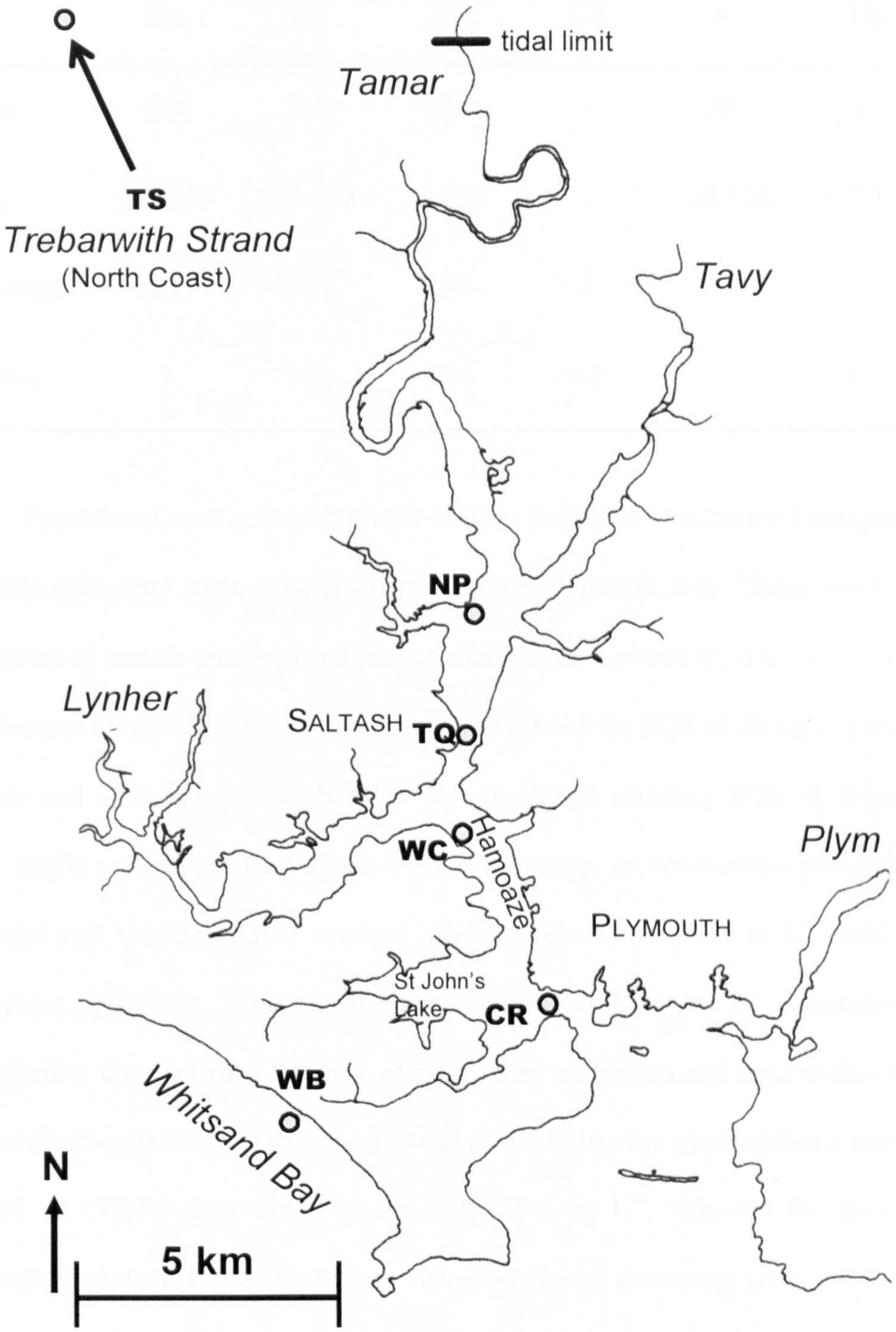


Figure 5.1: Sampling locations in the Tamar Estuary and coastal zone. Tidal cycle monitoring stations are marked CR: Cremyl, WC: Wilcove and TQ: Saltash Town Quay. Additional discrete samples were taken at the reference sites TS: Trebarwith Strand at Cornwall's north coast (off the map), WB: Whitsand Bay and at NP: Neal Point, the confluence between the Tamar and Tavy Estuaries.

Table 5.1: Metals in sediments (μg g^{-1} dry wt) from UK sites

Site	Cu	Zn	Pb	Cd	As	Hg
Lynher	274	317	150	-	50.7	2.1
Tamar	145-545	221-605	19-239	-	25-236	0.2-1.5
Restronguet	1690	1540	684	3	1080	-
Tees Bay	8	74.1	45.4	0.2	-	0.1

Provisional conclusions from the Marine Pollution Monitoring Management Group in 1998 highlighted some concerns regarding the Plymouth area. These concerns included high levels of metals (As, Hg) and polyaromatic hydrocarbons (PAHs) in Tamar sediments and elevated levels of lindane (sometimes in excess of the EQS of 20 ng L^{-1}) and pesticides atrazine and simazine (although below the combined statutory EQS of 2 μg L^{-1}) in the water. PAHs generally reflect inputs from a wide range of combustion processes involving industrial and vehicle related sources, where concentrations >1 μg L^{-1} (total PAHs) are considered significant. Principal 'hotspots' for several PAHs are the Hamoaze (Figure 5.1) and opposite the dockyard. Sources of pesticides, herbicides and insecticides include run-off and discharges from STWs. Langston *et al.* (2003b) also reported that concentrations of tributyl tin (TBT) often exceeded the EQS of 2 ng L^{-1}, although the data showed an apparent downward trend. There was also evidence of decreased usage of the insecticide lindane.

Despite the concerns, the Tamar Estuary supports a more diverse flora and fauna compared with parts of the Fal system. In the broader lower reaches, particularly around the mouth of the River Lynher and at St. Johns Lake, extensive tidal mud-flats are present which contain an infaunal community of bivalves and other invertebrates (Langston *et al.*, 2003b).

5.2.2. Sampling strategy and protocol

At three sampling stations, Cremyll (CR), Wilcove (WC) and Saltash Town Quay (TQ), surveys were carried out from a small vessel (16 ft wooden leisure yacht) anchored for 9.5-11 h on 15th,16th and 17th May 2006, respectively. The monitoring periods were chosen to cover low water (14:00 h, 14:40 h and 15:13 h GMT on 15, 16, 17th May, respectively) and near high water (20:07 h, 20:40 h and 21:22 h GMT, respectively) at each site. Additional samples were collected near the time of low water from the shore at the coastal reference locations of Trebarwith Strand (TS, North Cornwall) and Whitsand Bay (WB) (Figure 5.1), and at the confluence of the Tavy and Tamar Estuaries at Neal Point (NP).

Trace metal clean techniques were used for all sample processing and measurement steps. Sample bottles were rinsed three times with the sample before final sample collection. At Cremyll, Wilcove and Town Quay, the VIP was deployed to measure the *in situ* speciation of Cd, Pb and Cu at ca. 2 m depth with a temporal resolution of ca. 35 min. A CTD (YSI 6600 multiparameter sonde, OSIL, Havant, UK) was lowered to the same depth as the sample inlet to measure *in situ* temperature, pH, conductivity and turbidity (NTU) with a temporal resolution of 1 min. Due to technical problems the CTD data available for the tidal cycle at Cremyll was patchy. Discrete samples were obtained at regular intervals (1-1.5 h) by vacuum-pumping (Dymax 30, Charles Austen Pumps, UK) into a polycarbonate (PC) carboy (20 L) or glass bottle for laboratory dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) determinations. The pump tubing was attached to the VIP in close proximity to the sample inlet for dynamic metal analyses. Vacuum filtration, with collection into LDPE containers, was performed on-line using a filter cartridge (Swinnex-47, Millipore, US; 0.4 μm hydrophilic PC membranes, Cyclopore, Whatman UK)) to obtain water for trace metal determinations, bioassay exposures, salinity and nutrient determinations (N, P and Si), or into glass vials (I-chem, Techmate Ltd, Milton Keynes, 0.7μm GF/F, Whatman) for DOC and TDN analysis.

Samples were either acidified (Q-HNO_3 for total dissolved metal concentrations and Q-HCl for DOC and TDN) or immediately placed in the dark on dry ice awaiting transport and freezing in the laboratory each evening. Discrete bulk samples (10 L) were collected by hand from the shore and were filtered and stored in the same way.

DOC and TDN concentrations were determined simultaneously using high temperature catalytic combustion (Badr *et al.*, 2003) by X. Pan (National Oceanography Centre, Southampton). Nutrient analyses were undertaken on thawed samples using standard AutoAnalyzer techniques (Skalar, Flow Access, 2001) by R. Tuckwell (University of Plymouth).

5.2.3. Reagents and equipment

The preparation and treatment of reagents and equipment used in this study are as detailed in Section 4.3.3 with references to other relevant sections.

5.2.4 Trace metal analysis

5.2.4.1. Voltammetric *In situ* Probe

The reader is referred to Chapter 3 for a description of the VIP system (section 3.2.5) and the preparation of the voltammetric cell and gel-integrated microelectrode (GIME) (section 3.3.2.3). Operating conditions and data treatment for the simultaneous determination of Cd, Pb and Cu were the same as those detailed in Chapter 4, section 4.3.4.1, with slight differences in the deposition potential in the field (Table 5.2)

5.2.4.2 Hanging mercury drop electrode (HMDE)

Anodic stripping voltammetry (ASV) was used to determine total dissolved Cd and Pb and adsorptive cathodic stripping voltammetry (AdCSV) for Cu, using the methods and conditions detailed in sections 3.3.1.3 and 3.3.1.4.

Table 5.2: Conditions for the simultaneous determination of Cd(II), Pb(II) and Cu(II) using SWASV with the VIP system.

Step	Calibration conditions	Field conditions
Deposition potential (mV)	-1270	-1300 to -1200
Deposition time (min)	15	20 to 35
Equilibration time (deposition potential) (s)	30	30
Final potential (mV)	+175	-100 to -50
Cleaning time (final potential) (s)	60	60
Pump time (s)	90	90
Pause (mV ; s)	-80 ; 0	-80 ; 0
Pulse amplitude (mV)	25	25
Pulse step (mV)	8	8
Frequency (Hz)	200	200

Table 5.3: Analytical parameters used for Cu titrations. Modulation was square wave, depositon potential was -0.12 V, scan frequency 50 Hz, scan was from -0.12 to -0.55 V, step amplitude 25 mV, step potential 2.44 mV, Hg drop size # 3, purge time 180 s. Salicylaldoxime was used as competing ligand at 25 μM concentrations for total Cu concentrations.

Parameter	Whitsand			Cremyll			Wilcove			Town Quay			Neal Point		
SA (μM)	2.5	10	25	2.5	10	25	2.5	10	25	2.5	10	25	2.5	10	25
Cu_T (nM)	4.83	4.83	4.83	23.3	23.3	23.3	27.8	27.8	27.8	36.4	36.4	36.4	45.6	45.6	45.6
Upper limit of Cu added (nM)	40	40	40	140	140	140	160	160	160	200	200	200	160	160	160
Deposition time (s)	30	20	10	30	5	2	10	5	2	10	5	2	20	20	10
Stirrer setting (#)	6	6	6	6	2	1	4	2	2	5	5	5	6	6	4
Equilibration time (s)	8	8	5	8	5	5	8	5	5	8	5	5	5	5	5

Competitive ligand-exchange Cu titrations at different detection windows were carried out based on the procedure detailed in Campos and van den Berg (1994) and described in section 3.3.1.5. The working conditions for the Cu-ligand titrations are presented in Table 5.3. For each competition strength or detection window, the van den Berg/Ruzic linearization method was used to determine the concentration of the Cu-binding ligand concentration (C_L) and its conditional stability constant (log K'_{CuL}) (Ruzic, 1982; van den Berg, 1982, refer to Section 3.2.4.2).

5.2.5 Toxicity test

The toxicity of the water collected from the sample sites was assessed by means of the oyster-embryo larval bioassay and based on the method adopted by the Environment Agency (EA, 2004). See Sections 2.3.4.1 and Section 4.2.5 for the methods and details that relate to the field study, respectively.

5.3. Results and Discussion

5.3.1. Master variables

The physical parameters observed during the survey showed that the estuarine waters were dominated by marine influence (Table 5.4). The salinity measured in samples taken at low water ranged from S = 34.9 at the marine site at Whitsand Bay to S = 22.2 at Neal Point, the most landward site (Figure 5.1). At tidal cycle stations, the observed salinity range was S = 32.3-35.6 at Cremyll (Figure 5.2), S = 31.1-34.0 at Wilcove (Figure 5.3) and S = 29.1-33.4 at Town Quay (Figure 5.4), with the lowest salinities occurring ca. 1-2 h after low water. The water temperature ranged from 11.7°C at Cremyll to 14.3°C recorded at Town Quay. The turbidity measured *in situ* was lowest at Cremyll (<1 NTU) and highest during low water at Town Quay (29 NTU).

Table 5.4: The ranges of physical and chemical parameters determined at the *in situ* sampling sites of Cremyll (CR), Wilcove (WC) and Town Quay (TQ).

Site	pH	Temp (^{0}C)	Salinity (ppt)	Turbidity (NTU)	N (μM)	P (μM)	Si (μM)	DOC (μM)	TDN (μM)
CR	8.32-8.44	12.2-13.0	32.3-35.6	0.1-0.9	0.33-1.70	0.23-0.30	0.71-1.59	118-180	12.1-23.3
WC	8.40-8.55	12.7-14.4	31.1-34.0	0.2-5.4	0.17-0.55	0.22-0.25	0.71-8.81	127-170	10.3-33.6
TQ	8.39-8.49	13.1-14.3	29.1-33.4	0.7-29	ND	ND	ND	127-252	11.1-18.7

ND = not determined

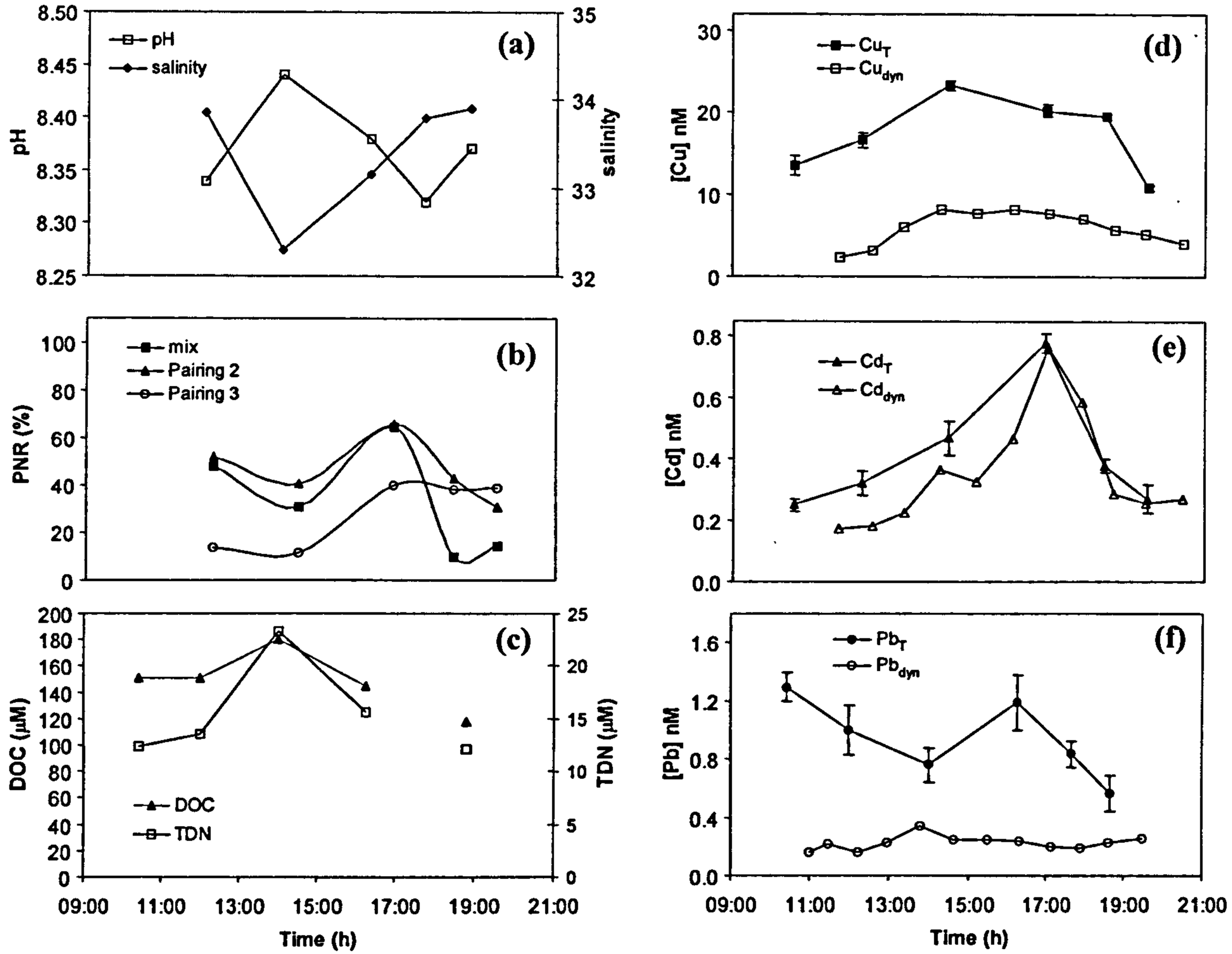


Figure 5.2: Cremyll: Time series of salinity (a), percent net response (PNR) of oyster larvae to discrete water samples (b) for three pairings (P2, P3 and mixed), dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (c), dynamic and total Cu (d), Cd (e) and Pb (f) concentrations (M_{dyn} and M_T, respectively). Dynamic metal concentrations were measured *in situ*, the remaining parameters were determined in discrete samples. Error bars represent ± 2σ.

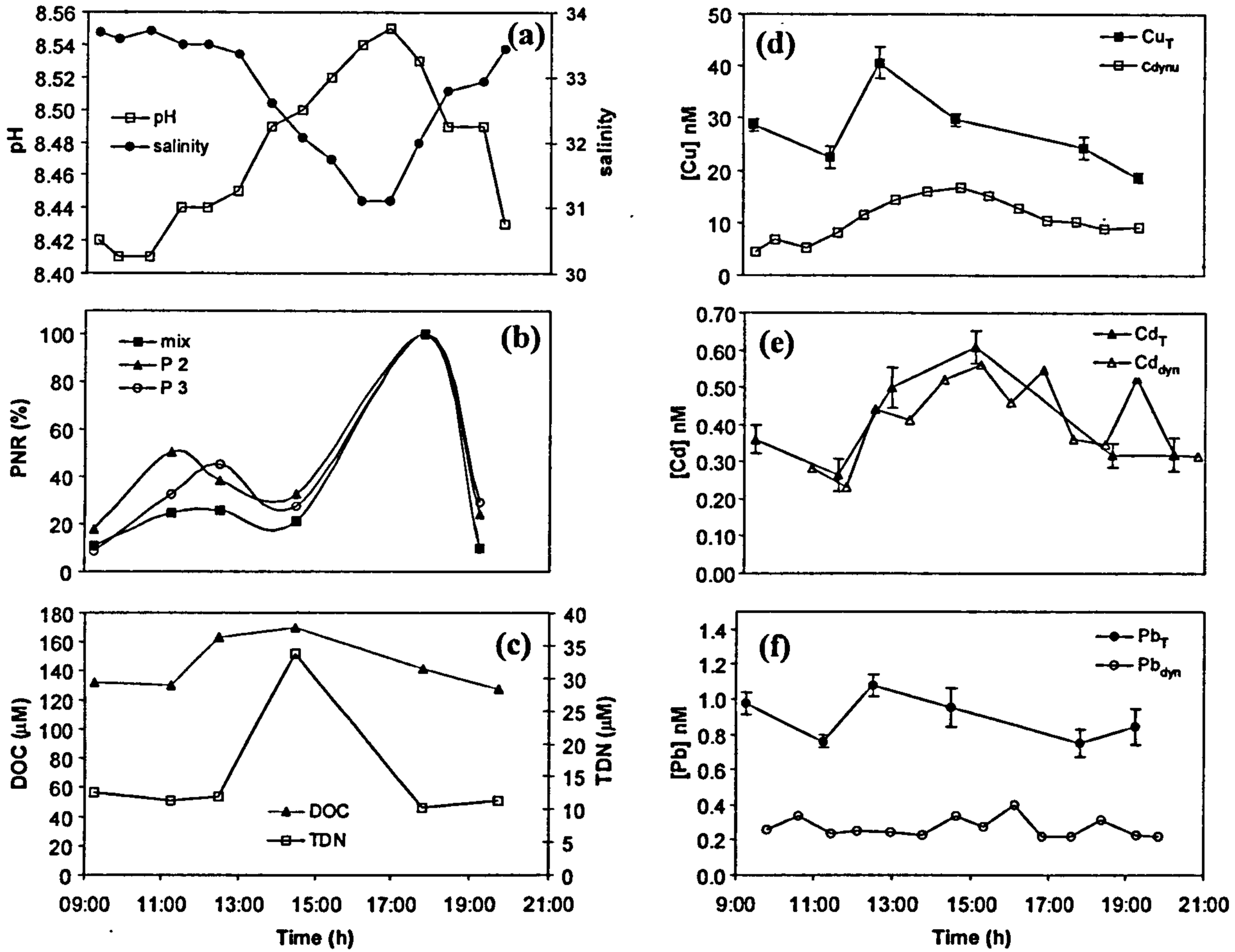


Figure 5.3: Wilcove: Time series of pH and salinity (a), percent net response (PNR) of oyster larvae to discrete water samples (b) for three pairings (P2, P3 and mixed), dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (c), dynamic and total Cu (d), Cd (e) and Pb (f) concentrations (M_{dyn} and M_T, respectively). Salinity, pH and dynamic metal concentrations were measured *in situ*, the remaining parameters were determined in discrete samples. Error bars represent $\pm 2\sigma$.

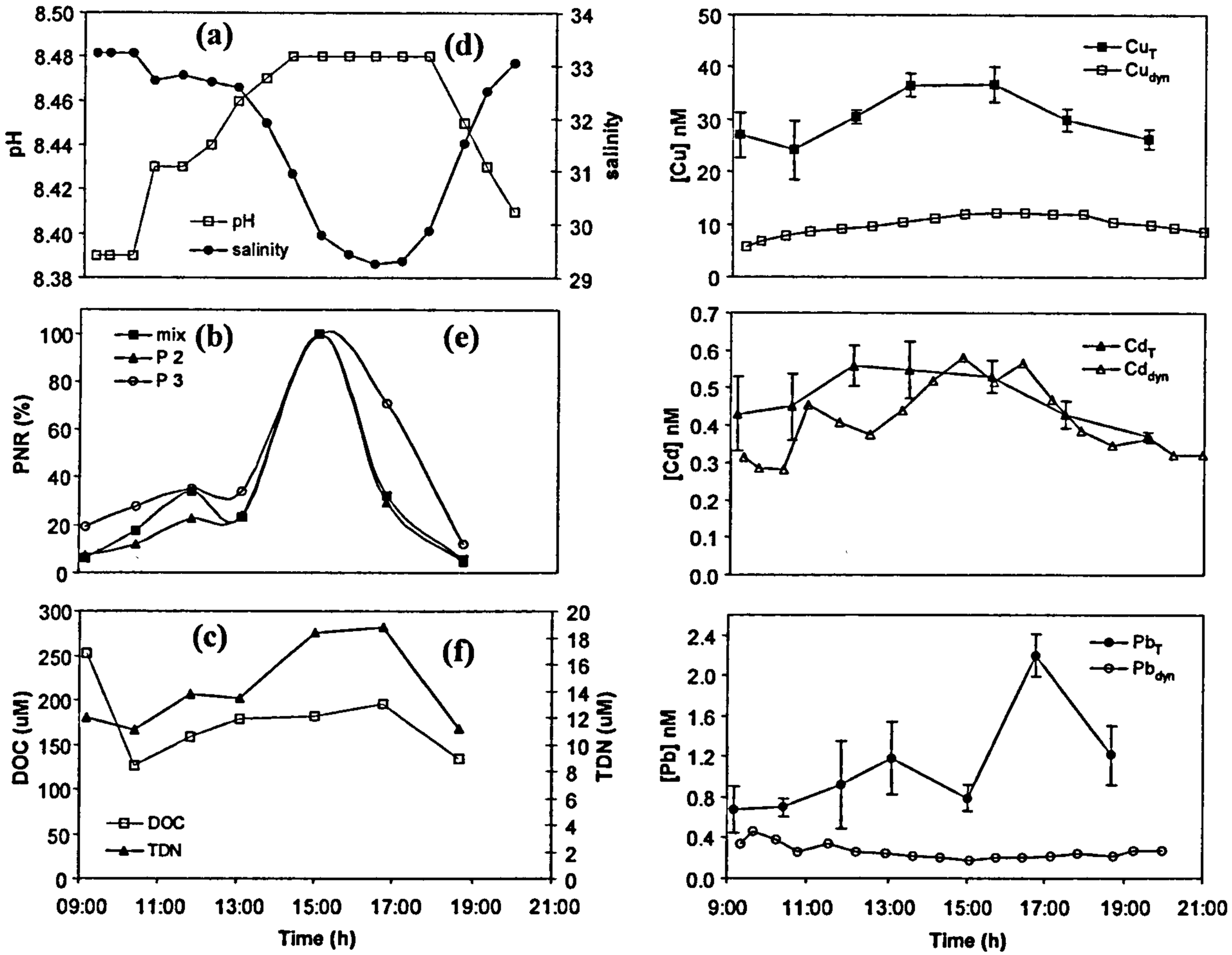


Figure 5.4: Saltash Town Quay: Time series of pH and salinity (a), percent net response (PNR) of oyster larvae to discrete water samples (b) for three pairings (P2, P3 and mixed), dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) (c), dynamic and total Cu (d), Cd (e) and Pb (f) concentrations (M_{dyn} and M_T, respectively). Salinity, pH and dynamic metal concentrations were measured *in situ*, the remaining parameters were determined in discrete samples. Error bars represent ± 2σ.

During tidal cycle studies at Cremyll, Wilcove and Town Quay, the pH was relatively constant (pH 8.44 ± 0.16) and slightly elevated compared with the average sea water pH (pH ~8.2, Chester, 1993), suggesting enhanced phytoplankton production (Howell et al., 2003), and this was supported by the observation of an algal bloom during the flood and ebb tides at Wilcove. Dissolved inorganic nutrient concentrations in the lower Tamar estuary were 0.17-1.7 μM N, 0.22-0.30 μM P and 0.71-8.81 μM Si, with lowest N concentrations at low water, and highest Si levels throughout the tidal cycle at Wilcove. Compared with previous studies in the Tamar estuary, where 5-570 μM N and 0.97-1.73 μM P were observed (Langston *et al.*, 2003b and references therein; David *et al.*, 2001), the relatively low concentrations determined in the present work indicate the utilisation of N and P by phytoplankton at the time of the survey. Dissolved organic nitrogen (DON) + ammonium, defined as the difference between total dissolved nitrogen and total oxidised nitrogen (nitrate + nitrite), predominated (> 86%) with concentrations that ranged between 10.1 and 33.4 μM N. In a year long study in the upper Tamar Estuary, Mankasingh (2005) found that TDN was typically dominated by total oxidised nitrogen (50–100%; mean 82%), except in the months of April and May. During the latter period, biological productivity intensified and this was indirectly linked to an increase in DON. No linear correlation between TDN and total oxidised nitrogen was observed by Mankasingh during April and May, which is in agreement with the current data (R^2 = 0.013). Furthermore, a strong positive correlation (R^2 = 0.98) between TDN and DON in the current study indicates that biological production was a major factor controlling the nutrient concentrations during the surveys. Dissolved organic carbon (DOC) ranged between 120 and 200 μM C, and this was consistent with levels reported for the Tamar estuary in previous studies (110-487 μM C, Miller, 1999). DOC and TDN levels (and DON, data not shown) largely co-varied during the tidal cycles (e.g. Town Quay: Figure 5.4c), with maxima coinciding with the lowest salinity at all locations, indicating that the

dominant source of organic nitrogen and carbon was located upstream of Saltash Town Quay.

5.3.2. Bioassays

The proportion of abnormal oyster larvae development (normalised percent net response, PNR) in response to exposure to discrete water samples from the Tamar Estuary is presented in Table 5.5 and Figures 5.2b, 5.3b and 5.4b for Cremyll, Wilcove and Saltash Town Quay, respectively. The coefficient of variation (CV) for replicate exposures (n=3) was $\leq 33\%$ and was obtained for replicate exposures to all samples from pairings P2, P3 and the mix. A CV > 40% was obtained for most sample replicates for P1 and therefore P1 has been excluded from further discussion. Since identical exposure conditions were used throughout, the response from P1 was most likely due to genetic variability, although positioning in the growth chamber, bacterial growth and exudate release by developing embryos and/or any bacteria present may be contributing factors. The difference in response between P2, P3 and the mix were not statistically significant between pairings (P >>0.05 in all cases, Fishers LSD, Statgraphics, Version 5.1) and no significant difference between sites was indicated (P>> 0.05).

Table 5.5: Results from exposure of oyster larvae to Tamar water samples in percent net response as a measure of abnormal development. Values are normalised to the respective control values for each pairing/mix of eggs. Number of samples at each site given as n. Negative values indicate a response superior to the control.

Site	n	P1	P2	P3	Mix
WB	1	3.2	35	-2.5	16
TS	1	13	31	17	22
CR	6	6.1-65	31-66	11-40	9.6-64
WC	6	-3.0-94	17.5-100	8.5-100	11-100
TQ	7	1.9-98	12-100	5.6-100	4.5-100
NP	2	10-17	97-100	54-99	47-100

The lowest PNR during tidal cycles (≤31%) occurred around high water at Cremyll, Wilcove and Town Quay (at 08:00, 08:30 and 09:11 h, respectively), when the seawater influence was at its greatest. The two coastal locations (Whitsand Bay and Trebarwith Strand) were sampled at low water and triggered a PNR between 0 and 35%, showing similar toxicity levels to high water conditions in the lower estuary. The larval response to the waters collected at Cremyll remained relatively uniform, with the maximum toxic response represented as PNR = 66%. Further upstream PNR = 100% was reached during tidal cycles at Wilcove and Town Quay. Complete abnormal development was also observed for the low water sample at Neal Point, suggesting that the main source of toxicity was located upstream. This is supported by data from the tidal cycle at Town Quay, where the maximum toxic response coincided with the sample taken just after low water with the lowest salinity (Figure 5.4a,b). At Cremyll and Wilcove, the highest PNR occurred in samples taken two hours after low water (Figures 5.2a,b and 5.3a,b, respectively), while low water samples triggered modest responses (mean PNR = 27% for Wilcove and 28% for Cremyll). This indicates that the main agent causing embryotoxicity was carried to these locations with the flood tide, and illustrates the complexity of estuarine dynamics and the importance of monitoring over at least one complete tidal cycle.

5.3.3. Trace metals in the Tamar Estuary

Total dissolved and dynamic Cd concentrations spanned 0.25-0.78 nM Cd_T and 0.17-0.77 nM Cd_{dyn}, respectively (Table 5.5). The colloidal fraction of Cd ($Cd_T - Cd_{dyn}$) was low, and ranged between 0 and 44% of the total dissolved concentration, and these findings were consistent with previous reports for the Tamar estuary (Howell *et al.*, 2003a). Consequently, most of the Cd was present in the dynamic fraction, and therefore potentially biologically available. While total dissolved concentrations were determined at longer time intervals (every 1-2 h), the dynamic Cd concentrations measured *in situ* (every

35 min) showed significant short-term in-water variability (e.g. Figures 5.2e, 5.3e and 5.4e), most likely a result of chemical and physical estuarine processes. Since the time intervals for total dissolved concentration analysis were not coincident with the measurements of the dynamic fraction, more meaningful interpretation of the data is difficult. Nonetheless, this observation further illustrates the advantage of higher resolution *in situ* measurements for observing short-term variability in dynamic estuarine systems, as well as the requirement for more rigorous (i.e. more frequent) sampling campaigns.

In contrast to Cd, the colloidal fraction dominated the Pb speciation (45-90%), with total and dynamic concentration ranges of 0.57-2.20 nM Pb_T and 0.16-0.40 nM Pb_{dyn}, respectively (Table 5.6). Lead is highly particle reactive (Elbaz-Poulichet *et al.*, 1984) and associations with colloids have been shown to be important (Howell *et al.*, 2006; Hart and Davies, 1981).

Total dissolved Cu concentrations ranged between 10.9 and 46.3 nM in the Tamar, with lowest and highest values observed at Cremyll and Neal Point, respectively. Dynamic Cu concentrations followed the same pattern, with lowest levels (2.3 nM Cu_{dyn}) at Cremyll and highest (24.4 nM Cu_{dyn}) at Neal Point. The non-dynamic fraction ranged between 46% and 85%, indicating the presence of an important fraction of Cu complexing ligands that reduced the biologically available dynamic concentration.

Metal concentrations at the reference site Trebarwith Strand were lower (0.20 nM Cd_T, 0.22 nM Pb_T and 2.81 nM Cu_T) than those observed in the Tamar. Within the estuary, the maximum concentrations of Cd and Pb were not observed furthest upstream, but at Cremyll (Cd) and Saltash TQ (Pb). Copper and Pb were actively mined up to the early 20th century in the catchments of the Tamar, Tavy and Lynher (Dines, 1956) and Cd is a common guest metal in the mineralisation. Dissolved and solid outputs from the abandoned mines continue to enrich estuarine water and sediment with metals, making the sediment a potential source of contamination to the water column. In addition, inputs of waters containing various concentrations of dissolved metals from the tributaries, sewage

Table 5.6: Dynamic and total Cu, Pb and Cd concentrations measured *in situ* and in discrete samples, respectively, during tidal cycle surveys at Cremyll (CR), Wilcove (WQ) and Saltash Town Quay (TQ). The colloidal fraction has been calculated as the difference between total and dynamic concentrations and is given in percent.

	Cd (nM)			Pb (nM)			Cu (nM)		
Site	$Cd_{dynamic}$	Cd_T	Colloidal (%)	$Pb_{dynamic}$	Pb_T	Colloidal (%)	$Cu_{dynamic}$	Cu_T	Colloidal (%)
CR	0.17-0.77	0.25-0.78	5-44	0.16-0.25	0.57-1.30	55-87	2.3-8.1	10.9-23.3	52-80
WC	0.27-0.61	0.27-0.61	0-17	0.22-0.40	0.75-1.08	65-78	4.4-16.7	18.7-40.7	46-85
TQ	0.37-0.56	0.37-0.56	2-38	0.18-0.34	0.68-2.20	45-90	5.7-12.3	24.2-36.7	60-79

treatment works and industry along the mid-and lower reaches of the estuary (Langston *et al.*, 2003b), as well as mobilisation from the sediment, will influence the dynamic and total metal concentrations encountered at the monitoring stations over a period of a tidal cycle and longer time scales.

The time series at Cremyll, Wilcove and Town Quay showed varying ratios of dynamic to total metal concentrations throughout the tidal cycle (e.g. Figures 5.2d-f, 5.3d-f and 5.4d-f), from which up-or downstream sources of dynamic and non-dynamic metals may be inferred. Dynamic and non-dynamic Cu was carried to the three monitoring stations on the ebb-tide (upstream source), and generally reached maximum concentrations near low water. Cd showed a similar trend, although at Town Quay, a secondary input of dynamic Cd occurred one hour after high water, which may well be related to remobilisation of particle-complexed Cd through chloride complexation at enhanced salinity (Comans and van Dijk, 1998), followed by an increase in the non-dynamic fraction (partial downstream source). Secondary inputs also occurred at Wilcove, one and four hours after low water when the non-dynamic fraction was decreasing, implying a downstream source. In contrast to Cu and Cd, no trend was discernable for dynamic Pb concentrations, which showed little variation. This behaviour is most likely the consequence of the particle reactive nature of Pb, resulting in scavenging and low dissolved concentrations (Hart and Davies, 1981). At Town Quay (Figure 5.4f) and Cremyll (Figure 5.2f), a sharp increase in the colloidal Pb fraction was observed two hours after low water, probably released from freshly suspended sediment on the advancing tide.

In general, the results from this study show similarities to the observations made over a tidal cycle at Restronguet Point (Fal Estuary) where the main source of dynamic and colloidal Cd and Cu was located upstream of the sampling point. No trend was observed for Pb where Pb_{dyn} gradually declined over the tidal cycle and colloidal Pb showed random variability that was attributed to intermittent inputs from mobilisation of sediments that may have been from up-or downstream sources. Highly variable colloidal metal fractions

of all three metals studied were also determined over the tidal cycle at Percuil Creek (where the dynamic metal concentrations remained relatively constant). Whether this variability was an artefact of the sampling methodology used in the Fal study is uncertain, although the method used in the Tamar study was preferred since similar water masses have been analysed.

5.3.4. Copper speciation in discrete samples

Total dissolved Cu concentrations in samples used for bioassays increased from the coastal location (Whitsand Bay: 4.83 nM) into the estuary (Cremyll: 23.3 nM) and upstream to Neal Point (NP: 45.6 nM) (Table 5.7). The labile concentrations determined using the weakest competition strength (2.5 μM SA) were lowest at Whitsand Bay (1.37 nM Cu_{lab}), intermediate at Wilcove and Town Quay, and highest at Cremyll and Neal Point (7.9 nM). Between 66% (Cremyll) and 90% (Wilcove) of the total Cu was complexed by SA, which corresponds to a relatively weak class (mean log K'_{CuL}=12.8±0.28) of natural ligands, for which the concentration increased with Cu_T between the coastal sample (Whitsand Bay: 9.39 nM C_L) and Neal Point (50.4 nM C_L). This trend has also been observed for other coastal waters (Braungardt *et al.*, 2007a; Kozelka and Bruland, 1998), and is related to the production of Cu complexing ligands by phytoplankton as a response to Cu stress (Croot *et al.*, 2000). At Neal Point and Saltash Town Quay, titrations at higher competition strengths (10 and 25 μM SA) showed that natural ligands with higher binding strengths were saturated with Cu, while further downstream (Wilcove, Cremyll and Whitsand Bay), non-saturated stronger ligands (log K'_{CuL}=13.0-15.3) were detected. As discussed in Chapter 3, section 3.2.4.1, the application of more than one competition strength allows different ligand classes to be determined with greater confidence, particularly when $\alpha_{CuAL} \approx \alpha_{CuL}$, where the alpha-coefficient for the Cu-natural ligand complex (α_{CuL}) is defined as $[C_{Lx}]/[Cu^{2+}]$. Mostly, at the lowest competition strength it was estimated $\alpha_{CuAL} < \alpha_{CuL}$ and therefore the values of C_L and $\log K'_{CuL}$ suffer some uncertainty.

Table 5.7: Total and labile Cu concentrations determined by AdCSV in the discrete samples used for the oyster larvae bioassays. Ligand titrations were carried out in all samples using three different concentrations of SA (see Section 5.2.4). Stability constants for Cu-ligand complexes (log K'_{CuL}), concentrations of natural ligands (C_L) and cupric ion concentrations [Cu^{2+}] are only reported for titrations in which the natural ligand L was not saturated with Cu. Numbers highlighted in bold refer to titrations in which the natural ligand L was saturated with Cu. Logα_{CuLx} is defined as [C_{Lx}]/[Cu^{2+}].

Site	[SA] µM	logα_{CuL1}	logα_{CuL2}	[Cu_{labile}] nM	[Cu_T] nM	Log K'_{CuL1}	C_{L1} (nM)	Log K'_{CuL2}	C_{L2} (nM)	[Cu^{2+}] pM
NP	2.5	4.77	4.93	7.89	45.6	12.6 ± 0.23	50.4 ± 1.09	11.8	72.5	0.86
	10	4.59		25.7	45.6	**13.5 ± 0.03**	**33.7 ± 0.16**			
	25	4.46		16.5	45.6	**14.7 ± 0.25**	**25.0 ± 0.36**			
TQ	2.5	4.87	4.87	5.40	36.4	13.3 ± 0.84	43.9 ± 1.12	12.4	44.1	0.59
	10	4.57		13.1	36.4	**14.4 ± 1.03**	**22.0 ± 0.94**			
	25	4.36		25.6	36.4	**14.7 ± 0.37**	**13.6 ± 0.39**			
WC	2.5	5.73	5.73	2.65	27.8	12.9 ± 0.58	48.1 ± 1.90	11.8	63.3	0.09
	10	5.52		1.67	27.8	14.5 ± 0.66	29.9 ± 0.53			
	25	5.50		2.51	27.8	15.3 ± 0.70	28.8 ± 0.54			
CR	2.5	5.11	5.12	7.9	23.3	12.7 ± 0.18	39.1 ± 0.52	12.4	42.4	0.30
	10	4.90		11.3	23.3	13.0 ± 0.06	23.6 ± 1.01			
	25	4.67		10.1	23.3	**14.3 ± 0.12**	**14.0 ± 0.47**			
WB	2.5	4.59	4.59	1.37	4.83	12.7 ± 0.03	9.39 ± 0.10	12.3	11.4	0.24
	10	4.35		2.49	4.83	13.7 ± 0.05	5.38 ± 0.10			
	25	4.06		2.60	4.83	**15.9 ± 0.99**	**2.78 ± 0.13**			

Despite this, at Wilcove, Saltash Town Quay and Whitsand Bay the values of α_{CuL} are within the detection window (considered to span a decade either side of the centre) and therefore are less uncertain. There was more confidence in the estimated values at higher competition strengths (when the natural ligands were unsaturated) where $\alpha_{CuAL} \approx \alpha_{CuL}$. By applying three competition strengths different ligand classes were determined and a more detailed description of Cu speciation was achieved. Indeed two ligand classes were determined at the lowest competition strength which further illustrates that a continuum of ligands with different binding strengths exists in natural waters.

The concentration of the most toxic Cu species to aquatic life, the free cupric ion [Cu^{2+}], was lowest at Wilcove (0.09 pM Cu^{2+}), due to the presence of excess concentration of ligands with stability constants between log K'_{CuL}=12.9 and 15.3, which was within the range of free ion concentrations observed in near-surface marine waters of the North Pacific (0.01-0.1 pM Cu^{2+}, Sunda and Huntsman, 1995; Coale and Bruland, 1990). At all other locations Cu^{2+} concentrations were of the same order of magnitude as that determined at Percuil Creek and high water at Restronguet Point. For locations where $C_L \approx Cu_T$ (e.g. the concentration of free complexing sites on ligands was relatively low) higher [Cu^{2+}] were calculated. The titrations were performed on samples that had previously been frozen and, as discussed in section 3.4.2.1, over-estimation of the cupric ion concentration may have occurred. However, the relationship between [Cu^{2+}] and Cu_T showed a positive linear relationship ($R^2 = 0.55$) across the sampling locations. This relationship was strongest ($y = 1.10^{-5} x + 9.10^{-5}$, $R^2 = 0.86$) when Wilcove was excluded, where the excess of ligands acted to maintain the low [Cu^{2+}] and the slope indicates that on average 0.001% of Cu_T is present as Cu^{2+} (Braungardt *et al.*, 2007a).

5.3.5. Embryotoxicity of Tamar Estuary waters

The concentrations of Cu^{2+} observed in the mid-Tamar Estuary were biologically relevant to phytoplankton and cyanobacteria (see Chapter 4, Section 4.4.5) but two to three

orders of magnitude lower than concentrations of [Cu^{2+}] that caused a toxic response in single metal exposures of the oyster larval bioassay (EC_{50} = 230 ± 80 pM Cu^{2+}, EC_{05} = 160 ± 50 pM Cu^{2+}, see Section 2.4.3). As for the Fal system, it is unlikely that dissolved Cu alone caused the toxic response in the bioassay. Nonetheless, since at Cremyll and Saltash Town Quay, the maximum toxic response coincided with the highest total Cd and Pb concentrations, and highest *in situ* Cd and Cu concentrations, it is probable that the combined effect of these metals (see Section 2.4.4) contributed to the overall toxicity through synergistic effects, as would the presence of metals (and metalloids) other than those studied here. A current study estimated annual fluxes of 130 t Zn, 60 t Cu, 70 t As, 2.2 t Pb and 700 kg Ag entering the estuary at Gunnislake (Braungardt *et al.*, 2007b), derived mainly from point and diffuse inputs from abandoned mines and spoil heaps. Although a review of water quality in the Tamar estuary (Langston *et al*, 2003b) showed no evidence of regular breaches of water quality standards for the protection of saltwater life, maximum Cu and Zn concentrations near or above the EQS (0.079 μM Cu, 0.612 μM Zn) have been observed.

In the Tamar Estuary, pesticides and herbicides, polychlorinated biphenyls and polycyclic aromatic hydrocarbons are present at detectable concentrations from agriculture, combustion processes and sewage works effluent (Langston *et al.*, 2003b). The combined effect of metals and organic contaminants on toxicity to aquatic life remains poorly constrained and requires further study. Furthermore, future surveys should coincide with the breeding season of indigenous species in order to include larvae obtained from local animals, as other studies have indicated that adaptive processes occur (Damiens *et al.*, 2006; Hoare *et al.*, 1995). Indeed, a multi-species range of bioassays that takes account of responses to different contaminants is recommended. Beiras *et al.* (2003) proposed sea urchin and bivalves which are sensitive to metals, an ascidian for neurotoxic compounds, a marine arthropod to detect the presence of insecticides and a photosynthetic organism for herbicides.

5.3.6 Comparison with the Fal Estuary

The reference sites for this study were selected on the basis that they are exposed to coastal marine waters (English Channel at Whitsand Bay and the Atlantic Ocean/Celtic Sea at Trebarwith Strand) and therefore less impacted by anthropogenic inputs compared with upper estuarine waters. The salinity values recorded were comparable to Percuil Creek at approximately 35, indicating a strong marine influence. The mean percent net response (n=3) observed in bioassays exposed to waters collected at Trebarwith Strand (PNR=13.1%) and Whitsand Bay (PNR=3.19%) produced similar responses to discrete samples obtained from Percuil Creek (PNR= -13 to11%, n=7) in the Fal Estuary. At Percuil Creek a relatively constant PNR was observed throughout the tidal cycle, whereas in this study only single discrete samples collected at low water were available which limits further comment.

At all sampling locations in the Tamar estuary the PNR of bioassays varied between minima of PNR= -2.5% (Whitsand Bay) and maxima of PNR=66% (Cremyll) and PNR=100% (upstream of Cremyll) during the tidal cycles and at Neal Point in the low water sample. This pattern of variability in response to changes in the water masses encountered during a tidal cycle was also observed at Restronguet Point (PNR range: -1.40 to 90%, Section 4.4.2.). However, in the latter case, the origin of the main source of toxicity was clearly related to a source upstream of the sampling location, in Restronguet Creek, and the PNR trend closely followed dissolved Cu and Cd concentrations over the tidal cycle (Section 4.4.3). In contrast, the Tamar appeared to be a more complex system with respect to the source of toxicity, with maximum PNR values offset from the timing of minimum salinity and maximum metal concentrations at some sites.

In the Tamar system, Cd was found primarily in the dynamic fraction where the range of Cd_{dyn} exceeded the concentrations observed in Percuil Creek (range 0.13-0.28 nM, Section 4.4.3), although mostly Cd_{dyn} was less than the concentrations determined at Restronguet Point (0.23-1.91 nM, Section 4.4.3). In contrast, Pb_{dyn} concentrations were

generally lower and the colloidal fraction higher than observed in the Fal system. The colloidal Cu fraction was comparable to that determined in the Fal system, with the Cu_{dyn} fraction determined at Cremyll similar to the concentrations determined at Percuil Creek (4.76-5.80 nM) with the maxima at the remaining sites (Wilcove 16.7 nM and Saltash Town Quay 12.3 nM, Section 5.3.3) below that determined at Restronguet Point (23.2 nM, Section 4.4.3). Total dissolved Cu concentrations were below the maximum determined at Restronguet Point but within the range determined for that system (19.6-88.0 nM). Despite some uncertainties in the determinations of C_L, $logK'_{CuL}$ and Cu^{2+}, the concentration of the most toxic Cu species, Cu^{2+}, were of the same order of magnitude as determined at Percuil Creek and during high water at Restronguet Point. Only at Neal Point and Saltash Town Quay were Cu^{2+} concentrations greater than Percuil Creek, with Neal Point comparable to Restronguet Point (high water). In addition, at the lowest competition strength, similar $logK'_{CuL}$ values were observed. Based on these observations, it is likely that the contribution of Cd to the larval responses was greater in the Tamar system compared to the Fal (higher dynamic concentrations and therefore more biologically available), and for Pb it was less. The effects of Cu are more difficult to assess since the dynamic and total dissolved concentrations were largely greater than determined at Percuil Creek (the reference site) but within the ranges determined at the more metal-impacted site of Restronguet Point.

Notably, PNR=100% was not reached at the metal impacted site of Restronguet Point, whereas total abnormal development occurred at Wilcove, Saltash Town Quay and Neal Point. The Fal field study was undertaken to test the validity of the developed approach. Parts of the Fal system are principally impacted with high concentrations of metal contaminants and consequently the speciation measurements could largely be related to toxic responses. However, the Tamar appears to be a more complex environment where up-and downstream sources of toxicity were inferred. Clearly, factors other than the metals studied are important in determining the toxicological impact of the Tamar waters, as

discussed in previous sections. Moreover, the Tamar supports a diverse assemblage that will have undergone adaptive processes, unlike the conditioned oysters used in both of these studies. Furthermore, *C. gigas* is not a native species although it has been transplanted to areas of the Fal Estuary previously.

5. 4. Conclusions

The dynamic and total dissolved metal concentrations of Cd, Pb and Cu and toxic response indicators increased from the coastal reference sites into the estuary and upstream to Neal Point. This showed that the sources of toxicity and metals were located upstream, although there was evidence for additional metal inputs to the water column from the sediment and/or land-based sources in the lower estuary. The concentration of the weakest class of natural Cu-complexing ligands detected was greater than the total Cu concentration, $[Cu_T]$, at all locations. This indicates that an efficient buffering system is present to counter additional inputs of copper. As a consequence, the concentration of the most toxic metal species present at environmentally relevant concentrations, Cu^{2+}, was unlikely to have solely caused the toxic effects observed in the bioassays. The larvae of the Pacific oyster have been shown to be a sensitive screening tool for metal contaminants, as observed in the Fal study (Chapter 4). The dissolved metal concentrations determined in the Tamar were below the concentrations determined at low water at Restronguet Point and largely similar to the reference site of Percuil Creek, where the waters were shown to be relatively pristine and moderately toxic to the developing larvae. The fact that a greater toxic response was achieved at some locations within the Tamar (above that observed at Restronguet Point) indicates that the larvae are exposed to additional stressors within this system, probably of anthropogenic origin. Anthropogenic influences from the City of Plymouth, naval dockyard, sewage treatment works and the significant water traffic will impact on this system. However, when synergistic effects between the studied metals are considered in combination with other contaminants, such as arsenic and organic

contaminants, it is probable that the metals measured in this work contributed significantly to the bioassay response.

This study used parent oysters from Guernsey that were not acclimatised to the Tamar estuary environment and the larval response excludes effects brought on by adaptation. Notwithstanding this, this approach allows the most sensitive response to toxicity to be measured and supports its use as an effective screening tool. The results showed that the waters in the mid- and lower estuary carry dissolved constituents that have the potential to impair ecosystem functioning, in particular during periods of the tidal cycle when the influence of marine waters is minimal.

Chapter 6:

Conclusions and Future work

6.1. General conclusions

This study has increased existing knowledge of trace metal speciation measurements by resolving high temporal variations and spatial distributions of Cu, Cd and Pb in contrasting estuarine environments. Furthermore, an improved understanding of the effects of trace metal species of Cu, Cd, Pb and Zn on the developing embryos and larvae of the Pacific oyster, *Crassostrea gigas*, has been achieved through controlled laboratory experiments, which showed the order of toxicity to free ion concentrations as $Cu^{2+} >> Cd^{2+} > Zn^{2+} > Pb^{2+}$. This contrasts with the findings for total metal concentrations reported by His *et al.*, 2000 which showed Cu > Zn > Pb > Cd and further supports the fact that chemical speciation is an important consideration in assessing the toxic impacts of trace metals. Based on the results from two contrasting estuarine systems, one highly perturbed with metal contaminants and the other subject to greater anthropogenic influences, it is proposed that the integrated approach used in this study is suitable for the investigative and surveillance monitoring purposes of the EU WFD. However, its practical application for routine monitoring purposes is less certain.

Within this study it was possible to investigate the effect of distinct metal species, computed with a thermodynamic equilibrium model (MINEQL+), on the developing embryos and larvae of *C. gigas*. The toxicity induced by biologically relevant trace metal species (e.g. free metal ions) of Cu, Cd, Pb and Zn on embryo-larval development in controlled laboratory experiments was shown to occur at concentrations in excess of those generally found in estuarine waters, except for Cu in areas perturbed by metal contamination. Notwithstanding this, the response to binary metal combinations showed important changes in the toxic impact of the investigated metals. Synergistic and antagonistic effects were observed for different metal combinations. Hence it is proposed that the interaction between metals and with other contaminants (e.g. organic chemicals of natural or anthropogenic origin, colloidal species and nanoparticles) and the role played by

interacting species needs to be considered in assessing their impact on the aquatic environment.

In order to investigate the speciation of trace metals of Cu, Cd and Pb, the quantification of the different forms present in the water column was required. The characteristics of voltammetric techniques made them particularly well suited for this task. Moreover, the recent advances in *in situ* voltammetric instrumentation have provided the methods necessary to examine the speciation of trace metals in estuarine waters in more detail. The VIP system performed well in both contaminated and more pristine coastal waters in the Fal and Tamar Estuaries, delivering near real-time metal speciation measurements of an operationally defined fraction (<4 nm) that is considered biologically relevant. The use of the gel-integrated microelectrode (GIME) greatly simplified the practicalities of metal speciation measurements and minimised contamination, compared with conventional methodologies involving discrete sample collection and laboratory analysis. This study showed that the integrity of discrete samples was affected by filtration processes, sample storage and treatments. The advantage of high resolution data compared with discrete sampling methods was evident and (1) demonstrated the complexity of estuarine dynamics, (2) illustrated the importance of monitoring over at least one complete tidal cycle, and (3) allowed for a more detailed assessment of the biogeochemical cycling of Cd, Pb and Cu. Varying ratios of dynamic to total dissolved metal concentrations were observed through the time series measurements and this was attributed to inputs of colloidal material of variable organic content potentially influencing the dynamic trace metal distribution within the water column. The mobilisation of metal contaminated sediments into the water column, as well as riverine inputs transported with the ebb tide have been identified as likely sources of this material. Although some variability was observed between the different locations investigated, largely, Cd was found in the dynamic form, Pb in the colloidal fraction and for Cu the presence of an important fraction of organic-complexing ligands was indicated. These observations are consistent with

previous reports that have examined the biogeochemical behaviour of these elements in estuarine systems.

The capability to resolve short-term changes in dynamic metal fractions facilitates rapid detection of pollutant events thus enabling prompt and appropriate actions to be taken. Accordingly, for monitoring purposes, the VIP system would be of particular use as an early warning system for waters subject to fluctuations in metal contaminants and those with recognised pressures, for example from past and/or present mining activities and large scale marine activities.

However, the VIP system requires practiced users for its operation and is less cost-effective than, for example, the DGT method. In addition, there were a number of issues that arose through this study that would require further investigation before its routine use would be recommended. For example, the integrity of the electrode surface and long-term stability of the reference electrode, both of which affect the sensitivity of the instrument towards analyte metals, have been compromised at times during laboratory applications. This could also potentially affect unattended and extended periods of *in situ* operation. However, advances and improvements continue in its design and it is expected that the developments may also provide a more user-friendly system that could be used routinely for monitoring purposes. Notwithstanding this, the capability to detect short-term in-water variability is a distinct advantage when compared with, for example, a time-integrated response that cannot fully characterise these changes.

The present study has also highlighted the potential advantages of combining *in situ* trace metal speciation measurements with biological indicators in order to improve our understanding of biogeochemical processes in estuarine environments. The rapid, simple and relatively low-cost bioassay was shown to be a useful screening tool for marine waters. Moreover, the rapid response of the embryo-larval bioassay in conjunction with the high temporal resolution of the VIP system, and high sensitivity of both techniques, underlines how well these two techniques complement each other for environmental studies.

Furthermore, since metals do not occur in isolation in the natural environment, methods that simultaneously determine *in situ* concentrations of trace metal species are important and in combination with biological measurements can provide a more robust methodology for examining the impact of metal contaminants in estuarine waters.

During all tidal cycle surveys very little change in salinity and pH was observed and this limits any conclusions with respect to their influence on the dynamic metal concentrations determined. Notwithstanding this, the variable embryo-larval response across tidal cycle surveys could be linked to the tidal dynamics of the estuarine systems investigated through the temporal and spatial variations in the dynamic metal concentrations determined *in situ*. This promoted a more detailed interpretation of the systems investigated. It is proposed that a partial interrelationship between the metals studied and the bioassay response was observed, although, the difficulty of de-coupling the bioassay response from individual contaminants remains. Largely, lower dynamic metal concentrations were observed in the Tamar system than concentrations determined in a heavily metal impacted site at Restronguet Creek in the Fal Estuary. Despite this, a higher toxicological response (PNR = 100%) was observed under low water conditions at specific locations within the Tamar catchment, namely Wilcove, Saltash Town Quay and Neal Point. This suggested that the Tamar Estuary was a more complex system and that the larvae were exposed to additional stressors. It is hypothesised that interactivity between metals and other contaminants was likely to contribute to the overall toxicity observed in these waters. These findings support the need for comprehensive surveys to be carried out by incorporating ancillary data (e.g. chlorophyll, suspended particulate matter, turbidity etc), as well as additional chemical (e.g. determining specific organic pollutants such as pesticides, herbicides and PAHs) and biological measurements (e.g. the inclusion of multi-species bioassays and effects on whole organisms with biomarker tests). Moreover, this approach has the potential to be developed for a wider application.

Copper-ligand titrations carried out at three detection windows provided a more complete assessment of the buffering capacity of these systems toward potential inputs of total dissolved Cu. The concentration of metal-complexing ligands at the lowest competition strength employed was higher than total dissolved Cu. This has been observed previously where it has been argued that these Cu-complexing ligands strongly buffer free Cu ion activity. The concentrations of Cu^{2+} determined at the sites investigated were variable and ranged from 0.09 to 0.86 pM in the Tamar system to 0.45 to 3.60 pM in the Fal Estuary, which corresponded to levels found in little to moderately perturbed systems, respectively, and indicated that the natural ligands present influenced the biological availability of Cu. Although greater tolerances to Cu^{2+} concentrations have been reported for marine invertebrate larvae, the concentrations determined in this study are relevant for a number of plankton species. Since the free ion is considered to be the most toxic metal species to biota, and few established techniques are available that combine speciation capabilities with high sensitivity, the competitive ligand exchange cathodic stripping voltammetry approach is an indispensable tool to investigate the relevant metal fractions.

The difficulty in extrapolating results from laboratory studies to natural systems is recognised and was highlighted in this study. For example, under controlled laboratory conditions the mean EC_{50free} for Cu^{2+} was determined as 230 ± 80 pM, whereas at three locations in the Tamar system in which total abnormal development (PNR = 100%) was observed, the Cu^{2+} concentrations were determined in the range 0.09 to 0.86 pM. This emphasises the complexity of natural waters and the importance of working in environmental systems. Indeed with respect to the legislative requirements of the WFD, the importance of field-based studies is evident both for monitoring purposes and for when relevant maximum standards are set for multi-compound contaminated systems.

The advantage of *in situ* measurements and/or samples collected over full tidal cycles for more complex systems, such as estuaries, have been demonstrated with more meaningful data generated compared with individual spot samples. Overall, an improved

quality of data interpretation of geochemical cycling and biological effects of elements in natural waters has been achieved with the integrated approach.

6.2. Future work

6.2.1. Controlled static bioassay exposures

It is uncertain by what mechanism trace metals are assimilated by the developing embryos and larva, although it has been suggested that transportation takes place across the entire biological membrane, particularly in the early divisional stages. Therefore further work is needed at the molecular level in order to gain a better understanding of (1) uptake processes, and (2) the role of Cu, Cd, Pb and Zn in biochemical processes in the developing larva. This would provide further insights into the biogeochemical cycling of these metals.

A number of studies have reported the release of metal-complexing ligands (e.g. exudates) by organisms under metal stress which have been shown to exert some control over the uptake of trace metals. To the authors' knowledge this has not been investigated with the larva of the Pacific oyster and this warrants further study, particularly for natural water samples where high metal concentrations are observed.

The effects of individual metals within binary metal mixtures showed enhanced and reduced sensitivities toward the combinations compared with single metal exposures. Metal mixture experiments are challenging not only because of the inherent variability of biological systems but also because, as the number of investigated elements increases an exponential rise in the number of exposure solutions to be tested occurs. Clearly, the planning stages and the experimental design are crucial if robust data is to be generated. Further work arising from this study needs to establish more clearly the effects of different proportions of trace metals on larval responses and to gain a better understanding of the mechanisms of interaction.

The possibility that developing embryos may be able to utilise metal-EDTA complexes, either through redox processes or surface membrane permeability changes warrants further investigation. This is important as metal buffers are widely used in controlled laboratory investigations that are carried out to predict biologically available metal species.

6.2.2. Estuarine biogeochemistry of trace metals

6.2.2.1. *In situ* trace metal speciation measurements

Estuarine processes that control trace metal distributions are still not fully understood, and long term high resolution data sets can help to improve the knowledge in this field. Seasonal variations and location of point sources of contaminants can be resolved with intensive monitoring programmes and by monitoring along axial transects of an estuary. For example, variations of Cd, Pb and Cu could be monitored at specific locations for a prolonged period of time (e.g. 1 y) by arranging fortnightly deployments of several days duration. Of particular interest would be the monitoring of the marine and freshwater end-members so that the flux of metals exiting into coastal regions could be better predicted. Similarly, sub-tributaries and localised point sources (e.g. harbours, marinas and effluent discharge sites) are suitable locations for more intensive monitoring in order to directly establish metal contaminant inputs.

Studying the effect of salinity and pH on *in situ* trace metal speciation of Cu, Cd and Pb by monitoring over extended periods along the length of the estuary would provide greater insight into their biogeochemical cycling in estuarine environments.

6.2.2.2. Biological measurements

Biological measurements are easily incorporated into monitoring programmes to assess both whole organism responses (e.g. biomarker tests) and/or at appropriate times (e.g. during the reproductive period) to assess the effect of contaminants on the early life

stage of indigenous marine species. Biomarkers of exposure and of effect would provide more detailed information on organism health and ecosystem functioning. Multi-species bioassays can provide information of specific pollutants as different species have shown variable tolerances to contaminants. Examining the effects of pollutants on different life stages can also provide information at the population level. It is suggested therefore that a range of tests using organisms at different life stages and from different trophic levels, as well as whole organism responses (e.g. biomarker tests) are used in order to provide a more comprehensive description of the ecological status of a water body. Also, with respect to cost-effectiveness for legislative purposes, it could be of benefit to use this type of approach at a specified location in order to establish the minimum test requirements needed for assessing the ecological status of a system.

To parallel the high resolution *in situ* data generated with the VIP system, the development of *in situ* bioassays and/or flow-through systems that could be situated on-site or on-board ship and this would provide complementary data that would enhance the interpretation of the biogeochemical cycling of contaminants in estuarine systems.

It would also be of interest to investigate the buffering capacity of estuarine systems, in particular the Tamar system, by exposing larvae to natural water samples spiked with metal aliquots. To complement this, the introduction of organic contaminants known to be present in the Tamar would also provide help to elucidate their role as interacting species in toxicological responses.

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APPENDIX I

APPENDIX I

Table A-1: Log K values for species distribution computed with MINEQL+

Species	Log K (MINEQL+ version 4.5)
OH^-	-13.998
$BF_2(OH)_2^-$	7.630
BF_3OH^-	13.220
BF^{4-}	19.912
$CaOH^+$	-12.697
$Cd(OH)_3^-$	-32.505
$Cd(OH)_4^{2-}$	-47.288
$CdOH^+$	-10.097
$Cd(OH)_2$ aq	-20.294
Cd_2OH^{3+}	-9.397
CdOHCl aq	-7.404
ZnOHCl aq	-7.480
$Cu_2(OH)_2^{2+}$	-10.594
$Cu(OH)_3^-$	-26.879
$Cu(OH)_4^{2-}$	-39.980
$CuOH^+$	-7.497
$Cu(OH)_2$ aq	-16.194
$CuOHEDTA^{3-}$	8.500
$MgOH^+$	-11.397
$Pb(OH)_2$ aq	-17.094
$PbOH^+$	-7.597
$Pb_4(OH)_4^{2+}$	-19.988
Pb_2OH^{3+}	-6.397
$PbOH_3^-$	-28.091
$Pb(OH)_4^{2-}$	-39.699
$Pb_3(OH)_4^{2-}$	-23.888
$SrOH^+$	-13.177
$Zn(OH)_4^{2-}$	-40.488
$Zn(OH)_3^-$	-28.091
$ZnOH^+$	-8.997
$Zn(OH)_2$ aq	-17.794
ZnOH[EDTA]	5.800
$H_2BO_3^-$	-9.236
$CaHCO_3^+$	11.599
$CaHEDTA^-$	15.900
$CdHCO_3^+$	10.686
$CdHEDTA^-$	21.500
H_2CO_3 aq	16.681
HCO_3^-	10.329
$CuHCO_3^+$	12.129
$MgHCO_3^+$	11.339
$NaHCO_3^+$	10.079
$PbHCO_3^+$	13.200

Species	Log K (MINEQL+ version 4.5)
$ZnHCO_3^+$	11.829
$CuHEDTA^-$	24.000
H_2F_2 aq	6.768
HF_2^-	3.750
HF aq	3.170
$MgHEDTA^-$	14.970
$PbHEDTA^-$	23.000
PbH_2EDTA	24.900
HSO_4^-	1.990
$SrHEDTA^-$	14.795
$ZnHEDTA^-$	21.400
$EDTAH^{3-}$	10.948
$EDTAH_2^{2-}$	17.221
$EDTAH_4$	22.500
$EDTAH_5^+$	24.000
$EDTAH_3^-$	20.340
$CdBr_3^-$	3.100
$CdBr_4^{2-}$	2.900
$CdBr_2$ aq	3.000
$CdBr^+$	2.150
$CuBr^+$	-0.030
$PbBr_2$ aq	2.660
$PbBr^+$	1.700
$ZnBr_2$ aq	-0.980
$ZnBr^+$	-0.070
$BF(OH)_3^-$	-0.399
$CaCO_3$ aq	3.200
CaF^+	1.038
$CaSO_4$ aq	2.360
$CaEDTA^{2-}$	12.042
$CdCl^+$	1.980
$CdCl_3^-$	2.400
$CdCl_2$ aq	2.600
$CuCl_3^-$	-2.290
$CuCl_2$ aq	-0.260
$CuCl_4^{2-}$	-4.590
$CuCl^+$	0.200
$PbCl_2$ aq	2.200
$PbCl^+$	1.550
$PbCl_3^-$	1.800
$PbCl_4^{2-}$	1.460
$ZnCl_3^-$	0.500
$ZnCl^+$	0.400
$ZnCl_4^{2-}$	0.199
$ZnCl_2$ aq	0.600
$CdCO_3$ aq	4.358
$Cd(CO_3)_2^{2-}$	7.228

Species	Log K (MINEQL+ version 4.5)
$CuCO_3$ aq	6.770
$Cu(CO_3)_2^{2-}$	10.200
$MgCO_3$ aq	2.920
$NaCO_3^-$	1.270
$PbCO_3$ aq	6.478
$Pb(CO_3)_2^{2-}$	9.938
$SrCO_3$	2.810
$ZnCO_3$ aq	4.760
CdF^+	1.200
CdF_2 aq	1.500
$Cd(SO_4)_2^{2-}$	3.500
$CdSO_4$ aq	2.370
$CdEDTA^{2-}$	18.200
CuF^+	1.800
$CuSO_4$ aq	2.360
$CuEDTA^{2-}$	20.500
MgF^+	2.050
NaF aq	-0.200
PbF_4^{2-}	3.100
PbF_2 aq	3.142
PbF^+	1.848
PbF_3^-	3.420
KSO_4^-	0.850
$MgSO_4$ aq	2.260
$MgEDTA^{2-}$	10.570
$NaSO_4^-$	0.730
$NaEDTA^{3-}$	2.700
$PbSO_4$ aq	2.690
$Pb(SO_4)_2^{2-}$	3.470
$PbEDTA^{2-}$	19.800
$SrSO_4$ aq	2.300
$Zn(SO_4)_2^{2-}$	3.280
$ZnSO_4$ aq	2.340
$SrEDTA^{2-}$	10.436
$ZnEDTA^{2-}$	18.000

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