PARTICULATE PHYTOCHELATINS AS AN INDICATION OF METAL STRESS IN NATURAL WATERS

by

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ABSTRACT

The aim of this study was to investigate the metal stress response of phytoplankton in the Fal, Plym and Tamar Estuaries (SW England, UK) by field measurements of particulate phytochelatins (PCs) and glutathione (GSH) and their relationships with Cu speciation. A high performance liquid chromatographic method was optimised for the determination of particulate PCs and GSH from estuarine waters. Dissolved Cu speciation was determined using well established cathodic stripping voltammetric methods. Glutathione was found throughout the estuaries ranging from 0.6 to 274 μmol (g chl a)^{-1}, and PCs were detected mostly in the Fal samples at concentrations up to 36 μmol (g chl a)^{-1}. Elevated PC production was observed in Restronguet Creek (Fal Estuary) and Gunnislake (Tamar Estuary), the most metal mine impacted sites. For the 2002 survey in the Fal Estuary, GSH and PCs presented strong positive correlations with total dissolved Cu and free Cu^{2+} (as log values). For this survey, it was observed that PC production was faster than GSH production under increasing total dissolved Cu concentrations. This indicated that PC production is a more specific response to metal stress than GSH production in natural waters. High variabilities in the particulate GSH and PC concentrations were likely caused by the heterogeneous phytoplankton composition within the estuaries and effects of metal interactions. Combined exposure of Cd and Zn, for instance, caused antagonistic effects on PC production by the laboratory diatom culture Phaeodactylum tricornutum, probably due to competition for cellular binding sites. Other mechanisms may be involved in the phytoplankton defence system against metal concentrations, as not all species reported in those areas appear to be able to synthesise PCs. Dissolved Cu-organic complexes with high conditional stability constants (K = 10^{11} - 10^{13}) dominated Cu speciation in most of the samples from the Fal, Tamar and Thau Lagoon (France), forming an important mechanism to reduce metal toxicity.

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### List of abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Source/maker</th>
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<tbody>
<tr>
<td>DTNP</td>
<td>2,2’-dithiobis(2-nitrobenzoic acid)</td>
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<tr>
<td>DTPA</td>
<td>diethylenetriaminepentaacetic acid</td>
<td>Sigma (99%)</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
<td>Sigma (99%)</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
<td>BDH</td>
</tr>
<tr>
<td>EQS</td>
<td>Environmental Quality Standard</td>
<td>-</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
<td>Sigma (98%)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidised glutathione</td>
<td>-</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethansulphonic acid</td>
<td>BDH (99%)</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
<td>Merck-Hitachi</td>
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<tr>
<td>LC-ESI-MS</td>
<td>Liquid chromatography-electron spray ionisation-mass-spectrometry</td>
<td>Thermo</td>
</tr>
<tr>
<td>mBrB</td>
<td>3,7-dimethyl-4-bromomethyl-6-methyl-1,5-diazobicyclo-[3.3.0] octa 3,6-diene-2,8-dione or monobromobimane</td>
<td>Fluka (&gt;95%)</td>
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<td>Me₄B</td>
<td>tetramethylbimane</td>
<td>Fluka (99.0%)</td>
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<td>MSA</td>
<td>Methanesulfonic acid solution</td>
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<td>tris (2-carboxyethyl) phosphine hydrochloride</td>
<td>Sigma</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoracetic acid</td>
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</tbody>
</table>
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“A rapadura é doce, mas não é mole...”

(Rapadura is sweet, but not soft...)
AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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Relevant scientific seminars and conferences were regularly attended at which work was presented and papers prepared for publication.

Publications to be submitted in October/November 2004:

- Determination of particulate glutathione and phytochelatins in natural waters using high performance liquid chromatography and fluorescence detection. Silvia K. Kawakami, Eric P. Achterberg and Martha Gledhill. Talanta


- Effects of metal combinations on phytochelatin and glutathione production by Phaeodactylum tricornutum. Silvia K. Kawakami, Eric P. Achterberg and Martha Gledhill. Biometals

Presentation and Conferences Attended:


• **14th Meeting of the Society of Environmental Toxicology and Chemistry, SETAC Europe** – Prague, Czech Republic (04/2004), oral: *Production of intracellular metal-binding peptides and copper contamination in the Fal Estuary (UK).* Silvia K. Kawakami, Eric P. Achterberg and Martha Gledhill.


• **Challenger Centenary Conference in Marine Science** – Plymouth, UK (09/2002), poster: *Metal-binding polypeptides produced by the diatom Phaeodactylum tricornutum under Cu, Cd and Zn exposure.* Silvia K. Kawakami, Eric P. Achterberg and Martha Gledhill.


• **IV Progress in Chemical Oceanography** – Bangor, UK (09/2001), oral: *Glutathione and phytochelatins produced by marine phytoplankton in cadmium exposure experiments and in waters from Plymouth Sound (UK).* Silvia K. Kawakami, Eric P. Achterberg and Martha Gledhill.

Signed ........................................

Date 30th Oct 2004
Chapter 1

1. Introduction

Phytoplankton are well-known to provide a major, direct food source for animals in the water column and sediments and are of paramount importance in the marine food web. Aquatic systems are subject to enhanced inputs of toxic substances from natural (rivers, atmosphere, hydrothermal venting) and anthropogenic sources (sewage, mines, industry, agriculture, anti-fouling paints from boats). It is possible that deleterious effects on phytoplankton composition and distribution occur as a result of the toxicity of these compounds. In spite of this, studies addressing the capacity of phytoplankton to cope with a mixture of contaminants, such as metals, in natural waters are scarce.

The main emphasis of this research is to examine the production of phytochelatins and their precursor glutathione by phytoplankton in metal contaminated estuarine waters. Phytochelatin production is one of the mechanisms adopted by a number of phytoplankton species against metal toxicity. The relationships between copper complexation in the dissolved phase and the production of particulate phytochelatins and glutathione were also investigated. The effects of combinations of toxic metals (Cu, Cd and Zn) on phytochelatin production were studied in short-term experiments using a laboratory phytoplankton monoculture.

This thesis is organised in chapters that complement each other, but can also be read and interpreted as individual pieces of research. Thus, in order to facilitate readability, some repetition was necessary to describe or explain the results.

Chapter 2 provides an overview of the evidence for the production of phytochelatins and glutathione as important intracellular metal-binding peptides in phytoplankton for metal detoxification mechanisms. Metal speciation in natural waters and
laboratory experiments to stimulate PC production in phytoplankton cultures are discussed, as they represent a valuable tool to further information on thiol production in the field.

Chapter 3 examines the current analytical methods for particulate phytochelatins in phytoplankton cells. It provides considerations on the practical problems and limitations encountered with the application of the methods to field studies. In the present work, a method was optimised and applied to phytoplankton from estuarine waters.

In Chapter 4 short-term metal exposure experiments using the marine diatom Phaeodactylum tricornutum to stimulate phytochelatin production are outlined. The main objective of this chapter is to discuss the effects of metal combinations (Cu, Cd and Zn) on phytochelatin and glutathione production.

Chapter 5 discusses the field measurements of phytochelatins and glutathione in the Fal Estuary and Plymouth Sound (SW England, UK). It describes and compares the results in terms of the production of metal-binding peptides, distribution of trace metals and other ancillary parameters, and relates the production of metal-binding peptides and metal contamination, with special reference to copper species.

In Chapter 6 a short study on copper speciation in the Thau Lagoon (France), an important oyster farming area, is presented. This study provides baseline measurements of copper species and their possible implications to phytoplankton species, which are an important food source for the oysters.

Finally, Chapter 7 presents the conclusions and final remarks of this research. The subjects that need further research and the recommendations for the use of phytochelatins as metal stress indicators in field assessment are highlighted.

This study also contributed to the European Union Project IMTEC (In-situ automated Monitoring of Trace metal speciation in Estuaries and Coastal zones, in relation with the biogeochemical processes, EVK3-CT-2000-000036).
2. Phytochelatins produced by phytoplankton as an indication of metal stress in natural waters

2.1. Abstract

Phytoplankton cope with metal toxicity by a variety of strategies including the production of glutathione and phytochelatins. Glutathione and phytochelatins are efficient metal-binding peptides produced by eukaryotic algae for several intracellular functions, notably as a protection against oxidative stress, a metal detoxification mechanism and metal homeostasis. The production of phytochelatins can provide an indication of metal stress for phytoplankton at a sub-lethal level. Despite these essential biological roles, very little is known about the field conditions capable of influencing the production of these compounds by natural phytoplankton assemblages. This chapter describes what is currently known about the chemistry of the metal-binding peptides, particularly phytochelatins, their biological roles and distribution in natural waters, and some of the environmental conditions thought to cause variations in the production of these peptides. The potential applications of phytochelatins for environmental monitoring and remediation are also outlined.
2.2. Introduction

Many trace metals, including Cd, Co, Cu, Fe, Ni, Mn, Mo and Zn, are important micronutrients and are able to influence productivity and species composition of marine algal communities (Sunda 1988; Butler 1998). However, some trace metal nutrients, such as Cd, Cu, Ni and Zn become extremely toxic to marine algae at elevated free aqueous ion concentrations. These trace metals can complex non-specifically with coordination sites of important biological molecules, such as enzymes, and alter normal metabolic functions (Sunda 1988). Trace metal ions such as Cu$^{2+}$ and Fe$^{3+}$, for instance, can interfere in the photosynthetic electron chain, generating reactive oxygen species responsible for the oxidative damage of proteins, lipids, and nucleic acids (Pinto et al., 2003 and references therein). Other metal ions, such as Cd$^{2+}$, Pb$^{2+}$ and Hg$^{2+}$, can enhance the pro-oxidant status of cells by reducing the antioxidant component pools, activate calcium-dependent systems, and affect iron-mediated processes (Okamoto et al., 2001 and references therein). Increased exposure to these metal ions can cause profound effects on organisms, resulting in inhibition of growth, reduced fecundity and even death (Bryan & Langston 1992).

In coastal waters, the concentrations of trace metals are often enhanced as a result of urban and industrial waste water discharges, run-off from operating and disused metalliferous mines, and leaching of anti-fouling paints from vessels. However, no evidence of metal toxicity on phytoplankton assemblages in the field has been reported (Ahner et al. 1997). Several studies have demonstrated that a number of phytoplankton species respond to metal toxicity using protective mechanisms which involve induction of several antioxidative compounds (Rijstenbil et al. 1994; Okamoto et al. 2001; Sigaud-Kutner et al. 2002; Sunda et al. 2002), exudation of organic ligands to complex the toxic metal ions (Moffett et al. 1997; Croot et al. 2000; Gordon et al. 2000), and production of intracellular
metal-binding peptides (Ahner et al. 1995; 1997). The compounds related to these protective mechanisms are naturally present or produced in most marine algae and can scavenge reactive oxygen species, promote reduction conditions for essential biomolecules, and/or decrease metal toxicity by forming stable metal-organic complexes (Grill et al. 1985; Ahner et al. 1995; Pinto et al. 2003).

A summary of compounds related to the mechanisms against metal toxicity and/or metal-induced oxidative stress, with their corresponding target and catalysed reactions is shown in Table 2.1. All these compounds, apart from the algal exudates and dimethylsulphoxide and its metabolites, have been suggested as biomarkers of metal stress in aquatic organisms (Stegeman et al. 1992). It has been acknowledged that a suite of biomarkers, rather than a single one, should provide a better indication of stress and be able to give a forewarning of ecosystem-level damage (Adams & Greeley 2000).

The production of metal-binding peptides by fission yeast and higher plants treated with metals has been investigated since the late 1970s (Rauser 1990). In vitro experiments have shown that metal-binding peptides, known as phytochelatins, protected metal-sensitive enzymes from inactivation and restored the activity of metal-poisoned enzymes (Kneer & Zenk 1992). Only a limited number of research groups, however, have undertaken field studies to investigate the production of these peptides by natural phytoplankton assemblages and still little is known about the environmental conditions capable of causing variations in their levels (Ahner et al. 1997; 1998; Matrai & Vetter 1988; Tang et al. 2000; Wei et al. 2003). In this study, special attention has been given to examining the production of these peptides by phytoplankton in natural waters. This chapter describes what is currently known about the chemistry of the metal-binding peptides in phytoplankton, their biological roles and occurrence in natural waters.
Table 2.1. Compounds related to protective mechanisms against metal toxicity/metal-induced oxidative stress in algae, their target or catalysed reactions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Algal exudates</td>
<td>Metals</td>
<td>Extracellular complexation of metals</td>
<td>Gledhill et al., 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Croot et al. 2000</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>O₂ (Δπ), OH, O₂, HO₂</td>
<td>Vitamin C; electron donor to free radicals</td>
<td>Noctor &amp; Foyer, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pinto et al., 2003</td>
</tr>
<tr>
<td>Dimethylsulphoniopropionate (DMSP)</td>
<td>OH</td>
<td>Most abundant sulphur compound in marine algae</td>
<td>Sunda et al., 2002</td>
</tr>
<tr>
<td>DMS, acrylate, DMSO, MSNA</td>
<td>OH</td>
<td>Enzymatic cleavage products of DMSP</td>
<td>Sunda et al., 2002</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>OH and HOCl</td>
<td>Higher plants</td>
<td></td>
</tr>
<tr>
<td>Glutathione</td>
<td>Non-specific</td>
<td>Eukaryotes; multifunctional metal-binding peptide</td>
<td>Rijstenbil et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Noctor &amp; Foyer, 1998</td>
</tr>
<tr>
<td>Metallothionein</td>
<td>OH, metals</td>
<td>Bacteria, animals and higher plants</td>
<td>Langston et al., 2002</td>
</tr>
<tr>
<td>Phytochelatin</td>
<td>Metals</td>
<td>Plants, algae, some fungi; polypeptide</td>
<td>Ahner et al., 1995</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>RO₂⁻</td>
<td>Vitamin E</td>
<td>Matsukawa et al., 2000</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Pinto et al., 2003</td>
</tr>
<tr>
<td>β-carotene</td>
<td>O₂ (Δπ), RO₂⁻</td>
<td>Phytoplankton pigment</td>
<td>Matsukawa et al. 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pinto et al., 2003</td>
</tr>
</tbody>
</table>

Continued on the next page
**Table 2.1. continuation**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction catalysed</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbate peroxidase</td>
<td>$\text{H}_2\text{O}_2 + \text{ascorbate} \rightarrow \text{H}_2\text{O} +$ monodehydroascorbate</td>
<td>Eukaryotes</td>
<td>Noctor &amp; Foyer, 1998</td>
</tr>
<tr>
<td>Catalase</td>
<td>$2 \text{H}_2\text{O}_2 + 2 \text{H}^+ \rightarrow 2 \text{H}_2\text{O}_2 + \text{O}_2$</td>
<td>Eukaryotes</td>
<td>Pinto et al., 2003</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>$\text{H}_2\text{O}_2 + 2 \text{GSH} \rightarrow 2 \text{H}_2\text{O} + \text{GSSG}$</td>
<td>Eukaryotes</td>
<td>Pinto et al., 2003</td>
</tr>
<tr>
<td></td>
<td>$\text{ROOH} + 2 \text{GSH} \rightarrow \text{ROH} + \text{GSSG}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>$\text{GSSG} + \text{NAD(P)H} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NAD(P)}^+$</td>
<td>Eukaryotes</td>
<td>Pinto et al., 2003</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>$2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$</td>
<td>Eukaryotes</td>
<td>Sigaud-Kutner et al., 2002</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>$\text{Prot-S}_2 + \text{Prot'(SH)}_2 \rightarrow \text{Prot(SH)}_2 + \text{Prot'-S}_2$</td>
<td>Eukaryotes</td>
<td>Pinto et al., 2003</td>
</tr>
</tbody>
</table>

$\text{O}_2 (^1\Delta_g)$, oxygen singlet; $\text{RO}_2$, organic radical; DMS, dimethylsulphide; DMSO, dimethylsulphoxide; MSNA, methane sulphonic acid; GSH, glutathione; GSSG, oxidised glutathione; ROOH, organic acid; Prot-S$_2$, oxidised sulphur protein; Prot'(SH)$_2$, reduced sulphur protein
2.3. Thiols as intracellular metal-binding peptides

Phytochelatins and glutathione are metal-binding peptides classified as sulphhydril compounds or thiols - analogous to alcohols in which the oxygen is replaced by sulphur, forming the SH functional group (Buckberry & Teesdale-Spittle 1996; Morrison & Boyd 1997). These peptides chelate metals through coordination with the SH groups. Phytochelatins and glutathione are considered low molecular weight compounds as they comprise structures of around 10 kD or less (Grill et al. 1985).

2.3.1. Glutathione

Glutathione (GSH), a tripeptide formed by glutamate (Glu), cysteine (Cys) and glycine (Gly), is the major thiol produced by animals, plants, algae, and bacteria (Ahner et al. 2002). The structure of GSH is presented in Fig. 2.1.

![Structure of glutathione](image)

**Figure 2.1. Structure of glutathione.**

The pathways of GSH synthesis, presented in Fig. 2.2, has been elucidated and appears to be common to all organisms that contain GSH (Noctor & Foyer 1998). Two steps catalysed by the enzymes \( \gamma \)-glutamylcysteine synthetase and glutathione synthetase, lead to the sequential formation of \( \gamma \)-Glu-Cys and GSH, respectively. The utilization of
photorespiration to provide biosynthetic intermediates for GSH synthesis has been considered less significant. GSH can be synthesised in the cytosol and chloroplast, where its constituent amino acids and required enzymes have been detected, and transported to other cell compartments (Noctor & Foyer 1998).

![Diagram of GSH biosynthesis from constituent amino acids]

**Figure 2.2.** Biosynthesis of GSH from its constituent amino acids. Glu, glutamate; Cys, cysteine; Ser, serine; Gly, glycine (Norton & Foyer, 1998).

The major processes involving GSH in eukaryotic algae are summarised in Fig. 2.3. Glutathione is related to several functions, such as the maintenance of reducing conditions for amino acids and proteins, protective functions against oxidative and radiation damage, and against enhanced concentrations of metals and xenobiotic organic compounds, and...
production of phytochelatins. The reduced form of GSH exists interchangeably with the oxidised form, GSSG. The multifunctionality of GSH is still not fully understood. (Buckberry & Teesdale-Spittle 1996; Noctor & Foyer 1998; Ahner et al. 2002).

Interests in the determination of GSH in biological systems have increased in recent years, as the role of sulphhydryl compounds in physiological processes has become more evident. Another potential relevant role for this tripeptide is as a dissolved metal ligand in surface seawater, possibly as phytoplankton exudates (Le Gall & van den Berg 1998; Vasconcelos & Leal 2001). Further studies, however, need to be undertaken to determine the chemical stability and sources of the dissolved GSH in natural waters, as the intracellular GSH produced by phytoplankton are at lower concentrations for the high rates of GSH efflux required to explain the dissolved GSH levels in seawater (Ahner et al. 2002).

Figure 2.3. Processes involving GSH in eukaryotic algal cells (adapted from Ahner et al., 2002).
2.3.2. Phytochelatins

Phytochelatins (PCs) are produced by plants, algae, and some fungi and have similar functions as metallothioneins in animals and cyanobacteria (Ahner et al. 1995). Phytochelatins are also known as class III metallothioneins, although they are not proteins as the metallothioneins (Rauser 1990). Phytochelatins are synthesised by the constitutive enzyme phytochelatin synthase which catalyses the transfer of γ-Glu-Cys from GSH to another GSH molecule. This process requires a metal ion for activity (Cobbett 2000). These peptides present the general formula \((\gamma\text{-Glu-Cys})_n\text{-Gly}\) with the Glu and Cys residues linked through a γ-carboxylamide bond, and \(n\) ranging from 2 to 11 (PC2 to PC11) (Grill et al. 1985). Phytochelatin polypeptide chains with \(n = 2, 3\) and \(4\) are predominant in phytoplankton (Ahner et al. 1995; Rijstenbil & Wijnholds 1996). A simplified structure of GSH and PCs is presented in Fig. 2.4.

![Phytochelatin structure](image)

**Figure 2.4.** Basic structure of phytochelatins, \((\gamma\text{-glutamyl-cysteiny})_n\text{-glycine, } n = 1\text{ for glutathione and } n = 2-11\text{ for phytochelatins.}**
The enzymatic pathway of PC biosynthesis seems to occur in the cytosol, and its complete elucidation still needs further study (Cobbett 2000). The transport of potentially toxic metals from one cell compartment to another, for example, from the membrane to the vacuole, appears to be facilitated by complexation with PCs (Rauser 1990).

2.3.2.1. Phytochelatins and metal tolerance

Because of the high affinity of the SH groups for metals, both GSH and PCs are efficient metal complexing ligands inside cells. The conditional stability constants for the various PC-metal complexes have not yet been determined, but PCs have proved to be more efficient for sequestering metals and to reactivate metal sensitive plant enzymes than other biological metal chelators such as GSH and citrate (Kneer & Zenk 1992). Phytochelatins have been related to protective mechanisms against enhanced metal concentrations and also to homeostasis process, i.e. regulation of metal concentrations inside cells (Grill et al. 1985; Ahner & Morel 1995; Ahner et al. 1995). Longer PC polypeptide chains should form more stable metal-complexes due to the advantageous conformational structure and number of SH groups (Mehra et al. 1995).

The kinetics of PC production have been investigated mainly in laboratory diatom cultures (Thalassiosira weissflogii, Thalassiosira pseudonana and Phaeodactylum tricornutum) under metal exposure. Phytochelatins in T. weissflogii are readily synthesised within 1 h after exposure to pCd = 10 (pCd = - Log [Cd^{2+}]). The removal of the conditions of Cd stress, by resuspension of the cells in a medium free of Cd ions, resulted in a rapid restoration of the low basal levels of intracellular PCs, which indicates that PC production is tightly regulated by metal ions (Ahner & Morel 1995; Lee et al. 1996; Morelli & Scarano 2001).
For most of the phytoplankton species investigated in laboratory metal exposure experiments, PC production increased with increasing dissolved metal concentrations in the medium. Furthermore, the increase in PC production was observed before any essential physiological parameters, such as growth rate, were affected (Ahner et al. 1995; 1997). However, the intracellular stoichiometry of PCs to metal ions has apparently no single rule. Some phytoplankton species seem to present a ratio between PC and intracellular Cd of 2:1, but others produce PCs at concentrations 80 times greater than the required concentration to chelate the intracellular Cd (Ahner et al. 1995; 2002).

Although a high PC-producing capacity is not necessarily a feature of tolerant plants (Deknecht et al. 1995), early, rapid formation and overproduction of PCs and PC-metal complexes could account for an enhanced metal tolerance (Rauser 1990). Indeed, phytoplankton species under Cd exposure exhibiting high ratios between PC production and intracellular Cd content had little or no effects on growth rate. In contrast, species more sensitive to Cd presented low PC to intracellular Cd ratios and a significant growth rate decline (Ahner et al. 1995; Rijstenbil & Wijnholds 1996).

2.4. Stimulation of phytochelatin production in laboratory phytoplankton monocultures

Most of the valuable information about PCs produced by phytoplankton has come from short-term metal exposure experiments conducted under controlled laboratory culturing conditions. These experiments consist of incubation of phytoplankton cells, usually monocultures, with metal ions, in an artificial medium with a well defined composition to allow a thorough calculation of metal speciation (Rijstenbil & Wijnholds 1996; Ahner et al. 1995; 2002).
2.4.1. Phytochelatin production by different phytoplankton species

The phytoplankton species investigated together with the metal ions to which they have been exposed are presented in Table 2.2. The concentrations of the metals ranged from low to growth inhibiting levels. The concentrations of GSH and PCs are given in different units in literature which hamper a direct phytoplankton interspecies comparison. The preference for one normalisation or another usually depends on the experimental conditions applied. For incubation experiments at high dissolved metal concentrations (μmol L⁻¹), for example, normalisation with chl a is not recommended because metal toxicity affects pigment content, especially for Cu (Cid et al. 1995; Rijstenbil & Wijnholds 1996). Normalisations of PCs using chl a, however, have been preferred for comparisons between field data or for incubations at low metal concentrations. In a number of laboratory studies, PCs are expressed using the concentration of SH groups and are given in biovolume (cell number x cell volume) to allow comparisons between different phytoplankton species (Rijstenbil & Wijnholds 1996; Ahner et al. 2002).

Despite the different units, it can be observed that chlorophytes and prymnesiophytes produce PCs, with the prymnesiophyte *Emiliania huxleyi* showing a pronounced thiol production under Cu and Cd exposure (Ahner et al. 2002). The only two reported dinoflagellate species, *Heterocapsa pygmaea* and *Prorocentrum micans*, showed low PC production under Cd exposure, and no production under Cu exposure, respectively, suggesting that they have developed adaptive detoxification mechanisms other than PC production (Ahner et al. 1995; Lage et al. 1996).
Table 2.2. Eukaryotic phytoplankton species used in short-term (22-24h) laboratory metal exposure experiments. Concentration ranges of GSH and PCs are given in μmol (g Chl a)^{-1} unless indicated otherwise.

<table>
<thead>
<tr>
<th>Species</th>
<th>GSH</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
<th>PC6</th>
<th>Metal exposure</th>
<th>References</th>
</tr>
</thead>
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<tr>
<td><strong>Bacillariophyceae</strong></td>
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<tr>
<td>(Diatoms)</td>
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</tr>
<tr>
<td><em>Ditylum brightwellii</em></td>
<td>80 – 270°</td>
<td>0 – 40°</td>
<td>0 – 10°</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Cd, Cu</td>
<td>Rijstenbil and Wijnholds, 1996</td>
</tr>
<tr>
<td>(Coastal)</td>
<td></td>
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<tr>
<td><em>Skeletonema costatum</em></td>
<td>400 – 640°</td>
<td>15 – 170°</td>
<td>0 – 14°</td>
<td>0 – 26°</td>
<td>-</td>
<td>-</td>
<td>Cd, Cu</td>
<td>Ahner et al. 1997</td>
</tr>
<tr>
<td>(Coastal)</td>
<td></td>
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<tr>
<td><em>Phaeodactylum tricornutum</em></td>
<td>1970 – 3380°</td>
<td>64 – 570°</td>
<td>0 – 240°</td>
<td>0 – 515°</td>
<td>0 – 270°</td>
<td>0 – 67°</td>
<td>Cd, Cu, Zn</td>
<td>Wei et al. 2003</td>
</tr>
<tr>
<td>(Estuarine)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Ahner et al. 1996</td>
</tr>
<tr>
<td><em>Thalassiosira pseudonana</em></td>
<td>800 – 1300°</td>
<td>0 – 290°</td>
<td>0 – 190°</td>
<td>0 – 50°</td>
<td>-</td>
<td>-</td>
<td>Cd, Cu</td>
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</tr>
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<td>(Coastal)</td>
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</tr>
<tr>
<td><em>Thalassiosira weissflogii</em></td>
<td>1000 – 2000°</td>
<td>3.2 – 730</td>
<td>0.8 – 500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Cd, Cu, Zn, Ag, Pb, Co, Ni, Hg</td>
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</tr>
<tr>
<td>(Coastal)</td>
<td></td>
<td></td>
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<tr>
<td><em>Thalassiosira oceanica</em></td>
<td>-</td>
<td>15 – 1200°</td>
<td>12 – 690°</td>
<td>5.9 – 160°</td>
<td>-</td>
<td>-</td>
<td>Cd</td>
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</tr>
<tr>
<td>(Oceanic)</td>
<td></td>
<td>4 – 112</td>
<td>2 – 60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>Ahner et al. 1995b</td>
</tr>
</tbody>
</table>

continued on next page
<table>
<thead>
<tr>
<th>Species</th>
<th>GSH</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
<th>PC6</th>
<th>Metal exposure</th>
<th>Reference</th>
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<tr>
<td><strong>Chlorophyte (green algae)</strong></td>
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<td>2-56</td>
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<td>-</td>
<td>Cd</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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<td>Cu, Cd, Zn (mixture)</td>
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<td></td>
<td>0.5-8.5</td>
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<td></td>
<td>-</td>
<td>23^e</td>
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<td><em>Tetraselmis maculata</em></td>
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<td>Cd, Cu, Zn, Pb</td>
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<td><em>Tetraselmis tetrathele</em></td>
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<td>Satoh et al. 1999</td>
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<tr>
<td><em>Stigeoclonium tenue</em></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>Cd, Pb, Zn (mixture)</td>
<td>Pawlik-Skowrońska 2001</td>
</tr>
<tr>
<td>(Freshwater)</td>
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<tr>
<td><em>Stigeoclonium sp</em></td>
<td></td>
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<td></td>
<td>Cd, Pb, Zn (mixture)</td>
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<tr>
<td><strong>Prymnesiophyte (coccolithophores)</strong></td>
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<tr>
<td><em>Pleurochrysis carterae</em></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
<td>Ahner et al. 1995b</td>
</tr>
<tr>
<td>(Coastal)</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Emiliania huxleyi</em></td>
<td>500-2400^a</td>
<td>46-430^a</td>
<td>58-490^a</td>
<td>17-560^a</td>
<td>-</td>
<td>-</td>
<td>Cd, Cu, Zn, Pb</td>
<td>Ahner et al. 2002</td>
</tr>
<tr>
<td>(Coastal/Oceanic)</td>
<td>25-450</td>
<td>13-375</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Cd, Cu, Zn, Pb</td>
<td>Ahner et al. 1995b</td>
<td></td>
</tr>
<tr>
<td><em>Pavlova lutheri</em></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td>Cd</td>
<td></td>
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<tr>
<td>(Estuarine)</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td><strong>Dinoflagellate</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Heterocapsa pygmaea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cd</td>
<td>Ahner et al. 1995b</td>
</tr>
<tr>
<td>(Estuarine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Prorocentrum micans</em></td>
<td>0^b</td>
<td>0^b</td>
<td>0^b</td>
<td>0^b</td>
<td>0^b</td>
<td>0^b</td>
<td>Cu</td>
<td>Lage et al. 1996</td>
</tr>
<tr>
<td>(Estuarine)</td>
<td>0^c</td>
<td>0^c</td>
<td>0^c</td>
<td>0^c</td>
<td>0^c</td>
<td>0^c</td>
<td>Cd, Cu</td>
<td>Pistocchi et al. 2000</td>
</tr>
</tbody>
</table>

^a μmol SH per liter biovolume; ^b amol cell^-1 (total PC as γ-Glu-Cys pools); ^c total PC concentration; ^d nmol SH g^-1 dry weight; ^e incubation for 48 h; ^f incubation for 3 h, with no PC synthesis; ^g incubation for 7 to 21 days.
The diatoms *Thalassiosira weissflogii* and *Phaeodactylum tricornutum* showed a high PC production in metal exposure experiments (Rijstenbil & Wijnholds 1996; Ahner et al. 2002). These species have been extensively used by many workers to assess the effects of a range of metals on PC production (Cid et al. 1995; 1997; Ahner et al. 1995; Ahner & Morel 1995; Morelli & Scarano 1995; 2001; Rijstenbil & Wijnholds 1996; Lee et al. 1996; Torres et al. 1997; 1998) because of their well-known physiology and relative ease of culturing.

Regarding GSH production, relatively constant concentrations (0.8-2.8 mM) have been observed in different phytoplankton monocultures upon long-term exposure to ambient Cu and Cd concentrations (Ahner et al. 2002). Glutathione was produced at similar concentrations by cultures of *T. pseudonana* and *Dunaliella sp.* even at different physiological states. Generalisations, however, cannot be made as other species, such as *E. huxleyi*, produced significantly higher PCs and GSH concentrations in the exponential growth phase than in the senescent phase. Acute metal exposure can cause a decrease in GSH levels. For instance, *P. tricornutum* underwent a decrease in GSH levels over the growth phase, which was linked to a biochemical cost for leading to an enhanced PC production (Rijstenbil & Wijnholds 1996). The concentrations of γ-Glu-Cys and cysteine, the precursors of GSH, increased significantly in some metal-stressed cultures in response to metal exposure, suggesting a thigh modulation in the synthesis of these thiols (Ahner et al. 2002; Wei et al. 2003).

### 2.4.2. Effects of various metals on phytochelatin production

The range of free aqueous metal ion concentrations used by Ahner et al. (1995) in short-term metal exposure experiments spanned from pM to nM, and reflected relevant ambient
metal ion concentrations. Most studies have employed metal ions at concentrations as high as μM (Rijstenbil & Wijnholds 1996; Morelli & Scarano 2001). The highest PC induction for some representative coastal phytoplankton species (T. weisflogii, T. maculata, E. huxleyi) has been obtained with Cd and to a lesser extend Cu, Zn and Pb (Ahner & Morel 1995; Ahner et al. 1997). The combination of these metals has caused antagonistic and suppressing effects on PC production, probably as a result of competition for cellular binding sites (Pawlik-Skowronska 2001; Wei et al. 2003). Synergistic effects on PC production have also been observed for combinations of Cd, Cu and Zn, but the reasons for such effects have not been fully elucidated (Wei et al. 2003).

For the experiments involving trace metals at low concentrations, it has been necessary to expose some phytoplankton species to the low metal levels for about 3 to 18 generations before PCs could be detected (Ahner et al. 2002; Wei et al. 2003). This continuous metal exposure simulates more closely the chronic metal contamination in the field.

2.5. Phytochelatins and glutathione produced by phytoplankton assemblages in natural waters

Intracellular phytochelatin concentrations produced in natural waters are over an order of magnitude lower than those in laboratory phytoplankton cultures under exposure to the same metal concentrations. This indicates that the interaction between phytoplankton and metals should not be the only factor involved in the thiol production in the field. Thus, the ability to replicate field conditions in the laboratory and the transfer of techniques to the field are essential to further our understanding of thiol production, particularly PCs, in natural waters. It is important to emphasise that environmental considerations from incubation experiments are best addressed using ambient metal concentrations as well as a
range of phytoplankton species which are naturally present in marine ecosystems, as each species responds differently to metal exposure. It is likely that organisms developed from metal-tolerant strains have an enhanced capability to synthesise PCs. Strains of the same phytoplankton species collected from metal-polluted and unpolluted sites produced different levels of PCs (Pawlik-Skowronska 2003). Thus a more realistic approach adopted in some laboratory studies considers the effects of combinations of metals on PC production by phytoplankton communities isolated from study areas. Results from these studies have helped to clarify findings in the field (Ahner et al. 1994; 1997; 1998; Knauer et al. 1998).

2.5.1. Field measurements of phytochelatins and glutathione

Only a few studies have been undertaken to determine the production of PCs and GSH by phytoplankton in natural waters. A summary of the field data for GSH and PC2 in the particulate phase of natural waters subject to varying free Cu²⁺ concentrations is presented in Table 2.3. Particulate PCs and GSH in these studies corresponded to the phytoplankton fraction retained on 0.7-0.8 μm pore size filters. The sampling areas comprised diverse environmental conditions, from pristine oceanic waters to highly metal polluted coastal/estuarine systems. Cyanobacterial blooms occurred during the collection of samples in the upper Galveston Bay (Tang et al. 2000). Little information regarding phytoplankton composition is available for the other sites that could be linked to PC production.
Table 2.3. Concentrations of PC2 and GSH in the particulate phase of natural waters, together with concentrations of aqueous Cu$^{2+}$.

<table>
<thead>
<tr>
<th>Sample location</th>
<th>GSH µmol (g Chl a)$^{-1}$</th>
<th>PC2</th>
<th>Cu$^{2+}$ M</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>New England, USA</td>
<td>-</td>
<td>2 – 50</td>
<td>$10^{-13}$ - $10^{-10}$</td>
<td>Various harbours</td>
<td>Ahner et al., 1997</td>
</tr>
<tr>
<td>Equatorial Pacific</td>
<td>-</td>
<td>2 – 50</td>
<td>$0.3 \times 10^{-9}$</td>
<td></td>
<td>Ahner et al., 1998</td>
</tr>
<tr>
<td>Switzerland and Italy</td>
<td>-</td>
<td>4 – 17</td>
<td>$10^{-16}$ - $10^{-10}$</td>
<td>Various freshwater lakes</td>
<td>Knauer et al., 1998</td>
</tr>
<tr>
<td>Galveston Bay, USA</td>
<td>10 – 40</td>
<td>Up to 6.3</td>
<td>$10^{-14}$ - $10^{-13}$</td>
<td>Coastal area not strongly metal contaminated</td>
<td>Tang et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Blooms of cyanobacteria</td>
<td></td>
</tr>
<tr>
<td>Saanich Inlet, USA</td>
<td>38 – 200</td>
<td>-</td>
<td>-</td>
<td>Anoxic fjord</td>
<td>Matrai and Vetter, 1988</td>
</tr>
<tr>
<td>Southern California Bight, USA</td>
<td>100 – 250</td>
<td>-</td>
<td>-</td>
<td>Coastal area</td>
<td></td>
</tr>
<tr>
<td>Elizabeth River Estuary, USA</td>
<td>1 – 178</td>
<td>1 – 19</td>
<td>$10^{-14}$ - $10^{-12}$</td>
<td></td>
<td>Wei et al., 2003</td>
</tr>
<tr>
<td>Eel Pond, USA</td>
<td>33 – 83</td>
<td>0.7 – 4.5</td>
<td>$10^{-11}$ - $10^{-10}$</td>
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</tr>
</tbody>
</table>
In general, the dominant phytoplankton groups in coastal and estuarine waters are diatoms and dinoflagellates, while other smaller but important groups include cryptophytes, chlorophytes (green algae), and chrysophytes (cyanobacteria) (Fogg & Thake 1987). It is then expected that most of the contribution on PC production in these studies has come from the dominance of diatoms, prymnesiophytes, and green algae, as the few dinoflagellate species investigated so far apparently have a different mechanism against metal toxicity than PC production.

From the field data, it can be observed that PC production provides a general indication of metal stress for phytoplankton. In areas with higher Cu$^{2+}$ concentrations, the production of PC2 was also higher. In small harbours in southeastern New England (USA), for example, PC2 concentrations ranged between 2 to 50 μmol (g chl a)$^{-1}$, increasing systematically with increasing free aqueous Cu$^{2+}$ concentrations, from $10^{-14}$ to $10^{-10}$ M. In Massachusetts Bay, PC2 concentrations were higher in the vicinities of harbours which received sewage and riverine inputs, and decreased seawards, farther from anthropogenic metal sources (Ahner et al. 1994). Phytochelatins have also been observed in freshwater phytoplankton in a number of metal contaminated Swiss lakes (Knauer et al. 1998), at comparable concentrations to those reported in marine phytoplankton. For the remote oceanic region of the Equatorial Pacific, however, where essential metals occur at limiting concentrations, PCs have also been reported at concentrations comparable to those from polluted coastal sites (Ahner et al. 1998). In such oceanic areas, PC production seems to function as a storage and supply mechanism of essential metals for some phytoplankton species and is stimulated during metal-limiting conditions, notably depletion in Zn which can be ameliorated by Cd through uptake involving PC induction (Ahner et al. 1998; Lee et al. 1996). This has been supported by results from laboratory experiments indicating that Cd can substitute Zn in some Zn-limited diatoms, chlorophytes and prymnesiophytes (Lee &
Morel 1995). High variabilities in the particulate PC and GSH concentrations for a same sampling site, at different times, have been associated to differences in the phytoplankton composition and nutrient limitations (Wei et al. 2003; Tang et al. 2000).

Phytochelatin polypeptide chains longer than \( n = 3 \) have not been reported for natural waters. This may be due to the limitations of the population and/or cellular constraints of natural phytoplankton assemblages. The dynamic nature of coastal ecosystems with rapid phytoplankton turn-over rates and short estuarine and coastal flushing times should also influence the production and stability of longer polypeptides. In addition, lack of sensitivity of the current analytical methods puts constraints on the determination of PCs, as PCs with longer chains typically occur at lower concentrations than the dimer PC2.

### 2.6. Factors affecting the production of GSH and PCs in natural waters

From the above considerations, it can be emphasised that the production of thiols, particularly PCs, is dependent upon phytoplankton species, the degree of toxicity of the trace metal ions, and competition among metals for cellular binding sites. A limited number of studies have addressed the environmental conditions influencing thiol production in natural waters. Metal speciation and dissolved organic matter in natural waters greatly influence the bioavailability and toxicity of metals to biota (Campbell 1995), and consequently should affect the intracellular GSH and PC production. Light intensity, nutrient supply or a combination of both also appear to play a role in the concentrations of the metal-binding peptides in the field (Matrai & Vetter 1988; Rijstenbil et al. 1998).
2.6.1. Influence of trace metal speciation on PC production

A variety of chemical species of trace metals are present in natural waters, differing in their bioavailability and toxic effects on organisms (Sunda 1988). Ionic metal species are thought to be more bioavailable and toxic because they can pass through cell membranes, in contrast to metal-organic complex forms (Campbell 1995). In laboratory experiments, a direct relation between free aqueous Cu$^{2+}$ species and Cu toxicity in marine algae has been observed (Ahner & Morel 1995; Gledhill et al. 1999). The production of PCs has shown to be proportional to the free aqueous metal ions rather than to the total dissolved metal concentration (Ahner et al. 1997). Based on laboratory observations, the metals most likely to induce PC production in coastal waters are Cu, Cd and Zn, since other toxic metals (Pb, Ag and Hg) typically occur at levels below the concentration which stimulates PC production (Ahner et al. 1997). Although the organic ligands dominate Cu and Zn speciation in coastal waters, free Cu$^{2+}$ and Zn$^{2+}$ ions can be found at elevated concentrations in areas subjected to anthropogenic influences, and acid mine run-off, where total dissolved Cu and Zn concentrations exceed the buffering capacity of the natural organic ligands (Kozelka & Bruland 1998).

2.6.2. Influence of light and nutrient supply on GSH and PC production

As the intracellular GSH is involved in the antioxidant system, oxidative stressors such as high light, UV exposure and metal intoxication can cause an increase in GSH levels, as a cellular acclimation response. Extreme exposure to these stressors, however, has lead to a decrease in GSH levels (Noctor & Foyer 1998; Okamoto et al. 2001). Phytoplankton exposed to lower than ambient light intensities showed a decrease in GSH concentrations, when nitrate levels were low (Matrai & Vetter 1988). These combinations of effects on
GSH concentrations could possibly result in a reduced PC production, since GSH is required for the PC synthesis. However, low GSH concentrations do not seem to affect PC production in the field, suggesting that an alternative biosynthesis pathway of PCs could occur through intracellular polymerization of γ-Glu-Cys (Wei et al. 2003).

A low PC production was observed in the diatom *T. pseudonana* under nitrate limitation, which was associated to the fact that inorganic nitrogen is required for the peptide synthesis (Rijstenbil et al. 1998). A limitation in nitrogen was also found to affect sulphate assimilation and hence GSH biosynthesis (Ahner et al. 2002). Some phytoplankton species were unable to produce PCs in the senescent phase (when the culturing medium becomes depleted in nutrients), also indicating possible effects of nutrient limitation (Ahner et al. 2002).

### 2.7. Application of PCs for environmental purposes

Intracellular PCs have been suggested as metal stress biomarkers for phytoplankton in coastal waters as they provide an early measure of metal toxicity (Ahner et al. 1997). The use of synthetic PCs has recently been considered as sensitive biosensors for toxic metals and for bioremediation.

#### 2.7.1. Use of phytochelatins as biomarkers of metal stress

The concept of the biomarker involves the use of a biochemical, cellular, physiological or behavioral parameter as diagnostic screening tools in environmental monitoring (Stegeman et al. 1992; Sanders 1990). The use of the biomarker approach has the advantage to be more sensitive, less variable, and easier to measure in comparison to stress indices such as
inhibition of growth, changes in rate of development, and reduced reproductive potential. The disadvantage is that it can be more difficult to relate the biomarker responses to the health of the organism and to adverse effects on the population (Sanders 1990).

Recently, a suite of biomarkers to assess metal stress has been proposed to be more adequate than the use of a single biomarker, as the effects of adverse environmental conditions can mask some biomarker responses (Adams & Greeley 2000). Thus the use of phytochelatins as biomarkers, as a complement to the biomarkers referred to in Table 2.1, allows us to identify subtle biochemical perturbations caused by a mixture of bioavailable toxic metals, as proposed for similar compounds, metallothioneins, in environmental monitoring (Langston et al. 2002; Mouneyrac et al. 2002).

2.7.1.1. The ratio PC:GSH in natural waters as a metal stress indication

The field data of PCs and GSH are usually presented in terms of chl $a$. Chlorophyll $a$ content provides a general indication of phytoplankton biomass and is relatively easy to measure. However, chl $a$ content varies with light intensity and phytoplankton species composition and this can compromise its use for data normalisation (Wei et al. 2003). Furthermore, prokaryotes produce chl $a$ but not PCs, and hence could cause an underestimation of PC normalised concentrations. Ratios of PC to total low molecular weight thiols may provide more accurate information on the physiological status of the phytoplankton assemblages regarding metal stress, than PC concentrations alone (Wei et al. 2003). As GSH is required for a number of biochemical functions, in addition to its use in PC synthesis, it is thought that the PC:GSH ratio is maintained at a stable level in
healthy phytoplankton cells (Rijstenbil & Wijnholds 1996; Ahner et al. 2002; Tang et al. 2000).

Based on the production of thiols of metal-stressed diatoms (Rijstenbil & Wijnholds 1996), PC:GSH ratios between 0.018-0.26 were considered an indication of healthy phytoplankton cells in Galveston Bay (USA), with enhanced ratios indicating metal stress (Tang et al. 2000). As highlighted before, a better understanding of the environmental effects on the thiol production is necessary for the adequate application of such ratios. Furthermore, a complicating factor is that bacteria, zooplankton and prokaryotic phytoplankton also produce GSH and therefore can interfere in the PC:GSH ratio approach.

2.7.2. Other applications for phytochelatins

Synthetic phytochelatins have recently been applied as sensitive biosensors for a range of heavy metal ions (Hg$^{2+}$, Cd$^{2+}$, Pb$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$) at concentrations in the fM to mM range (Bontidean et al. 2003). Genetically engineered long phytochelatin chains ($n = 20$) could provide an attractive strategy for developing high affinity bioadsorbents suitable for heavy metal remediation (Bae et al. 2000).

2.8. Conclusions and final remarks

1. Phytoplankton cope with metal stress in natural waters by using a range of protective mechanisms. These mechanisms can complement themselves or be more specific for situations of enhanced metal toxicity, such as with the production of the
metal-binding peptides phytochelatins. Coastal waters with elevated free metal ion concentrations have shown high particulate phytochelatin concentrations.

2. From laboratory experiments, it has been outlined that factors such as phytoplankton species composition, metal competition and effects of metal combinations in natural waters are to be considered when comparing laboratory and field data, as they affect phytochelatin production.

3. Despite the range of variables that can affect phytochelatin synthesis, the production of such metal-binding peptides by phytoplankton has been suggested as a valuable biomarker of metal stress.

4. It can be anticipated that a thorough characterisation of the environmental conditions under which phytochelatins and glutathione are produced by measurements of nutrients, phytoplankton species composition, trace metal speciation, and chlorophyll a should help assessing the effectiveness of phytochelatin production as a metal detoxification mechanism and also as a stress biomarker.

2.9. Reference List


Chapter 2

Phytochelatins as an indication of metal stress


Chapter 2  Phytochelatins as an indication of metal stress


Chapter 3

3. Determination of particulate glutathione and phytochelatins in natural waters using HPLC with fluorescence detection

3.1. Abstract

Phytochelatins and glutathione are present at low concentrations in natural waters. Very little is known about the variations in the concentrations of glutathione and phytochelatins produced by natural phytoplankton assemblages in the field. The current analytical methods for particulate thiols in natural waters are based on high performance liquid chromatography (HPLC) with detection of fluorescent derivatives, and comprise expensive time-consuming multi-step protocols. The aim of this chapter is: (i) to review the common analytical approaches, the limitations and main problems related to the current methods for the determination of GSH and PCs in phytoplankton from natural waters; (ii) to identify key strategies for the determination of PCs in the field based on the review; (iii) to discuss the application of a method based on fluorescence detection of derivatised GSH and PCs. An analytical method was optimised and is exemplified here for estuarine waters. The need for suitable analytical strategies to provide quality assurance and further the use of PCs as biomarkers is highlighted.
3.2. Introduction

Phytochelatins and GSH have been extensively investigated in plant cells and phytoplankton monocultures (Ahner et al. 1995; Cobbett 2000). However, there is a paucity of data on the variations in the concentrations of these compounds in natural phytoplankton assemblages. The determination of GSH is well-known for clinical applications and has been the subject of a number of recent reviews (Lock & Davis 2002; Rosenfeld 2003). A range of techniques is available for the determination of thiols (including PCs, GSH, cysteine, homocysteine, metallothioneins) in clinical and environmental samples. The performance characteristics of some of the analytical techniques for the determination of thiols are summarised in Table 3.1.

Methods based on electrochemical detection, such as cathodic stripping voltammetry and polarography (Nyberg & Zhou 1995; Winters et al. 1995; Zhang et al. 2002; Yosypchuk et al. 2003), are able to measure thiols directly without the need of a derivatisation step, but they lack in specificity. Sophisticated hyphenated techniques, such as HPLC with inductively coupled plasma-mass spectrometry (ICP-MS) and electrospray-mass spectrometry (ES-MS) (Vacchina et al. 1999a; 1999b; 2000) have provided information on the structure of PCs as metal-complexes and other thiols in plasma from different animal origins. HPLC and capillary electrophoresis can be coupled to different detectors and allow selectivity of a range of thiols in different redox states (Zhou et al. 1994; Kleinman & Richie 1995; Nozal et al. 1997; Chassaing et al. 1999; Ercal et al. 2001). Nevertheless, the detection limits of these methods are in the nM-μM range and not sufficiently low for the determination of thiols in the field, particularly PCs produced by natural phytoplankton assemblages.
Table 3.1. Characteristics of the instrumental methods for the determination of thiols. DL = detection limit; NM = not mentioned; CLC = capillary liquid chromatography; SEC = size-exclusion chromatography; CE = capillary electrophoresis

<table>
<thead>
<tr>
<th>Separation</th>
<th>Detection</th>
<th>Matrix</th>
<th>Analyte</th>
<th>DL (µM)</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>Polarography</td>
<td><em>P. tricornutum</em> (diatom) and <em>Agrostis capillaris</em> (graminae)</td>
<td>PCs, metallothioneins</td>
<td>NM</td>
<td>Cu electrode</td>
<td>Nyberg &amp; Zhou, 1995</td>
</tr>
<tr>
<td>-</td>
<td>Cathodic stripping voltammetry</td>
<td>Standard solutions</td>
<td>GSH, PC2, PC3</td>
<td>0.002</td>
<td>Cu solid amalgam electrode</td>
<td>Yosypchuk et al. 2003</td>
</tr>
<tr>
<td>CLC</td>
<td>Amperometry</td>
<td>Urine</td>
<td>GSH, cysteine, dopamine, thiopurine</td>
<td>0.2-0.5</td>
<td>Carbon electrode</td>
<td>Zhang et al. 2002</td>
</tr>
<tr>
<td>HPLC</td>
<td>Electrochemical</td>
<td>Tissue</td>
<td>Various</td>
<td>0.008</td>
<td>Dual Hg electrode</td>
<td>Kleinman &amp; Richie, 1995</td>
</tr>
<tr>
<td>HPLC</td>
<td>UV/Vis</td>
<td>Rabbit eye tissue</td>
<td>GSSG</td>
<td>0.08</td>
<td>Post-column DTNP derivatisation</td>
<td>Nozal et al. 1997</td>
</tr>
<tr>
<td>SEC</td>
<td>ICP-MS</td>
<td>Plants</td>
<td>PCs</td>
<td>10</td>
<td>PCs as Cd-complexes</td>
<td>Vacchina et al. 1999b</td>
</tr>
<tr>
<td>HPLC</td>
<td>ICP-MS ES-MS/MS</td>
<td>Plants</td>
<td>PCs</td>
<td>NM</td>
<td>PCs as Cd-complexes</td>
<td>Vacchina et al. 2000</td>
</tr>
<tr>
<td>HPLC</td>
<td>Fluorimetry</td>
<td>Various tissues</td>
<td>GSSG</td>
<td>0.005</td>
<td>thiolGlo / Pyrene labels</td>
<td>Ercal et al. 2001</td>
</tr>
<tr>
<td>CE</td>
<td>Electrochemical</td>
<td>Urine</td>
<td>GSSG</td>
<td>6</td>
<td>Ru oxide electrode</td>
<td>Zhou et al. 1994</td>
</tr>
<tr>
<td>CE</td>
<td>Fluorimetry</td>
<td>Plasma</td>
<td>Total thiols</td>
<td>3</td>
<td>Fluorescein label</td>
<td>Chassaing et al. 1999</td>
</tr>
</tbody>
</table>
Selective and very sensitive analytical methods are required, as PCs are at concentrations in the pM range in natural waters. The general routes that can be followed for the determination of thiol compounds are presented in Fig. 3.1.

The aim of this chapter is: (i) to review the common analytical approaches, the limitations and main problems related to the current methods for the determination of GSH and PCs in phytoplankton from natural waters; (ii) to identify key strategies for the determination of PCs in the field based on the review; (iii) to discuss the application of a reverse-phase HPLC method based on fluorescence detection of derivatised GSH and PCs. An analytical method was optimised and is exemplified here for estuarine waters. The need for suitable analytical strategies to provide quality assurance and further the use of PCs as biomarkers is highlighted.
Figure 3.1. Possible routes for the determination of thiol compounds (adapted from Lock & Davis, 2002). R-S-S-R = disulphide. R-SH = reduced thiol.
3.3. Analytical methods for particulate GSH and PCs in natural waters

In this work, particulate PCs and GSH refer to the fraction retained on 0.7-0.8 µm pore size filters. The few current analytical methods for particulate PCs and GSH in natural waters comprise multi-step procedures: 1) sampling, 2) sample filtration, 3) extraction of thiols, 4) reduction reactions, 5) derivatisation, and 6) analysis of thiol-derivatives by HPLC (Ahner et al. 1997; Tang et al. 2000; 2003).

All steps should be carried out in a careful manner due to the instability of thiol-peptides in aqueous solutions and their rapid oxidation (Winters et al. 1995). A general scheme of an analytical method for particulate PCs and GSH applied to estuarine water samples is summarised in Fig. 3.2. This method was originally optimised for thiols produced by phytoplankton monocultures (Rijstenbil & Wijnholds 1996), grown under controlled laboratory conditions, for which sufficient phytoplankton biomass can be obtained for PC detection.

3.3.1. Sample collection, handling and preparation

Phytochelatins and GSH are present at low concentrations in phytoplankton from natural assemblages and are subject to rapid oxidation once isolated from cells. Therefore, clean techniques and rapid sample preparation to avoid degradation and losses of the particulate compounds should be undertaken as part of the analytical protocol.
**Figure 3.2.** A summary of the optimised analytical method for particulate phytochelatins and glutathione (oxidised + reduced forms) in natural waters, adapted from Rijstenbil & Wijnholds (1996).
3.3.1.1. Reagents and laboratory ware

As organic and trace metal contamination can promote oxidation of reagents and thiol compounds, all laboratory ware were metal- and organic-free. Glassware and plastic bottles were soaked in 1 M HCl (Aristar) for at least 24 h, and then rinsed 3 times with de-ionised water (> 18.2 MΩ cm⁻¹). Reagents and solvents were HPLC or Aristar grade. All solutions were prepared with de-ionised water unless otherwise stated. Metal contamination was minimised during preparation and handling of the reagents and samples by working in a class 100 laminar flow hood.

3.3.1.2. Sample collection

Acid cleaned polycarbonate bottles were used to collect water samples. Large volumes of water samples (2-4 L) were needed to ensure collection of enough biomass (phytoplankton cells). Samples were typically collected from a depth of 0.3 m with the use of small boats. Samples were placed in cool boxes until return to laboratory and filtered within 8 h to minimise interferences from biological processes. Sub-samples were taken for chlorophyll a analysis, as this parameter is important to normalise PCs and GSH concentrations.

3.3.1.3. Filtration

The use of a manifold filtration system is highly recommended if a large number of samples are to be processed. In the method optimised here, samples were filtered under gentle vacuum pressure (< 5 psi) to avoid cell breakage and loss of material. The filtration step was carried out slowly to avoid back-pressure, which could clog the filter and lead to cell lysis.
Nitrocellulose filters (Whatman) of 47 mm diameter and 0.8 \( \mu \text{m} \) pore size were preferred here for the estuarine water samples. It was observed that 0.45 \( \mu \text{m} \) pore size filters clogged easily with particles other than phytoplankton, not allowing enough biomass (phytoplankton cells) for PC analysis. Other workers have employed GF/F filters (0.7 \( \mu \text{m} \) pore size) (Ahner et al. 1997). The use of nitrate cellulose filters has provided better recoveries than glass fiber filters for extraction based on ultrasonication (Rijstenbil & Wijnholds 1996). It may be necessary to combine 2 or more filters from the same sampling site in order to achieve sufficient biomass for particulate PC analysis (Wei et al. 2003). This is especially worthwhile, again, because the filters can easily clog with less than 1.5 L of estuarine water sample by particulate matter other than phytoplankton.

The filters containing the phytoplankton cells were then placed in microtubes (1.5 mL, Eppendorf) for the extraction process. Filters can be either stored under liquid nitrogen (Ahner et al. 1997) or immediately submitted to the extraction process.

3.3.1.4. Extraction of PCs and GSH

During the preparation of the phytoplankton extracts, the effects of enzymatic degradation of thiols and losses due to oxidation should be avoided (Fahey & Newton 1987). The use of HCl for extraction promotes denaturation of proteases, and the metal chelating reagent diethylenetriaminepentaacetic acid (DTPA) minimises oxidation of the SH groups. Therefore, the extraction of PCs and GSH from phytoplankton cells was carried out by addition of 1.2 mL of 0.1 M HCl containing 5 mM of DTPA. Phytoplankton cells were disrupted by ultrasonication (0°C, 5 min) and the cell extract was centrifuged at high speed (13000 g, 20 min, 4°C). Alternatively, the extraction can be performed using 10 mM methanesulphonic acid (MSA) at 70°C for 2 min, the sample can then be homogenised in a grinding tube and
centrifuged (Ahner et al. 1994). The resulting supernatant was immediately submitted to reduction and derivatisation reactions as described below.

3.3.1.5. Reduction reactions

Oxidised GSH and PCs (i.e. RS-SR, R = peptide with residual cysteic group) can be converted to free thiols (RS-H) by reduction using tris (2-carboxyethyl) phosphine hydrochloride (TCEP) or dithiothreitol (DTT) (Fahey & Newton 1987; Rijstenbil & Wijnholds 1996; Getz et al. 1999). The reduction of thiols by TCEP is indicated in reaction 1.

\[
(CH_3CH_2COOH)_3P: + RS-SR + H_2O \rightarrow (CH_3CH_2COOH)_3P=O + 2 RS-H \quad (1)
\]

\text{TCEP} \quad \text{oxidised thiol}

The reducing reagent TCEP is highly corrosive and is more expensive than DTT. However, the use of TCEP has advantages over DTT, as the latter is a thiol itself and therefore can also react with the derivatising reagents producing interfering chromatographic peaks for PCs (Ahner et al. 1995; Rijstenbil & Wijnholds 1996). In addition, the reaction time for the disulphide reduction using TCEP is shorter compared with DTT (Rijstenbil & Wijnholds 1996; Getz et al. 1999). Tributylphoshine (TBP) has also been successfully used to break disulphide bridges (Tang et al. 2000), but it requires careful handling due to its volatility. The use of fresh reducing agent solutions is recommended as oxidation of the reagent may occur during long-term storage (Getz et al. 1999).
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An alkaline pH of 8-9 is maintained by buffer solutions during the reduction reactions because the pKa of the thiol moiety is approximately 9 (Nekrassova et al. 2003). It means that at this pH, the predominant species is the thiolate anion RS\(^{-}\), a good nucleophile, which facilitates the subsequent derivatising reaction. The reagent HEPES is a commonly used pH buffer for biological analyses and can be used for this purpose. Chelating agents such as EDTA and DTPA can also be added in the reaction medium to minimise SH oxidation by metals (Rijstenbil & Wijnholds 1996; Getz et al. 1999).

In the method exemplified here (Fig. 3.2), GSH and PCs were obtained as the total thiol concentrations (oxidised plus reduced form). HEPES was added to 250 µL aliquot of the extract (final concentration of 0.1 M, pH = 9) together with TCEP or DTT (final concentration of 0.5 mM in the reaction medium). The reduction reaction was carried out for 5 min (TCEP) or 1 h (DTT). The concentrations of oxidised thiols can be determined by omitting the reduction step for a sample aliquot and subtracting the result from the total thiol concentration. Thus, the thiol redox ratios used to measure metal-induced oxidative stress, \([\text{RS-H}]/([\text{RS-H}] + 0.5 \times [\text{RS-SR}])\) can be determined (Rijstenbil & Wijnholds 1996).

3.3.1.6. Derivatisation of PCs and GSH

The derivatisation of PCs and GSH enhances sensitivity. The application of a derivatising reagent for PCs and GSH which leads to a minimum of interfering reagent peaks is required. A range of potential derivatising reagents for PCs and the corresponding thiol-derivatives are listed in Table 3.2. From these reagents only mBrB and SBD-F have been used for derivatisation of PCs produced by phytoplankton. Derivatising reagents for UV detection, such as DTNP, are not sensitive enough for determination of PCs produced by
phytoplankton, although they have been successfully applied for PCs produced by higher plants (Fahey & Newton 1987; Rijstenbil & Wijnholds 1996). MBrB is one of the most commonly used reagents, however the chromatographic methods for bimane-derivatives are time-consuming because of the need to eliminate fluorescent materials and to re-equilibrate the system. MBrB reacts preferentially with thiols, but amines, phosphates, and carboxylates at millimolar concentrations react slowly with MBrB and can interfere with the analysis (Fahey & Newton 1987).

Derivatisation of PCs using SBD-F requires very sensitive fluorescence detectors, with adequate optical filters (Tang 2000, pers. comm). OPA-derivatives are stable only for short periods (~ 3 h) and side-reactions of OPA with the amino groups of the peptides can occur (Mopper & Delmas 1984), limiting the application of OPA. The use of NPM, a maleimide, as a pre-column derivatisation may provide detection limits in the nM range for GSH, but can produce diastereomers (Winters et al. 1995).

The derivatisation of PCs and GSH with mBrB (in acetonitrile, final concentration of 1 mM) was carried out in dim light at room temperature. The derivatising reagent was in excess (1-2 mM stoichiometric excess) to ensure rapid and quantitative reaction (Fahey & Newton 1987). After 15 min, the reaction products were stabilised with MSA (final concentration of 0.1 M). The resulting derivatives were stored at 4°C, in the dark, until HPLC analysis (Rijstenbil & Wijnholds 1996). The blank of the method consisted of the same volume of the extracting medium, instead of sample extract, using the same analytical conditions.
Table 3.2. Reagents for derivatisation of thiols, their respective thiol products and the detection methods. UV = ultraviolet; FL = fluorescence.

<table>
<thead>
<tr>
<th>Reagent type</th>
<th>Structure</th>
<th>Thiol derivative and other products</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disulphide exchange, DTNP</td>
<td><img src="structure1.png" alt="Structure" /></td>
<td><img src="structure2.png" alt="Thiol derivative" /></td>
<td>UV</td>
</tr>
<tr>
<td>Isoindole, OPA</td>
<td><img src="structure3.png" alt="Structure" /></td>
<td><img src="structure4.png" alt="Thiol derivative" /> + H₂O</td>
<td>FL</td>
</tr>
<tr>
<td>Haloacetamide, MBrB</td>
<td><img src="structure5.png" alt="Structure" /></td>
<td><img src="structure6.png" alt="Thiol derivative" /> + HBr</td>
<td>FL</td>
</tr>
<tr>
<td>Maleimide, NPM</td>
<td><img src="structure7.png" alt="Structure" /></td>
<td><img src="structure8.png" alt="Thiol derivative" /></td>
<td>FL</td>
</tr>
<tr>
<td>Benzoaxidazole, SBD-F</td>
<td><img src="structure9.png" alt="Structure" /></td>
<td><img src="structure10.png" alt="Thiol derivative" /> + HF</td>
<td>FL</td>
</tr>
</tbody>
</table>
3.3.2. Sample analysis

The difficulties in determining particulate PCs in natural phytoplankton assemblages are clearly reflected by the few field data reported so far. Some of the factors that pose limitations to the sample analyses include the acquisition of high quality standards of PCs, the time spent with the current HPLC analyses and lack of procedures to assess analytical performance.

3.3.2.1. GSH and PC standards

Glutathione standard (GSH, 98%) and phytochelatins (PC2, \( n = 2 \)) were used for calibration purposes. GSH standard can be obtained from a number of companies at different purity levels, whereas commercial PC standards are not readily available. The syntheses and purification of PC standards are at request from a limited number of biochemistry laboratories, and are usually expensive. In the present study, fresh stock standard solutions and working solutions were prepared at concentrations of 0.01 M and 10-100 \( \mu \)M, respectively, in a solution containing 0.12 M HCl and 5 mM DTPA in order to minimise oxidation. Alternatively, standard solutions can be prepared in MSA (Fahey & Newton 1987).

3.3.2.2. Phytochelatins produced by diatom cultures (*Phaeodactylum tricornutum*)

Some workers have used the GSH calibration to quantify the concentrations of PCs, assuming that the fluorescence response is directly proportional to the number of SH groups (Ahner et al. 1995; Rijstenbil & Wijnholds 1996). As an alternative source for the
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PC standards synthesised in laboratory, PCs produced by algae or plants can be used for compound identification in samples. The marine diatom *Phaeodactylum tricornutum* is an efficient producer of PCs, presents high metal-tolerance and is relatively easy to culture. Furthermore, several studies have characterized PCs produced by this diatom under metal stress (Cid et al. 1996; Torres et al. 1997; Morelli & Pratesi 1997; Morelli & Scarano 2001; Morelli et al. 2002; Scarano & Morelli 2002).

Phytochelatins with short chains (PC2, PC3, PC4 and PC5, \( n = 2 \) to 5) have been obtained by extraction from *P. tricornutum* cultures exposed to single additions of \( \text{Cd}^{2+} \), \( \text{Cu}^{2+} \) or \( \text{Pb}^{2+} \) (Ahner et al. 1995; Rijstenbil & Wijnholds 1996; Scarano & Morelli 2002). For this purpose, cultures of *P. tricornutum* in the synthetic medium Aquil (Price et al. 1988) in exponential growth phase were exposed to total 10 \( \mu \text{M} \) \( \text{Cd} \) (or \( \text{Cu}, \text{Pb} \)) for 24 h under continuous light, at 15°C. Duplicate culture samples of 500 mL were filtered and the analysis of thiols from the diatom cells proceeded as described for natural water samples. Control experiments, without Cd addition, were performed in parallel to check for basal levels of PCs and GSH. If necessary, a thorough characterization of the medium conditions with calculation of metal speciation can be carried out using the software MINEQL+ (Schecher & Mcavoy 1992).

### 3.3.2.3. Stability of GSH and PCs in the derivatised form

After the labeling reaction with mBrB, oxidative loss of thiols can still occur (Fahey & Newton 1987). Therefore, it is important to assess the effects of storage on sample integrity if derivatised samples are to be stored for later HPLC analyses. SBD-F and mBrB derivatives have been reported to be stable for 10 days and 6 weeks, respectively (4°C, in the dark) (Rijstenbil & Wijnholds 1996; Tang et al. 2000).
In our laboratory conditions, the stability of a sample (algal extract) was checked under the same HPLC conditions. GSH as bimane derivative (Table 3.2) underwent 30% degradation after storage in the fridge (4°C) for 15 days. Phytochelatins as bimane derivatives were stable over a 30 day-period (at 4°C) without significant losses (Fig. 3.3).

![Figure 3.3. Stability of GSH and PCs in their bimane derivative form. Bars span the range from the average for triplicate injections.](image)

**3.3.2.4. Chromatographic conditions**

The separation of thiol-bimane derivatives can be performed using a reverse-phase HPLC system coupled to columns such as C-18. The use of an ion-pairing reagent, such as tetraoctyl ammonium bromide, in the mobile phase can help separation of PCs from many bimane interfering compounds by prolonging the retention times of PCs (Ahner et al. 1995). However, this does not allow separation of GSH, which co-elutes with other compounds at the beginning of the chromatogram. The separation of OPA-derivatives requires a guard-column (Mopper & Delmas 1984; Vairavamurthy & Mopper 1990),
because precipitation/flocculation of by-products occurs and can cause obstruction of the analytical column.

The chromatograms shown in Fig. 3.4 were obtained using an HPLC consisting of two pumps (Merck-Hitachi Models L-6200 and L-6000), a Rheodyne injection valve with a 100 µL loop and a Model FD-300 fluorescence detector (Dionex) operating at 380 nm (excitation) and 470 nm (emission) wavelengths. The separation was carried out using a 150 x 4.6 mm C-18 (Econosphere) HPLC column with 3 µm particle size. Solvent A was 0.1% trifluoroacetic acid (TFA) and solvent B was acetonitrile. The flow rate of the solvent was 1.0 mL min⁻¹. The gradient adapted from Rijstenbil & Wijnholds (1996) was: 0 to 13 min, 10 to 21% B; 13 to 33 min, 21 to 35 % B; 33 to 40 min, 35 to 100% B; 40 to 50 min, isocratic 100% B; 50 to 65 min, 100 to 10% B. This chromatographic condition allows the separation of derivatised GSH and PCs produced by phytoplankton in a single run. Calibration curves for the bimane derivatives of GSH and PC2 were obtained using standard solutions at concentrations ranging from 10 to 110 and 0.5 to 10 pmol, respectively. The detection limits for GSH and PC2, which were calculated using three times the standard deviation of the standard compound with the lowest concentrations, were 1.0 and 0.9 pmol for a 100 µL injection loop, respectively.

3.3.2.5. Reagent blank and samples

Although the current reverse-phase HPLC methods can provide good separation and relative high sensitivity for particulate PCs and GSH, some analytical problems were encountered. For the chromatograms exemplified here (Fig. 3.4), the broad peak at ~12.5 min, presenting an intense fluorescence response, was identified as tetramethylbimane (Me₄B) by using chromatography of a tetramethylbimane standard. This compound was
used as part of the synthesis of mBrB. The peaks at the beginning of the chromatogram were the result of fluorescent breakdown products.

Figure 3.4. Chromatograms for a) the reagent blank of the method; b) an estuarine water sample (Fal Estuary, Aug-2002); and c) an extract of the marine diatom *P. tricornutum* (after exposure to 1.4 μM Cu$^{2+}$).
The chromatogram for a blank of the method is presented in Fig. 3.4a. The blank of the method contained all reagents apart from the sample extract, which was substituted by the same amount of the extracting medium (HCl/DTPA solution). The addition of cysteine to react with the excess of mBrB may improve the blank (Ahner et al. 2002). The most problematic chromatographic peak, however, was caused by Me$_4$B, which does not react with cysteine and accumulates in the system, thus long elution times were needed to wash Me$_4$B off the column. A 200 μL sample injection loop has been used to increase the injected amount of analyte (Ahner et al. 1997) but this also leads to an increase of the interfering reagent peaks. Thorough rinsing with de-ionized water/acetonitrile was also necessary to clean the HPLC injector valve and sample microsyringe prior to the next sample injection.

Particulate GSH, PC2 and some non-identified peaks were observed in a water sample of a metal impacted estuary (Fig. 3.4b), in which PC production was highly pronounced. Longer PC chains have not been detected in natural phytoplankton assemblages, which was likely due to the influence of the environmental conditions on thiol production rather than analytical problems. Most laboratory phytoplankton monocultures under metal stress, for instance, produced PC2 at higher concentrations than longer PCs.

The method presented here is sensitive for PCs produced in metal impacted waters and by laboratory phytoplankton monocultures (Fig. 3.4c) as they are at optimal culturing conditions (light supply, nutrient and vitamin concentrations) and provide higher biomass than the natural phytoplankton assemblages present in the water samples.
3.3.2.6. Attempts to identify phytochelatins produced by *P. tricornutum* using LC-ESI-MS

Trifluoracetic acid (TFA) is the most commonly used mobile phase to separate peptides and proteins by reverse-phase liquid chromatography (LC). It forms ion-pairs with positive charged and polar groups on peptides and proteins to protect these sites from polar interactions and bring them to the hydrophobic reserved-phase surface of the column. However, the use of TFA in reversed-phase electrospray-mass spectrometry (ESI-MS) analysis for peptides has some incompatibilities. Trifluoracetic acid is a signal suppressor because the strong ion pairs between TFA and the peptides are not broken apart by the conditions in the electrospray ionisation (Garcia et al. 2002). A post-column addition of 50% acetic acid (or formic acid) in acetonitrile helps to minimise the signal supression by dilution of TFA (Garcia et al. 2002; Petritis et al. 2002). The use of acetic acid or formic acid at an equivalent concentration of TFA seems to be enough to keep the peptide side-chain carboxylates protonated (Apffel et al. 1995).

To investigate the possibility to identify PCs produced by *P. tricornutum*, extracts of this diatom exposed to Cd (10 μM) were analysed using LC-ESI-MS. The same reverse-phase HPLC system described above was coupled to the electrospray-mass-spectrometer (Thermo). A standard solution of GSH (5 μM) was also injected in the LC-ESI-MS system with and without post-column addition of 50% acetic acid in acetonitrile. A signal $m/z = 497.7-498.7$ was observed for GSH standard in the derivatised bimane form only after post-column addition of acetic acid (Fig. 3.5b). Phytochelatins were not identified in the algae extracts due to lack of sensitivity of the instrument. In order to identify PCs extracted
from microalgae by the LC-ESI-MS method, larger volumes of phytoplankton cultures (biomass) are needed for the incubation experiments.

Figure 3.5. Mass spectra for GSH standard (5.0 μM). a) without and b) with post-column addition of 50% acetic acid in acetonitrile.
3.3.2.7. Quality assurance

The analytical errors, given the low concentrations and multi-step analytical processes involved, have to be properly quantified. The collection of samples in duplicate is usually performed and the analyses of duplicate samples provide 10-20% variation in the concentration of thiols from the average (Tang et al. 2000; Wei et al. 2003). Triplicate samples are not carried out because of the length of the analysis. The addition of a thiol as internal standard to compensate for oxidation or other chemical reactions, and mechanical losses during sample processing is not adequate owing to differing rates of reaction for different thiols (Fahey & Newton 1987).

For a reliable application of PCs as biomarkers, important analytical aspects are still to be improved as a quality assurance, such as the development of adequate internal standards and certified reference materials for the concentrations of particulate GSH and PCs encountered over a range of natural waters. At present, the measurements of PCs and GSH have to rely on the analyses of replicates.

3.3.3. Application of the methods to natural waters

The current analytical methods for particulate PCs and GSH in the field consider the determination of total thiols (oxidised plus reduced). This may limit the interpretation of oxidative stress, but is acceptable because the thiol redox ratios do not appear to be a useful index for Cu-induced oxidative stress in several marine diatoms upon Cu exposure (Rijstenbil & Wijnholds 1996). Phytoplankton species respond differently to a mixture of metals, and the production of thiols, more specifically an increase in the concentration of particulate PCs, provides a general indication of metal stress in situ. The concentrations of
particulate GSH and PC2 in natural waters together with the analytical parameters of the methods applied are summarised in Table 3.3. Particulate GSH is found at concentrations in the nM range in natural waters and there are no major problems with the detection by the current methods. Particulate PCs, however, are present at pM concentrations and therefore require sensitive detection.

The analytical method exemplified here (Fig. 3.2) was successfully applied to water samples from the Fal Estuary (UK), a heavily metal impacted estuarine system. However, this method lacks in sensitivity for particulate PCs from pristine waters (where the production of PCs as a metal stress response is less pronounced) and is time-consuming. Another variation of this method, applied to waters from metal polluted harbours in New England (USA) (Ahner et al. 1997), produces lower detection limits for PCs by employing a 200 µL injection sample loop, but does not allow separation of GSH. Further optimisation of this method, also employing bimane derivatives, have separated other relevant particulate thiols, such as cysteine and γ-Glu-Cys. The quantification of such compounds may also be useful for field studies, as they are the precursors for the synthesis of GSH (Noctor & Foyer 1998). A more rapid method that takes 30 min for the chromatographic elution of GSH and PCs employs derivatives based on SBD-F (Tang et al. 2000); it is sensitive but not easy to reproduce as explained in section 3.5. A laborious method comprising derivatisation with DTNP followed by a second derivatisation with OPA has been developed for thiols (but not for PCs) in water samples (Vairavamurthy & Mopper 1990). In this method, the DTNP derivatives are suggested as a confirmation reaction for the compounds and also a mean to store thiols, as the OPA derivatives are not stable for long periods but enhance the sensitivity of the analysis.
Table 3.3. Particulate PCs and GSH in natural waters and the parameters of the analytical methods employed. DL = detection limit of compound between parentheses; nm = not mentioned.

<table>
<thead>
<tr>
<th>Sample location</th>
<th>GSH</th>
<th>PC2</th>
<th>DL (pmol)</th>
<th>Injection (µL)</th>
<th>Reduction</th>
<th>Derivatisation</th>
<th>Time (min)</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>New England (USA)</td>
<td>7 - 120</td>
<td>0.3 (PC2)</td>
<td>200</td>
<td>DTT</td>
<td>mBrB</td>
<td>80</td>
<td>Do not separate GSH</td>
<td>Ahner et al. 1997</td>
<td></td>
</tr>
<tr>
<td>Galveston Bay (USA)</td>
<td>65 - 720</td>
<td>0.01 (GSH)</td>
<td>150</td>
<td>TBP</td>
<td>SBD-F</td>
<td>30</td>
<td>Requires adequate optical filters</td>
<td>Tang et al. 2000</td>
<td></td>
</tr>
<tr>
<td>Black Sea</td>
<td>nm</td>
<td>0.05 - 0.1 µM (several thiols)</td>
<td>20</td>
<td>TBP</td>
<td>DTPN + OPA</td>
<td>15 - 30</td>
<td>Separates GSH, other sulfur compounds, but no PCs, pre-concentration in Sep-Pak columns</td>
<td>Varaivamurthy &amp; Mopper, 1990</td>
<td></td>
</tr>
<tr>
<td>Elizabeth Estuary (USA)</td>
<td>10 - 2390</td>
<td>nm</td>
<td>nm</td>
<td>DTT</td>
<td>mBrB</td>
<td>40</td>
<td>Separates GSH, PCs, cysteine and γ-Glu-Cys</td>
<td>Wei et al. 2003</td>
<td></td>
</tr>
<tr>
<td>Fal Estuary (UK)</td>
<td>850 - 2061</td>
<td>1.0 (GSH)</td>
<td>100</td>
<td>TCEP</td>
<td>mBrB</td>
<td>75</td>
<td>Not sensitive for all kind of natural waters</td>
<td>This study</td>
<td></td>
</tr>
</tbody>
</table>
The use of solid-phase extraction, with cartridges such as Sep-Pak C-18, to pre-concentrate PCs from the dissolved phase, or just after their extraction from phytoplankton cells, may be advantageous. Nevertheless, very little analytical details regarding pre-concentration and stability of PCs on such columns have been provided (Lee et al. 1996). Cartridge-adsorbed DTNP derivatives (not including PCs) have been reported to be stable at 0-5°C for long periods, and elution of these compounds from the cartridges using 1 mL methanol has provided quantitative results (Vairavamurthy & Mopper 1990). A combination of pre-concentration with the derivatisation reaction is ideal as it minimises the analytical steps, but can lead to problems with interfering/problematic reagent peaks. A method involving solid-phase extraction of thiols as bimane-derivatives (Tang et al. 2003), for example, has a detection limit for PC2 of 0.06 nM, but gives many chromatographic peaks of fluorescent breakdown products, and hence results in long HPLC chromatographic runs.

3.4. Conclusions

There is still a lack of standardised protocols for the analysis of thiols, particularly PCs, in natural waters despite their important roles in metal detoxification mechanisms and potential use as biomarkers of metal stress. Here, a method for the simultaneous determination of PCs and GSH was optimised and applied for estuarine waters. The use of filters of adequate pore size (0.7-0.8 μm) is required for the collection of sufficient phytoplankton biomass from estuarine waters, as the presence of particles other than phytoplankton in turbid waters can rapidly clog the filters of smaller pore sizes (0.4 μm). For the same reason, the combination of more filters from the same sampling site may be necessary in order to have enough biomass for PC analysis. The main analytical challenges identified were the low concentrations of these compounds in natural samples, their
instability in aqueous solutions, and requirement of multi-step procedures (filtration, extraction, reduction and derivatisation reactions prior to HPLC analysis). In addition, commercial PC standards were expensive as was the case for the reagents required (mBrB, TCEP).

The current analytical methods are time consuming, and limit the number of samples to be analysed. A cost-effective, rapid and sensitive analytical method is a crucial aspect to further the applications of PCs as indicators of metal stress in coastal waters. This is especially relevant if we consider that large sets of samples for environmental studies/monitoring are frequently needed, and time should also be required for determination of ancillary parameters such as chlorophyll a, trace metal speciation, phytoplankton composition, and nutrient levels in order to allow a full interpretation of the field data.

The need to further improve the analytical methods for particulate PC and GSH for field studies was highlighted. The aspects that need improvements and should enhance sensitivity are the pre-concentration step and derivatisation reaction. Sample enrichment using solid-phase extraction represents a viable alternative not yet properly optimised for PC analysis. Some of the derivatising reagents still not explored for particulate PC analysis should provide better reagent blanks with less interfering chromatographic peaks. The use of PCs produced by a well known phytoplankton species (P. tricornutum) can circumvent problems with the high costs of PC standards. Suitable procedures to provide quality assurance for the determination of low levels of particulate GSH and PCs are to be developed for their effective application in environmental monitoring.
3.5. References


Chapter 3

Determination of particulate glutathione and phytochelatins


Chapter 4

4. Effects of metal combinations on the production of phytochelatins and glutathione by *Phaeodactylum tricornutum*

4.1. Abstract

Copper, Zn and Cd have potential toxic effects on most phytoplankton species and can be found at elevated concentrations in contaminated coastal waters. In this chapter, the effects of these metals on the production of phytochelatins and glutathione by the marine diatom *Phaeodactylum tricornutum* in laboratory cultures are examined. Single additions of Cu and Cd (10 μM total concentration) to the culture induced the production of short-chained phytochelatins \((n = 2-5)\) in *P. tricornutum*, whereas single addition of Zn (10 μM total concentration) did not stimulate phytochelatins production. Combination of Zn with Cu resulted in similar phytochelatin production as for single Cu addition. Simultaneous exposure to Zn and Cd led to an antagonistic effect on phytochelatin production, which was probably caused by metal competition for cellular binding sites. Glutathione concentrations were affected only upon exposure to Cd (85% increase) or in combination with Zn (65% decrease), in relation to the control experiment. Ratios of phytochelatins to glutathione indicated a more pronounced metal stress for exposures to Cu and Cd combined with Zn. Although *P. tricornutum* is not a representative species in coastal waters, these findings indicate that variabilities in phytochelatin and glutathione production in the field can be explained in part as a result of metal competition for cellular binding sites.
4.2. Introduction

Laboratory phytoplankton cultures have been useful for providing information on phytochelatin and glutathione production under exposure to different trace metals, and also for optimising the multi-step analytical methods for sulphydryl compounds. Experiments using phytoplankton monocultures under stress of one single metal form a valuable tool to assess the primary implications and toxic metal effects on phytoplankton cells, but give a limited scenario for the interpretation of field observations. A range of laboratory experiments have indicated that phytochelatin production is dependent upon the phytoplankton species, the degree of toxicity of metal ions and interactions among metals (Ahner et al. 1995; Ahner & Morel 1995; Knauer et al. 1998; Wei et al. 2003).

The toxic effects of metals on the marine diatom *Phaeodactylum tricornutum* have been well documented. Exposure to Cu and Cd at concentrations of 0.1-0.5 mg L$^{-1}$ (1.6-7.9 μM) and 5-25 mg L$^{-1}$ (0.4-200 μM), respectively, can cause a strong decrease in growth, photosynthetic rate, chlorophyll a content, and intracellular ATP concentration in *P. tricornutum* cells (Cid et al. 1995; Torres et al. 2000). *Phaeodactylum tricornutum* is shown in Fig. 4.1. Its dimensions and shape depend on the culturing conditions. This diatom is considered one of the most metal-tolerant diatoms, presenting an EC$_{50}$ (Effective Concentration: concentration which reduces growth to 50% in relation to control) value for 200 μM Cd (Torres et al. 1997). This diatom species is an efficient producer of phytochelatins, capable of readily synthesising a wide range of phytochelatins under metal stress (Rijstenbil & Wijnholds 1996). Several studies have been undertaken to characterise phytochelatins produced by *P. tricornutum* (Morelli & Scarano 1995; Rijstenbil & Wijnholds 1996; Morelli & Pratesi 1997; Torres et al. 1997; Scarano & Morelli 2002). The effects of combinations of toxic metals on phytochelatin and glutathione production by this
diatom, however, have not yet been investigated. In coastal waters, a range of potential toxic metals can be present at varying concentrations which could cause different effects on organisms. Thus the study of the toxic effects of a mixture of metals is more relevant for environmental purposes than the effects of one single metal.

In this study, short-term metal exposure experiments using *P. tricornutum* cultures were performed with the aim to investigate the effects of single additions of Cu, Zn and Cd and combinations of these metals on phytochelatin and glutathione production. Algal extracts from Cd-treated cultures were furthermore used to assess the effectiveness of two reducing reagents for phytochelatin analysis.

![Image of algal cells](http://marine.rutgers.edu/ebme/html/docs/staff/aquigg.htm)

**Figure 4.1.** *Phaeodactylum tricornutum* cells.

4.3. Experimental

4.3.1. Culturing medium

Culturing medium was based on the synthetic medium Aquil, which was specially designed for studies examining the effects of trace metals on algae physiology (Morel et al.)
1979; Price et al. 1988). This medium comprises the major salts, nutrients, trace metals, vitamins and chelating agents (ethylenediaminetraacetic acid, EDTA) at sufficient low concentrations to avoid precipitations and allows an accurate calculation of metal speciation. The need for such a medium arose as it became apparent that the bioavailability and toxicity of trace metals were functions of their chemical speciation, specifically their free ion activities (Morel et al. 1979; Price et al. 1988; Campbell 1995). Natural seawater can successfully replace the synthetic ocean water provided that it is collected from areas relatively free of metal impurities and organic matter, such as the Gulf Stream or Sargasso Sea (Price et al. 1988).

In the present study, a stock solution of 20 L of synthetic ocean water (SOW) containing the major salts (AR, reagent grade) was prepared using de-ionised water Millipore (18.2 MΩ cm$^{-1}$). Stock solutions of vitamins, nutrients, the chelating agent EDTA, and trace metals were prepared separately in de-ionised water and added to the SOW in order to form the Aquil medium. All solutions were stored in acid cleaned high density polyethylene bottles. The concentrations of the components in the stock solutions and the final concentration in the culture medium are presented in Table 4.1. The culturing medium and bottles were microwaved prior to use (1 min at high setting) in order to sterilise them. Trace metals were added after sterilisation and then the medium was allowed to equilibrate overnight before being seeded with diatom cells. The pH of the medium was 8.1.
Table 4.1. Composition of the culture medium Aquil and stock solutions used for the short-term metal exposure experiments.

<table>
<thead>
<tr>
<th>Substances</th>
<th>Stock solutions (M)</th>
<th>Final concentration in culture medium (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reagent grade salts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>4.2 x 10^-1</td>
<td>2.1 x 10^-1</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>2.9 x 10^-2</td>
<td>1.5 x 10^-2</td>
</tr>
<tr>
<td>KCl</td>
<td>9.4 x 10^-3</td>
<td>4.7 x 10^-3</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>2.4 x 10^-3</td>
<td>1.4 x 10^-3</td>
</tr>
<tr>
<td>MgCl₂ . 6 H₂O</td>
<td>5.5 x 10^-2</td>
<td>2.8 x 10^-2</td>
</tr>
<tr>
<td>CaCl₂ . 2 H₂O</td>
<td>1.1 x 10^-2</td>
<td>0.6 x 10^-2</td>
</tr>
<tr>
<td><strong>Microelements</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KBr</td>
<td>8.4 x 10^-4</td>
<td>4.2 x 10^-4</td>
</tr>
<tr>
<td>KI</td>
<td>2.4 x 10^-4</td>
<td>2.4 x 10^-5</td>
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<tr>
<td>H₃BO₃</td>
<td>4.9 x 10^-4</td>
<td>2.5 x 10^-4</td>
</tr>
<tr>
<td>NaF</td>
<td>7.1 x 10^-5</td>
<td>3.6 x 10^-5</td>
</tr>
<tr>
<td>SrCl₂ . 6 H₂O</td>
<td>6.4 x 10^-5</td>
<td>3.2 x 10^-5</td>
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<tr>
<td><strong>Nutrients</strong></td>
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<td></td>
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<tr>
<td>NaH₂PO₄ . H₂O</td>
<td>20 x 10^-3</td>
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<tr>
<td>NaNO₃</td>
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<tr>
<td>Na₂SiO₃ . 9 H₂O</td>
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<td>1.0 x 10^-4</td>
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<td></td>
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<tr>
<td>FeCl₃ . 6 H₂O</td>
<td>1.0 x 10^-3</td>
<td>0.5 x 10^-6</td>
</tr>
<tr>
<td>ZnSO₄ . 7 H₂O</td>
<td>23 x 10^-6</td>
<td>2.3 x 10^-8</td>
</tr>
<tr>
<td>MnCl₂ . 4 H₂O</td>
<td>91 x 10^-6</td>
<td>9.1 x 10^-8</td>
</tr>
<tr>
<td>CoCl₂ . 6 H₂O</td>
<td>25.5 x 10^-6</td>
<td>2.6 x 10^-8</td>
</tr>
<tr>
<td>CuSO₄ . 5 H₂O</td>
<td>4.7 x 10^-6</td>
<td>0.5 x 10^-8</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂₄ . 4 H₂O</td>
<td>10.4 x 10^-6</td>
<td>10.4 x 10^-8</td>
</tr>
<tr>
<td>Na₂SeO₃</td>
<td>10.0 x 10^-6</td>
<td>1.0 x 10^-8</td>
</tr>
<tr>
<td><strong>Chelating agent</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na₂EDTA</td>
<td>5.0 x 10^-3</td>
<td>5.0 x 10^-6</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B₁₂</td>
<td>5.5 x 10^-7 g L⁻¹</td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>5.0 x 10^-7 g L⁻¹</td>
<td></td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.0 x 10^-4 g L⁻¹</td>
<td></td>
</tr>
</tbody>
</table>
4.3.2. Stock culture of *Phaeodactylum tricornutum*

Cells of the diatoms *Phaeodactylum tricornutum* Bohlin were obtained from the collection of the Marine Biological Association (UK). This species was collected from the English Channel (off Plymouth Sound, UK) and isolated by E. J. Allen in 1910. Stock cultures of *P. tricornutum* were grown in the Aquil medium (salinity of the SOW = 17), at 15°C under continuous light, until the late exponential growth phase and a cell density of about 8.0x10^8 cells L^-1. The culture was manually shaken once a day. Cells were counted daily using an Olympus microscope and an Improved Neubauer haemocytometer. A growth curve for the stock culture *P. tricornutum* is shown in Fig. 4.2.

![Figure 4.2](image.png)

**Figure 4.2.**
Growth curve for stock culture *P. tricornutum* in Aquil medium, May 2002 (average of triplicate counts).
4.3.3. Short-term metal exposure experiments

The conditions of the metal exposure experiments and concentrations of the metals employed in this study were based on Rijstenbil & Wijnholds (1996) and Morelli & Scarano (2001). Ionic metal concentrations were calculated from the total concentrations in the culture medium using the thermodynamic speciation programme MINEQL+ (Schecher & Mcavoy 1992). The experiments were performed in duplicate. Aliquots of 200 mL of the stock culture (at late exponential growth phase) were transferred to 1 L polycarbonate bottles, and diluted with the Aquil medium (salinity of SOW = 17) to 800 mL total volume, to allow a continuous cell growth for the short-term metal exposure experiments. The cultures were spiked with metal solutions, except the control experiment, as follows: 1) control – no addition of metals; 2) 10 μM Cd(NO₃)₂; 3) 10 μM Cu(NO₃)₂; 4) 10 μM Zn(NO₃)₂; 5) 10 μM Cu(NO₃)₂ + 10 μM Zn(NO₃)₂; 6) 10 μM Cd(NO₃)₂ + 10 μM Zn(NO₃)₂. The incubations were conducted under continuous light at 15°C for 24 h. After this period, the cultures were filtered and prepared for the analysis of phytochelatins and glutathione on the same day of filtration. A scheme of the incubation experiments is presented in Fig. 4.3.

![Figure 4.3](image)

**Figure 4.3.** Scheme of the short-term metal exposure experiments using *P. tricornutum* under exposure to single and combined metal. A stock culture was equally divided into 1 L polycarbonate bottles, diluted with Aquil medium and spiked with metal ions.
4.3.4. Analysis of phytochelatins and glutathione produced by P. tricornutum

The analysis of thiols produced by the metal-treated diatom cultures was based on Rijstenbil & Wijnholds (1996). The source of chemicals, abbreviations and analytical details are explained in Chapter 3. Briefly, 500 mL of cultures were filtered using 0.45 μm nitrocellulose membrane filter, under gentle vacuum pressure to avoid cell breakage. The filters were placed in an Eppendorf microtube and the extraction of thiols from the phytoplankton cells was carried out by adding 1.2 mL solution of 0.1 M HCl and 5 mM diethylenetriamine pentaacetic acid (DTPA), at 0°C by ultrasonication. The extraction was followed by centrifugation at high speed (1300 g / 20 min at 4°C). Aliquots of the extracts from the Cd-treatment experiments and control were submitted to two different reduction reactions in order to compare the reagent blanks.

4.3.4.1. Reduction with dithiothreitol (DTT)

Reagents were added to an Eppendorf tube (1.5 mL) according to the sequence: 250 μL algal extract (treated with 10 μM Cd(II)); 625 μL of 200 mM HEPES containing 5 mM DTPA at pH 9.0; and 25 μL 20 mM DTT in 200 mM HEPES/5 mM DTPA. After 1 hour of reduction, 10 μL 100 mM mBrB in acetonitrile was added and the derivatisation step was carried out in a dark room at room temperature for 15 min. The reaction was stopped by addition of 100 μL 1 M methanesulfonic acid and the samples were stored in the dark at 4°C until HPLC analysis.
4.3.4.2. Reduction with tributylphosphine (TCEP)

Aliquots of 250 μL algal extract (from incubation experiment with 10 μM Cd(II)) were treated with 25 μL 20 mM TCEP in 0.12 M HCl containing 5 mM DTPA, instead of DTT, for 5 min. Then 160 μL 200 mM HEPES/5 mM DTPA at pH 9.0 was added. After another 5 min, 10 μL 100 mM mBrB and 465 μL HEPES/DTPA were added and the protocol continued as described for DTT.

4.3.4.3. HPLC analysis

The HPLC system consisted of two pumps (Merck-Hitachi Models L-6200 and L-6000), a Rheodyne injection valve with a 20 μL loop and a Model FD-300 fluorescence detector (Dionex) operating at 380 nm (excitation) and 470 nm (emission) wavelengths. Separation was carried out using a 150 x 4.6 mm C-18 (Econosphere) HPLC column with 3 μm particle size. Solvent A was 0.1% trifluoroacetic acid (TFA, in de-ionised water) and solvent B was acetonitrile (ACN). The flow rate of the solvent was 1.0 mL min⁻¹. The gradient, adapted from Rijstenbil & Wijnholds (1996) was: 0 to 13 min, 10 to 21% B; 13 to 33 min, 21 to 35 % B; 33 to 40 min, 35 to 100% B; 40 to 50 min, isocratic 100% B; 50 to 65 min, 100 to 10% B.
4.4. Results and Discussion

Typical calibration curves for GSH and PC2 are presented in Fig. 4.4. The ratio between the slopes of the linear regressions is 1.7, indicating an approximately double fluorescence response for PC2 (which contains two SH groups) in relation to GSH (which contains one SH group). Quantification of phytochelatins PC3, PC4 and PC5 produced in the short-term Cd exposure experiments was based on the PC2 calibration curve, assuming that the fluorescence response for the polypeptides was proportional to the number of SH groups (Ahner et al. 1995; Rijstenbil & Wijnholds 1996).

![Calibration curves](image)

**Figure 4.4.** Calibration curves for: a) glutathione; b) phytochelin PC2.

4.4.1. Reproducibility of the short-term metal exposure experiments

The reproducibility of the short-term metal exposure experiments was assessed using Cd at total concentrations of 10 μM, for 5 replicates. Relative standard deviations (RSD) between 16 to 24% were observed for the chromatographic peak areas of GSH and PC2.
Chapter 4  Effects of combined metals on phytochelatins and glutathione

PC3, PC4 and 30% for PC5 (Table 4.2). This shows that the incubation experiments resulted in a reproducible thiol production.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>GSH</th>
<th>PC2</th>
<th>PC3</th>
<th>PC4</th>
<th>PC5</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>59206</td>
<td>20786</td>
<td>10022</td>
<td>17302</td>
<td>2736</td>
</tr>
<tr>
<td>2</td>
<td>45335</td>
<td>12370</td>
<td>7368</td>
<td>11175</td>
<td>1556</td>
</tr>
<tr>
<td>3</td>
<td>40389</td>
<td>19580</td>
<td>8581</td>
<td>13572</td>
<td>1506</td>
</tr>
<tr>
<td>4</td>
<td>53067</td>
<td>13395</td>
<td>10964</td>
<td>15120</td>
<td>2602</td>
</tr>
<tr>
<td>5</td>
<td>35161</td>
<td>14503</td>
<td>8292</td>
<td>16364</td>
<td>1690</td>
</tr>
<tr>
<td>Mean</td>
<td>46632</td>
<td>16127</td>
<td>9045</td>
<td>14707</td>
<td>2018</td>
</tr>
<tr>
<td>RSD%</td>
<td>20.7</td>
<td>23.6</td>
<td>15.9</td>
<td>16.5</td>
<td>29.7</td>
</tr>
</tbody>
</table>

4.4.2. Comparison between reducing reagents

Thiols, such as glutathione and phytochelatins, undergo rapid oxidation forming disulphide bonds (Getz et al. 1999). Therefore, the use of reducing reagents is important to break down such bonds and keep reducing conditions for the thiols during analysis. The reducing reagent DTT was initially used in the experiments; however, the reagent blank of the method was not satisfactory for phytochelatin analysis. An alternative reducing reagent, TCEP, has been reported to provide efficient recovery of oxidised thiols, faster reactions and less interfering chromatographic peaks than DTT (Rijstenbil & Wijnholds 1996; Getz et al. 1999). The interferences caused by the different reducing reagents (TCEP and DTT) in the analysis of phytochelatins can be observed in the chromatograms shown in Fig. 4.5.

The control experiment treated with DTT (Fig. 4.5c) showed several chromatographic peaks with similar retention times expected for PC3 and PC4. As outlined in Chapter 3, DTT is a thiol itself and can react with the derivatising reagent mBrB, forming fluorescent
products. Therefore, the use of TCEP as a reducing reagent was preferred throughout this work.

Figure 4.5. Chromatograms of extracts of *P. tricornutum* a) control treated with TCEP; b) exposure to 4.9 μM Cd^{2+} and treated with TCEP; c) control treated with DTT; d) exposure to 4.9 μM Cd^{2+} and treated with DTT.
4.4.3. Effects of combined metal additions on phytochelatin and glutathione production by *Phaeodactylum tricornutum*

The concentrations of the ionic metals and metal-EDTA complexes in the culture media were calculated from the total concentration of metals using the thermodynamic equilibrium speciation programme MINEQL+, and are presented in Table 4.3. The chelating agent EDTA forms metal complexes that are not taken up by phytoplankton, thus serving to control metal speciation and bioavailability in the medium (Price et al. 1988). The concentrations of Cu\(^{2+}\) and Cd\(^{2+}\) used in the experiments causes a 30% decrease in growth rate according to Rijstenbil & Wijnholds (1996), however, such a decrease was not observed in the present study, as the cell numbers in the metal-treated and control experiments did not show significant differences after the 24 h incubation (< 2% difference).

Table 4.3. Concentrations of ionic metals and EDTA-complexes calculated using MINEQL+ in the short-term metal exposure experiments. Cell numbers were counted before and after the incubation period.

<table>
<thead>
<tr>
<th>Metal species (M)</th>
<th>Control</th>
<th>Zn</th>
<th>Cu</th>
<th>Cu + Zn</th>
<th>Cd</th>
<th>Cd + Zn</th>
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</thead>
<tbody>
<tr>
<td>Zn(^{2+})</td>
<td>1.57E-09</td>
<td>3.40E-06</td>
<td>1.62E-08</td>
<td>7.71E-06</td>
<td>7.77E-09</td>
<td>5.07E-06</td>
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<tr>
<td>Cu(^{2+})</td>
<td>1.60E-12</td>
<td>1.69E-11</td>
<td>1.43E-06</td>
<td>1.43E-06</td>
<td>4.96E-09</td>
<td>4.96E-09</td>
</tr>
<tr>
<td>Cd(^{2+})</td>
<td>9.64E-20</td>
<td>5.15E-19</td>
<td>9.50E-19</td>
<td>5.15E-19</td>
<td>3.17E-06</td>
<td>5.32E-06</td>
</tr>
<tr>
<td>EDTA</td>
<td>4.70E-06</td>
<td>4.54E-07</td>
<td></td>
<td></td>
<td>6.73E-07</td>
<td></td>
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<td>Zn-EDTA</td>
<td>4.48E-06</td>
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<td></td>
<td>1.52E-08</td>
<td>2.78E-06</td>
<td></td>
</tr>
<tr>
<td>Cu-EDTA</td>
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<td>4.97E-06</td>
<td>4.85E-06</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>4.23E-06</td>
<td>1.99E-06</td>
<td></td>
</tr>
<tr>
<td>cells L(^{-1}) before incubation</td>
<td>2.00E+08</td>
<td>1.92E+08</td>
<td>1.58E+08</td>
<td>1.92E+08</td>
<td>1.25E+08</td>
<td>2.70E+08</td>
</tr>
<tr>
<td>cells L(^{-1}) after incubation</td>
<td>2.00E+08</td>
<td>1.92E+08</td>
<td>1.58E+08</td>
<td>1.92E+08</td>
<td>1.25E+08</td>
<td>2.75E+08</td>
</tr>
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</table>
4.4.3.1. Phytochelatins

The concentrations of phytochelatins produced by *P. tricornutum* in the experiments were normalised to cell numbers (amol cell\(^{-1}\) = 10\(^{18}\) mol cell\(^{-1}\)) (Fig. 4.6). The use of chl *a* to normalise the data is not recommended for incubation experiments performed at high metal concentrations (μM), as metal toxicity affects pigment content (Cid et al. 1995; Rijstenbil & Wijnholds 1996). The production of phytochelatins with *n* ranging from 2 to 5 (PC2 – PC5) were induced in all metal exposure experiments, ranging from 0.1 to 3.6 amol cell\(^{-1}\). Low concentrations of phytochelatins were also found in the control, which could be a result of metal contamination in the incubations or basal levels of intracellular phytochelatins.

![Figure 4.6. Phytochelatins produced by *P. tricornutum* under metal exposure. Bars span the range from the average for duplicate analyses.](image)

The dimer PC2 was found at concentrations higher than the other polypeptides in cells treated with Cd only and in combination with Zn. Exposure to Cd only resulted in a more pronounced phytochelatin production compared to exposure to Cu only. Exposure to Zn did not induce phytochelatin production in *P. tricornutum*, being similar to the control. The
combination of Zn and Cu resulted in concentrations of phytochelatins similar to the induction found by the addition of Cu only. This could mean that no competitive interaction between Cu and Zn occurred at the established metal concentrations.

Cultures treated with Cd only produced 50% more PC2 than the combination of Zn and Cd. Such an antagonistic effect of Zn on the induction of PCs by Cd is probably due to competition between these two metals for cellular binding sites. Cadmium was the most effective inducer of PC2, which is in agreement with previous studies including other phytoplankton species (Ahner et al. 1995; Ahner & Morel 1995; Lee et al. 1996). A decrease in effects of Cd on PC production in some diatoms has been observed at high levels of Zn$^{2+}$ and/or Mn$^{2+}$ (Wei et al. 2003). A plausible explanation for this is that Cd uptake takes place through the same channels as Zn and Mn, thus Zn and Mn competitively inhibits the induction of PC by Cd (Sunda & Huntsman 1996). Although Zn$^{2+}$ is able to activate PC synthase, it is a weak inducer of PC production in studied algae, plants and fungi (Morelli & Scarano 2001 and references therein). For example, in a 3 day exposure experiment, the growth rate of *P. tricornutum* was not affected by intracellular accumulation of Zn (Morelli & Scarano 2001). Deleterious effects of Zn on other physiological parameters of *P. tricornutum* have not been reported.

The synthesis of PCs in *P. tricornutum* cells have been reported to occur after 15 min of exposures to either 10 µM Cd or 10 µM Pb (total concentration, at salinity 35) (Morelli and Scarano 2001). In these experiments, PCs reached concentrations comparable to the present study in 7 h (approximately 6 amol cell$^{-1}$ for each of the oligomers $n = 2$, 3 and 4, calculated from graph), with concomitant decrease in glutathione concentrations (from 90 to 40 amol cell$^{-1}$, obtained from graph).
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Such rapid formation of PCs in \textit{P. tricornutum} cells, together with their capability to release GSH-like substances and surfactants (Croot et al. 2000) could account for the high metal tolerance of this diatom species. In addition, a recent study reported that \textit{P. tricornutum} cells can respond rapidly to metal-induced oxidative stress by activation of a suite of antioxidant enzymes (Morelli & Scarano 2004). In contrast, metal sensitive phytoplankton species such as \textit{Skeletonema costatum} have growth inhibited at 100 times lower Cd concentrations (Torres et al. 1997). This species has also shown less pronounced PC production than \textit{P. tricornutum} (Rijstenbil & Wijnholds 1996), but is able to release Cu-chelators with weak binding strengths under Cu stress (Croot et al. 2000), which could minimise metal toxicity in its surrounding medium.

The results presented here corroborates with those reported by Wei et al. (2003). They observed a range of effects on PC production, such as antagonism, synergism and suppression, caused by combinations of Zn, Cu and Cd (at concentrations between $10^{-10}$ and $10^{-7}$ M) on a natural phytoplankton assemblage and laboratory phytoplankton monocultures.

\subsection*{4.4.3.2. Glutathione}

The concentrations of GSH produced by \textit{P. tricornutum} in the incubation experiments ranged from 4.7 to 25.3 amol cell$^{-1}$ (Fig. 4.7) and were higher than the total concentrations of phytochelatins (PC$_{\text{Total}}$). GSH was produced in the control experiment, to which no enrichment with metals was made, and also occurred at similar concentrations in all experiments, except for incubations with added Cd only and with Cd in combination with Zn. These metal treatments caused an 85% increase and 65% depletion in GSH concentration, respectively, relative to the control.
For various phytoplankton species, the intracellular GSH was found at similar concentrations upon Cd or Cu exposure at ambient/low levels (Ahner et al. 2002). It has been considered that healthy phytoplankton cells maintain intracellular GSH at a constant level for essential functions, in addition to its use for PC production (Tang et al. 2000; Ahner et al. 2002). However, there appear to be two situations regarding metal stress that cause variations in GSH concentrations in phytoplankton cells. For example, an increase in intracellular GSH concentration can be a result of the cellular acclimation response for an initial metal stress (Okamoto et al. 2001). This could explain the higher GSH concentration observed here for the exposure to Cd only in relation to the control. The second situation is related to acute metal stress, when the synthesis of PCs by \textit{P. tricornutum} could imply a biochemical cost with depletion in GSH concentration (Rijstenbil & Wijnholds 1996; Morelli & Scarano 2004). This acute stress situation could explain the lower GSH concentration observed for the exposure to Cd combined with Zn in comparison to the control. However, the lower GSH concentration for the exposure to Cd combined with Zn contradicts the effects of the exposure to Cd only. It was expected that a competition
between the two metals would minimise Cd toxicity, leading to less effects on intracellular GSH concentration.

4.4.3.3. Ratios PC_{total}:GSH

The ratios of the concentrations of PC_{total} to GSH, in which PC_{total} corresponds to the sum of the γ-Glu-Cys units of the PC oligomers, have been suggested to provide an indication of the extent of metal stress (Tang et al. 2000). In the present experiments, the ratios ranged from 0.11 to 1.95 (Fig. 4.8). The lowest PC_{total}:GSH ratio was observed for the control experiment and the highest ratio for the combination of Cd with Zn. According to these ratios, exposure to Cu caused stronger stress than exposure to the combination of Cu with Zn or single Cd. Despite the competition between Cd and Zn for binding sites, which should ameliorate Cd toxicity, a higher PC_{total}:GSH value was observed for the exposure to the combination of Cd with Zn than to the single exposure to Cd. Wei et al. (2003) hypothesised that at certain Cd and Zn concentration thresholds, Cd could inhibit Zn uptake and the cell, experiencing limitation in Zn, would promote more intracellular Zn transporters. These transporters would allow for greater Cd accumulation and thus lead to an enhanced stress condition. As intracellular metal concentrations were not determined in that study nor in the present one, direct evidence is required to support this hypothesis.

A comparison of GSH and PC concentrations, together with PC_{total}:GSH ratios for P. tricornutum under different metal exposure conditions is presented in Table 4.4. Cultures grown in a medium similar to the one employed here and exposed to separate additions of 0.4 μM Cu and 9 μM Cd (Rijstenbil & Wijnholds 1996) resulted in PC_{total}:GSH values comparable to the present study. P. tricornutum cultures grown at higher salinity and
subjected to a shorter incubation period (7 h) with individual 10 µM Cd and 10 µM Pb produced the highest $PC_{\text{total}}$:GSH values (Morelli & Scarano 2001).

Table 4.4. Production of $PC_{\text{total}}$ (sum of $\gamma$-Glu-Cys units of the oligomers) and GSH, together with thiol ratios for laboratory $P. \text{tricornutum}$ cultures under metal stress. $p_{\text{Metal}} = -\log[\text{Metal}^{2+}]$.

<table>
<thead>
<tr>
<th>Metal concentration</th>
<th>GSH</th>
<th>$PC_{\text{total}}$</th>
<th>$PC_{\text{total}}$:GSH</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13.7</td>
<td>1.81</td>
<td>0.13</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>$p_{\text{Cu}} = 5.8$</td>
<td>10.9</td>
<td>11.0</td>
<td>1.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cu}} = 5.8$</td>
<td>12.8</td>
<td>9.09</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Zn}} = 5.0$</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Zn}} = 5.5$</td>
<td>16.0</td>
<td>1.73</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cd}} = 5.5$</td>
<td>25.3</td>
<td>18.0</td>
<td>0.71</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cd}} = 5.3$</td>
<td>4.68</td>
<td>9.12</td>
<td>1.95</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>780</td>
<td>14.6</td>
<td>0.019</td>
<td></td>
<td>Ahner et al., 2002</td>
</tr>
<tr>
<td>$p_{\text{Cd}} = 11$</td>
<td>560</td>
<td>2.90</td>
<td>0.052</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cd}} = 10$</td>
<td>680</td>
<td>53.4</td>
<td>0.079</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cd}} = 9$</td>
<td>710</td>
<td>51.6</td>
<td>0.073</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cu}} = 11$</td>
<td>260</td>
<td>81.1</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cu}} = 10$</td>
<td>860</td>
<td>116</td>
<td>0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$p_{\text{Cu}} = 9$</td>
<td>2100</td>
<td>247</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control [Cd] = 10 µM</td>
<td>40</td>
<td>65</td>
<td>1.6</td>
<td></td>
<td>Morelli and Scarano, 2001</td>
</tr>
<tr>
<td>[Pb] = 10 µM</td>
<td>45</td>
<td>80</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Zn] = 10 µM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control [Cu] = 0.4 µM</td>
<td>2359</td>
<td>379</td>
<td>0.16</td>
<td></td>
<td>Rijstenbil and Wijnholds, 1996</td>
</tr>
<tr>
<td>[Cd] = 9.0 µM</td>
<td>2173</td>
<td>1424</td>
<td>0.66</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Incubation exposure experiments using low ambient metal concentrations (pM to nM range) produced PC:GSH ratios around one order of magnitude lower than those for metals in the μM range (Ahner et al. 2002). In these low metal concentration exposure experiments, Cu-treated cells produced higher PC\textsubscript{total}:GSH ratios than Cd-treated cells. Such lower ratios are consistent with the role attributed to PCs in metal detoxification mechanisms, with higher metal concentrations inducing a more pronounced PC production.

It is worth noting that the *P. tricornutum* employed in the present study has been cultured for more than 90 years in the collection facilities of the Marine Biological Association. Thus, in addition to the natural high metal tolerance of *P. tricornutum* (Torres et al. 1997), the biological responses to metal toxicity could be altered through evolution of even more tolerant strains over these years. The different strains of *P. tricornutum* could also explain the variabilities in the production of GSH and PCs by the different workers.

### 4.5. Conclusions and final remarks

1. *Phaeodactylum tricornutum* cultures under exposure to Cu, Cd and Zn, singly and in pairs, synthesised a range of short-chained phytochelatins with \( n = 2-5 \). Simultaneous determination of GSH and PCs can be more useful to assess the physiological status of phytoplankton under metal stress. However, the proposed PC\textsubscript{total}:GSH ratios did not explain the level of metal stress within the experiments. Exposure to Cd only, for example, caused less stress than the Cd combined with Zn.
2. An antagonistic effect of Zn on PC induction by Cd was observed, which was likely due to competition between these two metals for cellular binding sites. Zinc did not induce PC production nor affect GSH concentrations. Copper combined with Zn induced similar PC concentration to the Cu exposure on its own. Although *P. tricornutum* is not considered a representative phytoplankton species for environmental studies, the results indicated that interaction among metals should influence PC production by natural phytoplankton assemblages, and probably cause variations in PC concentrations in the field.

4.6. References


5. Particulate phytochelatins and glutathione in the Fal Estuary and Plymouth Sound (UK)

5.1. Abstract

The aim of this chapter is to report field measurements of particulate phytochelatins (dimer PC2) and glutathione (GSH), together with copper complexation in two metal contaminated coastal systems in the UK: (i) the Fal Estuary and (ii) the Tamar and Plym estuaries (Plymouth Sound). Glutathione was found throughout the estuaries, but showed a wide of concentration range from 0.6 to 274 μmol (g chl a)$^{-1}$. Phytochelatins were observed mainly in the Fal Estuary at concentrations up to 36 μmol (g chl a)$^{-1}$, and were below the detection limit in most of the Plymouth Sound. Highest particulate PC2 and GSH concentrations were observed in the mine impacted sites Restronguet Creek (Fal Estuary) and Gunnislake (Tamar Estuary). In Restronguet Creek concentrations of total dissolved Cu exceeded those of Cu-complexing ligands. Free Cu$^{2+}$ and total dissolved Cu concentrations were positively correlated with PC2 and GSH concentrations in the Fal Estuary during 2002. High variabilities in GSH and PC2 concentrations (5-200 fold) could be due to the heterogeneous phytoplankton composition within the estuaries and antagonistic and suppressing effects on PC2 and GSH production caused by metal interactions. The production of PC2 showed to be faster than that of GSH in the Fal Estuary (2002) under increasing total dissolved Cu concentrations, suggesting that PC2 is a more specific response to metal stress than GSH in natural waters.
5.2. Introduction

Metal bioavailability and toxicity in aquatic systems are related to the kinetically labile species, including free ionic metals, inorganic hydroxide, chloride, and carbonate metal complexes, and also metals complexed by organic ligands with weak binding strengths. The degree of toxicity of a metal species depends on the metal itself and the organism, but it is usually higher for the free ionic metal species (Gledhill et al. 1997). In this respect, dissolved organic ligands ubiquitously found in natural waters play a key role in modifying the metal bioavailability and toxicity. These ligands are thought to be derived from natural sources (algae exudates and humic substances) and anthropogenic inputs (e.g. sewage) and have been classified according to their conditional stability constants as strong \((K_1 = 10^{12-10^{15}})\) and weak \((K_2 = 10^{7-10^{11}})\) ligands (Moffett et al. 1997). Strong organic ligands complex metals forming non-bioavailable species, and therefore minimise the potential for the toxic effects of metals.

In aquatic systems subject to acid mine run-off, agricultural, industrial and urban discharges, and leaching of metals from antifouling paints, the total metal concentrations can approach or exceed those of the organic ligands, resulting in high aqueous inorganic metal concentrations. It has been suggested that even low ambient concentrations of inorganic metal species in the aquatic environment could have adverse effects on the distribution and composition of phytoplankton communities (Sunda 1988; Rijstenbil et al. 1991). The effects are difficult to assess as phytoplankton composition and spatial distribution are functions of various environmental factors, including salinity, turbidity, nutrients, turbulence, and depth (Fogg & Thake 1987a). Furthermore, these variables can be perturbed by natural forcing or human activities. The production of intracellular metal-
binding peptides, more specifically phytochelatins, is one of the mechanisms used to reduce the toxic effects of metals by many phytoplankton species, and is possibly significant in metal affected environments. Phytochelatins have been suggested to provide a sub-lethal measure of metal stress to phytoplankton in natural waters (Ahner et al. 1997). Few field studies, however, have been undertaken to support this hypothesis.

The aim of this chapter is to describe and interpret field measurements of particulate metal-binding peptides in two metal impacted systems in the UK: (i) the Fal Estuary and (ii) the Tamar and Plym estuaries (Plymouth Sound). Only a handful of studies have focused on the adaptive mechanisms against metal toxicity by organisms living in these metal impacted areas (Langston et al. 2003b). Baseline levels of particulate metal-binding peptides have not yet been determined in these systems. Here, the relationships between metal-binding peptides with dissolved (< 0.45 μm) copper speciation are considered. Copper is essential for photosynthetic organisms playing a vital role in electron transport in photosynthesis, but it is also one of the most toxic metals to marine algae (Gledhill et al. 1997; Pinto et al. 2003). Copper is found at elevated concentrations in the Fal and Plymouth estuarine systems.

5.3. Study areas – environmental setting

The Fal Estuary and Plymouth Sound and its estuaries, are located in the SW England (UK) and are candidates for special areas of conservation. Langston et al. (2003a, b) have recently reviewed the water and sediment quality of these areas, in order to provide a baseline for assessing the present and future effects of anthropogenic activities on these
environments. Most of the following discussions on metal distribution in these areas are based on these reviews.

5.3.1. Fal Estuary

The Fal Estuary lies in a highly mineralised area and is considered one of the most metal impacted estuaries in the UK (Fig. 5.1). Mining activities for deposits of tin, copper, lead, iron, arsenic, tungsten, uranium, and silver reached their apogee in the 19th century, together with ore processing in the Carnon Valley (Bryan & Gibbs 1983; Pirrie et al. 1997). Most studies in this area have concerned trace metal association with sediments, and the effects of long-term metal exposure on benthic organisms (Bryan & Gibbs 1983; Warwick 2001). Restronguet Creek is the most metal affected branch of the Fal Estuary as it is directly fed by the Carnon River, which receives drainage from inactive metalliferous mines with high concentrations of Zn, Mn, Cu and Cd (Bryan & Gibbs 1983; Rijstenbil et al. 1991). Metal contaminated sediments and mine tailings (sources of metals to the water column) are transported from Restronguet Creek to Mylor, Pill (adjacent to Restronguet) and St Just creeks, where they accumulate (Langston et al 2003b).
5.3.2. Plymouth Sound—Tamar and Plym estuaries

The Plymouth Sound and its estuaries (Fig. 5.2), also located in the SW England, are subject to several anthropogenic influences such as shipping activities, including naval, commercial and pleasure craft (Langston et al. 2003a).


The Tamar and Plym Valleys have around nine centuries of mining activities for deposits of tin, copper, lead, silver, iron, arsenic, zinc, tungsten, and manganese. The catchment area of the upper Tamar Estuary today is influenced by agriculture and old mines, whilst the lower estuary is subjected to substantial urban and industrial development. As a
consequence, the waters and sediments of the Tamar Estuary are enriched with metals and nutrients through point and diffuse sources. The quality of the waters of the Plym Estuary is subjected to the influence of the two large consented discharges by volume (excluding sewage treatment works), which could have effects on other parts of the system (Langston et al. 2003a).

5.3.3. Other contaminants and environmental quality of the Fal Estuary and Plymouth Sound

Other contaminants and physical changes have also been introduced in the Fal Estuary and Plymouth Sound mainly through sewage and industrial discharges and agricultural run-off from the catchment area. Table 5.1 summarises the major contaminants, physical factors, the most affected areas and consequences to the water quality of the Fal Estuary and Plymouth Sound in relation to the Environmental Quality Standard (EQS) values. The EQS was based on laboratory ecotoxicological studies using a range of organisms. Values above the EQS indicate that deleterious effects on biota/feature might occur, although there is a paucity of data on sub-lethal effects to guarantee a safe threshold for these values. Furthermore, it is possible that synergistic and/or antagonistic effects of a mixture of the various contaminants present in these estuaries occur, altering the expected biological responses for one stressor.

Specific responses to metal impact have been reported for a limited number of organisms in the Fal and Tamar estuaries. These include adaptive mechanisms such as production of the metal-binding proteins metallothioneins in gills of mussels and crabs (Pedersen & Lundebye 1996; Langston et al. 2003b), changes in permeability in macroalga to reduce
metal uptake (Langston et al. 2003b), and cyto- and genotoxic biomarkers in molluscs (Cheung & Jha 2004).

Table 5.1. Summary of contaminants, physical factors, sources, affected areas, and most vulnerable features/biota in the Fal Estuary and Plymouth Sound and estuaries (adapted from Langston et al. 2003a; b). EQS = Environmental Quality Standard.

<table>
<thead>
<tr>
<th>Contaminant/ Physical factor</th>
<th>Source</th>
<th>Affected area</th>
<th>Most vulnerable species/feature</th>
<th>Water quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organotin</td>
<td>Falmouth Docks, shipping sediments, sewage discharges</td>
<td>Widespread in both systems, especially Falmouth Docks</td>
<td>Molluscs</td>
<td>Chronic inputs not systematically investigated</td>
</tr>
<tr>
<td>Metals (As, Cu, Cd, Fe, Zn)</td>
<td>Historic mining activities, sediments, discharges Also industry in the Plymouth Sound</td>
<td>Restrongouet and Mylor Creeks, upper Fal</td>
<td>Tamar and Tavy Estuaries</td>
<td>Invertebrates, birds and fish, species composition</td>
</tr>
<tr>
<td>Nutrients</td>
<td>Sewage discharges, run-off from land, mine drainage</td>
<td>Upper Fal (Truro area), Helford</td>
<td>Upper Tamar, Yealm, Lynher</td>
<td>Invertebrates, fish, seabirds, mammals</td>
</tr>
<tr>
<td>Turbidity</td>
<td>Sewage discharges, aggregate extraction</td>
<td>Upper Fal (Truro, Tresilian area)</td>
<td>Tamar</td>
<td>Benthic communities, fish</td>
</tr>
<tr>
<td>Microbiological parameters</td>
<td>Sewage discharges, farm animals (run-off from land)</td>
<td>Upper Fal (Tresilian) Penryn River (flushing)</td>
<td>Lynher, Tamar, Plym</td>
<td>Bivalves, birds, marine organisms</td>
</tr>
<tr>
<td>Hydrocarbon oils, polycyclic aromatic hydrocarbons</td>
<td>Discharges, shipping, urban run-off, atmospheric deposition</td>
<td>Lower Fal and Helford</td>
<td>Tamar and Plymouth sound sediments</td>
<td>Bivalves, fish, seagrasses and shoreline communities, species composition</td>
</tr>
<tr>
<td>Pesticides, herbicides and other synthetic organics</td>
<td>Agricultural run-off and sewage discharges</td>
<td>Poorly defined, silty sediments are probably main reservoir</td>
<td>Plym, Tamar, Plymouth Sound</td>
<td>Invertebrates, food chain bioaccumulation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.4. Experimental

5.4.1. Sample collection

Surface water samples were collected from different branches of the Fal Estuary and some points of the Caron River (Fig. 5.1) during surveys on 08\textsuperscript{th} August 2002, 30\textsuperscript{th} April 2003 and 1\textsuperscript{st} July 2003, using 1 or 2 L acid washed polycarbonate bottles. Axial transects along Plym and Tamar estuaries (Fig. 5.2) were undertaken on 24\textsuperscript{th} September 2002 and 25\textsuperscript{th} April 2003, respectively. All samples were collected from a depth of around 0.3 m with the use of small boats. The samples were placed in a cool box until returned to laboratory (within 8 h). Samples for trace metal and chlorophyll \(a\) analyses were collected separately using 250 mL acid cleaned high density polyethylene bottles. Sub-samples were collected for the determination of salinity. \textit{In-situ} measurements of pH, temperature and conductivity were also taken using portable instruments (Hanna instruments).

5.4.2. Particulate phytochelatins and glutathione

The analysis of the particulate metal-binding peptides phytochelatins (PCs) and glutathione (GSH) was based on Rijstenbil & Wijnholds (1996) with a few modifications, and is fully described in Chapters 3 and 4. The water samples collected from selected sites in Plymouth Sound and Plym Estuary were filtered using 0.45 µm pore size nitrocellulose filters, instead of 0.8 µm, as part of the initial adaptations of the analytical method for field application.
5.4.3. Total dissolved copper

Determination of total dissolved Cu (Cu_{Total}) in water samples was based on adsorptive cathodic stripping voltammetry (AdCSV), using salicylaldoxime (SA, 2-hydroxybenzaldehyde oxime) as added ligand (Campos & van den Berg 1994). Samples were filtered using acid washed polycarbonate membrane filters (0.4 μm, Whatman), then acidified to pH ~ 2 using quartz distilled HCl and irradiated with ultraviolet light at 70°C in quartz tubes for 6 h using a 400 W mercury vapour lamp. The pH of the sample was neutralized with ammonia and subsequently a 10 mL aliquot was pipetted into the voltammetric cell. The buffer N-2-hydroxyethylpiperazine-N' -2-ethansulphonic acid (HEPES, pH = 8.2 adjusted with NH₃) and SA solutions were added to the cell to achieve 10 mM and 25 μM (final concentrations), respectively.

The measurements were carried out using a potentiostat (Autolab Ecochemie) interfaced with an automated hanging mercury drop electrode (Model VA 663, Metrohm), with potentials measured against an Ag/AgCl/KCl reference electrode. The sample solution was purged for 4 min, and the CuSA complex was deposited on a fresh Hg drop at -1.1 V for 30 s. The potential was scanned from -0.1 to -0.6 V with Cu reduction peak occurring at -0.35 V (square-wave voltammetry, 50 Hz, pulse amplitude 0.025 V, scan rate 25 mV s⁻¹). The concentration was measured by addition of Cu from 10⁻⁵ M stock standards. Duplicate Cu measurements produced less than 2% difference, and analysis of certified reference material (SLEW2) were in good agreement with certified values for Cu. Sample handling was carried out in a Class 100 laminar flow hood.
5.4.4. Natural organic ligands and copper species

Determination of natural organic ligands (L), free Cu$^{2+}$ and inorganic Cu (Cu') concentrations in filtered samples was carried out using competitive ligand titrations with SA as described in Campos and van den Berg (1994). Eleven sample aliquots of 10 mL each were pipetted into 30 mL polystyrene cups together with 2 $\mu$M SA and 10 mM HEPES (pH = 8.5). Copper solution was added in ten of the cups in increasing increments (100-800 nM Cu for samples from Restronguet Creek and Camon River, and 10-200 nM Cu for the other sample locations), and then allowed to equilibrate for at least 15 h at room temperature. The voltammetric analysis was performed using the same conditions as for the determination of Cu$_{Total}$. Triplicate titrations resulted in standard deviations of 0.7 and 0.2 for the ligand concentrations and Log $K_{CuL}$, respectively.

5.4.5. Chlorophyll a

Chlorophyll a analysis was performed using a well-established fluorimetric method (Parsons et al. 1984). Water samples were filtered onto 0.8 $\mu$m nitrocellulose filters, under gentle vacuum. The filters with retained particles were wrapped in aluminium foil and kept frozen until extraction. The extraction of chl a from the particles was carried out using 90% acetone (HPLC grade) in MilliQ water and ultrasonication in an ice bath for 15 min, followed by overnight storage (4°C, protected from light). Fluorescence of the samples was measured using a spectrofluorimeter (Merck-Hitachi) at 680 nm (emission) and 436 nm (excitation) wavelengths. A linear response was observed for chl a standard (Sigma) solutions (made up in 90% acetone) between 2 to 500 $\mu$g L$^{-1}$. Pheophytine concentrations were determined after acidification of the samples by addition of 0.1 M HCl to the sample cuvette (4 mL) and discounted from the chl a measurements.
5.5. Results & Discussion

5.5.1. Fal Estuary

5.5.1.1. Phytoplankton composition of the Fal Estuary

Chlorophyll $a$ concentrations and salinities for the Fal Estuary samples are presented in Fig. 5.3. Chl $a$ concentrations ranged from 3.1 to 69.1 $\mu$g L$^{-1}$ and indicated the occurrence of phytoplankton blooms for a number of sample locations ($> 10$ $\mu$g L$^{-1}$). Salinities were relatively similar and higher than 23 for most of the sampling sites. The only exceptions were some of the Carnon River and Restronguet Creek samples which were under a greater riverine influence and showed the lowest chl $a$ concentrations.

The phytoplankton species composition for August 2002 is summarised in Table 5.2 (Environment Agency, pers. comm.). Not all the sampling sites chosen by the Environment Agency coincided with those from the present study, and data for phytoplankton composition for 2003 was not available. Apart from this data set, recent studies concerning phytoplankton composition/distribution in the Fal Estuary are scarce. A study conducted in the Fal Estuary in 1989 showed that the phytoplankton species composition in the metal contaminated Restronguet Creek deviated from that in other branches (Rijstenbil et al. 1991). Rijstenbil et al. (1991) observed that in the riverine parts of Restronguet Creek, the dinoflagellate $E. mutabilis$ occurred at $\mu$M Cu and Zn, whereas in clean waters presenting nM Cu and Zn, $Chlamydomonas$ sp. and $Oocystis$ sp. were the common species.
Figure 5.3. Salinity and chlorophyll a for the Fal Estuary surveys. a) 8th August 2002 and 7th October 2002 (latter samples marked with *); b) 30th April 2003; c) 1st July 2003.
Table 5.2. Common dominant phytoplankton species reported in the Fal Estuary (Environment Agency data, August 2002). * Autumn 1989 data from Rijstenbil et al. 1990; + indicates 6-50 cells mL\(^{-1}\); ++ indicates > 50 cells mL\(^{-1}\).

<table>
<thead>
<tr>
<th>Species</th>
<th>River</th>
<th>St Just</th>
<th>St Mawes</th>
<th>Falmouth Yacht Marina</th>
<th>Mylor Creek</th>
<th>Restronguet Creek</th>
<th>Carnon River</th>
<th>Carrick Roads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diatoms</td>
<td>Fal/Truro</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dactyliosolen fragilissima</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudonitzschia spp.</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizosolenia setigera</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minidiscus sp.</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Skeletonema sp.</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>++</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Thalassiosira sp.</td>
<td>++</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td></td>
<td>++</td>
<td></td>
</tr>
<tr>
<td>Dinoflagellates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>Euglena mutabilis*</td>
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<td>Criptomonas spp*</td>
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</table>
Bloom of the dinoflagellates *K. mikimotoi*, *P. triestinum* and *P. micans*, and the diatoms *Minidiscus sp.*, *Thalassiosira sp.* and *S. costatum*, were common in the estuary during 2002. Among these phytoplankton species, only the diatoms *S. costatum* and *Thalassiosira spp.*, the dinoflagellate *P. micans* and the protozoa *E. mutabilis* have been exposed to metals under laboratory conditions to evaluate their metal detoxification responses. *S. costatum* and *Thalassiosira pseudonana* were found to be metal tolerant and good producers of PCs under metal stress (Ahner et al. 1995; Rijstenbil & Wijnholds 1996). *P. micans* did not produce PCs as a metal detoxification mechanism, and instead, responded with a Cu efflux system and Cu sequestration via polymeric substances within the cell (Lage et al. 1996). There are no literature data for metal-binding peptides produced by the metal tolerant flagellate *E. mutabilis*. This species has been reported as present in acidic mine drainage waters, and can tolerate extremely high concentrations of As without apparent production of methylated species, a common pathway for As detoxification in most algae (Casiot et al. 2004).

Freshwater phytoplankton species have been reported to produce PCs at similar concentrations as marine species (Knauer et al. 1998; Pawlik-Skowronska 2001). Thus, it can be expected that the phytoplankton species most likely to contribute to the production of particulate PCs are the numerous diatoms and green algae, either marine or freshwater species, as the abundant dinoflagellates in the Fal system have not yet been reported to produce PCs.

### 5.5.1.2. Particulate metal-binding peptides and total dissolved Cu in the Fal Estuary

The chl *a*-normalised concentrations of particulate GSH and PC2, together with the concentrations of dissolved Cu<sub>Total</sub> for the different branches of the Fal Estuary, are
presented in Fig. 5.4. It was preferred to plot the data for metal-binding peptides using the geographical locations (sampling sites) instead of salinity, because the salinity values were relatively similar for most of the branches. In addition, the branches were subject to different metal inputs and a comparison among them was more appropriate to highlight the relations between the metal contamination and the production of metal binding peptides. In this study, only Cu was determined because of 3 aspects: 1) Cu is one of the most toxic metals to phytoplankton; 2) Cu is present at high concentrations in the Fal Estuary; 3) and time constrains to analyse other metals and metal speciation.

Phytochelatin polypeptide chains longer than \( n = 2 \) were not observed in the Fal samples. The only field measurement of PC3 \((n = 3)\) was reported by Ahner et al. (1997) at concentrations approximately three times lower than those of PC2. Longer chained PCs, produced by some phytoplankton species under controlled and prolonged laboratory metal exposure experiments (Ahner et al. 1995; 1997; Rijstenbil & Wijnholds 1996; Morelli & Pratesi 1997), should be able to bind metals more efficiently due to the number of SH groups and advantageous conformational structures. The dynamic nature of the estuarine system with rapid phytoplankton turnover rates and estuarine flushing times are likely to influence the production and stability of such longer polypeptides. Thus, the absence of longer chained PCs in the samples could be due to population and/or cellular constraints of natural phytoplankton assemblages living in these dynamic environments, in addition to limitations in the sensitivity of the analytical method.
Figure 5.4. Particulate PC2, GSH and dissolved Cu_{Total} in the Fal Estuary. a) PC2 vs Cu_{Total} for Aug-2002 survey; b) GSH vs Cu_{Total} for Aug-2002 survey; c) PC2 vs Cu_{Total} for April-2003 survey; d) GSH vs Cu_{Total} for April-2003 survey; e) PC2 vs Cu_{Total} for July-2003 survey; f) GSH vs Cu_{Total} for July-2003 survey. *collected in October 2002. Bars span the range from the average for duplicate analyses. Dotted histogram columns are below detection limit.
For the first survey (August 2002), GSH and PC2 were observed throughout the estuary at concentrations ranging from 58 to 274 μmol (g chl a)$^{-1}$ and 2 to 36 μmol (g chl a)$^{-1}$, respectively. The highest GSH and PC2 concentrations were found in Restronguet Creek (site 8). A significantly higher production of metal-binding peptides, particularly PC2, was also found in Mylor Creek sample (site 6). Phytochelatins were not detected in St Mawes (site 3), situated at the marine end of the estuary and further from the influence of the mine discharges.

A relatively high dissolved Cu$_{\text{Total}}$ concentration was measured (250 nM) in Restronguet Creek during October 2002 (samples marked with * in Fig. 5.4a), at low tide, but PC2 and GSH concentrations did not show enhanced levels accordingly. With the exception of this last sample, strong correlations were observed between the concentrations of dissolved Cu$_{\text{Total}}$ and metal-binding peptides (Fig. 5.5a and 5.5b). Even stronger positive relationships were observed for free aqueous Cu$^{2+}$ (as log values) with GSH and PC2 (Fig. 5.5e and 5.5f). Dissolved Cu$_{\text{Total}}$ and Cu$^{2+}$ concentrations also showed a close and significant correlation (correlation coefficient = 0.94; $P < 0.01$; $r^2 = 0.86$).

For the other surveys (April and July 2003), GSH concentrations were found between 0.6 and 9 μmol (g chl a)$^{-1}$, and PC2 concentrations were below detection limit for many samples. A maximum PC2 concentration of 7 μmol (g chl a)$^{-1}$ was observed in Restronguet Creek for April 2003 (Fig. 5.4c). High correlations between the concentrations of metal-binding peptides and dissolved Cu$_{\text{Total}}$ and Cu$^{2+}$ were not observed in the data set from 2003 (Fig. 5.5c,d,g,h), in spite of the similar Cu levels observed in the 2002 surveys.
Figure 5.5. Fal Estuary a) GSH vs CuTotal for 2002; b) PC2 vs CuTotal for 2002; c) GSH vs CuTotal for 2003; d) PC2 vs CuTotal for 2003, circled values are below detection limit (not included in the regression equation); e) GSH vs Log[Cu^{2+}] for the 2002 survey; f) PC2 vs Log[Cu^{2+}] for the 2002; g) GSH vs Log[Cu^{2+}] for the 2003; h) PC2 vs Log[Cu^{2+}] for the 2003 survey. Triangles correspond to data from April 2003.
The interaction between the dissolved and the particulate phases was observed by the positive relationships between the concentrations of dissolved Cu species and particulate metal-binding peptides. The y-intercept in the graph presented in Fig. 5.5a shows a relatively high residual value for GSH, which indicated that 108 μmol (g chl a)^{-1} of GSH were produced for a theoretical zero concentration of dissolved Cu_{Total}. It is consistent with the fact that GSH is a naturally thiol produced by all eukaryotes (Noctor & Foyer 1998). From the equations of the curves shown in Fig. 5.5a and 5.5b, it can be noted that for the 2002 survey the production of PC2 was faster than that of GSH. For example, 5 times increase in dissolved Cu_{Total} concentration resulted in 8 times increase in PC2 production in comparison to the 2 times increase in GSH production. Although free Cu^{2+} is the most toxic Cu species for phytoplankton (Gledhill et al. 1997), considerations regarding the relationship between the concentrations of free Cu^{2+} and metal-binding peptides are more limited here, as the Cu^{2+} concentrations were transformed into log values.

Samples collected between Carnon River and Restronguet Creek at varying salinities (1 to 28) during the 2003 surveys also showed low concentrations of particulate GSH and PC2, ranging from 3.1 to 8.3 μmol (g chl a)^{-1} and 0.2 to 3.3 μmol (g chl a)^{-1}, respectively (Fig. 5.6), in comparison to the 2002 surveys. Dissolved Cu_{Total} concentrations for samples C4 and C5 were about 300 nM. Free aqueous Cu^{2+} and organic ligands were not determined for these samples due to saturation of the electrodes.

For the 2002 surveys, particulate PC2 concentrations in the Fal Estuary were comparable to those in New England (USA), where PC2 concentrations above 10 μmol (g chl a)^{-1} were typical in heavily metal polluted harbours (Ahner et al. 1997). Contrasting results, however, were observed for the 2003 surveys for which the metal-binding peptides were at...
5 to 200-fold lower concentrations, in spite of the similarities in Cu complexation (see further on). The most metal impacted site (Carnon River) did not exhibit high production of metal-binding peptides during any survey. Restronguet Creek showed low concentrations of metal-binding peptides for some surveys. No statistically significant relationships were observed between GSH and PC2 concentrations for any of the surveys ($P > 0.05$). Furthermore, neither PC2 nor GSH presented strong correlation with chl $a$, contrary to what has been reported for less metal affected coastal systems (Matrai & Vetter 1988; Rijstenbil et al. 1998; Tang et al. 2000).

![Figure 5.6. Concentrations of GSH and PC2, together with pH values and salinity for samples collected between Carnon River-Restronguet Creek. C1, C2 and C3 were collected in April 2003. C4 and C5 were collected in July 2003.](image)

High variabilities in the concentrations of metal-binding peptides were also observed within Elizabeth River Estuary (Wei et al. 2003) and in heavily metal contaminated lakes in Switzerland (Knauer et al. 1998) and were associated with a heterogeneous phytoplankton species composition of the sampling areas. The heterogeneity in phytoplankton composition in the Fal Estuary may explain the lack of correlation between metal-binding peptides and chl $a$ concentrations. For instance, dinoflagellate species common in the Fal Estuary ($P. micans$) and Carnon River ($E. mutabilis$) seem to have alternative metal detoxification mechanisms to PC production; consequently their high abundances could have lead to relative low levels of PC production. The assumption that
low concentrations of particulate PCs in the field indicate a lack of metal stress should therefore be taken with caution. Furthermore, as the Fal Estuary has been contaminated with metals for centuries, the biological responses to metal toxicity could be altered through evolution of metal tolerant strains with detoxification mechanisms other than PC production.

Another contributing factor to the high variabilities in the concentrations of the metal-binding peptides for the surveys could be a limitation in nutrients, particularly nitrogen, as it is required for peptide synthesis (Rijstenbil et al. 1998; Wei et al. 2003). Nutrient levels in the Fal system, however, have lead to regular episodes of algal blooms, including toxic ones (Langston et al. 2003b), and therefore were unlikely to be affecting the production of thiols in the Fal system.

5.5.1.3. PC:GSH ratios

The PC2:GSH ratios together with the PC2 concentrations for the different branches of the Fal Estuary are plotted in Fig. 5.7. Restronguet Creek (April 2003) and Carnon River (C1, April 2003) showed the highest PC2:GSH values, 0.89 and 0.40, which were more indicative of metal stress than their respective PC2 concentrations alone. Samples from the Carnon River, C2 and C3, (July 2003) were collected at lower salinities, therefore the dominating phytoplankton were likely dinoflagellate species, which have not yet been reported to produce PCs. This could explain the low PC2 concentrations and low PC2:GSH values for these metal contaminated samples.
Figure 5.7. Comparison between PC2 concentrations and PC2:GSH ratios for the Fal Estuary samples. a) August 2002; b) April 2003; c) July 2003.
Most of the PC2:GSH ratios in the present study were lower than 0.05, yet within the same range found in Elizabeth River (Wei et al. 2003). Higher ratio values were observed for a number of sample locations in Galveston Bay (0.018-0.52), a coastal system not considered metal polluted (Tang et al. 2000). Apart from the Restronguet Creek and Carnon River samples, these ratios suggest that phytoplankton in the Fal Estuary branches are not under strong metal stress. It is important to highlight again that PC production is phytoplankton species dependent and the development of tolerant strains could mask the biological responses to metal stress. Furthermore, the disadvantage of using the PC2:GSH ratios is the limitation caused by the lack of data on how GSH concentrations vary in the field. For instance, some studies have associated variations in GSH production with parameters other than metal stress, such as the presence of xenobiotics, light availability, nitrate and phosphate levels (Matrai & Vetter 1988; Rijstenbil et al. 1998; Tang et al. 2000). The Fal Estuary has been subjected to several other contaminants (Table 5.1) that could have effects on the intracellular GSH concentrations, by means of GSH S-transferases in the detoxification of xenobiotics (Noctor & Foyer 1998). Furthermore, zooplankton and bacteria, also retained in the particulate phase, can cause an overestimation of GSH production by phytoplankton. Nevertheless, the advantage in using PC:GSH ratios to examine metal stress relies primarily on the elimination of the uncertainties caused by chl a normalisation, as chl a levels vary greatly as a function of light intensities, stage of phytoplankton life cycle, and phytoplankton species (Wei et al. 2003).

5.5.1.3. Copper complexation in the Fal Estuary waters

Representative Cu-ligand titration and linearization curves are shown in Fig. 5.8. The curvature at the beginning of the titration curve (Fig. 5.8a) indicates the presence of natural
Chapter 5 Field measurements of particulate phytochelatins and glutathione

Cu-complexing ligands in the water sample. Linear treatment of the titration data (Fig. 5.8b) produced a straight line for all samples, indicating that Cu-complexation was controlled by a single class of ligands (Campos & van den Berg 1994).

![Typical titration curve for the estuarine samples (Mylor Creek, July 2003); van den Berg linearization for the titration data. Cu_total = Cu in the sample plus Cu added for the titration; Cu_L = Cu complexed by natural organic ligands; Cu_1abl = Cu complexed by SA.](image)

The concentrations of dissolved Cu_total, organic ligands (L), free Cu^{2+}, inorganic Cu (Cu'), together with the conditional stability constants of the Cu-organic complexes (Log K_{CuL}) and the degree of Cu-complexation (%CuL) for the separate field campaigns, are shown in Table 5.3. The dissolved organic ligands were at concentrations higher than or close to those of Cu_total for most of the samples. The high conditional stability constants for the Cu-organic complexes found in the water samples (Log K_{CuL} = 10.8-13.5) were in the range for the strong ligands. Copper was complexed by strong ligands (100% CuL), as the major species for most of the sites. Complete saturation of the organic ligands by Cu was only observed in Restronguet Creek during low tide ([Cu_total] > [L]). For this sample, the titration curve produced a straight line, without the initial curvature shown in Fig. 5.8a. Such ligand saturation resulted in elevated free Cu^{2+} and Cu' concentrations.

125
Table 5.3. Copper complexation in the Fal Estuary waters. The concentrations of Cu<sub>total</sub> are means obtained from duplicate measurements. %Cu<sub>L</sub> = ([Cu<sub>total</sub> - [Cu<sup>2+</sup>] - [Cu'])/([Cu<sub>total</sub>])x100.

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<tr>
<th>08-Aug-02</th>
<th>Cu&lt;sub&gt;total&lt;/sub&gt;</th>
<th>L</th>
<th>Log K&lt;sub&gt;Cat&lt;/sub&gt;</th>
<th>Cu&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Cu'</th>
<th>%Cu&lt;sub&gt;L&lt;/sub&gt;</th>
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<td>Fal/Truro</td>
<td>13.4 ± 1.5</td>
<td>73.4 ± 2.0</td>
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<td>6.7E-15</td>
<td>9.3E-15</td>
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<tr>
<td>St Just</td>
<td>10.9 ± 0.7</td>
<td>34.8 ± 3.8</td>
<td>12.5 ± 0.6</td>
<td>1.3E-13</td>
<td>1.9E-13</td>
<td>100.0</td>
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<tr>
<td>St Mawes</td>
<td>6.7 ± 0.5</td>
<td>24.4 ± 0.4</td>
<td>13.2 ± 0.5</td>
<td>2.2E-14</td>
<td>3.0E-14</td>
<td>100.0</td>
</tr>
<tr>
<td>Falmouth Yacht</td>
<td>19.2 ± 1.5</td>
<td>65.1 ± 2.3</td>
<td>13.1 ± 0.7</td>
<td>3.6E-14</td>
<td>5.0E-14</td>
<td>100.0</td>
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<tr>
<td>Mylor</td>
<td>36.2 ± 1.5</td>
<td>95.1 ± 7.9</td>
<td>13.0 ± 0.4</td>
<td>6.0E-14</td>
<td>8.5E-14</td>
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<td>Mylor/Restronguet*</td>
<td>51.2 ± 1.0</td>
<td>52.3 ± 1.5</td>
<td>12.3 ± 0.1</td>
<td>2.0E-11</td>
<td>2.8E-11</td>
<td>99.9</td>
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<td>Restronguet1</td>
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<tr>
<td>Restronguet2</td>
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<td>63 ± 30</td>
<td>10.8 ± 0.03</td>
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<td>3.8E-08</td>
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<th>Cu&lt;sup&gt;2+&lt;/sup&gt;</th>
<th>Cu'</th>
<th>%Cu&lt;sub&gt;L&lt;/sub&gt;</th>
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<td>Fal/Truro</td>
<td>33.9 ± 0.5</td>
<td>39.9 ± 0.8</td>
<td>12.9 ± 0.3</td>
<td>7.5E-13</td>
<td>1.1E-12</td>
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</tr>
<tr>
<td>St Just</td>
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<td>48.4 ± 1.1</td>
<td>12.6 ± 0.2</td>
<td>3.8E-13</td>
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<td>100.0</td>
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<td>Percuil/Mawes</td>
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<td>41.6 ± 0.3</td>
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<tr>
<td>Falmouth Yacht</td>
<td>17.0 ± 2.6</td>
<td>27.7 ± 2.4</td>
<td>13.1 ± 0.8</td>
<td>1.2E-13</td>
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<td>Restronguet</td>
<td>500</td>
<td>620 ± 18</td>
<td>11.7 ± 0.2</td>
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<th>Cu'</th>
<th>%Cu&lt;sub&gt;L&lt;/sub&gt;</th>
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<td>Fal/Truro</td>
<td>31.8 ± 0.6</td>
<td>32.3 ± 1.2</td>
<td>12.3 ± 0.1</td>
<td>2.8E-11</td>
<td>4.0E-11</td>
<td>99.8</td>
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<tr>
<td>St Just</td>
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<td>21.4 ± 2.4</td>
<td>11.9 ± 0.1</td>
<td>4.0E-11</td>
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<td>99.5</td>
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<tr>
<td>St Mawes</td>
<td>25.2 ± 0.7</td>
<td>36.0 ± 1.0</td>
<td>13.4 ± 0.5</td>
<td>9.4E-14</td>
<td>1.3E-13</td>
<td>100.0</td>
</tr>
<tr>
<td>Falmouth Yacht</td>
<td>25.8 ± 0.7</td>
<td>37.0 ± 4.0</td>
<td>12.1 ± 0.1</td>
<td>2.0E-12</td>
<td>2.8E-12</td>
<td>100.0</td>
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<tr>
<td>Mylor</td>
<td>43.7 ± 1.4</td>
<td>70.5 ± 4.0</td>
<td>12.6 ± 0.5</td>
<td>4.4E-13</td>
<td>6.2E-13</td>
<td>100.0</td>
</tr>
<tr>
<td>Restronguet</td>
<td>38.5 ± 0.9</td>
<td>44.7 ± 1.4</td>
<td>12.8 ± 0.2</td>
<td>1.0E-12</td>
<td>1.4E-12</td>
<td>100.0</td>
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</tbody>
</table>

Higher concentrations of dissolved strong ligands observed for some of the branches (mostly during the 2002 survey) were likely due to anthropogenic inputs of organic matter and run off of humic substances from the catchment area. No obvious relationship was observed between the concentrations of dissolved organic ligands and chl<sub>a</sub> for the surveys (Fig. 5.9). This is consistent with the fact that the dissolved organic ligands in coastal waters are derived from a range of sources and are not only associated to phytoplankton exudates, as is likely the case for oceanic waters (Croot et al. 2000).
Figure 5.9. Dissolved organic ligands and chlorophyll a concentrations for the Fal Estuary samples. a) August 2002; b) April 2003; c) July 2003.
The highest chl $a$ content in Mylor Creek for July 2003, indicative of a phytoplankton bloom, coincided with the highest ligand concentration. Again, it could be simply due to inputs of organic matter and nutrients (favouring phytoplankton blooms) associated to anthropogenic sources and run off from the catchment area. The Cu complexation in the Fal Estuary followed the trends observed in other aquatic systems for which the molar ratios of strong organic ligands to Cu$_{\text{Total}}$ were approximately 1:1. This relation is believed to be tightly modulated by the concentrations of dissolved Cu$_{\text{Total}}$ (Kozelka & Bruland 1998). Weak dissolved Cu-complexing ligands, not detected by the established analytical conditions, should be at concentrations higher than the strong ligands (Moffett et al. 1997) and could also be important for the total metal buffering capacity of the system, despite their weaker binding strengths.

The abundant presence of phytoplankton species such as $P$. micans and $S$. costatum, able to produce exudates with different binding strengths (Croot et al. 2000), may also be a key characteristic for phytoplankton adaptation in such metal affected systems. This feature is ecologically relevant as the released ligands could benefit other metal-sensitive zoo- and phytoplankton species by decreasing the concentration of bioavailable toxic metals in the surrounding waters, and balancing the low production of phytochelatins.

The structure and exact composition of the dissolved Cu complexing ligands remain unknown in spite of several attempts at elucidating their identity (Gordon et al. 1996; 2000; Ross et al. 2003). Potential metal complexing ligands include sulphide and thiols like GSH and cysteine (Gordon et al. 1996; 2000; Leal & van den Berg 1998). The conditional stability constants for GSH and cysteine, on the basis of Cu$^{2+}$, are $10^{12.4}$ and $10^{13.0}$, respectively (Leal & van den Berg 1998), which are within the range for the strong
ligands found in the present study. Dissolved GSH, determined by voltammetric
techniques, was found at concentrations up to 100 nM in the Mersey Estuary (Le Gall &
van den Berg 1993). Dissolved GSH and PC2, determined by chromatography, were
observed ranging from 0.2 to 6.2 nM and 1.2 to 3.0 nM, respectively, in Galveston Bay
(Tang et al. 2000). Tang et al. found that dissolved GSH and PC2 were at 10 and 100 fold
higher concentrations than in the particulate phase, respectively. It seems unlikely that PCs
participate in the bulk of the dissolved metal-organic complexes, as PC-metal complexes
undergo rapid dissociation and degradation once exported from cells (Lee et al. 1996). A
high rate of GSH efflux would be necessary considering the lower levels of GSH in
phytoplankton cells (Ahner et al. 1997; Ahner et al. 2002). Therefore, dissolved GSH in
natural waters is likely originating from other sources in addition to phytoplankton.

5.5.1.4. Other dissolved metals and effects on the production of metal-binding
peptides in the Fal Estuary

Apart from the Restronguet Creek and the Carnon River, the rest of the Fal system has
presented dissolved metal concentrations in the water column that mostly fall within the
Environmental Quality Standard (EQS) values (Table 5.4). The tidal regime and degree of
water stratification are responsible for the great variabilities observed for the metal
concentrations in Restronguet Creek (Langston et al. 2003b). Zinc and Cu concentrations
in other parts of the Fal system occasionally exceed the EQS values due to the influence of
the sewage treatment works in the upper Fal Estuary and activities in the Falmouth
dockyard area (Langston et al. 2003b).
### Table 5.4. Dissolved metal concentration ranges (nM) in the Fal Estuary (data from Environmental Agency 1990-2001, estimated from graphs in Langston et al. 2003b; EQS = Environmental Quality Standard for annual average).

<table>
<thead>
<tr>
<th></th>
<th>Fal/Truro</th>
<th>Percuil</th>
<th>Fal off Mylor</th>
<th>Penryn Dock</th>
<th>Falmouth Marina</th>
<th>Fal off Restronguet</th>
<th>Restronguet mouth</th>
<th>EQS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>32-47</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>79-157</td>
<td>80</td>
<td>425</td>
<td>16-441 d</td>
</tr>
<tr>
<td>Zn</td>
<td>77-918</td>
<td>77-382</td>
<td>275-1223</td>
<td>290-306</td>
<td>275-459</td>
<td>183-6500</td>
<td>122-1911 tt</td>
<td>612 d</td>
</tr>
<tr>
<td>Cd</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.8-80</td>
<td>8.9 t</td>
<td>22 d</td>
</tr>
<tr>
<td>As</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>13-67</td>
<td>444 t d</td>
<td>334 d</td>
</tr>
</tbody>
</table>

1 European Community Dangerous Substances Directive for freshwater
2 European Community Dangerous Substances Directive for saltwater

_d_ = dissolved (i.e. involving filtration through 0.45 μm membrane filter before analysis)

_t_ = total (i.e. without filtration)

As discussed in the previous chapter, there is evidence that combinations of the free ions Mn\(^{2+}\), Pb\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\) and Cd\(^{2+}\) cause antagonistic, synergistic, and suppression effects on PC production, by metal competition for cellular binding sites and mechanisms still not fully elucidated (Pawlik-Skowronska 2001; Wei et al. 2003). Free Cd\(^{2+}\) has been shown to be the most effective inducer of PC production for many phytoplankton species under laboratory conditions (Ahner & Morel 1995). However, the high levels of dissolved Zn and Mn in Restronguet Creek could have decreased Cd\(^{2+}\) effects on PC production, as Cd\(^{2+}\) uptake takes place through the same cellular channels as Zn and Mn (Sunda & Huntsman 1998). Thus, the variabilities observed in the concentrations of metal-binding peptides for samples from Restronguet Creek and Carnon River could be in part a result of the metal interactions.
Despite the complexity of the estuarine systems regarding to the phytoplankton composition and its variety of possible strategies to cope with metal toxicity, enhanced production of metal-binding peptides, particularly PC2, were observed at the sites with high dissolved Cu$_{\text{Total}}$ and free Cu$^{2+}$ concentrations. This supports the role attributed to the particulate metal-binding peptides in protective mechanisms against metal toxicity and as a sub-lethal response in impacted aquatic systems.

5.5.2. Plymouth Sound: Tamar and Plym Estuaries

5.5.2.1. Phytoplankton composition of the Tamar Estuary

Chlorophyll a concentrations were similar for most of the samples from the Tamar transect, ranging from 5.2 to 14.1 $\mu$g L$^{-1}$, and indicated phytoplankton blooms only for the two samples at the saline end of the transect (Fig. 5.10). As for the Fal Estuary, little information concerning phytoplankton composition is available for the Tamar and Plym estuaries. A compilation of data by Langston et al. (2003b) indicates that the upper Tamar Estuary can be dominated by freshwater phytoplankton species, including the diatom Cyclotella atomus, green algae Nannochloris sp., and dinoflagellates Heterocapsa triquetra and Amphidinium sp. (with dinoflagellates contributing less to the biomass). In addition, marine species such as the diatoms Rhizosolenia delicatula, Nitzchia closterium, and Skeletonema costatum, and the dinoflagellate Prorocentrum micans, can be transported by the flood tide from Plymouth Sound into the brackish waters within the Tamar to survive in the short term. Low light availability, as a result of the high turbidity of the Tamar estuarine waters (Langston et al. 2003a), could limit the development of phytoplankton species that are more light-dependent, such as diatoms.
A continuous increase in phytoplankton diversity can be expected down estuary, with progressive gain in marine species (Fogg & Thake 1987b; Langston et al. 2003a). Therefore, as for the Fal Estuary, the phytoplankton species most likely to contribute to the production of metal-binding peptides in the Tamar and Plym estuaries are diatoms and green algae, either marine or freshwater species.

5.5.2.2. Particulate metal-binding peptides and Cu complexation in the Tamar Estuary

The chl a-normalised concentrations of particulate PC2 were below the detection limit for most of the samples of the Tamar transect (Fig. 5.11). The highest PC2 concentration, 16.5 μmol (g chl a)\(^{-1}\) was observed in Gunnislake at the tidal limit of the Tamar. Particulate GSH was found at concentrations ranging from 18.8 to 244 μmol (g chl a)\(^{-1}\) with highest value in Morwelham. The PC2:GSH ratios were the highest in Gunnislake, which receives inputs of metals from old mine workings, spoil heaps and adits (Langston et al. 2003a).
Dissolved Cu\text{Total}, free Cu\textsuperscript{2+}, inorganic Cu (Cu'), and organic ligand concentrations were determined for selected sites in the Tamar transect from a separate field survey. Dissolved Cu\text{Total} concentrations were between 34.0 and 91.0 nM, and slightly lower than those of organic ligands (34.4 to 92.3 nM), resulting in free Cu\textsuperscript{2+} concentrations of 10\textsuperscript{-11} to 10\textsuperscript{-12} and Cu-organic complexes with conditional stability constants from 10\textsuperscript{12} to 10\textsuperscript{13} (Table 5.5). These data indicate a decrease in Cu\textsuperscript{2+} concentrations down estuary with increasing salinity. As for the Fal Estuary and other aquatic systems (Kozelka & Bruland 1998), an approximately 1:1 molar ratio was observed for the Tamar samples.

Table 5.5. Copper complexation in the Tamar Estuary. Dissolved copper species, organic ligands and conditional stability constants for the Cu-organic complexes. \%CuL = ([Cu\text{Total}] - [Cu\textsuperscript{2+}] - [Cu'])/([Cu\text{Total}]) * 100

<table>
<thead>
<tr>
<th>28\textsuperscript{th} March</th>
<th>Cu\text{Total}</th>
<th>L</th>
<th>Log K\text{CuL}</th>
<th>Cu\textsuperscript{2+}</th>
<th>Cu'</th>
<th>CuL</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Halton Quay</td>
<td>34.0 ± 0.6</td>
<td>34.4 ± 1.8</td>
<td>12.9 ± 0.2</td>
<td>3.0E-11</td>
<td>4.2E-11</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Cargreen</td>
<td>91.0 ± 1.6</td>
<td>92.3 ± 1.4</td>
<td>12.8 ± 0.08</td>
<td>1.1E-11</td>
<td>1.6E-11</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Saltash</td>
<td>71.2 ± 1.1</td>
<td>72.5 ± 1.4</td>
<td>13.0 ± 0.2</td>
<td>1.4E-12</td>
<td>1.9E-12</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Devil's Point</td>
<td>30.0 ± 0.6</td>
<td>35.3 ± 7.4</td>
<td>12.5 ± 0.3</td>
<td>7.7E-12</td>
<td>2.2E-11</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>
5.5.2.3. Other metals in the Tamar and Plym estuaries

As in the Fal Estuary, other potential toxic metals such as Cd, Zn and As can be found at elevated concentrations in the Tamar Estuary (Table 5.6). In general, dissolved metal concentration averages were below the EQS values, thus, acute biological and ecological effects were not expected to occur (Langston et al. 2003). Nevertheless, in the present study PC production was highly pronounced in Gunnislake, indicating a sub-lethal metal effect on phytoplankton. Another phytoplankton detoxification response reported for these estuarine waters was linked to arsenic with the presence of dissolved biomethylated arsenic species (monomethylarsenic and dimethylarsenic) at the seaward end (Howard et al. 1988). These results also indicated that the phytoplankton in the Tamar Estuary were under metal stress.

Table 5.6. Dissolved metal concentration ranges (nM) in freshwater and tidal waters of Tamar Estuary. Data from 2001, except Halton Quay (1997) and Tamar off Lynher (1993). STW = sewage treatment work; EQS = Environmental Quality Standard for annual average (all data from Langston et al. (2003a) obtained from graphs, except EQS).

<table>
<thead>
<tr>
<th></th>
<th>Gunnislake</th>
<th>Halton Quay</th>
<th>Ernesettle STW</th>
<th>Shellfish water</th>
<th>Off Lynher</th>
<th>Plymouth STW</th>
<th>Plym (Marsh Mills)</th>
<th>EQS (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>79-220</td>
<td>43-79</td>
<td>8-39</td>
<td>32-87</td>
<td>8-79</td>
<td>24-87</td>
<td>16-441</td>
<td>79^d</td>
</tr>
<tr>
<td>Zn</td>
<td>77-275</td>
<td>31-214</td>
<td>61-122</td>
<td>61-184</td>
<td>31-92</td>
<td>122-612</td>
<td>122-1911^d</td>
<td>612^d</td>
</tr>
<tr>
<td>Cd</td>
<td>0.3-0.7</td>
<td>0.4-1.2</td>
<td>1.3</td>
<td>0.9</td>
<td>1.3</td>
<td>1.2</td>
<td>8.9^e</td>
<td>22^d</td>
</tr>
<tr>
<td>As</td>
<td>27-80</td>
<td>27-67</td>
<td>27-71</td>
<td>33-40</td>
<td>20-33</td>
<td>27-67</td>
<td>444^d</td>
<td>334^d</td>
</tr>
</tbody>
</table>

^1 European Community Dangerous Substances Directive for freshwater
^2 European Community Dangerous Substances Directive for saltwater
^d dissolved (i.e. involving filtration through 0.45 μm membrane filter before analysis)
^t total (i.e. without filtration)
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Whether the dissolved metal concentrations in the Tamar Estuary are at a threshold that causes metal interactions capable of affecting PC production is an issue that requires further investigation.

5.5.2.4. Particulate metal-binding peptides in the Plym Estuary and Plymouth Sound

Chlorophyll $a$ and particulate GSH concentrations in the Plymouth Sound were found to be in the range 1.9 and 14.4 $\mu$g L$^{-1}$ and 111 and 419 $\mu$mol (g chl $a$)$^{-1}$, respectively (Fig. 5.12). Chlorophyll $a$ concentrations in the Plym Estuary ranged from 1.5 to 3.0 $\mu$g L$^{-1}$, with highest value of 37.5 $\mu$g L$^{-1}$ at the marine end of the estuary (Oreston, 2001), indicative of a phytoplankton bloom. Particulate GSH concentrations in the Plym Estuary increased seaward from 18.0 to 130 $\mu$mol (g chl $a$)$^{-1}$. The low chl $a$ values were consistent with other data (Langston et al. 2003b), and were probably low due to the high turbidity of the waters.

Particulate PCs were not detected in any of the samples collected in the Plym and Plymouth Sound. As part of the initial adaptations of the analytical method for thiols, these samples were filtered using 0.45 $\mu$m pore size filters, instead of 0.8 $\mu$m, which limited the collection of sufficient biomass, as the filters clogged with less than 1 L of the filtered sample, probably by particles other than phytoplankton cells.
A linear regression for the particulate GSH and chl a concentrations showed a strong positive linear correlation for the Plymouth Sound samples (Fig. 5.13). Particulate GSH and chl a concentrations for the Tamar transect, however, did not show any obvious relationship (Fig. 5.14). These contrasting results can reflect the heterogeneity composition of the samples from the Tamar transect (likely composed of a mixture of diatoms and dinoflagellates, either marine or freshwater species) in relation to the Plymouth Sound (dominated by marine diatoms) and Plym Estuary.

As outlined in section 5.5.1.3, GSH can be produced by most eukaryotic organisms and production may be affected by high light intensity, nutrient supply and organic xenobiotics (Matrai & Vetter 1988; Rijstenbil et al. 1998; Noctor & Foyer 1998). Lack in inorganic nitrogen supply, however, should not have affected GSH production in the Tamar and Plym estuaries, as the upper estuaries are subject to nutrient enrichment from agricultural run-off and sewage discharges (Langston et al. 2003a). The lack of correlation between GSH and Chl a concentrations could also indicate that the production of particulate GSH in the Tamar transect was not entirely derived from eukaryotic phytoplankton sources.
Metal speciation was not determined for the Plymouth Sound and Plym Estuary. Recent literature data from the Environment Agency (Langston et al. 2003) indicate that the dissolved metal concentrations for the Plym Estuary (Marsh Mills) are within the EQS values (Table 5.3). It is important to note, however, that these EQS values do not guarantee a safe threshold for the sub-lethal chronic effects on biota and their consequences. Therefore, the determination of biological responses such as PC production to provide stress indication could be an additional tool to the environmental assessment of the water quality.
5.6. Summary: particulate metal-binding peptides in the Fal Estuary, Plymouth Sound and other natural waters

The results presented here exemplify the challenges to investigate biological responses in complex environments such as estuaries, for which the dynamic nature involving tidal systems has great effects on the distribution of organisms and contaminants. The river/tidal flow can influence phytoplankton production and taxonomic distribution in an estuarine system through a number of mechanisms, including changes in: nutrient inputs, dilution rates or advection of phytoplankton cells out of the estuary, and light availability through stratification, gravitational circulation, and longitudinal positioning of turbidity maximum (Day et al. 1989).

The few field studies undertaken so far attributed the variations in the concentration of the metal-binding peptides not only to metal stress but also to the heterogeneity in phytoplankton species composition (Knauer et al. 1998; Tang et al. 2000; Wei et al. 2003). The sum of the estuarine abiotic and anthropogenic factors can hamper the interpretation of the variabilities in PC production, and the direct use of PCs as a metal stress indication to phytoplankton. Knauer et al (1998), for example, investigated PC production in metal contaminated Swiss lakes, which have different hydrodynamic characteristics than estuaries, and did not observe particulate PCs in the most metal polluted lake. In contrast, Tang et al. (2000) found that PCs were ubiquitous in the estuarine waters off of Galveston Bay (USA), showing different patterns (e.g. different slopes for PC2 vs chl a) for the upper and lower bay. Such patterns were possibly a result of the occurrence of different phytoplankton blooms and/or phytoplankton species abundances. Likewise, Wei et al (2003) found high variabilities in particulate GSH and PC2 concentrations for a same
sampling site during different sampling surveys. They also suggested interactions among metals (Cu, Zn and Cd) as a contributing factor to the variabilities in the production of thiols.

The Fal and the Plymouth Sound estuaries have long metal contamination histories and are subject to other anthropogenic contaminants that also pose deleterious effects on phytoplankton health (Table 5.1). The most common important aspects of the Fal and Plymouth Sound estuaries in relation to metal contamination noted here are summarised below:

\( (i) \) the EQS values were rarely exceeded, except at the most metal affected sites, Restronguet Creek (Fal Estuary) and Gunnislake (Tamar Estuary) (Langston et al. 2003a; b);

\( (ii) \) the concentration of dissolved Cu-complexing organic ligands were higher than the concentrations of total dissolved Cu for most of the sites, meaning that the Cu-complexing capacity of the waters probably reduced Cu bioavailability, and thus Cu toxicity to phytoplankton;

\( (iii) \) particulate GSH and PC2 were found at high concentrations at the most metal affected sites, where the concentrations of the free Cu\(^{2+}\) species were high and the dissolved organic ligands were saturated with Cu.

The high production of PCs and GSH in these estuaries was indicative of areas where Cu species were present at high concentrations in the bioavailable form (Cu\(^{2+}\)) for most phytoplankton species. These results indicate that the production of metal-binding peptides is an important mechanism against metal toxicity, especially at the most affected sites.
(Restronguet and Gunnislake). Again, phytoplankton species composition should be considered, as not all species present in the riverine parts of Restronguet Creek appear to be able to produce PCs. It can be concluded that the production of particulate PCs in the Fal and Plymouth Sound estuaries represents a general stress indication for a mixture of dissolved metals.

5.7. Conclusions and final remarks

- This study contributed to establish the first field measurements of particulate metal-binding peptides in European estuaries, using the Fal Estuary and the Plymouth Sound (Tamar and Plym Estuaries) in the UK as exemplars.

- The surveys in the Fal Estuary for two consecutive years showed distinct results for the production of metal-binding peptides and its relation with Cu speciation. The 2002 surveys presented higher concentrations of particulate PC2 and GSH than the 2003 surveys. For the 2002 surveys, the production of PC2 showed to be a faster response than that of GSH under increasing dissolved CuTotal concentrations. Although other dissolved toxic metals could have influenced the production of metal-binding peptides, only Cu speciation was investigated in these systems due to constraints to develop this project within the available time.

- High production of PC2 and GSH in the most metal impacted sites, Restronguet Creek (Fal Estuary) and Gunnislake (Tamar Estuary), indicates that these metal-binding peptides are part of an important adaptive mechanism against metal stress. However, phytochelatin concentrations did not reach high levels in Restronguet Creek and Camon River for all surveys. These could be mainly due to:
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i. phytoplankton species composition which were dominated by non-phytochelatin producers

ii. antagonistic and/or suppressing effects on PC production caused by the interaction among the metals present at high concentrations

- At present, a combination of ancillary parameters, including metal speciation, determination of chlorophyll $a$, an estimation of phytoplankton species composition in the samples, are required for a full interpretation for the variations in PC and GSH concentrations in the field.

5.8. References


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Chapter 6

6. Copper complexation in the Thau Lagoon (France), a shellfish farming area

6.1. Abstract

The quality of the waters in the Mediterranean Thau Lagoon (south of France) is of great concern as this is an important area for oyster farming. The northern portion of the lagoon is subject to metal inputs from urban and industrial activities. Organic ligands can bind metals and thus ameliorate metal toxicity to biota by decreasing the concentrations of bioavailable metals. In this chapter, the implications of Cu complexation in the surface waters of the Thau Lagoon are briefly considered. Copper complexed with organic ligands presented conditional stability constants in the range for the strong ligands (Log $K_{CuL} = 12.5 - 13.5$). The concentrations of the strong ligands (16.2 - 30.5 nM) were close to those of the total dissolved Cu (13.2 - 25.5 nM), resulting in free Cu$^{2+}$ concentrations typical for coastal areas ($10^{-11} - 10^{-13}$ M). Weaker ligands, not detectable by the established conditions, should also play an important role in the metal buffering capacity of the waters in the Thau Lagoon. The oyster farms appeared to influence Cu speciation, as higher concentration of free Cu$^{2+}$ was observed in the vicinities of the oyster beds. The effects of the farmed oysters on Cu speciation and the alterations induced in phytoplankton composition by Cu toxicity demand further investigations, as phytoplankton form the main food source for the bivalves.
6.2. Introduction

The water quality of the Mediterranean Thau Lagoon, in the southern France, is of great concern as this is an important shellfish farming area, particularly for oysters. This area accounts for an estimated standing stock of the oyster *Crassostrea gigas* of around 40,000 tons, which is one of the highest production in Europe (Dupuy et al. 2000). The lagoon, shown in Fig. 6.1, is 15 km long, 5 km wide and has an average of 4 m depth. It comprises a catchment area of 280 km² and is drained by various small streams with intermittent flows (Plus et al. 2003).

![Map of Thau Lagoon](image)

**Figure 6.1.** Thau Lagoon (France). Sampling sites are indicated by numbers. 1. Sewage discharge; 2. Crique de L'Angle; 3. Pointe de Balaruc; 4. Thau 4; 5. Thau 5; 6. Canal; 7. Middle of the lagoon (C4); 8. Oyster bed (C5).

Most of the studies in the Thau Lagoon are concerned with metal exchanges between the water column and sediments (Amouroux et al. 2003), the influence of the oyster farming...
activities on the water column (Souchu et al. 2001), the impact of the watershed and interactions with the Mediterranean sea (Plus et al. 2003). The Thau Lagoon is characterised by a lack of tides and sediment resuspension, with the shellfish farming area acting as a sink of particulate matter and source of nutrients (Souchu et al. 2001). Large interannual variation in precipitation (from 200 to 1000 mm per year) and strong wind (118.5 days per year above Beaufort force 5) are important for the lagoon hydrodynamics (Plus et al. 2003).

The lagoon is subjected to anthropogenic activities and receives inputs of metals, nutrients and organic matter from agriculture (vineyards), fertilizer industries, and urban discharges (Pena & Picot 1991). A view of the oyster farm is shown in Fig. 6.2. Metals do not appear to cause deleterious effects on cultivated bivalves but can accumulate in their flesh, and render them unpalatable and unhealthy for consumption. Depuration of the oysters is a process that can efficiently reduce such metal problems (Laing & Spencer 1997). The composition and physiology of phyto- and zooplankton assemblages, however, can be affected by metal inputs (Moffett et al. 1997). Although many metals, such as copper, are considered trace nutrients, they can be toxic to phytoplankton at elevated concentrations (Gledhill et al. 1997; Okamoto et al. 2001). Therefore, metal contamination could compromise the bivalve’s food source, as the oysters feed mainly by filtering phytoplankton species and organic detritus during all growth stages.

In this respect, dissolved organic matter ubiquitously found in natural waters play a significant role in controlling metal speciation and toxicity to biota. Several studies have shown that Cu and other metals are reversibly bound by dissolved organic ligands (Kozelka & Bruland 1998; Kogut & Voelker 2003), forming metal-complexes which are less bioavailable to phytoplankton. These organic ligands are thought to consist of a
continuous spectrum of ligands spanning complexing properties with 1:1 co-ordination sites to polyfunctional chelators (Gerringa et al. 1995). Such organic ligands include fulvic and humic acids and phytoplankton and bacterial exudates, organic breakdown products of these organisms, and constituents of sewage effluents (Kozelka & Bruland 1998). These ligands are classified as weak ($K = 10^9 - 10^{11}$) and strong ($K = 10^{11} - 10^{14}$), according to the conditional stability constants of the metal-complexes formed.

The aim of this study was to determine Cu complexation in the Thau Lagoon and nearby the oyster beds using competitive ligand exchange-adsorptive cathodic stripping voltammetry (CLE-ACSV). This technique is commonly used to examine the speciation of Cu in natural waters because of its sensitivity (Moffett et al. 1997).

6.3. Theory

A titration technique is employed in order to quantify the free ionic Cu concentration, estimate the system’s capacity to complex additional Cu inputs and determine the strength
of the complexes involved. The titration data is then transformed by a linearisation method such as van den Berg/Ruzic. For this purpose, the following relationships are used:

\[ [L] = [L'] + [CuL] \]  \hspace{1cm} (6.1)

where L is the ligand that form non-labile complexes, L’ is the ligand not complexed with Cu and CuL is the Cu-organic complex. The conditional stability constant for the formation of the complex CuL \( (K_{CuL}) \) is defined as:

\[ K_{CuL} = \frac{[CuL]}{[Cu^{2+}] \times [L]} \]  \hspace{1cm} (6.2)

A linear relationship is obtained by substitution of L’ in equation (6.1) with equation (6.2), providing that Cu complexation is predominantly controlled by a single class of organic ligand (Campos & van den Berg 1994):

\[ \left( \frac{[Cu^2+]}{[CuL]} \right) = \left( \frac{[Cu^2+]}{[L]} \right) + \left( \frac{1}{K_{CuL} \times [L]} \right) \]  \hspace{1cm} (6.3)

The titration of a sample with the metal of interest (Cu), and in the presence of the added ligand (for example, salicylaldoxime, SA), yields a series of labile Cu concentrations, \([Cu_{labile}]\).

\[ [CuL] = [Cu_{Total}] - [Cu_{labile}] \]  \hspace{1cm} (6.4)

where \([Cu_{Total}]\) is the total dissolved Cu concentration in the respective sample aliquot, which is calculated from the total dissolved Cu concentration in the original sample (after
UV irradiation of the sample) plus de added Cu concentration during the titration. In the presence of the added ligand SA, \([\text{Cu}_{\text{Total}}]\) is:

\[
[\text{Cu}_{\text{Total}}] = [\text{Cu}'] + [\text{CuSA}] + [\text{CuL}] \tag{6.5}
\]

where \([\text{Cu}']\) is the inorganic Cu concentration. The \([\text{Cu}_{\text{labile}}]\) is that which equilibrates with the added ligand (SA), and this is measured by CSV. Combining equations (6.4) and (6.5) results in:

\[
[\text{Cu}_{\text{labile}}] = [\text{Cu}'] + [\text{CuSA}] \tag{6.6}
\]

The CSV peak height is related to the \([\text{Cu}_{\text{labile}}]\) via the sensitivity, \(S\) (\(S = \text{peak current} / \text{Cu concentration} (\text{nA/nM})\)).

\[
I_p = S \times [\text{Cu}_{\text{labile}}] \tag{6.7}
\]

\(S\) is calibrated by standard additions of Cu to the sample. The \([\text{Cu}_{\text{labile}}]\) includes \([\text{Cu}']\), as a small constant of the added Cu remains uncomplexed by SA. \([\text{Cu}^{2+}]\) is directly related to the \([\text{Cu}_{\text{labile}}]\) by \(\alpha'\):

\[
[\text{Cu}^{2+}] = [\text{Cu}_{\text{labile}}] / \alpha' \tag{6.8}
\]

\(\alpha'\) is the overall \(\alpha\)-coefficient, excluding complexation by L (Campos & van den Berg 1994). Substitution for \([\text{Cu}^{2+}]\) in equation (6.3) using equation (6.6) results in:
\[
([\text{Cu_labile}] / [\text{CuL}]) = ([\text{Cu_labile}] / [L]) + (\alpha'/([L] \times K_{\text{CuL}})) \quad (6.9)
\]

This equation is used for the van den Berg/Ruzic plot as it is \([\text{Cu_labile}]\) which is measured by cathodic stripping voltammetry (CSV) rather than \([\text{Cu}^{2+}]\). \(\alpha'\) is obtained from:

\[
\alpha' = \alpha'_{\text{Cu}} + \alpha_{\text{CuSA}} \quad (6.10)
\]

where \(\alpha'_{\text{Cu}}\) is the \(\alpha\) coefficient for inorganic complexation of \(\text{Cu}^{2+}\) and \(\alpha_{\text{CuSA}}\) is the \(\alpha\) coefficient for the complexation of \(\text{Cu}^{2+}\) by \(\text{SA}\):

\[
\alpha_{\text{CuSA}} = \beta'_{\text{CuSA}} [\text{SA'}] \quad (6.11)
\]

where \([\text{SA'}]\) is the concentration of \(\text{SA}\) not complexed by \(\text{Cu}\), and \(\beta'_{\text{CuSA}}\) is the conditional stability constant of \(\text{CuSA}\) in seawater:

\[
\beta'_{\text{CuSA}} = [\text{CuSA}] / [\text{Cu}^{2+}] [\text{SA'}] \quad (6.12)
\]

values for \(\alpha_{\text{CuSA}}\) were obtained from Campos & van den Berg (1994).

The values for \([\text{Cu_labile}]\) in equation (6.9) are obtained directly from the CSV peak current as \([\text{Cu_labile}] = i_p/S\) (6.7), and the concentration of \(\text{CuL}\) from (6.4). The sensitivity \(S\) is obtained from the linear portion of the titration where all the ligands were saturated; ligand saturation is verified by comparison with the slope obtained from further Cu standard additions to a sample-aliquot in which the Cu-complexing ligands were previously saturated by a Cu increment greater than the ligand concentration. The sensitivities
obtained by these two methods should be equal, providing a means to estimate whether the end of the titration has been reached. Values of [L] and $K_{CuL}$ are calculated by linear least-squares regression from $1/slope$ and $\alpha/(y$-intercept $x [L])$ of a plot of $[\text{Cu}_{\text{labile}}]/[\text{CuL}]$ as a function of $[\text{Cu}_{\text{labile}}]$. Many titrations of natural samples produce a linear van den Berg/Ruzic plot, suggesting the presence of only one group of ligands, while a curvature would indicate the presence of more than one class of complexing sites (Campos & van den Berg 1994). The range of detectable ligands is restricted by the detection window of the method applied. The detection window is set by the relative magnitudes of the $\alpha$ coefficient of the added ligand (Campos & van den Berg 1994).

6.4. Experimental

6.4.1. Sampling

Surface water samples were collected during spring 2003 in the Thau Lagoon (Fig. 6.1), using clean sampling techniques. All samples were collected using Niskin bottles from a depth of around 0.3 m with the use of a small boat. Samples were transferred to acid cleaned 250 mL low density polyethylene bottles (Nalgene). Sample handling and preparation were carried out using a portable laminar flow hood. All samples were filtered using acid clean 0.4 $\mu$m polycarbonate filters (Whatman). Samples for the determination of total dissolved Cu were acidified with quartz distilled HCl to pH 2. All samples were double bagged and frozen. Analyses were performed on return to laboratory in Plymouth. Samples for phytochelatins and glutathione analysis were also collected and filtered as described in chapters 3 and 4. The filters were frozen and transported to Plymouth. Glutathione and phytochelatins were not observed in any of the samples, probably because
of degradation of the compounds as the filters were thawing when they arrived in Plymouth.

6.4.2. Determination of copper complexation in the Thau Lagoon

Determination of total dissolved Cu (Cu_{Total}) and Cu speciation was performed using adsorptive cathodic stripping voltammetry as described in Campos & van den Berg (1994). A detailed description of the analysis and instrumentation was given in Chapter 5, items 5.4.3 and 5.4.4.

6.4.3. Determination of chl a and salinity in the Thau Lagoon

Determination of chl a in the samples was based on Parsons et al. (1984), as described in Chapter 5, item 5.4.5. Salinity was determined using a salinometer.

6.5. Results and Discussion

Salinities were between 32.8 and 33.7 and pH values for surface waters were generally 8.2. Chlorophyll a contents ranged from 2.9 to 7.9 μg L\(^{-1}\) and were higher than the literature values for the system (< 2 μg L\(^{-1}\)) (Dupuy et al. 2000). The higher chl a levels found in this study were consistent with the occurrence of dinoflagellate blooms (Alexandrium sp.) during the sampling season. Low chl a contents in the lagoon has been associated with the abundance of picophytoplankton species, which is considered a paradox because of the high growth rates of the oysters (Dupuy et al. 2000).
Ligand titrations were carried out in order to determine the Cu species, natural organic ligands, and the conditional stability constants of the Cu-organic complexes. Representative Cu-ligand titration and linearisation curves for the Thau Lagoon samples are shown in Fig. 6.3. The curvature at the beginning of the titration curve (Fig. 6.3a) indicates the presence of natural Cu-complexing ligands. The straight line produced by the van den Berg linearisation treatment of the titration data (Fig. 6.3b) indicates that the complexation was controlled by a single class of ligands (Campos & van den Berg 1994).

![Figure 6.3.](image)

**Figure 6.3.** *a)* Typical titration curve for the estuarine samples (Sewage discharge, site 1, March 2003); *b)* van den Berg linearization for the titration data. Cu<sub>Total</sub> = Cu in the sample plus Cu added for the titration; Cu<sub>L</sub> = Cu complexed by natural organic ligands; Cu<sub>labile</sub> = Cu complexed by SA.

The parameters obtained from the titration and linearisation, together with salinity and chl a concentrations are presented in Table 6.1. The highest dissolved Cu<sub>Total</sub> concentration was found in the Canal sample (25.5 nM, site 6), which was probably due to the proximity to the urban and industrial sources of metals. Copper-organic complexes presented high conditional stability constants (Log K<sub>CuL</sub> = 12.5-13.5), in the range for the strong ligands. The concentrations of these ligands (16.2-30.5 nM) were close to the concentrations of the dissolved Cu<sub>Total</sub> (13.2-25.5 nM) for most of the sampling sites, approaching a molar ratio...
Chapter 6 Copper complexation in the Thau Lagoon

This is in accordance with the trends observed in other aquatic systems (Kozelka & Bruland 1998). Thau 5 and Canal samples (sites 5 and 6, respectively) showed somewhat higher concentrations of ligands compared to the dissolved Cu\textsubscript{Total} concentrations. Anthropogenic organic matter and run-off of humic substances from the small streams draining the surrounding area could have contributed to the bulk of dissolved organic ligands. Indeed, both extremities of the lagoon (East-West) have been reported to be rich in organic matter that lead to a risk of anoxia during summer (Plus et al. 2003).

Table 6.1. Copper complexation in the Thau Lagoon (19th May 2003). Concentrations of dissolved Cu\textsubscript{Total}, total organic ligands (L), Cu\textsuperscript{2+}, Cu\textsuperscript{+}, together with the conditional stability constants of the Cu-organic complexes (CuL), salinity (S) and chlorophyll \textit{a} contents.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cu\textsubscript{Total} (nM)</th>
<th>L (μg L\textsuperscript{-1})</th>
<th>K\textsubscript{CuL} (M\textsuperscript{-1})</th>
<th>Cu\textsuperscript{2+} (M)</th>
<th>Cu\textsuperscript{+} (M)</th>
<th>S (‰)</th>
<th>Chl \textit{a} (μg L\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sewage discharge</td>
<td>13.2 ± 0.3</td>
<td>16.2 ± 1.0</td>
<td>13.5 ± 0.5</td>
<td>1.36E-13</td>
<td>1.90E-13</td>
<td>33.3</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>Crique de L'Angle</td>
<td>18.6 ± 0.4</td>
<td>20.2 ± 0.9</td>
<td>13.4 ± 0.4</td>
<td>4.32E-13</td>
<td>6.05E-13</td>
<td>33.5</td>
<td>5.4 ± 0.3</td>
</tr>
<tr>
<td>Pointe de Balaruc</td>
<td>17.4 ± 0.3</td>
<td>18.3 ± 2.1</td>
<td>12.5 ± 0.2</td>
<td>6.06E-12</td>
<td>8.48E-12</td>
<td>33.3</td>
<td>-</td>
</tr>
<tr>
<td>Middle of lagoon</td>
<td>17.4 ± 0.3</td>
<td>18.9 ± 0.6</td>
<td>12.7 ± 0.1</td>
<td>2.15E-12</td>
<td>3.02E-12</td>
<td>32.8</td>
<td>7.9 ± 0.4</td>
</tr>
<tr>
<td>Near oyster bed</td>
<td>17.7 ± 0.4</td>
<td>17.7 ± 1.0</td>
<td>12.8 ± 0.2</td>
<td>4.71E-11</td>
<td>6.60E-11</td>
<td>33.7</td>
<td>5.2 ± 0.3</td>
</tr>
<tr>
<td>Thau 4</td>
<td>22.2 ± 0.4</td>
<td>24.4 ± 0.8</td>
<td>13.5 ± 0.4</td>
<td>3.01E-13</td>
<td>4.21E-13</td>
<td>-</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Thau 5</td>
<td>22.5 ± 0.5</td>
<td>30.5 ± 0.8</td>
<td>12.8 ± 0.1</td>
<td>4.43E-13</td>
<td>6.21E-13</td>
<td>-</td>
<td>6.3 ± 0.3</td>
</tr>
<tr>
<td>Canal</td>
<td>25.5 ± 0.5</td>
<td>29.8 ± 0.8</td>
<td>12.8 ± 0.1</td>
<td>9.99E-13</td>
<td>1.40E-12</td>
<td>33.5</td>
<td>4.3 ± 0.2</td>
</tr>
</tbody>
</table>

The Cu\textsuperscript{2+} concentration range (10\textsuperscript{-13}-10\textsuperscript{-11} M) found in this area is typical for coastal waters (Gledhill et al. 1997; Kozelka & Bruland 1998). The highest Cu\textsuperscript{2+} concentration (10\textsuperscript{-11} M) was observed near the oyster beds (site 8), where the strong organic ligand concentration was close to the dissolved Cu\textsubscript{Total} concentration. A saturation of organic ligands implies that small increases in the dissolved Cu\textsubscript{Total} results in important increases in Cu\textsuperscript{2+} and Cu\textsuperscript{+} concentrations. This is further illustrated in Fig. 6.4 with a maximum Cu\textsuperscript{2+} concentration for the sample collected near the oyster beds. Nevertheless, the results indicated that Cu
was complexed by strong ligands (%CuL = 100, \%CuL = \[Cu_{\text{Total}}\] - \[Cu^{2+}\] - \[Cu'\] / \[Cu_{\text{Total}}\] \times 100) as the major form within the lagoon. Other dissolved organic ligands, not detectable by the established conditions, may have some importance in the metal buffering capacity of the waters. Weaker organic ligands are usually at concentrations higher than the strong ligands (Moffett et al. 1997). These ligands could be detected by varying the detection window of the method (by either using other added ligand or changing the concentration of the added ligand SA) (Campos & van den Berg 1994).

Figure 6.4. Copper speciation in the Thau Lagoon, showing effects of the relation Cu_{\text{Total}}: Organic ligand.

Even the low Cu$^{2+}$ concentrations (10^{-11} M) found in this study could cause deleterious effects on the growth rate of some larvae of copepods, cyanobacteria and planktonic ciliates, as indicated by laboratory studies (Stoecker et al. 1986; Moffett et al. 1997). It has been suggested that the dominance of eukaryotic picophytoplankton over prokaryotic picophytoplankton in the Thau Lagoon could be linked to the tolerance to Cu (Vaquer et al.
1996). A defense response from most organisms can be expected for chronic or elevated 
Cu stress in natural waters. Mechanisms involving the production of intracellular metal-
binding peptides, such as phytochelatins and glutathione (Ahner et al. 1997) and exudation 
of organic ligands (Croot et al. 2000) have been reported for natural phytoplankton 
assemblages under metal stress. For instance, phytoplankton species such as the diatoms 
*Skeletonema costatum* and *Pseudo-nitzschia*, and the dinoflagellate *Prorocentrum sp*, 
common in the Thau Lagoon (Dupuy et al. 2000), were able to release metal-complexing 
ligands under Cu stress (Lage et al. 1996; Rue & Bruland 2001; Croot et al. 2000), with 
characteristics comparable to the dissolved ligands determined in the present study.

In the present study, the oysters appeared to affect the dissolved Cu speciation in the 
surface waters of the lagoon. Indeed, natural population of bivalves are known to control 
phytoplankton composition, reduce total suspended solids through filter feeding, and 
recycle and remove organic nutrients in the water column (Pietros & Rice 2003). Filter 
feeders are efficient vehicles of particulate matter from the water column to sediments by 
filtration coupled with biodeposition, leading to accumulation of organic matter in 
sediments (Souchu et al. 2001). It is then possible that the alterations caused by the farmed 
oysters in their surrounding water column were responsible for the increase in the 
concentrations of free Cu$^{2+}$ and inorganic Cu$^+$. It is important to note, however, that the 
hydrodynamics of the lagoon is highly influenced by wind (Plus et al. 2003), which could 
have altered the composition of the water column during sampling. Therefore, more studies 
are required in order to link dissolved Cu species and oyster activities.

A shift of phytoplankton species dominance from one species to another in response to the 
feeding rate of the oysters is unlikely to cause large variations in the total dissolved ligand
concentrations, and with consequent changes in Cu complexation. This is supported by results from laboratory experiments which demonstrated that phytoplankton blooms did not impact Cu speciation (Beck et al. 2002). At present, further investigations are required in order to establish how dissolved Cu species in the Thau lagoon could be affected by the farmed oysters and how Cu species could affect the phytoplankton composition.

6.6. Conclusion

This study provided baseline measurements for dissolved Cu species in the Thau Lagoon, an important shellfish farming area. The concentrations of total dissolved Cu and free Cu$^{2+}$ in the lagoon were in the range observed for other coastal areas. Higher free Cu$^{2+}$ and inorganic Cu$^{+}$ concentrations were observed in the vicinities of the oyster beds in comparison to the other sites. This could imply that the oysters were affecting the dissolved Cu speciation. The relationships between the effects of oysters on Cu speciation and the alterations in phytoplankton composition due to Cu toxicity demand further investigations, as phytoplankton form the main food source for the bivalves.

6.7. References


Chapter 7

7. Conclusions and Future Work

This research contributed to establish the first baseline levels for the production of the particulate metal-binding peptides phytochelatins and glutathione in European estuaries. The production of phytochelatins by phytoplankton showed to be a general indication of metal stress in the Fal and Tamar estuaries (southwest of the UK). Particulate phytochelatins were present at higher concentrations in Restronguet Creek (Fal Estuary) and Gunnislake (Tamar Estuary), the most metal affected sites. For the 2002 survey in the Fal Estuary, it could be observed that the production of phytochelatins was a faster response than the production of glutathione for same increases in the total dissolved Cu concentrations. This is an indication that the production of phytochelatins is a more specific response to metal stress in the field than the production of glutathione.

The estuarine conditions that cause variations in the concentrations of thiols, as exemplified by the data set presented here, require further investigation. There is still a paucity of laboratory and field data addressing this issue. The short-term metal exposure experiments using the diatom culture Phaeodactylum tricornutum indicated that phytochelatin production is influenced by metal combinations, probably due to competition among metals for cellular binding sites. Effects on phytochelatin production can be expected, as a mixture of metals such as Cd, Zn and Cu are usually found in contaminated waters. At the present stage, a characterisation of the study area, with the determination of a range of parameters, including chl a content, phytoplankton species composition,
nutrients, metals and organic xenobiotics, is needed for a full interpretation of the production of thiol compounds.

In addition to field work, laboratory metal exposure experiments using natural phytoplankton assemblages or representative species will be helpful for the control and assessment of the variables that influence the production of thiols. Further investigations on the effects of a mixture of contaminants on representative phytoplankton species, particularly species isolated from Restronguet Creek and Gunnislake, could provide a better understanding on the chronic stress response and the variations in phytochelatin and glutathione production.

It has been acknowledged that the measurements of biological responses of metal stress provide a better indication of the environmental quality than the measurements of total dissolved metals or even metal speciation data. This is explained by the fact that adverse or toxic effects produced by a chemical on a biological system do not manifest, unless that chemical or its biotransformation products reach specific sites in the organism, at a certain concentration and for a sufficient length of time. There is a vast field to be explored regarding the biological responses caused by a mixture of contaminants in estuarine systems. Work on these responses would then further our understanding on how organisms such as phytoplankton cope with a mixture of stressors, and consequently would help the assessment of the environmental quality.

The current methods for the simultaneous determination of phytochelatins and glutathione comprise multi-step time-consuming protocols that pose constraints to routine analyses. This is a relevant aspect to take into account due to the large sets of samples required for environmental studies/monitoring. Future work on the development of cost-effective, rapid
and sensitive analytical methods is a crucial aspect to further the applications of phytochelatins in field assessment. Work on the application of a pre-concentration step and an alternative derivatising reagent for the analysis of particulate PCs, which provides less interfering compounds, is needed in order to improve the sensitivity of the analytical method.

The study undertaken in the Thau Lagoon provided baseline levels for dissolved Cu species. Previous data on dissolved metal speciation and metal complexing ligands were unavailable for the system during this investigation. The present results indicated that the oyster farm could be affecting the dissolved Cu species in the surface waters. The relationships between the effects of oysters on Cu speciation and the alterations in phytoplankton composition due to Cu toxicity demand further investigations. Understanding the factors that influence phytoplankton composition is an important aspect in the Thau Lagoon system, as phytoplankton form the main food source for the farmed bivalves.

7.1. The use of phytochelatins in environmental quality assessment

The research requirements to assess the environmental quality of the Fal Estuary and Plymouth Sound were recently reviewed by Langston et al. (2003a, b), and included the need for the determination of metal stress indicators. The use of phytochelatins as a metal stress indication for environmental monitoring has not been applied yet. This is mainly because biological responses to toxicants in dynamic aquatic systems, such as estuaries, are difficult to interpret and a stress indication can be masked, as observed for phytochelatin
production during some field situations. Therefore, as highlighted above and in Chapters 2 and 5, the use of a suite of stress indicators is believed to be the best approach to assess biological impacts in individuals. In this respect, the determination of particulate phytochelatins can represent an additional tool to provide a stress indication for mixtures of toxic metals.

The general recommendations for the use of phytochelatins in environmental quality assessment can be summarised:

1. As a first step for a feasible application of phytochelatins in field monitoring, improvements to the analytical method for phytochelatins are needed (emphasised above and in Chapter 3);

2. An adequate sampling strategy is necessary for screening the system for potential sources of contaminants to allow identification of the most affected areas. This can be then followed by a refined monitoring of the system, with the determination of particulate phytochelatins as a metal stress indication;

3. Elevated particulate phytochelatin concentrations indicate where dissolved metals are more bioavailable for most of the phytoplankton species (and/or where dissolved organic ligands are saturated). It is important to take into account the limitations of this approach, as some phytoplankton species do not employ phytochelatin production as a metal detoxification mechanism. This could provide a better understanding on the extent of the major impacts on phytoplankton and the phytoplankton capacity to cope with metal stress.