UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING, SCIENCE AND MATHEMATICS

School of Ocean and Earth Science

The Influence of Viruses on Trace Metal Speciation during the Life Cycle of *Emiliania huxleyi*

By

Turki Al-Said

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ABSTRACT

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The main objective of this study is to examine the effects of phytoplankton growth, senescence and decay on concentrations and the physico-chemical speciation of trace metal. The role of coccolithovirus induced lysis was examined on trace metal (Cu, Co, Ni) speciation in laboratory *Emiliania. huxleyi* culture experiments and in natural blooms in the Western English Channel (summer of 2005 and 2006). Depth profiles of trace metals (Cu, Co, Ni, Zn) were obtained and in particular the speciation of Cu was investigated in detail.

In the laboratory, *E. huxleyi* was grown in seawater enriched only with N and P, without the control of free metal concentrations using EDTA. Dissolved metal speciation was determined using well established adsorptive cathodic stripping voltammetric (Ad-CSV) methods. Total dissolved Cu was constant in the control and virus (EhV-86) infected cultures, in agreement with reported observations. Viral lyses of *E. huxleyi* caused a sharp increase in the concentration of Cu per cell reaching a maximum of 4.78 10^{-17} mol/cell. Ligand production in both cultures resulted in a decrease in the concentration of free aqueous Cu (Cu^{2+}) with time. The Cu-organic complexes observed in all samples were classed as weak ligands. Cu^{2+} became more abundant in the dissolved fraction relative to particulate Cu, following cell lyses. However, no increase in the dissolved/labile Cu fraction was observed in response to the decrease in the particulate Cu in the virus infected culture. Measuring the incremental effect of EhV-86, additions on the Cu assay showed enhanced Ad-CSV peaks in the absence of EhV-86. The subsequent removing virus fractions from the samples (days 8 & 16) in the culture experiments showed an increase in labile Cu, confirming the inhibitory effect EhV-86 has on Ad-CSV based Cu measurements and thus Cu speciation. The ratio of labile to total Ni was almost constant throughout the experiments (control and virus). High labile fraction of Ni observed in our experiment indicated limited influence of the *E. huxleyi*/EhV-86 life cycles and/or on Ni speciation. In the control culture, the ratio of labile to total Co concentrations decreased over time, which indicated accumulation by the healthy *E. huxleyi* cells; the ratio decreased in the virus infected culture until day 4, following an immediate increase until day 8 due to virus infection. The relationship between phytoplankton and metal speciation in viral lyses affected systems has not been investigated before and our findings serve as a basis for future research.

In the Western English Channel, none of the viruses detected during the 2005-2006 surveys exhibited typical coccolithovirus analytical flow cytometry (AFC) signatures. The investigated *E. huxleyi* bloom was in the process of being succeeded by *Synechoccocus*, and the virus community was dominated by small viruses (most likely bacteriophage). Coccolithoviruses were present at concentrations below the limit detection for AFC. *Synechoccocus* dominated the phytoplankton community during both surveys. Total dissolved Cu concentrations in the depth profiles in the Channel ranged between 1.87-3.73 nM in 2005, and between 2.11- 4.43 nM in 2006. Furthermore, the ligand concentrations (3.62-5.98 nM in 2005, 6.10-9.76 nM in 2006) exceeded total dissolved Cu concentration, resulting in low [Cu^{2+}]. Copper organic ligands in both surveys presented a high conditional stability constant (Log K_{CuL} 12.20-13.77 M), which is characteristic of the strong Cu-binding L_1 ligand class. The Cu^{2+} concentration range was higher in 2005 (0.14-1.69 pM) than in 2006 (0.01-0.73 pM), when slightly higher ligand concentrations were observed. The synchronicity of the appearance of L_1 and *Synechoccocus* abundance points strongly to these cyanobacteria as a strong ligand source. A comparison between Cu speciation in the culture experiments and the findings of coastal surveys is difficult as the survey studies looked at different 'snap shots' of bloom dynamics. In both years, the non-labile fractions of Co and Zn were the dominant species. This indicates that these elements were strongly complexed by organic ligands. Collection of samples from the same stations during different seasons is highly recommended to provide a seasonal picture of metal speciation in these shelf waters.

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This PhD dissertation by

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has been produced under the supervision of the following persons;

Supervisor/s

Professor Eric Achterberg
Dr Declan Schroeder
Dr Alison Taylor

Chair of Advisory Panel

Dr Duncan Purdie

Declaration

This thesis is the result of work done wholly while under registered postgraduate candidature.

Associated studies and professional development

- Oral presentation entitled 'Time series of dissolved trace metal species during the life cycle of coastal phytoplankton *Emiliania huxleyi*' at school of ocean, earth and environmental sciences, University of Plymouth, 18th of Jun 2003.

- Poster presentation entitled 'Time series of dissolved trace metal species during the life cycle of coastal phytoplankton; *Emiliania huxleyi*' progress in Chemical Oceanography Conference, University of East Anglia, Norwich, 17-18 July 2003.

- Attended 'Learning and Teaching for Graduate Teaching Assistants' course, University of Plymouth, Oct-Dec 2002.

- Attended 'ENV 5101 course (Assessed Practical Demonstration)' at school of ocean, earth and environmental sciences, University of Plymouth Oct-Dec 2002. (70% was the over all mark).

- Poster presentation entitled 'Time series of dissolved trace metal species during the life cycle of coastal phytoplankton; *Emiliania huxleyi*'4th European Meeting on Environmental Chemistry Conference, Plymouth, 10-13 December 2003.

- Poster presentation entitled 'The role of viruses in the speciation of Cobalt, Copper and Nickel during the life cycle of *Emiliania huxleyi*' Challenger conference for Marine Science, Liverpool, 13-17 September 2004.

- Oral presentation entitled ' The role of viruses in the speciation of Cobalt, Copper and Nickel during the life cycle of *Emiliania huxleyi*' at school of ocean, earth and environmental sciences, University of Plymouth, 17th of Dec 2004.

- Oral and poster presentation entitled 'The role of viruses in the speciation of cobalt, copper, and nickel during the life cycle of *Emiliania huxleyi*'. Post Graduate Research Conference, University of Southampton, UK, 2005.

- Oral presentation entitled 'The role of viruses in the speciation of cobalt, copper, and nickel during the life cycle of *Emiliania huxleyi'*. AMBIO 1 Conference, University of Southampton, UK, September 2005.

- Oral presentation entitled 'Life cycle and virus infection of *Emiliania huxleyi*: the influence on trace metal speciation'. American Society of Limnology and Oceanography (ASLO) summer conference, Victoria, Canada, 04-09 June 2006.

THESIS CONTENT

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Chapter 1

1.General Introduction

The distribution and chemical speciation of trace metals in the upper water column plays an important role in the community structure and physiology of phytoplankton (Sunda, 1994). Speciation of trace metals is important because only particular chemical forms of given metals are biologically available. In the upper water column, the speciation of many biologically active trace metals is controlled by complexation with strong organic ligands (Bruland *et al.,* 1991). In general, when a metal is complexed by an organic ligand, the metal becomes less biologically available, because the free metal ions are the most available to the biota (Campbell, 1995; Sunda, 1994; Morel *et al*., 2004; Bruland and Lohan, 2004; Leao *et al*., 2007).

Emiliania huxleyi is the most abundant of the coccolithphores on a global basis and it is extremely widespread (Winter *et al*., 1994). Viruses are known to infect a range of phytoplankton species. Viruses have been responsible for the demise of *E. huxleyi* blooms in the English Channel (Wilson *et al*., 2002 b). Information regarding the effects of the viral lyses of *E. huxleyi* on trace metal speciation is not readily available in the literature.

The main objective of this study is to examine the effects of phytoplankton growth, senescence and decay on dissolved trace metal concentrations and their physico-chemical speciation. To this end the role of viral induced senescence (lysis) was examined on trace metal speciation in *E. huxleyi* culture experiments and natural blooms in the Western English Channel in the summers of 2005 and 2006. Trace metal (Cu, Co, Ni and Zn) depth profiles were obtained; in particular the speciation of copper was investigated. The following section highlights the thesis structure.

Chapter 2

This chapter provides an overview of the importance for the phytoplankton community of trace metal speciation in marine waters. The findings from culture experiments and

field surveys focusing on the relationship between phytoplankton and trace metals speciation are reviewed. The overview includes trace metal speciation studies, in particular those involving the life cycle of *E. huxleyi* (the species of interest in this study) and the role of viruses in the marine environment.

Chapter 3

Chapter 3 describes all the methods used for sampling, storing and the determination of dissolved trace metals: cleaning procedures, the equipment used for trace metal sample collection and the techniques for analysis. This will include related measurements of dissolved organic carbon, nutrients, graphite furnace atomic absorption spectroscopy (GFAAS) for particulate trace metals and flow cytometry. The important technique of Stripping Voltammetry for trace metal speciation studies will be discussed in detail.

Chapter 4

Chapter 4 will describe the role of viruses on the speciation of cobalt, copper and nickel during the life cycle of *E. huxleyi* and will detail the findings from culture experiments. Experimental design, experiments and all precautions taken for the research will be outlined and the results will be discussed. The objective of the experiments was to examine the effects of *E. huxleyi* growth, senescence and decay on Cu speciation. Furthermore, the influence on particulate copper concentrations and the concentrations and binding strengths of copper complexing ligands was determined. An important focus was to document the effects of viral infection of *E. huxleyi* on metal speciation.

Chapter 5

This chapter will present the study on the speciation of metals (Cu, Co, Ni and Zn) during an *E. huxleyi* bloom in the English Channel (summer 2005 & 2006). This is the first reported study carried out to investigate the speciation of trace metals during an *E. huxleyi* bloom in the Western English Channel. Sampling, all related measurements and findings will be described. Trace metal (Cu, Co, Ni and Zn) depth profiles during the sampling period will be interpreted, in particular focussing on the speciation of copper.

Chapter 6

This chapter will present conclusions and final remarks on the current research. Aspects that need further research and investigation are highlighted in this chapter.

The thesis is presented in chapters that complement each other, but can also be read and interpreted as individual research studies. Thus, in order to promote readability, some repetition was necessary in describing and explaining the results.

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Chapter 2

2.Interaction of Phytoplankton, Trace Metals and Viruses in the Marine Environment.

2.1 Abstract

This chapter highlights the importance of trace metal speciation in marine waters. It presents the findings of culture experiments and field surveys. Speciation studies related to copper and other metals during the life cycle of the *E. huxleyi* are reviewed. The importance of viruses in the marine food web and the correlation with *E. huxleyi* are also discussed.

The free metal ion of many metals have been reported to be the most bioavailable and toxic, indicating the importance of speciation studies. Studies in the English Channel and the North Sea have confirmed a correlation between the presence of viruses and shifts in phytoplankton communities following crashes of *E. huxleyi* blooms. Results from *E. huxleyi* culture experiments revealed that they can produce organic ligands in response to elevated free Cu ions. Cyanobacteria are also able to release strong trace metal binding compounds into seawater. The high abundance of viruses in the oceans and their small size suggest that they may serve as nucleation centres for iron adsorption and precipitation in the ocean.

2.2 Introduction

Phytoplankton play a major role in the cycling of trace metals in oceanic waters. Our understanding of the processes involved is reasonably well advanced (Bruland *et al*., 1991; Whitfield, 2001; Bruland and Lohan, 2004; Morel *et al*., 2004). In contrast, little is known about the influence of phytoplankton related processes on trace metal speciation and distribution in coastal waters.

Biological processes can strongly influence the chemistry of trace metals; in turn trace metals can influence plankton production and community structure (Sunda, 1989).
Metals are of environmental interest both as limiting nutrients and as toxicants (Sunda and Huntsman, 1998). At enhanced concentrations, metals are toxic when entering cells through known essential metal transport systems (Bruland *et al*., 1991), and metal toxicity often occurs when toxic metals displace essential metals from their metabolic sites (Sunda and Huntsman, 1998).

Metal complexes with organic ligands dominate the chemical speciation of essential metals such as Cu, Zn, Co and Fe (Elwood and van den Berg, 2001; Gledhill and van den Berg, 1994). Their biological availability is determined by the concentration of free metal ions or of kinetically labile organic species (free ions plus weak inorganic and organic complexes) (Sunda, 1989; Sunda and Huntsman, 1995 (a); Bruland *et al.*, 1991).

This chapter provides an overview of the importance of trace metal speciation in marine waters. Findings of culture experiments and field surveys focusing on the correlation between phytoplankton and trace metals will be reviewed. This will include trace metal speciation studies, in particular the speciation of copper and other metals during the life cycle of *E. huxleyi* (species of interest in this study).

Although their existence has been known for years, marine viruses have recently been recognised as an important factor influencing the community dynamics (Poorvin *et al*., 2004). They play an important role in marine geochemical cycles (Fuhrman, 1999; Suttle, 2005). It is the current opinion that the primary result of cell lysis is the release of dissolved organic matter and nutrients into the surrounding water, thereby directly

promoting bacterial production (Brussaard, 2003). Consequently, the importance of viruses in marine food webs and its effect on *E. huxleyi* will be also discussed.

2.3 Trace metals in marine waters; the importance of metal speciation

2.3.1 Sources of trace metal to marine waters

Trace metals are generally defined as those metals present in concentrations of less than 1 μM (Bruland, 1983). Understanding of the marine chemistry of trace metals has been enhanced since the 1970s (Libes, 1992). Sample contamination has decreased due to improved analytical procedures, as a result of advances in trace metal "clean" sampling techniques and analytical protocols (Connelly, 1997).

Metals are introduced into the sea by rivers, the atmosphere, vent fluids associated with hydrothermal activity and benthic release (Bruland, 1983; Libes, 1992 and Martin *et al.,* 1993). Riverine and hydrothermal inputs are considered to be point sources, and their impact can be localised (Connelly, 1997). However, over the last 20 years, it has been recognised that the atmosphere is an important source of trace metals to the surface ocean in both oceanic and coastal regions (Chester *et al*., 1997, Chester *et al*., 2000, Herut *et al*., 2001). Atmospheric inputs can also be responsible for the long range transport of trace metals away from their source i.e. to the open ocean (Chester *et al*., 2000). Most atmospheric metals are associated with aerosols, which are fine particulate matter. Particles are divided into two broad groups, fine particles (< 2 μm) and coarse particles (> 2 μm) (Chester, 2000). Table 4.1 in (Chester, 2000) defined a number of aerosol types on the basis of their composition and sources. Components of aerosols originate from two different types of processes: (1) the direct formation of particles (crustal weathering, seasalt generation, volcanic emissions) and (2) the indirect formation of particles in the atmosphere (Chester, 2000). In the dry deposition mode, aerosols are nearly continuously delivered to the sea surface. Chester *et al.* (2000) found that the trace metal composition of aerosol particles transported to coastal sites reflects the air mass source areas. Close to the source, the composition of a specific aerosol component, such as mineral dust, will be related closely to that of the parent material (Chester *et al*., 1994). Both coarse and fine particles can undergo physical and chemical modifications, and the charcter of an aerosol will change with increasing distance from the source (Chester *et al*., 2000). Therefore, the physical and chemical composition of the marine aerosol is variable in both space and

time and aerosol charecterstics are governerd by a combination of processes that are involved in the generation, conversion, transport and removal of particles (Chester, 2000). The rate and the quantity of the trace metal dissolution from particulate mode is constrained by particle-seawater reactivity (Biscombe *et al*., 2004). Both atmospheric inputs are influnced by the aerosol metal loadings which vary spatially and seasonally depending on dominating aerosol types, emission strengths and physical and chemical modifications to the aerosol population during atmospheric transport (Kocak *et al*., 2007). The processes that influence seawater and rainwater dissolution of trace metals from aerosol material are varied and act in a complex manner (Kocak *et al*., 2007). Chemical, biotic and physical factors such as pH, presence of dissolved organic complexing ligands, particle concentrations, bacteria, phytoplankton, and temperature may influence the extent of metal dissolution (Chester *et al*., 1994, Kocak *et al.,* 2007).

2.3.2 Distributions of trace metals in coastal waters

Since the early 1970s, marine chemists have gained a first-order understanding of the concentrations, distributions, and chemical behaviours of trace metals in seawater (Bruland and Lohan, 2004). The revolution in our knowledge of the distribution of trace metals in sea water was a result of the advances in instrumental analysis and control of contamination (Bruland, 1983). As mentioned earlier, the distribution of trace metals in coastal waters is controlled by either input of metals by rivers, atmosphere, sediment and benthic inputs (Connelly, 1997). Processes removing trace metals include active biological uptake and particle scavenging (Bruland, 1983; Bruland *et al*., 1991; Bruland and Lohan, 2004).

Tappin *et al*. (1993) studied the concentrations, distributions and variability of dissolved Cd, Co, Cu, Mn, Ni, Pb and Zn in the English Channel. The most important factor controlling metal concentrations in the English Channel according to these workers was the mixing of Northeast Atlantic Ocean surface water with fresh water with higher metal concentrations. Other inputs (atmospheric and benthic), the transfer of metals between dissolved and particulate phases in the water column and variations in the end member of river inputs affected the correlations between metal and salinity (Tappin *et al.,* 1993). The rivers Seine, Tamar and Fal founded to be most important interm of their inputs to the English Channel (Tappin and Reid, 2000).

Statham *et al*. (1999) investigated dissolved and particulate metals at five stations across the English Channel between Cherbourg and the Isle of Wight. For Cu, Ni, Mn and Co coastal inputs from the UK were shown to be significant. Dissolved Ni and Cu were higher in concentration on the English side of the Channel, which reflected the riverine sources. Also dissolved fluxes of Mn and Co were significantly influenced by east to west fluxes associated with a gyre feature to the southeast of the Isle of Wight.

2.3.3 The distribution of trace metals in oceanic waters.

The horizontal and vertical distributions of dissolved trace metals are controlled by their relative supply and removal. The balance determines the depth profiles of the metals, which can be classified into the following categories: 1- conservative metals; 2- metals that have a nutrient-like distribution; 3- metals that are rapidly removed from the water column by scavenging processes (Bruland, 1983; Libes, 1992).

Conservative profiles show constant concentrations relative to salinity. Libes (1992) suggests that this behaviour is controlled by physical processes such as advection and mixing. Metals that show a nutrient-like profile exhibit surface depletion and enrichment at depth as a result of the involvement of the element in the biogeochemical cycle. This group is subdivided by Bruland (1983) into labile or refectory nutrient types and those that have a combination of both. The labile nutrient type profile is similar to nitrate and phosphate; surface depletion follows a mid-depth concentration maximum produced when there is regeneration of metal at shallow depths (Libes, 1992). Cadmium is an example of a labile nutrient-type metal.

A deep-water maximum is exhibited by metals that undergo regeneration below the thermocline. These metals (e.g. Zn) have a distribution similar to silicate and are thought to be exported to deep waters by the components of shell and skeletal material and hence have a refactotry nutrient profile. Nickel demonstrates a mixed behaviour (bio-intermediate metals), with both shallow and deep water regeneration (Libes, 1992).

Scavenged metals exhibit a surface enrichment and depletion at depth. These metals are delivered to the oceans via the atmosphere and scavenged throughout the water column,

the best examples being lead and aluminium. Mid-depth minima can result from surface input and regeneration at or near bottom waters; aluminium, lead and copper are reported to show this type of profile (Bruland, 1983).

2.3.4 Trace metals in seawater; separation techniques

Seawater samples can be divided into different physical fractions by filtration. Filtration is a widely used technique for separating dissolved and particulate forms of trace metals in natural waters. The definitions for dissolved and particulate forms are purely operational (Connelly, 1997).

The definition of truly dissolved metal species is determined as those metals that are in solution after the sample has passed through a 0.2-0.45 μm filter. This pore size prevents the passage of particulate matter (Lerman, 1979; Lohan, 2003; Connelly, 1997) and most biological particles, with the exception of bacteria and viruses. According to Muller (1996), this permeates with associated trace metals can be defined as dissolved. The filtrate contains particles that are colloidal in nature and have size range of 1 nm- 0.2 μm (Buffle, 1990). Some colloids of Fe and Mn oxides and macromolecules can pass through a 0.45 μm filter. They are not truly dissolved and not biologically available (Martin *et al*., 1995; Guo *et al*., 1995). There are various different size classifications reported and the size class used is dependent on the methods used for the collection of particles. For example, Moran *et al*. (1996) used cross flow filtration resulting in a 0.2 μm size. As described earlier, marine colloids are mostly organic in nature, they can strongly bind trace metals and thus play important role in the biogeochemical cycling of trace metals in natural waters (Martin *et al.,* 1995). Martin *et al*. (1995) indicated that 50% of iron, lead and manganese were present in association with colloidal material. The range of metals in colloidal phases in different marine systems is large (Santschi *et al*., 1999), which indicates that the nature and composition of organic and inorganic ligands that bind trace metals vary greatly in different systems. Wang and Guo (2001) demonstrated that the decomposition of biogenic particles may contribute considerably to the production of colloids in the marine environment.

In summary, trace metals are present in seawater in different physical-chemical forms or species. The speciation of an element represents its individual physico-chemical form.

The forms together make up the total composition in a sample. Trace metals occur as aqueous metal ions and dissolved inorganic or organic complexes. They may associate with colloids and particulate matter.

2.3.5 The importance of speciation studies

Total dissolved trace metal concentrations do not reveal sufficient information about the toxicity, bioavailability and geochemical behaviour of trace metals in seawater (Achterberg and Braungardt, 1999). Dissolved trace metals in seawater exist in different forms - free hydrated ions, inorganic complexes and organic complexes (Bruland *et al.*, 1991). For many metals, including Cu, Ni and Zn the free aqueous metal ions have been reported to be the most bioavailable and toxic (Campbell, 1995; Gledhill *et al*., 1997; Leal *et al.,* 1999). This is because of their ability to pass through the cell membranes of phytoplankton. However, metals complexed by organic ligands are not able to pass through membranes; organic ligands in natural waters hence reduce the availability of metals to organisms (Hunter *et al*., 1997; Sunda and Huntsman, 1998; Sunda and Huntsman, 1995 (b); Leal *et al.* 1999). However, little is known about the source and the structure of organic ligands (Laglera and van den Berg, 2003; Croot *et al*., 2000). There is some evidence that ligands may include sulphide species (Rozan *et al*., 2000), including thiol compounds specifically complexing copper (Leal and van den Berg., 1998; Leal *et al*., 1999), but also colloidal ligands have been observed (Tang *et al*., 2001). The Cu-binding ligands in the colloidal fraction have relatively higher stability constants than soluble ligands (Muller, 1996; Tang *et al*., 2001). The calculated stability constants $LogK_{CuL}$ for the colloidal ligands was 12.9 compared to 12.3 and 11.1 for the filter passing fraction (dissolved) and ultrapermeat fraction respectively for samples collected from Galveston Bay (Tang *et al*., 2001).

Organic complexation and the particulate binding of metals therefore decrease the concentration of free metal ions and labile inorganic complexes (Sunda and Huntsman, 1998; Bruland *et al*., 1991). Consequently, it is necessary to study the specific forms of each trace metals in order to gain more knowledge about the role of metals in biogeochemical processes.

Electrochemical techniques (e.g ASV and Ad-CSV) have been widely used in trace metal speciation studies. The electrode system a typically includes the hanging mercury drop

electrode which is popular due to its reproducible surface (Achterberg and Braungardt, 1999, Bruland and Lohan, 2004). The electrochemical labile fraction of metals is the fraction that is kinetically labile in the boundary layer of a hanging mercury drop during stripping voltammetry analysis and is thought to include the biological available fractions of metal in the solution (Achterberg and van den Berg, 1997; Achterberg and Braungardt, 1999; Whitfield, 2001). Therefore it is more relevant than total metal concentrations when assessing their biological impacts and involvement in biogeochemical processes in the marine environment.

2.4 Phytoplankton and Trace Metals

Trace metals have a range of functions in prokaryotes (cells without membrane-bound nuclei, e.g. bacteria) and eukaryotes (organisms with membrane bound nuclei, e.g. coccolithophores). They are used for a variety of catalytic and electron transfer functions. Specific roles may differ between different groups of organisms. It is generally inferred from previous studies that biological metal uptake is a function of free metal ion concentration (Campbell, 1995; Sunda and Huntsman, 1995 b; Croot *et al*., 1999).

Williams (1981) stated four conditions that must be met for an element to perform an effective role in processes in cells:

1- Sufficient abundance to provide a reliable resource.
2- Ready availability for uptake, i.e. the metal chemistry in the solution should allow this.
3- Capability to be taken up and held by the cell in a suitable kinetic trap; to be transferred and used in the cell.
4- The capability to perform an efficient function within the cell.

The uptake of trace metals by phytoplankton cells can be summarised in three steps (Whitfield, 2001), bearing in mind the differences in characteristics of prokaryotes and eukaryotes (Table 2.1).

1. **Diffusion**: the transport of metal species to the cell surface
2. **Sequestration or capture:** binding to a biologically-produced ligand.
3. **Internalization**: transfer of complexes into the cell membrane.

The links between the functions of nutrients (elements) within cells are shown in Figure 2.1.

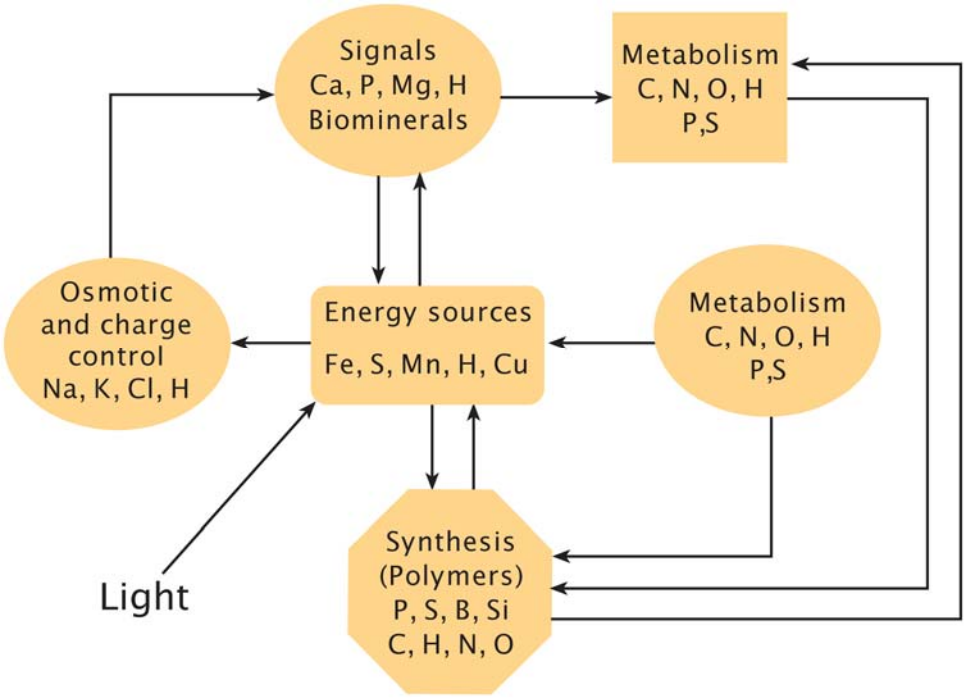

Figure 2.1: The links between the functions of nutrients (elements) within the cell (redrawn from Whitfield, 2001).

Table 2-1: Characteristics of prokaryotic and eukaryotic phytoplankton, taken from Whitfield, 2001.

Small cells (prokaryotes) (eg. Cyanobacteria*)*	**Large cells (eukaryotes) (eg. Diatoms)**
Large surface to volume ratios; efficient assimilation of nutrients, etc.	Smaller surface to volume ratios; less efficient assimilation of nutrients, etc.
Low total requirement per cell of nutrients; reaching reproductive stage (division) quickly	High total requirement per cell of nutrient; reaching reproductive stage (division) slowly
Rapid division (hours)	Slow division (days)
High non-chlorophyll pigment: Chlorophyll *a* ratios are efficient at absorbing incident radiation	Low non-chlorophyll pigment: Chlorophyll *a* ratios are inefficient at absorbing incident radiation
Function most effectively in nutrient poor conditions	Function most effectively in nutrient rich conditions
Grazed by small protozoa; short multiplication time	Grazed by large copepods; long multiplication time

Sunda and Huntsman (1998) suggested that metal ions are taken up into cells by membrane proteins designed for intracellular transport and regulation of nutrient metals. This transport is related to the external concentration of either free metal ions or kinetically labile inorganic and organic species.

Trace metals are essential to phytoplankton metabolism, because of their catalytic roles in enzymes (Sunda, 1989). However, a number of trace metals are toxic, and may compete with nutrients for the same binding sites on enzymes and membrane transport sites (Bruland *et al*., 1991). When competition occurs and the wrong metal is bound, a toxic effect can be observed (Sunda and Huntsman, 1998; Bruland *et al.,* 1991).

In the case of copper and zinc, the largest fraction of the metal present in the upper layer of the ocean is generally considered to be unavailable for uptake by phytoplankton. This may be due to organic complexation, occlusion of the metal in particulate material or formation of an insoluble oxide phase. Therefore the physicochemical speciation of these metals is an extremely important consideration in any attempt to describe and quantify their biogeochemical cycles (Muller *et al*., 2001, Hunter *et al*., 1997, Leal *et al*., 1999).

Phytoplankton affect trace metal chemistry in natural waters by surface reactions, by direct uptake and by production of extra cellular organic matter with metal complexing properties. A number of studies have illustrated that several trace metals are strongly complexed by natural organic ligands, which has important implications for geochemical cycling, biological uptake and toxicity (Bruland *et al*., 1991; Hunter *et al*., 1997; Sunda and Huntsman, 1998). Indeed, complexation of trace metals with organic ligands often ameliorates trace metal toxicity and influences the biogeochemistry of these elements in the aquatic environment by preventing metal scavenging and precipitation (Moffett *et al*., 1990).

Enhanced metal concentrations may result in toxic effects. In contrast, low metal availability may restrict the critical cell physiological responses. For example, low Cu in the oxygen minimum zone was hypothesized to be responsible for the release of NO_2 to the atmosphere (Granger and Ward, 2003). Also, low Ni concentrations may limit phytoplankton ability to assimilate urea, which is an important source of nitrogen (Price and Morel, 1991). The Zn metallo-enzyme carbonic anhydrase is present in all marine

phytoplankton and is involved in the carbon concentration mechanism, catalysing the equilibrium between HCO_3^- and CO_2 (Buitenhuis *et al*., 2003). Cobalt and cadmium can replace Zn as a metal centre and function with the enzyme carbonic anhydrase. The acquisition of carbon dioxide will depend to some extent on the availability of these metals, especially under conditions of low pCO_2 (Sunda and Huntsman, 1992).

In summary, trace metals influence marine algae, and conversely the algal community affects trace metal concentrations and chemical speciation (Sunda, 1989). Thus, phytoplankton and bacteria are not only influenced by the availability of trace elements (Whitfield, 2001) but can actively alter the composition of their environment to optimise the use of these elements (Muller *et al*., 2005). To understand the interactions between phytoplankton and trace metals, several levels of complexity must be considered (Figure 2.2).

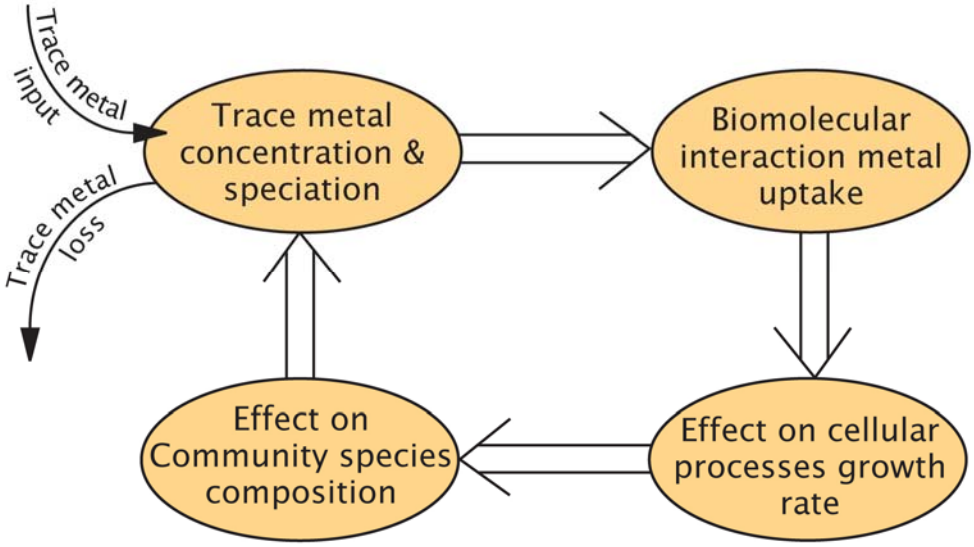

Figure 2.2: Interaction of trace metals with marine algae (Sunda 1989).

2.4.1 Copper (Cu)

Plastocyanin is a single strand protein and is considered to play an integral part in electron transfer proteins (Redinbo *et al*., 1994). Cu plays a major role in photosynthesis as a structural and electron exchange component of plastocyanin (Whitfield, 2001). In eukaryotes, the most common use of copper is in electron transfer (oxidative enzymes and energy capture) (Sunda, 1989). Copper is an essential, required element but can be toxic at relatively low concentrations. In addition, there is no evidence of copper being a limiting growth factor for phytoplankton (Morel *et al*., 2004).

Total copper (Cu_T) concentrations in the North Pacific range between 0.5 nM at the surface to 5 nM at 5000 m (Coale and Bruland, 1990). Cu_T concentrations in the North Atlantic are higher in surface waters (1.2 nM) than in the Pacific, but lower in deep waters (2 nM). Aeolian sources influences greatly the distribution of total copper in the Indian Ocean, with surface concentrations ranging from 2-4 nM (Saager *et al*., 1992). Deep water scavenging has been reported, with total dissolved copper ranges from 0.5 to 1 nM at 500 m (Saager *et al*., 1992). Tappin *et al*. (1993) reported that average total dissolved copper in the English Channel was around 3.2 nM, and distributions remained relatively uniform over the period of their observations (July-August 1986).

Culture studies of diatoms and a coccolithophorid, *E. huxleyi*, have included observations of copper exposures. Interesting levels of interaction between phytoplankton physiology and copper have been observed (Sunda and Huntsman, 1995a). The results indicated that the Cu: C ratio within the cells of all study species was related to the free Cu^{2+} in the culture medium.

Copper has been reported to be > 99% complexed by organic ligands, resulting in low Cu^{2+} concentrations (Croot, 2003; Muller *et al*., 2003; Croot *et al*., 2000; Mofett *et al*., 1997; Leão *et al*., 2007). Cu speciation is dominated by low concentrations of very strong chelators, log K'_{CuI} is 12-13, where K'_{CuI} is the conditional stability constant for the CuL interaction and L is the natural complexing ligand. The strong ligands are designated as class 1 ligands. Also, weaker ligands have been observed in seawater. These ligands become important in Cu complexation when stronger ligands become fully saturated. The weaker ligands (class 2) of lower stability constant 3 to 4 orders of

magnitude lower than those of L1 (Leão *et al*., 2007) tend to occur in greater concentration in coastal and estuarine waters (Moffett *et al*., 1997, 2000 Muller *et al*., 2003; Leal *et al*., 1999; Croot, 2003). 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*., 2000; Ross *et al*., 2003). These ligands may be produced by organisms by excretion or cell lysis or breakdown of exisiting organic material (Leal *et al*., 1999). There is some evidence that the ligands may be sulphide species (Rozan *et al*., 2000) or thoil compounds (Leal and van den Berg, 1998; Dupont *et al*., 2004) and also colloidal (Tang *et al*., 2001). Dupont *et al*. (2004) used precolumn derivatization high-performance liquid chromatography electrospray ionization ion-trap mass spectroscopy to identify thiols (organic ligands) produced and exuded by *E. huxleyi*. They found that *E. huxleyi* constitutively produced two thiols, arginine-cysteine and glutamine-cycteine in high intracellular concentrations (Dupont *et al*., 2004). In addition, *E. huxleyi* exudes these novel thiols in response to increased Cu concentration in their growth media. Laglera and van den Berg (2003) used a different approach to quantify thiol compunds in estuarine waters using Ad-CSV in the presence of SA as this lowers the free copper concentration thus realising thiols making them available for detection.

2.4.2 Cobalt (Co)

Cobalt is an essential growth factor for phytoplankton, as it is an active metal centre of vitamin B_{12} (Whitfield, 2001); few cobalt metalloproteins are known (Kobayashi and Shimizu, 1999). When marine phytoplankton are cultured under zinc limiting conditions, cobalt has been reported to substitute for zinc in the enzyme carbonic anhydrase (Sunda and Huntsman, 1995b; Yee and Morel, 1996). Laboratory cultures have shown that marine cyanobacteria *Synechococcus* and the coccolithophore *E. huxleyi* have a strong requirement for cobalt (Sunda and Huntsman, 1995 b).

In ocean waters, Co concentrations are generally low in surface waters (10-40 pM) with an increase to a maximum in the upper thermocline (30-100 pM) and then a decrease to 10-30 pM in deep waters (Martin *et al*., 1993). In a transect from the ocean to coastal waters, Co concentrations increased from about 25 pM in open waters (NE Atlantic Ocean) to 103 pM in the English Channel (Ellwood and van den Berg, 2001).

A number of workers have reported that Co is strongly complexed by naturally occurring complexing ligands (Zhang *et al*., 1990; Ellwood and van den Berg, 2001). Zhang *et al*. (1990) reported that a variable fraction (average 73%) of dissolved cobalt was very strongly complexed by organic ligands.

The low free Co^{2+} concentrations determined in open ocean waters suggest that certain phytoplankton, such as cocccolithophores and cyanobacteria, may be cobalt limited (Ellwood and van den Berg, 2001). High nitrogen, low chlorophyll regions tend to be iron-limited, but are also characterised by low cobalt concentrations, which suggests that they could possibly be Co-limited for certain phytoplankton species.

2.4.3 Nickel (Ni)

Nickel is not affected by redox cycling and it shows a typical nutrient profile, with surface depletion and deep water enrichment (Haraldsson & Westerlund, 1988). However, surface concentrations always remain higher than 1 nM, and the deep water concentrations are typically < 10 nM. Nickel concentrations observed in the Pacific Ocean (Bruland, 1980), the Indian Ocean (Saager *et al*., 1992) and the Atlantic Ocean (Bruland and Franks, 1983) showed an increase in deep water concentrations (from 7 to 10 nM) moving from the Atlantic to the Pacific Ocean, along the ocean conveyor belt. The profiles do not show very strong correlations with P or Si (Saager *et al*., 1992). In the English Channel, total dissolved nickel concentrations were around 3.8 nM (Tappin *et al*., 1993), with no spatial differences.

Nickel is an essential co-factor in the enzyme involved in urea uptake (Price and Morel, 1991). Urea can form an important source of nitrogen, following the release of waste products by grazing organisms (Whitfield, 2001; Sunda, 1989). Low free Ni concentrations can limit phytoplankton growth in culture experiments if they are grown on urea as a nitrogen source (Harrison *et al*., 1985). No such limitation has been reported for natural waters. This may be due to the relatively high background levels of Ni. There is some evidence of organic complexation by relatively small concentrations of strong binding ligands, which are capable of complexing ca 50% of the total nickel (van den

Berg and Nimmo, 1987). Achterberg and van den Berg (1997) reported that speciation of Ni is dominated by an electrochemically labile fraction.

2.4.4 Zinc (Zn)

Zinc is a cofactor in nearly 300 enzyme systems, such as alcohol dehydrogenase, carboxypeptidase and carbonic anhydrase (Sunda and Huntsman, 1995b; Ellwood and van den Berg, 2000; Buitenhuis *et al*., 2003). These enzymes are involved in nearly all aspects of metabolism. This makes zinc an essential micro-nutrient for organism growth (Anderson and Morel, 1978). Zinc also has a role in exported hydrolytic enzymes, which break down external organic debris. Low dissolved Zn concentrations, observed in the open ocean, have the potential to limit phytoplankton growth and its ability to acquire carbon dioxide (Sunda and Huntsman, 1992; Brand *et al*., 1983; De La Rocha *et al*., 2000).

Zinc exhibits vertical profiles characteristic of nutrient like elements with surface depletion and deep water regeneration. In the North Atlantic, Collier and Edmond (1984) reported total surface Zn concentrations around 0.1 nM, with levels increasing to 1.5 nM in deep waters. Similar surface concentrations have been observed in the North Pacific (Bruland, 1980), but deep water concentrations reached 8 nM. Saager *et al*., (1992) reported that Zn in the Indian Ocean showed a surface value of 1-3 nM, with deep water values 9-12 nM. Zinc ranged from 0.04 nM in the North East Pacific surface waters, to 0.9 nM at the station near to the Canadian shelf (Lohan *et al*., 2002). The overall mean concentration of Zn in the English Channel was reported to be around 7.6 nM (Tappin *et al*., 1993).

Growth at low Zn^{2+} concentrations was achieved by oceanic species of *Emiliania huxleyi*; the species reduced their internal Zn requirements (Sunda and Huntsman, 1992). This suggests that phytoplankton can adjust their requirements to match the Zn^{2+} levels available.

The influences of zinc and iron enrichments on phytoplankton growth in the north-eastern subarctic Pacific were studied by Crawford *et al*. (2003). The addition of Zn slightly increased chlorophyll concentrations relative to the control ($p<0.05$). Diatom and

coccolithophore abundance were higher in the seawater treated with added Zn, compared to the control. However the researchers observed that Zn additions had limited influence on conventional indices of phytoplankton growth compared to additions of Fe in the study region.

Results from the incubation experiments by Ellwood (2004), which were designed to investigate potential Zn limitation in the algal community, indicated that Zn additions had little effect on growth rates, chlorophyll *a* production and nutrient drawdown, compared to the control. Incubation speciation results suggested that phytoplanktons in subantarctic waters either have low requirements of Zn, lower than algae grown in laboratory culture experiments or that these organisms are able to use other metals such as cadmium and cobalt to satistfy their metabolic requirements (Ellwood, 2004).

2.5 Emiliania huxleyi

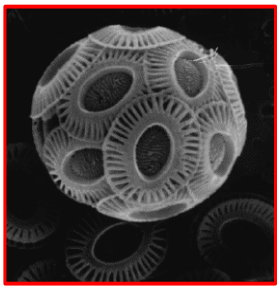

Figure 2.3: Transmission electron micrograph depicting *E. huxleyi*

Emiliania huxleyi is the most abundant of the coccolithphores on a global basis and it is extremely widespread, occurring in all oceans except the polar oceans (Vasconcelos *et al*., 2002). Massive blooms have been observed in many coastal seas when water conditions are favourable. *E. huxleyi* tend to occur in highly stratified water where the mixed layer depth is usually 10-20 m, and is always ≤ 30 m (Nanninga and Tyrrell 1996).
The *E. huxleyi* cells in a bloom outnumber all other species combined, accounting for 80-90% or more of the phytoplankton cells in the water (Brand, 1994). The armoured appearance of the *E. huxleyi* cell is due to its calcium carbonate platelets (coccoliths) (Figure 2.3).

E. huxleyi has a global significance as blooms of *E. huxleyi* are highly reflective, resulting in reflection of light and cooling of surface waters (Tyrell *et al*., 1999). The production of the coccoliths is strongly, but not completely, light dependent (Brand, 1994). The growth of huge numbers of *E. huxleyi*, with associated uptake of calcium and carbon, and their subsequent sinking to the ocean floor, has implications for marine biogeochemistry. For example it affects the ocean carbon system, decreasing alkalinity and CO_2 storage in the ocean (Malin *et al*., 1994; Nobel *et al*., 2003). Production of

calcium carbonate by *E. huxleyi* and its role in CO_2 cycling and dimethyl sulphide (DMS) production makes *E. huxleyi* a key species with respect to past and present marine primary productivity, sediment formation, global biogeochemical cycles and climate modelling studies (Schroeder *et al*., 2003; Malin *et al*., 1994; Iglesias-Rodrigues *et al*., 2002).

Vast coastal and midocean populations of *E. huxleyi* can be viewed by satellite imagery (Holligan *et al*., 1993) due to their reflective calcium carbonate coccoliths, which often disappear suddenly (Ziveri *et al*., 2000). Until recently, the mechanisms of *E. huxleyi* bloom disintegration were poorly understood, but recently it has been confirmed that viruses are intrinsically linked to those sudden crashes (Wilson *et al*., 2002a, Schroeder et al., 2002).

2.6 The role of viruses in the marine environment

Viruses are small particles, about 20-200 nm long, consisting of genetic material (DNA or RNA) surrounded by a protein coat (Fuhrman, 1999). Viral abundances are typically around 10^7 viral particles ml^{-1} in seawater (Suttle, 2005) and in ocean waters generally exceed those of bacteria by about one order of magnitude (Marie *et al*., 1999; Wienbauer and Suttle, 1999).

Viruses are ubiquitous in the marine environment and they exert a significant control on bacteria and phytoplankton populations, influencing diversity, nutrient flow and biogeochemical cycling (Fuhrman, 1999; Wommack & Colwell, 2000). It was first established by Suttle *et al*, (1990) that viruses were responsible for lysis of algal cells in the sea. Viral termination of algal blooms has significant biogeochemical implications, due to the conversion of cells to dissolved organic matter (Fuhrman 1999; Wilhelm and Suttle, 1999). Several studies have investigated the role of viruses in controlling the bloom forming *E. huxleyi* in the North Sea area (Bratbak *et al*., 1993; Brussaard *et al*., 1996; Wilson *et al*., 1998; Wilson *et al*., 2002 a) and Western English Channel (Wilson *et al*., 2002 b). It is clear from these investigations that viruses are intrinsically linked to the decline of *E. huxleyi* blooms and a correlation between the presence of viruses and a shift in phytoplankton communities following the crash of an *E. huxleyi* bloom has been demonstrated.

The liberation of carbon and nutrients by viral lysis may be important in supplying nutrients and carbon to photosynthetic and heterotrophic organisms (Gobler *et al*., 1997 and Wilhelm and Suttle, 1999). Viral lysis of microorganisms within sinking aggregates may effectively dissolve the particles converting some sinking particulate matter into non-sinking dissolved material and colloids at the specific depth at which lysis occurs (Fuhrman, 1999). The viral lysis of an *Aureococcus anophagefferens* bloom in the field released 40 µM dissolved organic carbon and rapidly transferd released metals to bacteria (Gobler *et al*., 1997).

Simple models (Wilhelm and Suttle, 1999; Fuhrman, 1999) have demonstrated that viruses are catalysts that accelerate the transformation of nutrients (which may include trace metals used in metabolism) from particulate (living organisms) to dissolved states, in which they can be incorporated by microbial communities (Suttle, 2005). Figure 2.4 shows the viral role in the marine food web.

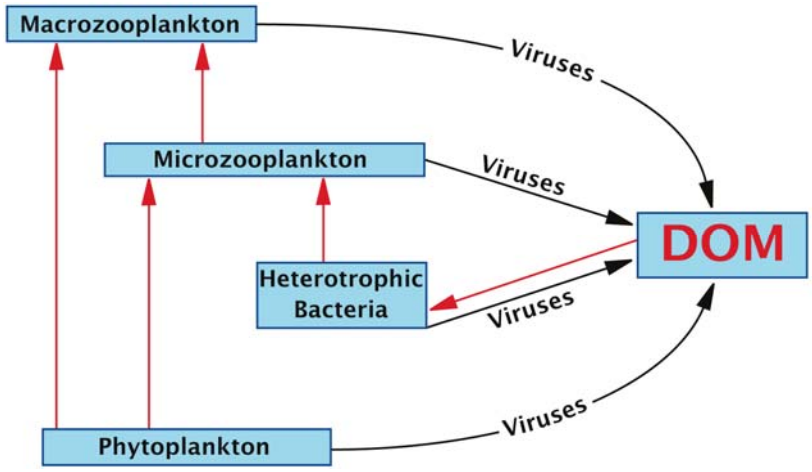

Figure 2.4: The viral short-circuit in marine food webs adapted from (Gobler *et al*., 1997 and Wilhelm and Suttle, 1999).Viruses divert the flow of carbon and nutrients from secondary consumers (red arrows). They destroy cells and release the content of these cells into the pool of dissolved organic matter (black arrows). DOM is then used as a food source for bacteria, which return it back to the food web.

Viruses are abundant in the aquatic environment and most microbial organisms have viruses that infect them (Wilson *et al*., 2002 a). Viral infection affects the dynamics of

microbial loops and has demonstrated impacts (sometimes dramatic) on both bacterial and phytoplankton populations (Nobel *et al*., 2003).

Virus abundance in the ocean is dynamic; in surface waters viruses are rapidly destroyed or damaged by sunlight and other factors such as grazing or sinking via attachment to marine aggregates (Wilhelm *et al*., 1998). New viral progeny must be continuously produced to replace the destroyed viruses. High production of viruses will result in significant lysis in host cells. The destruction of host cells can represent a significant source of organic carbon, nutrients and trace metals in the marine microbial food web (Wilhelm and Suttle, 1999; Suttle, 2005; Gobler *et al*., 1997; Fuhrman, 1999). Different cellular fractions released by lysis will include soluble cytoplasmic components and structural materials.

Significant advances have been made in understanding the dynamics of viruses and their effects on marine phytoplankton communities. Several studies (Wilson *et al*., 2002 a & b and Schroeder *et al*., 2002) found a correlation between the presence of viruses and a shift in phytoplankton communities following the crash of an *E. huxleyi* bloom. The viruses responsible for infection of *E. huxleyi* have been isolated from a number of locations. They are typical of the family Phycodnaviridae, being large double standard DNA viruses (Castberg *et al*., 2001; Wilson *et al*., 2005). Phylogenetic analysis of the DNA Polymerase genes of *E. huxleyi* viruses suggest they belong to a new genus within the family Phycodnaviridae, which has been designated *Coccolithovirus* (Schroeder *et al*., 2002).
Figure 2.5 show an example of a transmission electron microscopy (TEM) image of *EhV*-86 *Emiliania huxleyi* specific virus isolates which is about 170-175 nm in diameter (Wilson *et al*., 2002 b).

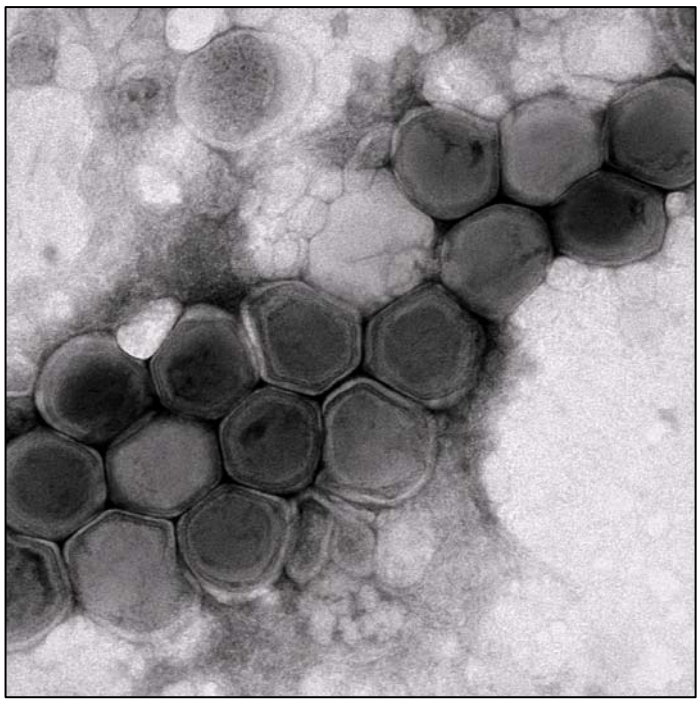

Figure 2.5: Transmission electron microscope (TEM) analysis of *Emiliania huxleyi*-specific virus isolates (Wilson *et al*., 2002 b)

Viral infection and host lysis are a continuous process; if host productivity increases, there will be a concurrent increase in viral activity (infection of hosts to produce viruses) (Wilson and Mann, 1997). Therefore, if there is an increase in host production there will be an initial decrease in virus numbers as they infect, then propagate within the hosts. During this continuous process, virus numbers will eventually increase as more hosts lyse (Wilson *et al*., 2002 b).

Viruses may also be important in shaping global climate (Fuhrman, 1999) because they induce the release of dimethyl sulphide (DMS). Culture studies by Malin *et al.* (1998) confirmed the release of DMS during the viral lysis of *Phaeocystis pouchetii*. Recent research by Evans (2004) provides the first conclusive evidence that the viral induced mortality of *E. huxleyi* directly resulted in DMS production. This was also observed in mesocosms containing natural seawater (Evans, 2004).

Gobler *et al*. (1997) examined the release of C, N, P, Se and Fe following viral lysis of the *Aureococcus anophagefferens*. Only 5% of the Fe was released into the dissolved phase during vial lysis. It has been suggested that virally lysed cells release high

molecular-weight proteins and nucleic acids that can promote particle formation (Proctor and Fuhrman, 1991).

Recent work by Poorvin *et al*. (2004) reported the activity of naturally occurring viral populations in high-nutrient low-chlorophyll (HNLC) regimes; can regenerate sufficient concentrations of dissolved Fe to support the growth of the phytoplankton community. Their results demonstrated that viral activity is of vital importance and, along with grazing regenerators, supplied organically complexed Fe.

The high abundance of viruses in the oceans and their small size suggest that they may serve as nucleation centres for iron adsorption and precipitation in the ocean (Daughney *et al*., 2004). Their results clearly demonstrated the potential of marine bacteriophage to serve as nuclei for iron adsorption and precipitation and models developed suggested that they may represent a significant reservoir of iron in seawater. However, as these experiments utilize a limited range of iron-to-viruses ratios relative to natural seawater, the results of their study do not prove that viruses affect the marine iron cycle. Their study might serve to justify continued research into iron-virus interactions.

2.7 Emiliania huxleyi and Trace Metals

Leal *et al*. (1999) undertook *E. huxleyi* incubation experiments that revealed that Cu complexing ligands were produced in response to copper additions. Glutathione and other unidentified thiols were produced by the alga in these incubations (Leal *et al*., 1999). The results indicated that thiols may account for an important part of the copper-complexing ligands produced by this alga. Production of the ligands was stimulated by an increase of $[Cu^{2+}]$ from 0.4 to 1.5 pM. The authors suggest that this release of ligands might be a general defence mechanism to cope with excessive metal levels. Moreover, this study reported that *E. huxleyi* constitutively produced two uncharacterised thiol compounds at high Cu intracellular concentrations. Recent research by Dupont *et al*. (2004) suggested that *E. huxleyi* appears to utilize ligands in nitrogen storage and assimilation as they are synthesised upon nitrogen addition to nitrogen-depleted cultures. Vasconcelos *et al*. (2002) studied the effect on growth, trace metal uptake and exudation of *Emiliania huxleyi* cultures of different exudates released by a range of phytoplankton, grown in natural seawater. The exudates of diatom *Phaeodactylum tricornutum* were able

to inhibit *E. huxleyi's* growth rate and final cell yield. In contrast, *Enteromorpha* (green macroalgae) exudates enhanced the final cell yield and growth of *E. huxleyi.* This was related to the presence of a high concentration of the glutathione-like compounds produced by *Enteromorpha.* The inhibition of growth of *E. huxleyi* as a result of *P. tricornutum* exudates appeared to be a result of the production of cysteine-like compounds. The nature and concentrations of the organic compounds present in the culture medium also influence directly or indirectly trace metal uptake, and also affect the concentration and composition of the exudates produced by *E. huxleyi* (Vasconcelos *et al*., 2002).

The speciation of Cu, Zn and Mn was investigated over the course of a bloom of *E. huxleyi* (Muller *et al*., 2003) in a mesocosm. The labile fraction of Cu decreased from 1.1 to 0.3 nM during the formation of *E .huxleyi* bloom. This was likely to be due to the active release of organic ligands by phytoplankton, which stopped as soon as the level of labile Cu reached 0.3 nM, $[Cu^{2+}]$=0.02 nM. Organic Zn ligand production coincided with a 15-fold increase in the cell numbers of dead *E. huxleyi.* This suggests that Zn-binding ligands may have originated from dead or decaying *E. huxleyi* cells.

Marine cyanobacteria are an important phytoplankton group with the ability to release trace metal-binding compounds into the seawater medium (Leão *et al*., 2007). Croot *et al*. (2000) and Croot (2003) observed that prokaryotes such as the cyanobacteria *Synechococcus* are the source of class 1 ligands. Those ligands have log K_{CuL} values within the range of 12-14. Their survey (Croot *et al.,* 2000) showed that there are many biological sources of copper binding ligands, but cyanobacteria are more plausible sources of class 1 ligands in the open ocean than eukaryotes. This is because other eukaryotes such as *E. huxleyi* is known to be copper tolerant (Brand *et al*., 1986) and other eukaryotes have other detoxification mechanisms such as intracellular metal sequestration (Leáo *et al*., 2007; Croot *et al*., 2000). It was observed recently during a nutrient-stimulated summer bloom (Muller *et al*., 2005), that copper speciation was controlled by the formation of very strong organic complexes. The cyanobacteria *Synechococcus* was the source of such complexes, whereas weak Cu-binding ligands were produced by metabolic production or decomposition of diatoms.

In culture experiments, *E .huxleyi* can release class 2 ligands (log K_{CuL} 11-12), as observed in response to Cu addition (Leal *et al*., 1999). Toxic effects of *E. huxleyi*

occurred at [Cu^{2+}] levels around 0.025 nM (Leal *et al*., 1999; Muller *et al*., 2003). The ligands produced in Muller *et al* (2003) had different Cu-complexing strengths (log K_{CuL} = 9.9-10.5) to the class 2 ligands (log K_{CuL} 11-12) observed by Leal *et al.* (1999) and Vasconcelos *et al.* (2002) in axenic *E. huxleyi* culture experiments. These differences might also be due to the differences in the physiological conditions of *E. huxleyi* in laboratory cultures and mesocosms.

2.8 Aim and objectives of this study

The overall aim is to study trace metal speciation through the life cycle of phytoplankton by specifically addressing the following:

- To use the coccolithophorid *E. huxleyi* in laboratory experiments as a model organism.
- Examine the effects of *E. huxleyi* growth, senescence and decay on dissolved trace metal (Cu, Co, Ni) concentrations and their physico-chemical speciation.
- To investigate the effect of viral induced lysis of *E. huxleyi* on metal (Cu, Co, Ni) speciation.
- To determine the trace metal speciation (Cu, Co, Ni, Zn) composition *in situ* during or post a natural *E. huxleyi* bloom event in the Western English Channel.

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Chapter 3

3.Experimental Procedures

3.1 Introduction

Trace metals are generally defined as those metals present in concentrations of less than 1 μM (Bruland, 1983). Understanding of the marine chemistry of trace metals has improved since the 1970s (Libes, 1992; Achterberg and Braungardt, 1999) as contamination has been minimised due to improved analytical procedures, and advances in "clean" sampling and analytical protocols (Connelly, 1997; Bruland *et al.,* 1979; Lohan, 2003). Detection limits of measurements have dropped as a result of improvements in the handling and analysis of seawater samples for dissolved trace metal (Lohan *et al*., 2002). Most often contamination occurs during the stages of sampling, filtration and storage (Hill, 1997).

Trace metal speciation studies have become very important during the last two decades. This is because of the recognition that total dissolved trace metal concentrations do not reveal sufficient information about the bioavailability, toxicity and geochemical behaviour of metals (Achterberg and Braungardt, 1999). This makes speciation studies very important, in order to improve our understanding of the role of metals in biogeochemical processes. According to Sunda, (1991), stripping voltammetry is one of a few sufficiently sensitive techniques to determine labile/free metal fractions directly in natural waters.

The aim of this chapter is to describe all methods and cleaning protocols used to control metal contamination, and to detail the methods and instruments used to measure total dissolved and labile metals. This includes stripping voltammetry for metal speciation measurements and the digestion of filter membranes to obtain total particulate copper concentrations using graphite furnace atomic absorption spectroscopy. The sample collection and analysis for dissolved organic carbon, inorganic nutrients and chlorophyll is also explained.

All biological growth experiments concerning *E. huxleyi*, including viral infection and associated measurements are explained in detail. Finally, sampling procedures during an *E.huxleyi* bloom in the Western English Channel are described.
The chapter is divided into two main sections:
1. Chemical analysis and measurements, 2. Biological experiments and measurements.

3.2. Chemical Analysis and Measurements

3.2.1 Cleaning Processes

3.2.1.1 Sample storage and the cleaning process for trace metal analysis

Low density polyethylene (LDPE, Nalgene) bottles were used for sampling and storage of samples. Trace metal clean polystyrene vials (30 ml) were used for storage of standard and reagent solutions. All plasticware used in the study was acid cleaned to remove trace metal contamination. Typically, bottles were soaked in Decon 90 (2% v/v, 24 h), rinsed using ultra-pure water, supplied by reverse osmosis (Milli-RO, Millipore) followed by ion exchange with a conductivity <0.1µS cm^{-1}(18 MΩ/cm) (Milli-Q water; Millipore), and then soaked in hydrochloric acid (HCl) (Aristar grade; Fisher Scientific) (50% v/v, 1 week). The bottles were rinsed with Milli-Q water and then submerged in nitric acid (HNO_3) (Aristar grade; Fisher Scientific) (50% v/v, 1 week). Lastly, bottles were rinsed thoroughly with Milli-Q water, filled with Milli-Q water, acidified to pH 2 using sub-boiled HCL (10 µl per 10 ml sample) and then double bagged and stored until needed.

The filtration unit (polysulfonic, Nalgene) was also soaked in Decon 90, rinsed with Milli-Q water and then soaked in 10% HCl, rinsed again with Milli-Q water, and double bagged ready to be used. Polycarbonate filters (Whatman) (0.4 µm 47 mm diameter) were soaked in (HCl), (1% v/v, 24 h) and rinsed with Milli-Q water prior to filtration of trace metal samples.

The quartz test tubes fitted with Teflon screw lids and collars, used for U.V. digestion, were washed twice in HCL. First, the tubes were soaked in HCl (10% v/v, 24h), rinsed with Milli-Q water, soaked again in HCl (10% v/v, 24h) and thoroughly rinsed with Milli-Q water. Screws and lids were acid washed in a similar way as the test tubes in separated HCl (2% v/v, 24h) baths (Lohan *et al*., 2002; Ellwood and van den Berg, 2001; Achterberg and Braungardt, 1999; Achterberg *et al*., 1999; Achterberg *et al*., 2003).

In order to obtain a bottle blank, cleaned bottles were filled with Milli-Q water and left for 2-4 days. Blank measurements on this MQ water were conducted. Negligible blanks (< 0-0.2 nM) were observed which indicated the efficiency of the cleaning process conducted in order to decontaminate bottles for any metal under study.

The 20 L poly carbonate (Nalgene) carboys were used for the culturing experiments and were acid washed to assure decontamination. The carboys were first filled with a Decon solution (2% v/v, 24 h), then rinsed with Milli-Q water, filled with HCL (2% 24 h), then rinsed three times with Milli-Q water; finally they were autoclaved. Note that the carboys were double bagged using auto clave bags to prevent contamination during autoclaving. The plastic tubes, carboy venting cabs and connections used in the experiments were decon washed (2%, 24 h), rinsed with Milli-Q water, then soaked in HCL (10%, 24 h), and rinsed 3 times with Milli-Q water. They were double bagged and autoclaved ready for the experiments.

3.2.1.2 Cleaning Processes for DOC and TDN analysis

All glassware, plastic and glass sample bottles used for the DOC and TDN samples were washed thoroughly. All glass and plasticware and filtration units were soaked in 2% Decon for 24 h, and then rinsed with UV-irradiated Milli-Q water. They were then soaked in 10% HCl for 24 h and subsequently rinsed with the UV irradiated ultrapure water three to five times. All the glassware used for sample collection and the filtration units were combusted at 450^{o}C for 6 hours. This was to remove any remaining organic contaminants (Badr *et al*., 2003).

3.2.2 Analytical procedures

3.2.2.1 Preparation of chemicals:

Sub-Boiling Distilled water (SBDW): Sub-Boiling Distilled Water was produced in the clean room at NOCS using a quartz system similar to the one described by Lohan (2003) and Connelly (1997). Milli-Q water was poured into a quartz flask and heated with two infra-red lamps. Water evaporates and condenses on a quartz cold finger and then drips through a collection tube and into a clean Teflon bottle. This system was placed in a

laminar flow hood in a clean room environment to eliminate contamination problems. Sub-Boiling Hydrochloric Acid (SBHCl) and Sub-Boiling Nitric Acid ($SBHNO_3$) were produced in a similar way.

Isothermally Distilled Ammonia solution (ID-NH_4OH):

The ammonia solution used for neutralisation of all acidified seawater samples was purified from analytical grade ammonia solution. Fluorinated ethylene propylene (FEP) beakers containing SBDW and analytical grade ammonia were placed in an airtight container and left for approximately 2 days. During this time, the ammonia partitioned the SBDW and ammonia solution and formed ID-NH_4OH. This was transferred into an FEP bottle and stored.

3.2.2.2 Reagents and Standards

All handling steps for reagents, standards, solutions and samples were carried out in a Class 100 laminar flow cabinet situated in a clean room. All reagents and standards were prepared in ultra-pure water. All samples and reagents were stored in LDPE bottles, placed in re-sealable plastic bags. Table 3.1 shows the Adsorptive Cathodic Stripping Voltammetric ligands used in the experiments and their final concentrations in the voltammetric cell. All reagents, samples and standards were prepared using monthly-calibrated auto-pipettes (Finnpipette, Labsystems Oy, Finland).

Metal standards were prepared from Spectrosol grade standards BDH (1000 mg L^{-1}), by serial dilution and acidification to pH 2, using sub-boiled HCl (10 µl per 10 ml standard). Standards were prepared on a weekly basis and stored at ca. 4^oC.

Boric acid was used as a pH buffer. The final concentration for the buffer in the 10 ml cell was 0.01 M. The buffer was cleaned in a Chelex column (Ellwood and Van den Berg, 2001). $NaNO_2$ (0.25 M) was used as a catalyst in the measurement of Co to enhance sensitivity. $NaNO_2$ was cleaned using a mercury pool electrode, as described by Vega and van den Berg (1997).

Table 3-1: The Ad-CSV ligands used in this study. The amount added and final concentrations in the cell are provided.

Element	Complexing Ligand & concentrations in (M)	Amount added (µL)	Final concentration in cell (10ml)
Co	Nioxime		
	5.10^{-3} Total	20	10 µM
	5.10^{-4} Labile	40	2 µM
Cu	Salicylaldoxime (SA)		
	1.10^{-2} Total	25	25 µM
	1.10^{-3} Labile	30	3 µM
Ni	Dimethylglyoxime(DMG)		
	1.10^{-1} Total	20	0.2 mM
	1.10^{-2} Labile	20	0.02 mM
Zn	Ammonium Pyrrolidine Dithiocarbamate (APDC)		
	1.10^{-1} Total	20	0.2 mM
	1.10^{-2} Labile	20	0.02 mM

3.2.3 Samples for Chemical Analysis

3.2.3.1 Culture Experiments in the Laboratory

Culture experiments were conducted at the Marine Biological Association (MBA, Plymouth). Samples were collected from the culture carboys on day 0, 2, 4, 6, 8, 10 and 14 for the March 2004 experiments and days 0, 2, 4, 6, 8, 10, 12 and 16 for the May and August 2004 experiments.

For trace metal analyses, samples were gently filtered in a Class 100 laminar flow cabinet using 0.4 µm polycarbonate membrane filters (Whatman) with a hand-operated pump (Nalgene) not exceeding 0.3 bar vacuum. A microscopic check of the algae on the filters revealed that they did not rupture or break during filtration. The filter membranes were stored in individual Petri dishes at -20°C until analysis. The membranes were retained for use for quantifying total particulate metal concentration in the algae (extracellular algal adsorption plus intracellular uptake).

Samples were transferred into acid washed low density polyethylene bottles. Samples for Cu organic ligand titration measurements were stored frozen at -20°C after filtration.

Samples for total trace metal analysis were acidified to pH 2 (addition of 10µl of sub-boiled HCl 6 M per 10 ml sample). Acidification was essential in order to stabilize the total dissolved concentration of metals (Tappin *et al*., 1995; van den Berg, 1989) and to prevent trace metal adsorption onto bottle walls (van den Berg, 1988). Labile dissolved trace metals were measured as soon as possible, without previous acidification or UV digestion, following storage at 4^{o}C.

3.2.3.2 DOC, TDN, and chlorophyll a; sample filtration, preservation of samples

Samples (125 ml from each 20 L carboy) were collected in Pyrex glass bottles. The bottles were rinsed twice with the sample to reduce contamination and adsorption onto the walls of the bottles. The DOC and TDN samples were filtered immediately after collection. This was to reduce loss of TDN or DOC through any biological activity. An acid washed glass filtration unit (Millipore) was used to filter all samples related to the organic measurements in the experiments. Glass fibre filters of 0.7 µm nominal pore diameter (47mm diameter, Whatman GF/F) were used. The filters were combusted at 450^{o}C for 6 hours prior to the filtration.

Following filtration, two 30 ml plastic bottles were filled and stored at -20^{o}C for DIN (NO_3^- and NO_2^-) and PO^{3-} analysis. The samples for TDN/DOC were transferred to clean and combusted glass ampoules (10ml/20ml). Then the samples were acidified to pH 2 using 50% HCl 10µL per 10 ml of sample. Acidification stops any biological activity and removes inorganic carbon as carbon dioxide. After acidification of the sample, the ampoules were flame sealed using a butane burner and stored at 4^{o}C until analysis. Chlorophyll *a* samples from cultures and the English Channel samples were filtered using GF/F filters. The filters were folded, placed into foil envelopes and stored in a freezer at -20^{o}C until further analysis.

3.2.3.3 Sampling in the English Channel

A field study was conducted on the RV 'Squilla' (Plymouth Marine Laboratory research vessel) during *E. huxleyi* blooms in the western English Channel, August 2005 and July 2006. Full station positions are shown in Chapter 5. At each station hydrographic data were collected using a CTD. Trace metal clean GoFlo (General Oceanis, 10 L) seawater samplers were used for collecting ultra clean samples for trace metal analysis and

biological analysis (van den Berg, 1999; Achterberg and Braungardt, 1999). The GoFlo samplers (supplied by National Marine Facilities Division in the National Oceanography Centre) were cleaned one week prior to the sampling in the laboratory using HCl (2% v/v, 24 h) rinsed 3 times by Milli-Q water and then covered with polyethylene sheets until used. Intial check experiments were carried out by measuring blanks for Cu, Co, Ni, and Zn (0.13, 0.05, 0.10, 0.25 nM repectively). Low blanks indicates the efficiency of the cleaning process. Kevlar wire (8 mm) was mounted on a winch for collection of seawater samples using the GoFlo bottles. The bottles were tripped by a Teflon messenger. Water samples were collected from depth profiles at three stations in both 2005 and 2006. Trace metal samples were collected into clean 500 ml LDPE (Nalgene) bottles, and nutrients and organic compounds sampled into acid washed Pyrex glass bottles. Bottles were always rinsed twice with the sample and gloves were worn during sampling. Samples were placed in a cool box until returned to the laboratory. Filtration was conducted at MBA in a laminar flow hood using similar procedures and filtration units as used for the culture samples.

3.2.3.4 Ultra Violet Digestion of Organic Complexes

Total dissolved trace metal concentrations analysis by voltammetric measurements require the use of ultraviolet (U.V.) digestion of the samples. This is because natural organic ligands form strong and co-ordinated bonds with trace metals and could compete with the added Ad-CSV ligand for the analyte. Surfactants could also foul the mercury drop and thus prevent the metal-ligand complex from adsorbing on the mercury drop. These factors would result in a lower observed dissolved trace metal concentration (van den Berg, 1988). Therefore, U.V. digestion and acidification of the sample is essential to break down interfering organic substances and metal complexing organic ligands. (Achterberg and van den Berg, 1994; Lohan, 2003).

U.V digestion was performed using a purpose built system (Achterberg and van den Berg, 1994; Achterberg *et al*., 2003). This system was housed in an aluminium box and fitted with a medium pressure (400W) mercury lamp (Photochemical Reactors Ltd, Reading, UK). Fan assisted cooling of the lamp was essential to maintain a sample temperature of between 60 and 70^{o}C. The system can digest 8 samples during each digest. The samples were placed in quartz tubes (ca. 30 ml) around the lamp and were

exposed to U.V light (4-5 h). Acidified samples (pH 2, ca. 30 ml) were transferred to acid washed quartz tubes, and hydrogen peroxide (H_2O_2) (27% v/v; 60 µl) was added. The H_2O_2 was added to enhance sample digestion. The samples were stored for at least 24 h after UV digestion, at laboratory temperature, in order to remove any interference from hypochlorite ions (Achterberg *et al*. 2003; van den Berg 1988).

3.2.4 Analytical Measurements*:*

3.2.4.1 Introduction

Few analytical methods can be utilised for the direct determination of trace metals in seawater, due to interference from major ions (Na^{2+},Mg^{2+}, Ca^{2+} and Cl^-) (Hill, 1997) . The methods most regularly used for measuring trace metals in marine samples include graphite furnace atomic absorption spectroscopy (GF-AAS) and inductively coupled plasma mass spectrometry (ICP-MS). Both methods require sample pre-treatment prior to analysis (Hill, 1997; Achterberg and Braungardt, 1999). It is important for the GF-AAS technique to remove interfering compounds and preconcentrate the metals of interest.

The GF-AAS technique is based on the fact that free atoms absorb light at a wavelength characteristic of the element of interest. The amount of light absorbed is linearly correlated to the concentration of analyte present. Free atoms of any element can be produced from samples by the application of a high temperature (Atkins, 1999).

In GF-AAS, samples are deposited in a small graphite or pyrolytic carbon coated graphite tube, which is heated to vaporize and atomize the analyte. Atoms absorb ultraviolet or visible light using a specific lamp for each metal of interest and make transitions to higher electronic energy levels. Concentrations can be determined from a working curve after calibrating the instrument with standards of the metal concentrations (Connelly, 1997). GF-AAS is widely used to measure trace metals in seawater (Bruland, 1983; Hill, 1997) and is a very selective technique, in which control of the temperature profile can be utilised to separate the analyte from the interfering matrix prior to atomisation (Atkins, 1999).

ICP-MS is a type of mass spectrometry that is highly sensitive and capable of determining a range of metals at concentrations below one part in 10^{12} (Hill, 1997). ICP-

MS requires sample dilution, as it must be introduced into the instrument as a liquid with less than 0.1% dissolved solids, to prevent a build up of solids on the nickel cones (Ebdon *et al.,* 1998).

The disadvantages of any analytical technique requiring pre-treatment of samples are: 1) the sample preparation requires more time and 2) there is an increased risk of sample contamination or change through sample handling (Hill, 1997).

The stripping voltammetry technique does not have the multi-element capabilities of ICP-MS. However, it has the advantage of measuring trace metals directly in the sample and the pre-concentration step is performed in the cell (Achterberg and Braungardt, 1999).

The following section will include background and details of measurements conducted to measure dissolved, labile and particulate trace metals. Stripping Voltammetry is used for the determination of trace metal speciation in water and (GFAAS) for total particulate copper on filters.

3.2.5 Dissolved Trace Metal Measurements:

3.2.5.1 Voltammetric Methods:

Voltammetry is based on the measurement of a current response as a function of the potential applied to an electrochemical cell. Stripping voltammetry techniques include anodic stripping voltammetry (ASV) and adsorptive cathodic stripping voltammetry (Ad-CSV).

Voltammetric instruments include a potentiostat and a three electrode cell; containing a:

1- Working electrode Hanging Mercury Drop Electrode (HMDE)
2- Reference electrode Ag/AgCl/KCl
3- The counter electrode platinum wire or carbon graphite rod

In stripping voltammetry, a pre-concentration step (isolating the metal of interest from the matrix) is combined with a stripping step. During the pre-concentration step, the trace metal of interest is collected onto or in a working electrode and, during the stripping step, the collected metal is either oxidised or reduced back into solution. This enhances the selectivity of the analysis. The advantages of the use of a HMDE are its reliability; with

the formation of each new drop, a new electrode surface is produced, which decreases any possible contamination.

The determination of trace metal species in the nano to pico molar ranges has been made possible by the development of Ad-CSV, which accommodates small sample volumes, allows different metal species to be determined and has very low detection limits (Achterberg and van den Berg, 1997; Achterberg and Braungardt, 1999). Ligands used in Ad-CSV should have the ability to form a complex with an element of interest. Also it should be electroactive i.e. capable of adsorbing onto the surface of the HMDE working electrode. The resultant current-potential stripping voltammogram provides quantitative information; the height of the peak is proportional to the metal concentration. The quantification of metal concentration in samples during voltammetry analysis is by the use of the standard addition method. A buffer is used in the measurement of samples to control pH, because the formation of metal complexes is pH dependent (van den Berg, 1988; Braungardt, 2000).

3.2.5.2 Theory of ASV

The technique of ASV involves the formation of a mercury-metal amalgam. A small fraction of metal is deposited during the deposition step {Eq. (1)}. Sensitivity is enhanced by using an Hg film instead of an Hg drop as a result of higher surface to volume ratio. Following this initial pre-concentration stage, the metal is stripped out of the mercury by application of a voltammetric scan towards a more positive potential. The metal is oxidized back into the solution {Eq (2)} and the oxidation current produced is determined. ASV has been applied successfullt for trace metal measurements of Cu, Cd, Pb and Zn (Achterberg and Braungardt, 1999). The number of of trace metals that can be determined by ASV however, is limited to those that can be reduced and reoxidized at appropriate potentials and that are soluble in a mercury amalgam (Bruland and Lohan, 2004).

$$\mathbf{M^{n+} + Hg + ne^{-} \rightarrow M\ (Hg)} \qquad \text{Equation 1}$$

The pre-concentration process of a complex formation and adsorption

$$\mathbf{M\ (Hg) \rightarrow M^{n+} + Hg + ne} \qquad \text{Equation 2}$$

The stripping process

3.2.5.3 Theory of Ad-CSV

Ad-CSV makes use of ligand metal complexation. A specific ligand (AL) is added to a sample and electrochemically labile metals (M^{n+}) form a complex with the ligand $M_y(AL)_z$ {Eq. (3)}. This metal-ligand complex is adsorbed on the surface of the working electrode during the pre-concentrate stage and a potential scan is subsequently carried out during which the metal is stripped from the surface of the mercury by the application of negative potential scan {Eq. (4)}. This is carried out under carefully controlled conditions. The scan direction is towards more negative potentials. The metal is reduced back into the solution during the potential scan {Eq. (5)}, and the reduction current is measured (Achterberg and van den berg, 1997; Achterberg and Braungardt, 1999). Range of metals detected by Ad-CSV are Fe, Cu, Zn, Co, and Ni (Achterberg and Braungardt, 1999)

$$\mathbf{yM^{n+} + zAL^{m-} \leftrightarrow M_y(AL)_z^{(y.n-z.m)}} \qquad \text{Equation 3}$$

The pre-concentration process involving the formation of the metal-ligand complex .

$$\mathbf{M_y(AL)_z^{(y.n-z.m)} \leftrightarrow M_y(AL)_z^{(y.n-z.m)}} \quad \textbf{adsorbed} \qquad \text{Equation 4}$$

The adsorption step.

$$\mathbf{M_y(AL)_z^{(y.n-z.m)}\ adsorbed + e^- \leftrightarrow yM^{(n-1)+} + zAL^{m-}} \qquad \text{Equation 5}$$

The stripping process.

3.2.5.4 Measurements using Adsorptive Cathodic Stripping Voltammetry (Ad-CSV)

Adsorptive cathodic stripping voltammetry (Ad-CSV) was used to measure trace metals in seawater during this study. The use of Ad-CSVmethods does not require the metal to be soluble in a mercury amalgam (ASV) and thus can be used for a wide variety of trace metals (Bruland and Lohan, 2004). The advantages of using Ad-CSV are lower detection limits and shorter pre-concentration time (reducing the interferences from organic compounds present in the sample) compared to ASV. However, manual handling of samples using Ad-CSV can be a source of contamination and it is mostly limited to single element analysis. The voltammeter system used consisted of an Auto lab

potentiostat (Eco Chemie, the Netherlands) and a hanging mercury drop electrode (663 VA stand, Metrohm) interfaced with an ALT 386 SX, Amstrad compatible PC. The reference electrode was a double junction Ag/AgCl, KCl (3 M) electrode, with a saturated AgCl internal solution and a salt bridge filled with 3 M KCl. The counter electrode was a carbon graphite rod.

Measurements were made by introducing a 10ml sample (acidified to pH 2 and UV digested in case of total metal analysis) into the voltammetric cell. Distilled ammonia was used to neutralise the pH. Different ligands for the various metals and a buffer (borate) were added. The sample, buffer and ligand solution were purged with nitrogen to remove dissolved oxygen, which interferes with voltammetric measurements. The sample was continually stirred during the deposition stage. This is to enhance transport of the metal of interest to the surface of the mercury drop and to prevent a significant concentration gradient occurring at the electrode surface. All voltammetric measurements were repeated three times, for each aliquot of the sample, until peak heights gave (<5%) RSD.

Quantification of the metal (Co, Cu, Ni and Zn) in each sample was undertaken by introducing a known quantity of metal standard into the cell and measuring the resultant increase in peak height. The metal added was always sufficient to increase the peak height by at least a factor of two. Repeated voltammetric measurements were recorded for each aliquot of sample until peak heights were <5%. The linearity of the signal was checked by adding a second standard addition to the same cell in a similar manner. The concentration of the metal of interest was calculated using extrapolation. Two to three aliquots were analysed for each sample. The precision relative standard deviation (RSD) for Cu, Co, Ni and Zn was (1.17, 2.33, 0.43, and 5.90 % respectively).

Both cell and electrode were rinsed three times using Milli-Q water between aliquots. Measurements of blanks were performed using Milli-Q water and the same reagent concentrations and experimental conditions as for the samples. This also helps to assess the carry over between aliquots. Figure 3.1 summarises the steps undertaken during Ad-CSV analysis.

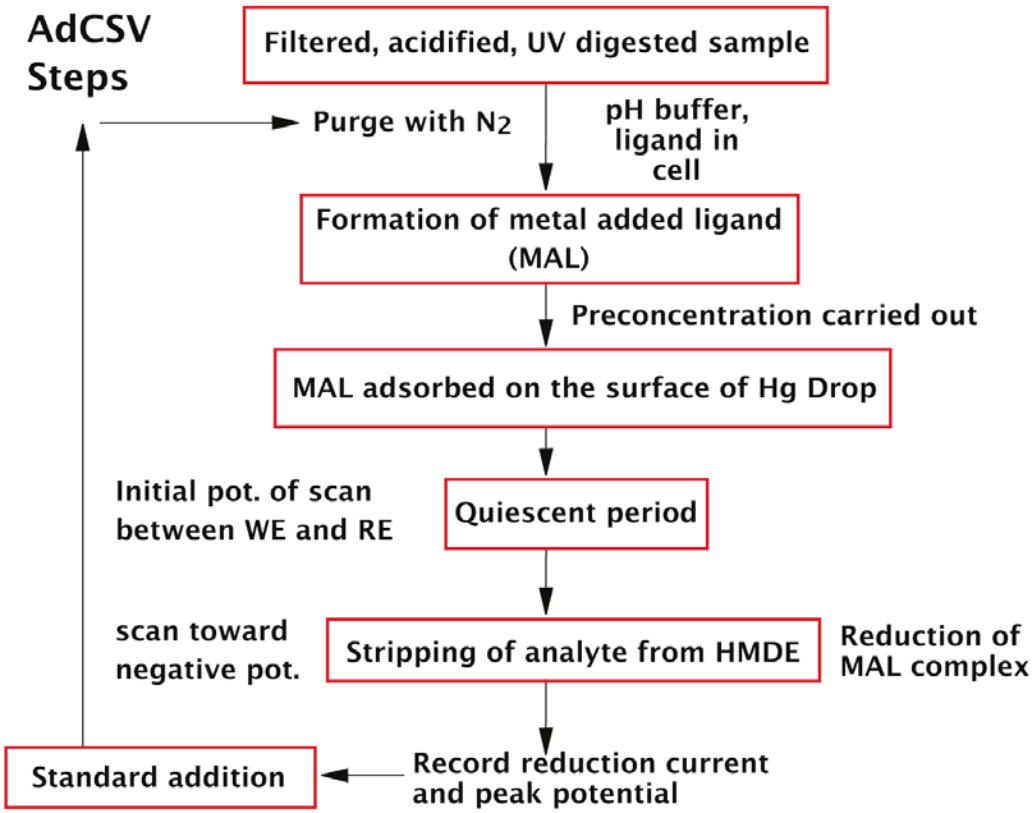

Figure 3.1 Schematic diagram of Ad-CSV steps used for the determination of dissolved trace metals redrawn from Achterberg and Braungardt (1999) and Braungardt (2000).

Experimental conditions for Ad-CSV analysis of Co, Cu, Ni and Zn were optimised prior to sample analyses. The optimisation experiments investigated the effect of different experiment conditions (scan speed, deposition potential, deposition time, and buffer and ligand concentration) on metal measurements using Ad-CSV. All experiments were performed using Milli-Q water and seawater samples collected from the Plym estuary. Table 3.2 shows the conditions selected for experiments following the optimisation experiments. Calibration curves of Co, Cu, Ni and Zn were produced after optimising measurements to assess the linearity of the method used (Figure 3.2). This was conducted every month to asses the reducibility of Ad-CSV. To assess the accuracy of the entire analytical procedures, prior to every set of analyses, estuarine water (SLEW-2, 1999) and nearshore seawater (CASS-4, 2002) (National Research Council, Canada) reference material was analysed in triplicate. It was analysed as a sample and all procedures were similar as for samples measured using Ad-CSV. Blanks were determined on a daily basis using Milli-Q water and the limit of detection was calculated as three times the value of the standard deviation of the value for the blank (Miller and Miller, 1993). The detection

limits for Co, Cu, Ni and Zn was 0.08, 0.09, 0.16 and 0.05 nM, respectively. The low detection limit observed, confirmed the sensitivity of the method used for trace metal speciation studies.

A summary of data for the quality control measurements for this study can be found in Table 3.3. Certified reference material findings indicate close agreement between the observed and certified values. This enhances confidence in the methods and instruments used.

Table 3-2: Voltammetric experimental conditions used for the determination of trace metals.

Element	**Co**	**Cu**	**Ni**	**Zn**
Voltammetric Experiment conditions				
Purge (S)	240	300	100	240
Initial potential (v)	-0.8	-0.15	-0.75	-0.85
Final Potential (v)	-1.2	-0.6	-1.1	-1.3
Deposition potential (v)	-0.8	-0.15	-0.75	-0.85
Speed (Hz)	100	20	50	50
Deposition time (s)	60	60	30	60
Equilibration time (s)	8	8	8	8

Table 3-3:Certified reference material and blanks analyses for the quality control of the measurements.

Dissolved metal	Blank (nM)	SLEW-2 Certified Value (nM)	SLEW-2 Observed (nM)	CASS-4 Certified Value (nM)	CASS-4 Observed (nM)
Cu	0.13±0.03	25.44 ± 1.73	25.65 ± 0.30	9.31 ±0.80	8.91± 0.26
Ni	0.07±0.05	12.00 ± 0.92	12.53 ± 0.06	5.53 ± 0.50	5.74 ± 0.03
Zn	0.22±0.01	16.82 ± 2.14	15.38 ± 1.29	5.82 ± 0.87	6.41 ± 0.42
Co	0.03±0.02	0.933 ± 0.135	0.951 ± 0.0250	0.44± 0.05	0.39± 0.01

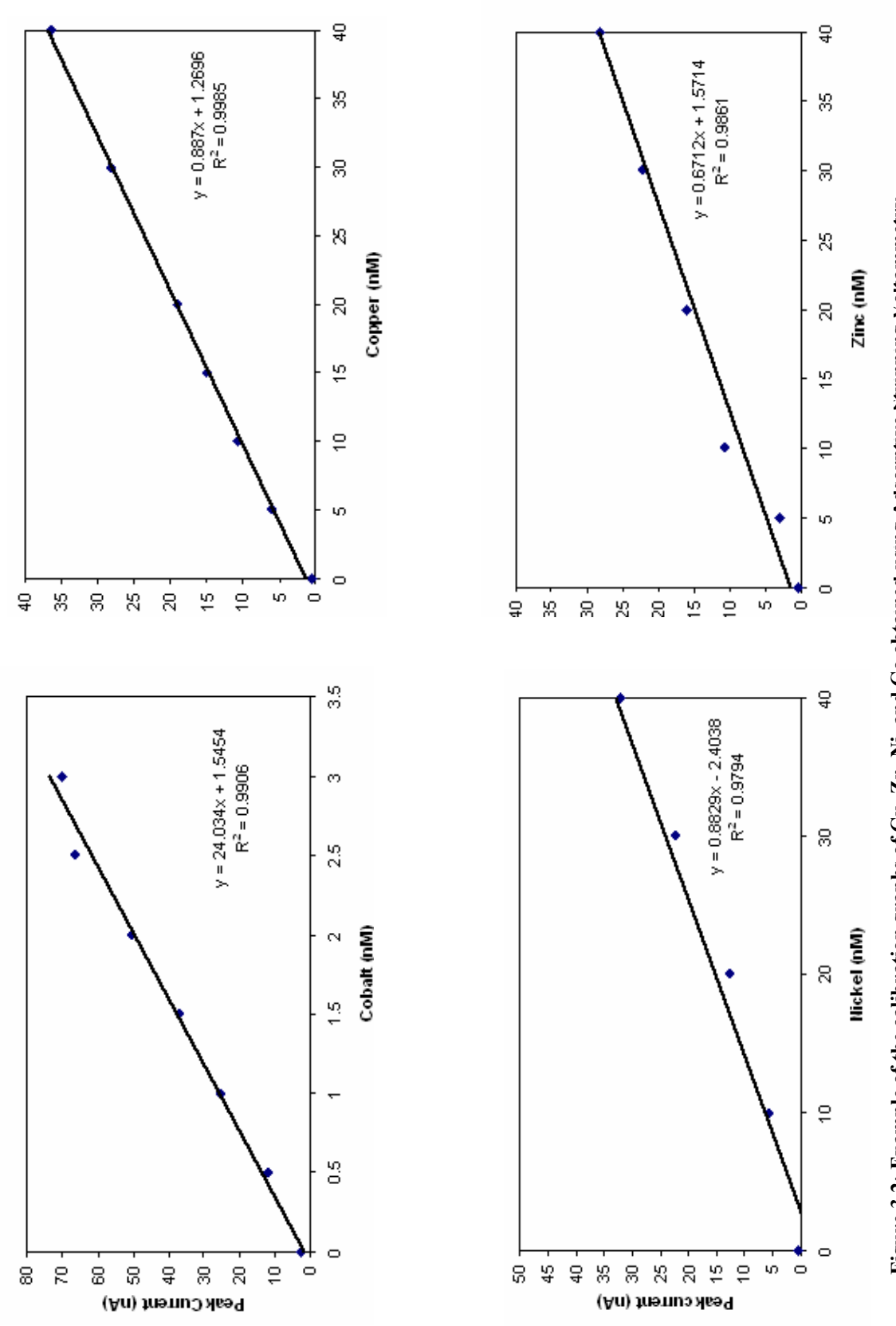

Figure 3.2: Example of the calibration graphs of Cu, Zn, Ni, and Co obtained using Adsorptive Stripping Voltammetry.

3.2.5.5 Copper Complexing Ligand Titration

Several studies have shown that Cu and other metals are bound by dissolved organic ligands, which form metal complexes that are less available to phytoplankton (Croot *et al*., 2000; Ellwood and van den Berg, 2001; Gledhill and van den Berg, 1994; Tang *et al*., 2001). There is evidence that the Cu^{2+} ion induces the release of complexing ligands by cyanobacteria (Moffett and Brand, 1996) and other species in seawater (Croot *et al*., 2000; Moffett and Brand, 1996).

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). Organic ligands may include phytoplankton and bacteria exudates, products produced by the organic breakdown of these organisms and also fulvic and humic acids and sewage effluents (Kozelka and Bruland, 1998; Hill, 1997; Whitfield, 2001).

Theory of titration:

The concentrations of complexing organic ligands (L), free Cu^{2+}, inorganic Cu (Cu') and the respective conditional stability constant (log K_{CuL}) were determined by titration of the filtered culture media and samples collected from the English Channel. This was carried out using competitive ligand titration with SA as the added Ad-CSV ligand described in Campos and van den Berg (1994). A full description of this titration is given in Chapter 4.

A titration technique was employed in order to quantify the free ionic Cu concentration and dissolved Cu complexing ligands and their binding strengths. The titration data was transformed by the linearisation method of van den Berg (1982) /Ruzic (1982).
The following relationship was used:

$$[L] = [L'] + [CuL] \qquad \text{Equation 6}$$

where L is the ligand that forms 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:

$$K'_{CuL} = [CuL] / [Cu^{2+}] \times [L] \qquad \text{Equation 7}$$

A linear relationship is obtained by substitution of L' in equation 6 with equation 7, providing that Cu complextaion is predominantly controlled by a single class of organic ligand (Campos and Van den Berg, 1994):

$$([Cu^{2+}] / [CuL]) = ([Cu^{2+}] / [L] + (1 / K'_{CuL} \times [L]) \qquad \text{Equation 8}$$

The titration of a sample with the metal of interest (Cu) is made in the presence of an added ligand (salicylaldoxime SA). This will result in a series of labile Cu concentrations $[Cu_{labile}]$.

$$[CuL] = [Cu_{Total}] - [Cu_{labile}] \qquad \text{Equation 9}$$

$[Cu_{Total}]$ is the total dissolved Cu concentration in the sample of interest which should be calculated from the total dissolved Cu concentration in the original sample. This sample was UV irradiated in addition to the Cu concentration added during the titration. In the presence of added ligand (SA), $[Cu_{Total}]$ is:

$$[Cu_{Total}] = [Cu'] + [Cu\ SA] + [CuL] \qquad \text{Equation 10}$$

Where [Cu'] is the inorganic Cu concentration. $[Cu_{labile}]$ is the fraction which equilibrates with the added ligand (SA), and this is measured by Ad-CSV. The combination of equations 9 and 10 will result in:

$$[Cu_{labile}] = [Cu'] + [Cu\ SA] \qquad \text{Equation 11}$$

The Ad-CSV peak height is related to $[Cu_{labile}]$ via the sensitivity, S, which is defined as peak current/ Cu concentration (nA/nM)

$$I_p = S \times [Cu_{labile}] \qquad \text{Equation 12}$$

S is calculated by standard additions of Cu to the sample. The [Cu_{labile}] includes [Cu'] as a small constant of the added Cu, which remains uncomplexed by SA. [Cu^{2+}] is related to the [Cu_{labile}] by α':

$$[Cu^{2+}] = [Cu_{labile}] / \alpha' \qquad \text{Equation 13}$$

α' is the overall α-coefficient, excluding complexation by L (Campos and van den Berg 1994). When [Cu^{2+}] is substituted in equation 8 using equation 11 will result in:

$$([Cu_{labile}] / [CuL]) = ([Cu_{labile}] / [L]) + (\alpha' / [L] \times K'_{CuL}) \qquad \text{Equation 14}$$

This equation is used for the van den Berg (1982) /Ruzic (1982) plot, as it is [Cu_{labile}] which is measured by cathodic stripping voltammetry (AdCSV) rather than [Cu^{2+}]. α' is obtained from:

$$\alpha' = \alpha'_{Cu'} + \alpha_{CuSA} \qquad \text{Equation 15}$$

Where $\alpha'_{Cu'}$ is the α coefficient for inorganic complexation of Cu^{2+} and α_{CuSA} is the α coefficient for the complexation of Cu^{2+} by SA:

$$\alpha_{CuSA} = \beta'_{CuSA} [SA'] \qquad \text{Equation 16}$$

[SA'] is the concentration of SA not complexed by Cu and β'_{CuSA} is the conditional stability constant of CuSA in seawater:

$$\beta'_{CuSA} = [CuSA] / [Cu^{2+}] [SA'] \qquad \text{Equation 17}$$

The values for [Cu_{labile}] in equation 14 are obtained from the Ad-CSV peak current as = I_p/S (equation 12) and the concentration of CuL from equation 9. The sensitivity S should be obtained from the linear portion of the titration, where all the ligands are saturated; ligand saturation is verified by comparison with the slope obtained from further Cu standard additions to a sample. This is when Cu-complexing ligands were previously saturated by increments greater than the ligand concentration. This should provide an estimation of whether the end of the titration had been reached. [L] and K'_{Cul} values will

be calculated from the linear squares regression from 1/slope and α'/ (y-intercept x [L]) of a plot of $[Cu_{labile}]$ / [CuL] as a function of $[Cu_{labile}]$ (Campos and Van den Berg, 1994). Titration of natural samples typically produces a linear Van den Berg/Ruzic plot. This suggests that one group of ligands is present in the sample (saturation). However, a curvature would indicate the presence of more than one class of complexing sites (Croot *et al*., 1999; Muller *et al*., 2003., Moffett *et al*., 1997). The range of detectable ligands is restricted by the detection windows of the method applied. The detection windows is set by the relative magnitudes of the α coefficient of the added ligand (Campos and van den Berg, 1994).

The free Cu^{2+}, inorganic Cu' concentrations in the sample were estimated by speciation calculations based on the values of total dissolved copper, the overall inorganic side-reaction coefficient, total dissolved ligand concentration and the respective conditional stability constant.

3.2.6 Total Particulate Copper:

Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS) was used to determine total particulate Cu. A Perkin-Elmer 1100B AAS equipped with an HGA-700 graphite furnace and an AS-70 autosampler were used. All analyses were performed using pyrolytically coated L'Vov platforms and tubes. The programme used in the determination of copper is shown in table 3.4.

Table 3-4: Temperature programme used for the GFAAS analysis

Wavelength	324.8
Lamp energy	15
Drying ^{o}C	140
Ramp (sec)	15
Hold (sec)	15
Ashing ^{o}C	1000
Ramp (sec)	20
Hold (sec)	10
Standing ^{o}C	20
Ramp (sec)	5
Hold (sec)	4
Atomisation ^{o}C	2300
Ramp (sec)	0
Hold (sec)	4
Clean ^{o}C	2500
Ramp (sec)	2
Hold (sec)	3

Copper stock standard (Sigma) 100 µg L^{-1} was used and referred to as the stock standard. A working stock standard was prepared on a daily basis (by dilution of the stock standard) to a concentration of 10 µg L^{-1} and increased to 20 µg L^{-1} for the culture samples when high particulate copper concentrations were expected. This working standard was used for the primary standard on the GFAAS. The autosampler was programmed to produce serial dilutions of the working standard. All analyses were performed with deuterium arc lamp background correction. All results were quantified in µg L^{-1}, determined according to the dilution factor and volume of seawater initially filtered and then reported in nM. Each concentrate was analysed in triplicate on the GFAAS. Samples with high relative standard deviation RSD (10%) were re-analysed. In fact, all results were maintained below ± 6% RSD. Examples of copper standard curves are shown in Figure 3.

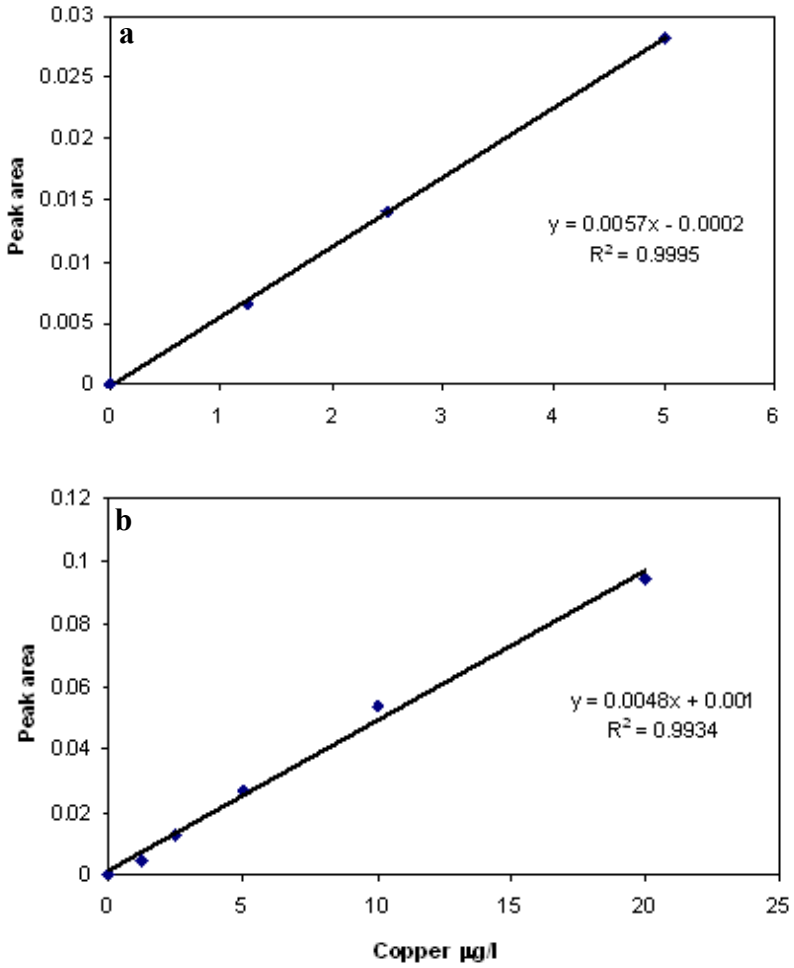

Figure 3.3: Examples of calibration curves obtained in GFAAS for determination of total pariticulate copper: (a) low copper range, (b) higher copper range.

Digestion of the 0.4μm polycarbonate membranes was performed using high-pressure Teflon digestion bombs. Prior to any digestions, the bombs were thoroughly cleaned using the following steps:

1- A rinse in SBDW
2- 1 ml SBDW and 1 ml $SBHNO_3$ were added sealed and heated at 70°C
3- A rinse with SBDW and the 1 ml $SBHNO_3$ was added to each Teflon bomb sealed and heated at 70°C. This step was conducted twice.
4- A rinse in SBDW and then air dried under a laminar flow hood in the clean room.

Total copper concentrations in the algae were determined after the digestion of membranes using 2 ml sub boiled nitric acid in the Teflon bombs, heated on a hot plate at 70°C for 24 hours. Membranes were always submerged in the $SBHNO_3$ to ensure full digestion. After the digestion, the bombs were removed from the hot plate and allowed to cool at room temperature. The solution containing the membrane was transferred to trace metal clean polystyrene vials and diluted with 2 ml sub boiled distilled water. This gave a total sample volume of 4 ml. Samples were stored in the clean room until analysis. Filter blanks were carried through the same procedures for every digestion run conducted. The digestions were placed in the laminar flow hood in a clean room environment to eliminate possible contamination problems.

Reference standard Saragasso Seaweed CRM 279 was used to check the accuracy of the digestion and analysis procedures. The certified value for Cu is 4.9± 0.02 μg/g and the Cu content found was 4.7± 0.08 μg/g where 0.08 μg/g was the standard deviation on separate measurements of the same sample. The concentration obtained in this study is within the specified range. The precision of the digestion technique is ± 2% RSD.

3.2.7 Nutrient Measurements:

Seawater samples for nutrient analysis were collected in acid-washed Pyrex glass bottles. The bottles were rinsed two times with sample to reduce contamination and adsorption on to the walls of the bottles. Samples were filtered using acid washed glass filtration units (Millipore), through 0.7 μm nominal pore diameter glass-fibre filters (47mm diameter, Whatman GF/F). Samples were transferred to two 30 ml acid cleaned high

density polyethylene (HDPE) bottles (Nalgene), frozen at -20°C until analysed. Samples were analysed within 2-4 months.

Inorganic nutrients were determined using a Burkard Scientific (model SFA-2) autoanalyser. This system had an autosampler, a chart recorder and computer. The analytical system was linked to digital-analysis Microstream logging software and a reduction system. The colorimetric methods used for nutrient analysis were those described by Hydes (1984) and Hydes and Wright (1999).

The system was calibrated with potassium nitrate for nitrate and potassium dihydrogen phosphate for inorganic phosphate. Stock standards of nitrate (10 mM) and phosphate (10 mM) were prepared every month. Variable working standard concentrations were prepared prior to every measurement according to the expected range of concentrations. Milli-Q water was used to prepare all reagents, and stock standards. The instrument was set up to measure nitrate concentrations up to 200 μM and phosphate concentrations up to 8 μM for culture samples. A calibration was run at the beginning of each batch of 63 samples, and drift standards every 30 samples. Artificial seawater (NaCL 40 g L^{-1} (0.7 M)) was used as the wash, blank and the matrix for the working standard. Dilutions using the artificial seawater were conducted for samples that showed high nitrate concentrations. Drift standards of intermediary concentrations, low nutrient seawater and blanks were used in the middle and at the end of each run.

The analysis of nitrate requires the reduction of nitrate to nitrite. This was performed by passing the sample through a cadmium column and the sample was then mixed with sulphanilamide and naphthylethelynedihydrochloride (NED) to produce a pink compound with peak absorbance at 540 nm (Grasshof *et al*., 1999). Absorbance was measured in a 15 mm flow cell. Final values are the sum of nitrate and nitrite concentrations in the original sample.

Phosphate reacts with a molybdate reagent in an acidified medium forming a phosphomolybdate complex, which is reduced to a highly coloured blue compound (Grasshof *et al*., 1999). Ascorbic acid was used as a reducing agent and antimonyl tartrate speeded up the reaction. Absorbance was measured at 880 nm in a 50 mm flow

cell. Concentrations of nutrients (µM) were calculated by the computer programme. However, in some cases, manual calculations were performed from the chart peaks. Also the calibration plot was used to recalculate concentrations when there was uncertainty concerning the computer generated readings. Figure 3.4 shows an example of the calibration curves of nitrate and phosphate.

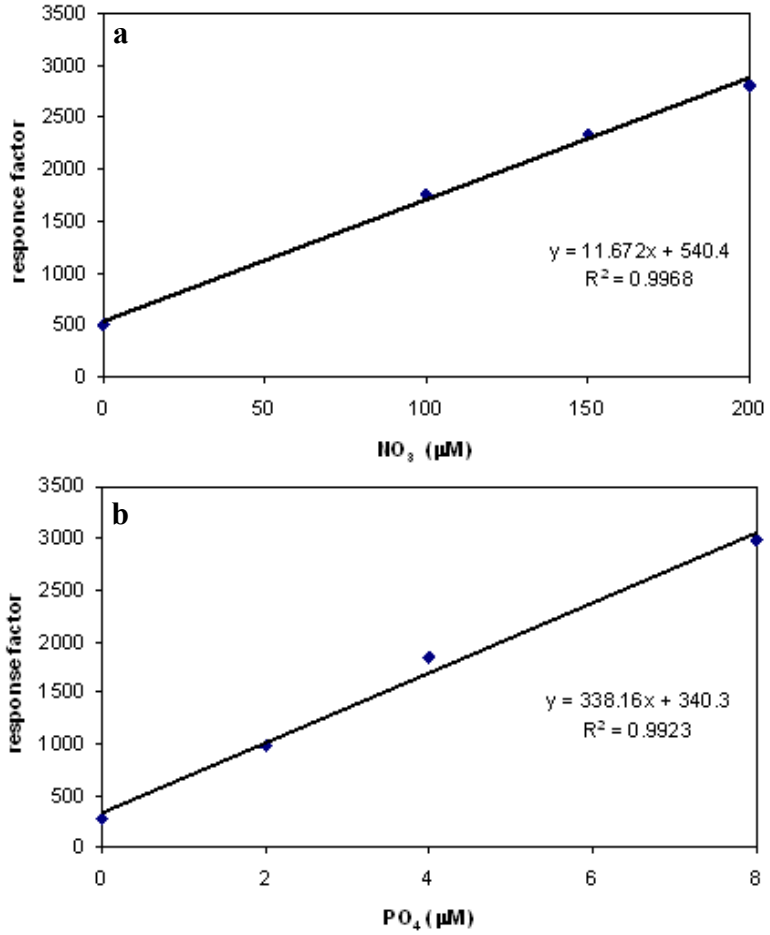

Figure 3.4: Calibration graphs for nitrate and phosphate.

3.2.8 Determination of TDN and DOC

DOC and TDN were measured using a high-temperature catalytic oxidation (HTCO) system with single injection of the sample. The system used comprised of a Shimadzu TOC 5000A coupled to a Sievers nitrogen chemiluminescence detector (NCD). After sample acidification, sparging was performed using high purity carbon free oxygen which results in removing all dissolved inorganic carbon (DIC) from the sample. Oxygen was also used as a carrier gas.

The sample was injected (100 µL) onto the combustion column, which contained platinum on an aluminium oxide catalyst; DOC and TDN were oxidized to carbon dioxide, nitrogen oxide and water. This was undertaken at 680°C. Carbon dioxide was determined using a non-dispersive infrared detector (NDIRD). The quantification of DOC concentrations was achieved using data integration system and peak-area measurement. This system recorded the signals created from the NDIRD. A vacuum pump pulled the combusted gases from the NDIRD into the NCD. TDN peak areas were quantified using Lab View software (Badr *et al*., 2003). Water blanks (CRM Deep Ocean Water Hansell Laboratory, University of Miami) (44-46 µM DOC and 21.5µM TDN) were determined for each analysis to assure high quality measurements. In addition, daily calibration of the system using easily oxidized standard compounds (potassium hydrogen phthalate and glycine mixture (C: N atom 6:1)) were conducted. The calibration curves showed a high correlation coefficient (R^2), which indicated high precision. The analysis of the CRM (44.3 µM DOC and 21.2 µM TDN) were in good agreement with the certified values. Figure 3.5 (a,b) shows an example of the calibration graphs obtained form DOC and TDN. The limit of detection was 0.8 µM for C and 1.2 µM for N.

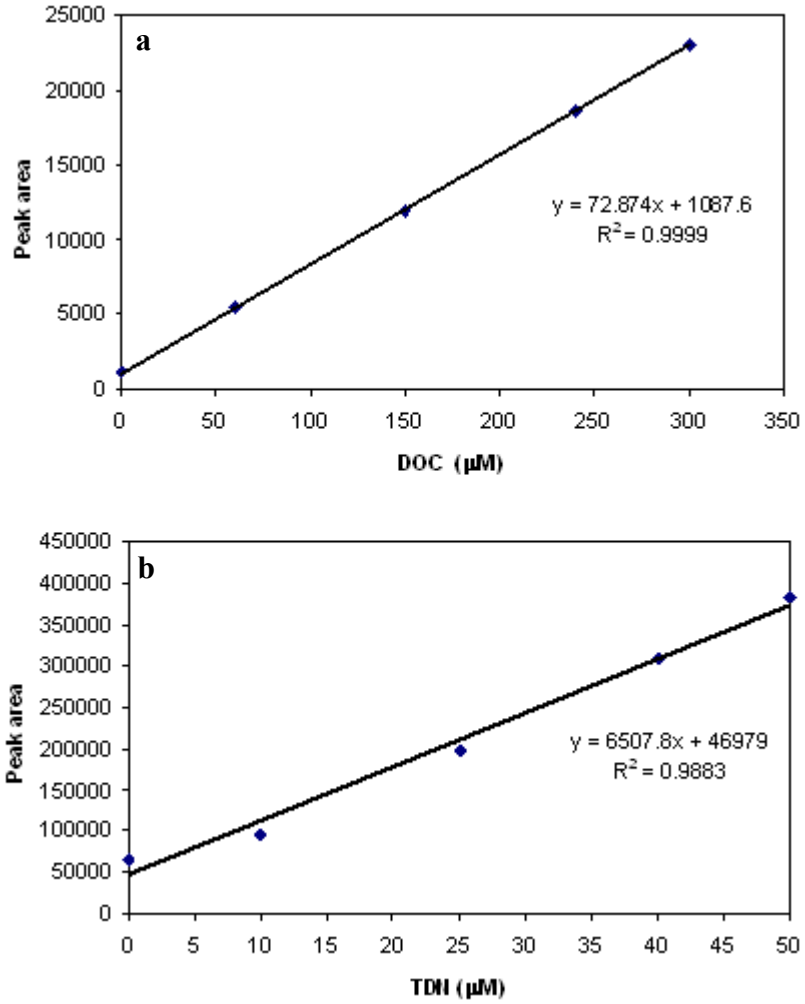

Figure 3.5: DOC and TDN calibration figures.

3.2.9 Chlorophyll *a*

Chlorophyll-*a* was determined by measurement of fluorescence, according to the methods of Welschmeyer (1994). The culture and the English Channel samples were filtered through 0.7 µm nominal pore diameter glass-fibre filters (47mm diameter, Whatman GF/F). The filters were folded, placed into foil envelopes and stored in a freezer at -20 ^{o}C until further analysis. Chlorophyll-*a* was extracted from the filters with 90% v/v acetone (5 ml). The extracts were carefully sonicated for 30 seconds using a Vibra Cell sonicator. This is to detach cells from membranes. Samples were then centrifuged (separate filters and debris) in a MSE Mistral 2000 centrifuge at 3000 rpm for 10 minutes. The fluorescence of each extracted sample was measured using a Turner Designs model 10AU fluorometer fitted with a F4T41/2B2 lamp, a 436 nm excitation filter and a 680 nm emission filter. This optical combination resulted in the greatest

discrimination of chlorophyll-*a* against chlorophyll-*b*, phytin-*a* and phytin-*b*, providing a sensitive method for the determination of chlorophyll-*a* (Welschmeyer, 1994). 90% acetone was used as a blank and in the preparation of the working standard solution of chlorophyll-*a* (Sigma) which has been used to calibrate the fluorometer (Jeffrey and Humphery, 1975). The concentration of chlorophyll-*a* standard was determined by spectrometry according to the equation by Jeffrey and Humphery, (1975):

Chl*a* = 11.85 E664 - 1.54 E647 – 0.08 E630

Where E= the absorbance readings of the spectrometer at 664, 647 and 630nm, using a 1 cm cell, after correction for turbidity by subtracting the reading at 750nm. The working standard solution was prepared (diluted according to the expect range of concentration) and the fluorescence measured prior to all analyses. The fluorometer was recalibrated if the concentration of the diluted standard was 10% higher than the value displayed by the flurorometer. Chlorophyll-*a* concentrations (µg/L) were calculated according to the following equation: C= f*v/V,

where f is the fluorescence reading, v is the volume of the extract (in 90 % acetone) in ml and V is the volume of the sample filtered in ml.

3.3 Biological Experiments and Measurements:

3.3.1 Stock Culture of *Emiliania huxleyi*

Cells of an axenic *E. huxleyi* (strain 1516) were obtained from the collection of the Marine Biological Association (MBA). Stock cultures of *E. huxleyi* were grown in F/2 medium. Seawater collected from station L4 (which is located 10 nautical miles south-west of Plymouth) was filtered using Sartorius cartridges (high capacity filter 0.2 µm cartridges). The filtered seawater was autoclaved for 30 minutes and kept in the culture room until it reached room temperature. Nitrate, phosphate, silicate, trace metals, Fe/EDTA and vitamins were added. This is the F/2 complete media preparation as described by Guillard and Ryther (1962). Finally, after addition of the recipe, the media was filtered again using a new Sartorius filter. This F/2 media was used to build the *E. huxleyi* stock culture to be used in the experiments.

3.3.2 Biological Experiments

Preliminary growth experiments with *E. huxleyi* strain CCMP 1516 in one litre bottles (Nalgene) were conducted using two different culture media. The main objective of the experiments was to gain knowledge of phytoplankton culturing techniques and any other related measurements. The two media used in the biological experiments were:

(1) F/2 media (N, P, Si trace metals, Fe/EDTA and vitamins), as described by Guillard and Ryther (1962).

(2) Filtered sea water media (FSW) with the addition of nitrate and phosphate only (final concentrations (176 µM N and 7.26 µM P respectively) according to Vasconcelos *et al*. (2002), Vasconcelos and Leal (2001).

Experiments with the F/2 media were conducted on 13.03.03 to verify the growth of *E. huxleyi* and to build a viral stock (specific for this species) which would be used for future experiments. Growth rates were comparable and reproducible with published rates for this species (Wilson *et al*., 2002 b; Schroeder *et al*., 2002). 500 µL of EhV86 virus (provided by Declan Schroeder) was added to the culture during the exponential phase of growth. Viruses were used to infect the *E. huxleyi* cultures. This was to observe the role of viruses in the speciation of metals during the life cycle of *E. huxleyi*. This growth experiment indicated a clear effect of viral induced lysis on cell numbers of *E. huxleyi* (Figure 3.6). The preliminary experimental results confirmed that virus lysis effectively controlled the cell numbers. This work also served to build up a viral stock.

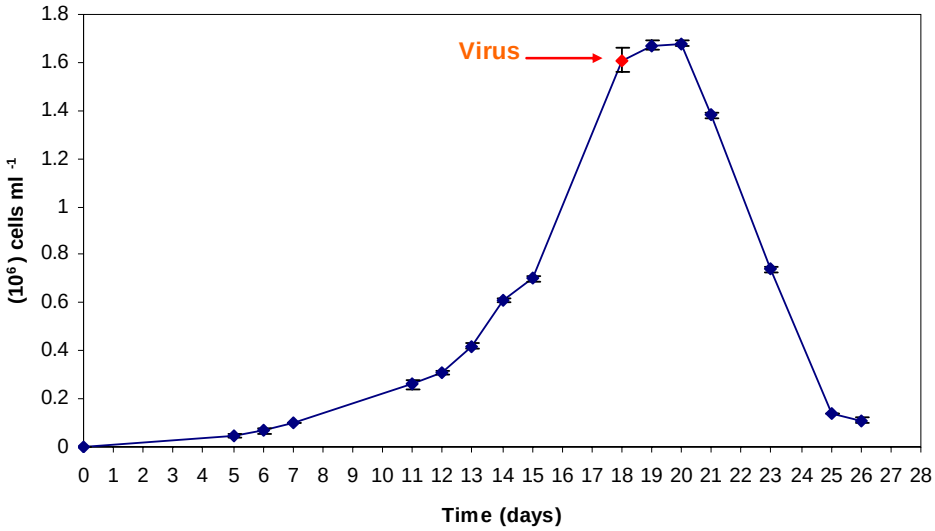

Figure 3.6: Growth curve of *E. huxleyi* (experiment conducted on 13.03.03). Error bars indicate standard error of cell counts.

In addition, viruses were added to a FSW experiment 28.03.03. Incubation, growth and the death process were monitored by cell counting using a Zeiss compound microscope and an Improved Neubauer haemocytometer. Initially *E. huxleyi* was grown in FSW (1 L) and subdivided into four sub-samples at the exponential phase. Growth rate was comparable and reproducible with the F/2 growth rate. Different amounts of virus (50, 20, and 10 µL) were added at day 17 to three bottles. A bottle with no virus added was considered as a control. Figure 3.7 shows that the viral induced lysis also strongly affected cell numbers in this medium (FSW) compared with the control. Although viruses were added in different quantities, a similar effect was observed. This indicates that larger volumes of the virus suspension contain viruses in excess for the infection. Triplicate bottles showed excellent reproducibility.

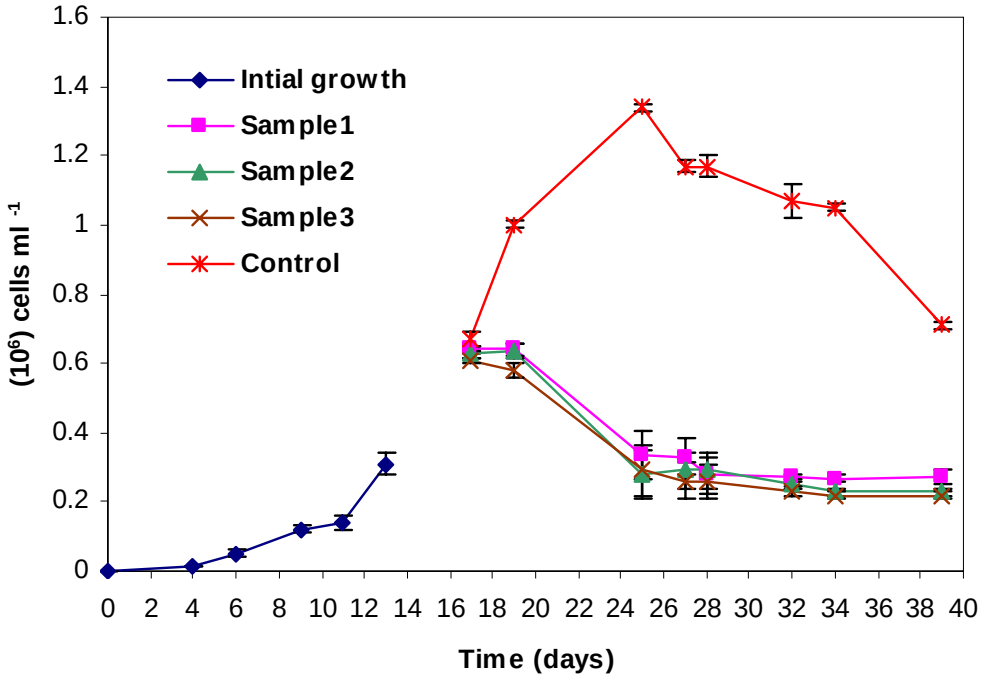

Figure 3.7: The growth curve of *E. huxleyi* (experiment conducted on 28.03.03). Different amounts of virus (50 , 20, and 10 µL) were added at day 17 to three bottles. A bottle with no virus added was considered as a control. Error bars indicate standard error of cell counts.

Culture experiments were conducted where by *E. huxleyi* was grown in FSW media (10.02.03) to monitor the growth and decay process. The one litre bottle was subdivided into three sub-samples and a control. No viruses were added in this experiment. Another growth experiment (08.04.03) was undertaken to confirm and document the effect of the new virus produced as a result of the 13.03.03 experiment. The new virus (10 µL) was added to 20 ml of a well-grown *E. huxleyi* culture. Cell numbers decreased from (1.93 to 0.12 x 10^6 cells ml^{-1}) within 6 days.

All culture experiments were conducted at the Marine Biological Association, Plymouth, at constant temperature (14^{o}C) in a clean culture room with a constant light flux (150 µmol photons m^{-2} s^{-1}) and a photo-period of 12 h light and 12 h dark. Zondervan *et al.* (2002) reported that photosynthesis by *E. huxleyi* was saturated at 140-150 µmol photons m^{-2} s^{-1}.

Cultures were gently shaken once a day. All seawater filtration sets, bottles and glass-ware used in the biological experiments were soaked in hydrochloric acid (HCl) (10% v/v, 1 week), rinsed with Milli-Q water and then dried in the clean laminar flow hood, after sterilising using an auto clave (Touch Autoclave-LAB, K150) .

3.3.3 Stock of Virus EhV86

Infection kinetics of *EhV86* were determined following a method described by Schroeder *et al*. (2002). A culture of exponentially growing *E. huxleyi* in F/2 medium (1 L) (approx. 1.61 x 10^6 cells ml^{-1}) was inocculated with 0.5 ml of virus lysate (approx. 1 x 10^5 pfu ml^{-1}). Six days later, once clearing of the host culture was observed, the lysate was passed through a 0.2 μm syringe filter (Gelman) to remove large cellular debris (Schroeder *et al*., 2002). Lysis was evident when the culture turned from a normal healthy milky-green colour to a milky white colour. In addition, a characteristic sulphurous odour was also indicative of culture lysis. The indications were similar to those described by Wilson *et al*. (2002 a&b). A virus clone was obtained by serial dilution to extinction three times. The highest dilution to lyse the host culture was passed through a 0.2 μm syringe filter (Gelman) for use in subsequent inoculations. Virus filtrates were concentrated by tangential flow ultrafiltration with a 50 K MW size cut-off unit (Vivaflow 50, Sartorius) to a final volume of 20 ml. Virus stock was subsequently stored at 4 ^oC in the dark. The effectiveness of this virus was tested on an *E .huxleyi* culture (08.04.03).

Virus provided by Schroeder was obtained from a stock previously prepared by his group. Viruses were isolated by these researchers using a dilution, extinction and plaque assay from seawater samples. These samples were collected at different stations and depths during the later stages of *E. huxleyi* blooms in the Western English Channel, during July 1999 and 2001. One ml of 0.45 μm filtered seawater collected during the late stages of a bloom was added to an exponentially growing host culture of *E.huxleyi* strain 1516. Filtration similar to the one outlined above was performed. In addition, plaque purification assays were performed. The plaque assay is a classical virological technique, originally developed for bacteriophages and it is still widely used for virus isolation and purification (Schroeder, pers. Comm.). Full details of virus isolation and plaque assays are described in Wilson *et al*. (2002) (a&b) and Schroeder *et al*. (2002&2003).

3.3.4 Preliminary Emiliania huxleyi cultures (20 L carboys)

The first attempt to grow *E. huxleyi* in a 20 L FSW carboy (Nalgene) was performed on 17.09.03. The culture was grown in 20 L because a large volume of sample was needed for trace metal analyses and all related measurements for the life cycle studies. Firstly the FSW was prepared by filtering L4 seawater using Sartorius cartridges (high capacity filter 0.2 µm). Nutrients were added (similar to the final concentrations stated earlier). Cells were added to the 20 L carboy; 0.0005 x 10^6 cells ml^{-1} was the initial cell density. The photo-period was 16 h light and 8 h dark. Samples were collected for trace metal analyses. This was also the first attempt to conduct biological and chemical measurements on a single culture. The experimental growth curves indicated longer lag and exponential phases of growth compared to previous experiments in a similar medium with 1 L bottles (Figure 3.8). Cells did not reach the same cell concentration as observed in previous experiments. The highest cell numbers observed were 0.22 x 10^6 cells ml^{-1} compared to 1.34 x 10^6 cells ml^{-1} in the 28.03.03 control bottle. This is perhaps due to the low inoculation of 0.0005 x 10^6 cells ml^{-1}, which did not seem sufficient to properly start the 20 L growth experiments. A bloom stage was not observed. Viruses were not added to this experiment due to low cell numbers.

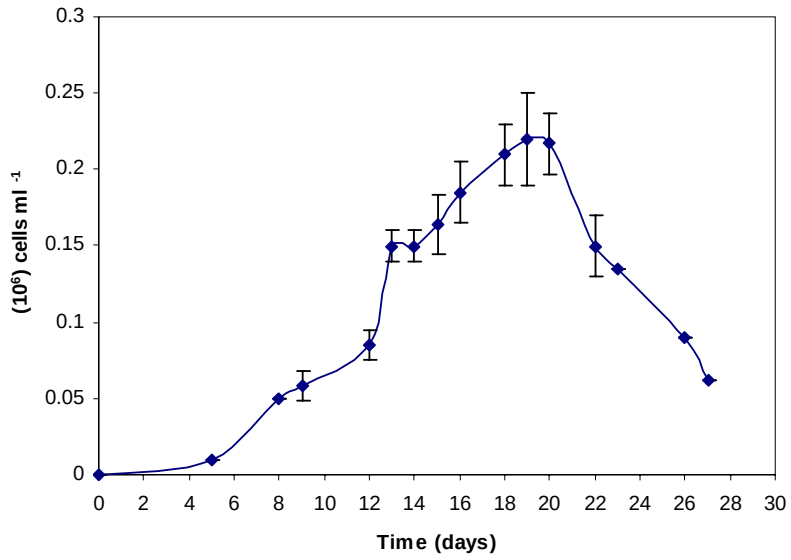

Figure 3.8: *E. huxleyi* growth curve for the experiment performed on 17.09.03. Error bars indicate standard error of cell counts.

This single 20 L carboy experiment was repeated on 23.02.04. The main objective of this was to grow *E. huxleyi* with a higher starting cell density (0.035 x10^6 cells ml^{-1}) compared to the starting cell numbers for the experiment described earlier. Figure 3.9 shows the growth curve for this experiment, and viruses being added on day 7. The exponential phase was shorter this time, because the cell starting density was higher and the viral induced lysis affected cell numbers. The experiment was shorter in time (16 days). The findings formed the basis for all culturing experiments in terms of starting cell numbers, and expected time needed for culturing *E. huxleyi* in a 20 L carboy.

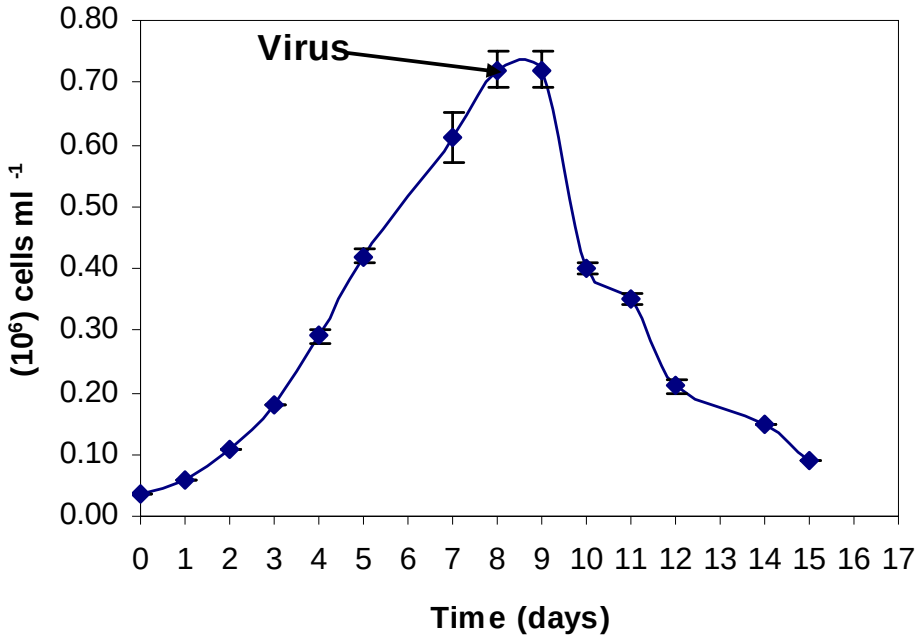

Figure 3.9: Growth curve of *Emiliana huxleyi* grown in 20 L using FSW media (23.02.04). Error bars indicate standard error of cell counts.

3.3.5 Experimental Design

Two controls and three virus infected 20 L experiments were undertaken in, May and August and four virus infected for March (2004) using *E. huxleyi*. The experimental design is shown in Figure 3.10. *E.huxleyi* was grown for the experiments in l L bottles and sub-cultured to five 1 L bottles (50 ml of the healthy cultured added to each bottle). The sub-culturing was conducted at the exponential phase of growth. The cells were subsequently added to the 20 L carboys, following washing (see below).

The seawater used to prepare the FSW media was collected from L4 and filtered twice using Sartorius cartridges (0.2 μm). After filtration, the seawater was enriched with

nitrate and phosphate to final concentrations of 176 μM and 7.26 μM (Vasconcelos *et al*., 2002; Vasconcelos and Leal, 2001).

3.3.5.1 Cell Concentration by Centrifugation:

E.huxleyi cells were centrifuged before the start of the experiments, using a Megafuge Heraeus centrifuge at 6000 rpm for 5 minutes. Cells were washed three times after spinning, using the FSW media. Note that sterile tubes were used for centrifuging.

This process was conducted to remove remnants of the trace metal, EDTA, and the nutrient-rich F/2 medium. After that, cells were added to five 20 L carboys and the experiment was started (Day 0).

3.3.5.2 Virus dialyses

Dialyzed viruses (*EhV-86*) (50 ml each) were added to 3 carboys, with 2 carboys treated as control. This took place at the exponential phase on day 4 for the March and August 2004 experiments. Viruses were dialyzed prior to the infection using dialyzing tubes (Medicell Visking). These are high purity membranes with extremely thin walls (cellulose membranes). This step was taken because virus stock had been built from the lyses of *E.huxleyi* grown in F/2 medium. All particles < 1200 Daltons would pass through the dialyzing tubes. Viruses >1200 Daltons should remain in the tubes.

To remove the remnants of the F/2 media, tubes were filled with viruses, sealed at the end using membrane closures and placed in an acid washed beaker filled with filtered seawater media (FSW) (media used for the incubation experiment), then left for 1 hour to equilibrate. The media was changed 3 times every hour. During the last step, the tubes were left in the beaker overnight to equilibrate. The viruses in the tubes were transferred into sterilised containers ready for infection.

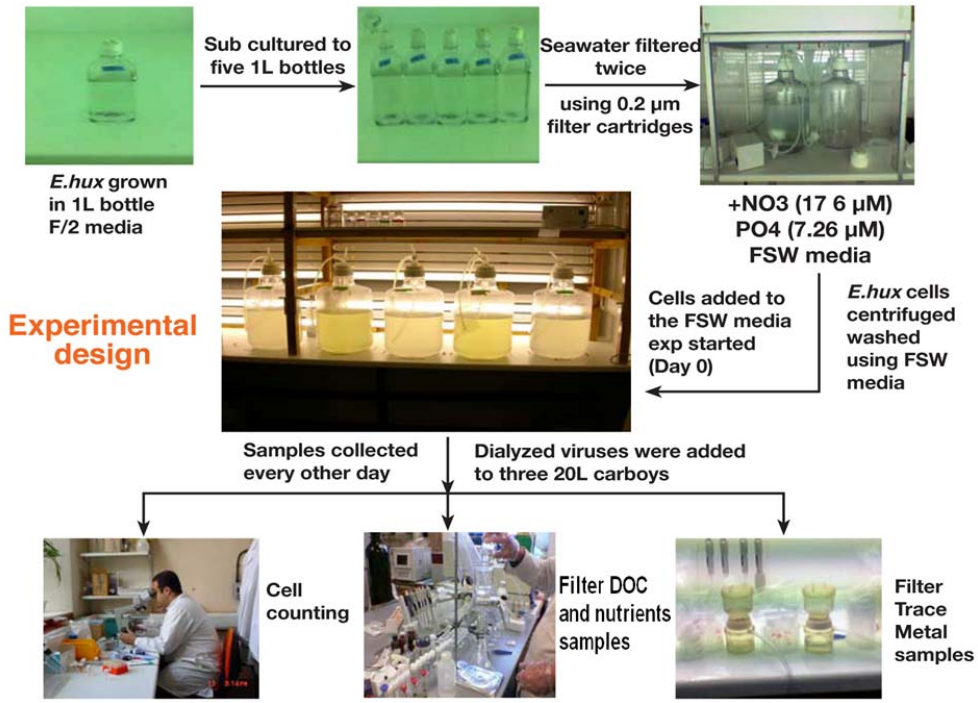

Figure 3.10: Schematic diagram of the culture experiments obtained at the MBA.

3.3.5.3 Samples collected from culture experiments for flow cytometry measurements

Daily samples were collected for biological measurements. *Emiliania huxleyi* in the cultures were enumerated using a Neubauer improved haemocytometer (Schroeder *et al.* 2002).

Samples for viral analysis using Analytical Flow Cytometery (AFC) were collected on a daily basis. Firstly, a one ml sample was collected from each 20 L carboy and samples were fixed in 0.5% (final concentration) glutaraldehyde. This sample can be used for *E. huxleyi*, virus and bacteria counts using AFC. Secondly, a one ml sample was collected from the carboys and centrifuged using a Sorval Pico centrifuge at 13000 rpm for 1 minute, and the supernatant was preserved using 0.5% (final concentration) glutaraldehyde. This was to be used for quantifying the concentration of the virus (EhV-86). This method requires a different staining regime, and the count of the virus is more accurate when *E. huxleyi* is removed. Samples were stored at -80 ^{o}C until analysis. Flow cytometry analysis was conducted for the second sample only and the first sample was stored as backup.

3.3.6 Viral and Bacteria Enumeration

Flow cytometry is commonly used in marine science as an analytical technique to analyse and sort cells on the basis of their optical characteristics (viruses and bacteria) (Marie *et al*., 1999; Brussaard *et al*., 2000). However, Epifluorescence Microscopy (EM) is a commonly used method for counting viruses (Suttle, 2005). This is because of higher accuracy and precision (Noble and Fuhrman, 1998).

Flow cytometry involving the analysis of cells in a flow-through mode. Flow cytometry is a technique whereby a stream of cells is passed through a laser beam at high speed (10,000 cells/second) (Sobti and Krishan, 2003). As each cell passes through the laser beam, the light scatter and fluorescence of each particle are recorded simultaneously and the information is processed by computer. Cram (2002) emphasised that flow cytometry has been rapidly evolving into a technique for rapid analysis of DNA content, cellular markers, expression and electronic sorting of cells of interest.

Analyses were conducted using a Becton Dickinson FACSort flow cytometer at Plymouth Marine Laboratory. This flow cytometer is equipped with an air-cooled 15 mW laser providing 488 nm with a standard filter set-up. Virus and bacteria enumeration was performed on each sample at the same time using methods adapted from Marie *et al*. (1999) and Wilson *et al*. (2002 a). Fixed frozen samples were defrosted at room temperature. Fluorescent nucleic acid stain SYBR Green I was used, as recommended by Brussaard *et al*. (2000). This SYBR Green stain was purchased from Molecular Probes at 10,000 times concentrate in Dimethyl sulfoxide (DMSO). On a monthly basis, the SYBR stain was diluted (1:10) with sterile distilled water and stored at -20 °C (main stock).

Working stock was produced as required by the dilution 1:100 of the main stock in sterile distilled water. The defrosted samples were diluted with TE buffer (10 mM Tris-HCL pH 7.5, 1 mM EDTA) which had been pre filtered through a 50-kDa VivaFlow 200 system (Sartorius) and then autoclaved. For samples from cultures, dilutions of 1 in 100 to 1 in 200 (samples after day 10) were used. Dilutions were applied to samples collected from the English Channel according to the expected virus concentrations. Diluted samples were mixed with the SYBR Green 1 at a final concentration of 10^{-4} of the commercial dilutions and heated at 65 ^{o}C for 10 minutes in the dark. According to Brussaard (2003)

many phytoplankton viruses exibit a relatively high green fluorescent signal after staining.

Heating was essential to allow the SYBR stain to adsorb on to the cells. Samples were analysed by AFC for 2 minutes at high flow rate (42-51.5 µl min^{-1}). The system was calibrated prior to every analysis using a concentration of 1.0157 x 10^6 ml^{-1} Beckman Coutler Flowset Beads. The beads were diluted 10 times in distilled water and analysed by AFC 6 times. Flow rate was calculated from the beads analysis. Data acquisition was triggered on green fluorescence and side scatter and collected on CellQuestPro™ software (Becton Dickinson) with log amplification on a four-decade scale. The amount of noise was reduced by analyses of blanks and subtracted from the samples. To prepare the blank, 10 kDa filtered seawater was diluted 10-fold with TE buffer, which contained 0.1 % Tritonx-100 and SYBR Green I. Data analysis was carried out using WinMDI 2.8 software. Scatter-plots of side-scatter vs. green fluorescence were used to detect viruses and bacteria groups.

This chapter has shown and summarised all methods (sampling and trace metal cleaning procedures) used in the current study. The voltammetric technique (Ad-CSV) and GF-AAS were described in detail. They have proved to be cost effective and sensitive analytical methods for the speciation of trace metals. The certified reference seawater samples analysed were close to certified values, which provided confidence in the methods and instruments used. The low detection limits and high precision levels observed in this study indicate the accuracy of the handling and the analysis of trace metal procedures.

The initial biological experiments and all biological measurements used in later experiments have been detailed in this chapter. Growth of *E. huxleyi* in FSW media was comparable and reproducible with F/2 growth rate. Virus additions strongly affected cell numbers in the medium. Accordingly, an appropriate experimental design was set and successfully tested. The methods for counting *E. huxleyi* cells and viruses, using a Zeiss compound microscope and AFC, respectively, have been explained in the chapter.

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Chapter 4

4.Life Cycle and Virus Infection of *Emiliania huxleyi*; Influence on Trace Metal Speciation

4.1 Abstract

Phytoplanktons influence the cycling and distribution of trace metals in oceanic and coastal waters. Viruses are known to infect a range of phytoplankton species and thus may influence trace metal speciation in these waters. This work aims to look at trace metal speciation during the life cycle of the coccolithophore *Emiliania huxleyi* in uninfected (control) and infected cultures. *E. huxleyi* was grown in seawater enriched only with nitrogen and phosphorus, and without the control of free metal concentration using EDTA. Cathodic Stripping Voltammetry was used to measure labile and total metals, and Cu complexing ligands. Total particulate Cu in algal cells was determined using GFAAS.

A typical AFC signature of *E. huxleyi* specific virus was observed 1 day post virus addition in the virus addition element. The increase in virus numbers directly related with the decline in the *E. huxleyi* cell numbers, indicating that they crashed as a result of the virus infection.

Total dissolved Cu concentrations were near constant in the control and virus infected cultures. However, labile Cu concentrations decreased with time in both control and virus infected cultures. Particulate Cu in the control cultures increased with time and therefore could be correlated with increasing algal biomass. Similarly, in the virus infected cultures the particulate Cu increased with time, until the cell number decreased due to viral infection. In the control culture the amount of Cu per cell decreased exponentially during algal growth, although the overall cellular Cu content increased. Viral lyses of *E. huxleyi* caused a sharp increase in Cu per cell, reaching a maximum of 4.78 x 10^{-17} mol/cell.
Ligand production in the control culture resulted in a decrease in the free aqueous Cu concentration [Cu^{2+}] with time. The lowest [Cu^{2+}] in the control culture was between 6.73-5.15 x 10^{-13} M, coinciding with the highest organic ligand concentrations. Also in the virus infected culture, [Cu^{2+}] decreased with enhanced organic ligand concentrations, while no increase in the dissolved Cu fraction in response to the decrease in the particulate concentration of Cu were observed. It is hypothesised that the virus particles played an important role in the Cu cycling.
Labile Ni accounted for 70-80% of the total Ni concentration in the virus infected culture, and 76-91 % in the control culture. In the control cultures the ratio of labile to total Co decreased with time which indicates the production of strong Co complexing ligands by the healthy *Emiliania huxleyi* cells. The ratio decreased in the virus infected culture until day 8 and then increased following the virus infection.

4.2 Introduction

Trace metals such as Co, Cu, Fe, Mn and Zn are essential elements and perform key biochemical functions in prokaryotic and eukaryotic cells (Whitfield, 2001). Whilst at low concentrations, these elements may be growth limiting, at enhanced levels they are potentially toxic (Muller *et al*., 2005). The uptake of elements by algal or bacterial cells depends on their chemical form (Hudson and Morel, 1993). Trace metals are usually taken up by algae via the formation of coordination complexes with specific transport ligands in their outer membranes (Sunda, 1989; Moffett and Brand, 1996). The biological availability of trace metals is determined by the concentration of free metal ions or of kinetically labile inorganic species (Sunda, 1989; Bruland *et al*., 1991). Trace metals (including Fe, Mn, Zn, Cu, Co) play a key role in the metabolism of cells, making for example essential contributions in metalloenzymes (Sunda, 1989, Sunda and Huntsman, 1998). However, trace metals may also inhibit metabolism (Bruland *et al*., 1991), when they bind to a metabolic site normally occupied by essential metal ions. In order to understand the impact of trace metals, the study of their chemical and phase speciation forms an important aspect in aquatic biogeochemical cycles research (Tang *et al*., 2001).

Complexation by organic matter dominates the chemical speciation of the Cu, Zn, Co and Fe in seawater (Ellwood and van den Berg, 2001; Gledhill and van den Berg, 1994; Moffett, 1995). Copper is one of the most extensively studied trace metals in natural waters, as it is an essential micro-nutrient (Leão *et al*., 2007), but is toxic at concentrations only slightly higher than the limiting levels. It has been reported that pico- to nanomolar concentrations of Cu^{2+} in seawater caused a reduction in the cellular division rates of cyanobacteria (Brand *et al*., 1986), and Cu^{2+} concentrations higher than tolerance levels may lower photosynthetic rates and cause interference with the uptake of other metals (Sunda and Huntsman, 1998). Strong organic ligands are largely responsible for Cu complexation in open ocean surface water (Coale and Bruland, 1988; Moffett *et al*., 1990), whereas weaker ligands often dominate Cu speciation in coastal waters (Donat *et al*., 1994). A nickel speciation study conducted in Southwest Spain (Braungardt *et al*., 2007) indicated that an average of 80% Ni was labile throughout the observed concentration range and this lies with in the range (40-80%) reported for other estuaries and coastal waters (Nimmo *et al*., 1989; van den Berg *et al*., 1987). There is some

evidence to suggest that cobalt may be strongly complexed by natural organic ligands in estuarine waters (Zhang *et al*., 1990; Ellwood and van den Berg, 2001). The presence of non-labile voltammetric fractions of cobalt indicates that it was at least partialy complexed in the Mediterranean (Vega and van den Berg, 1997).

The presence of viruses in the marine environment has been acknowledged for many years, and it is now well established that viruses are abundant in marine ecosystems (Fuhrman, 1999; Wilhelm and Suttle, 1999; Suttle, 2005). Viruses are known to infect a wide spectrum of hosts, including prokaryotes and eukaryotes (Suttle *et al*., 1990; Brussaard *et al*., 1996). Several studies have documented a correlation between the presence of viruses and a shift in phytoplankton communities following the crash of *E. huxleyi* blooms observed in the western English Channel (Wilson *et al*., 2002 a-b; Schroeder *et al*., 2002). Viral lysis of single species dominated phytoplankton blooms has been shown to significantly affect the transfer and the cycling of energy and matter within the pelagic food web (Gobler *et al*., 1997; Fuhrman, 1999; Wilhelm and Suttle, 1999).

Several culture and mesocosm experiments have been conducted to study the speciation of trace metals during the life cycle of *E. huxleyi.* These studies were conducted under exposure to metals, and/or under different nutrient regimes, and investigated the effects of the exudates released by phytoplankton on metal speciation and the growth of *E. huxleyi* (Vasconcelos *et al.,* 2002; Vasconcelos and Leal, 2001; Leal *et al*., 1999; Muller *et al*., 2003). However, effects of the viral lyses of *E. huxleyi* on trace metal speciation has yet to be determined. The objective of the experiments reported in this chapter is to examine the effects of *E. huxleyi* growth, senescence and decay on dissolved trace metal (Cu, Co and Ni) concentrations and their electrochemical lability. Furthermore, the influence on particulate Cu concentrations and the concentrations and binding strengths of Cu complexing ligands was determined. Consequently, the aim was to document for the first time the effect of viral infection of *E. huxleyi* on trace metal speciation. Here data is presented from two culture experiments using 20 L carboys.

4.3 Methods

4.3.1 Reagents and Standards

The water used for reagent preparation and vessel rinses was Milli-Q water ((Millipore, UK); >18 MΩ cm^{-1}). All reagents were AnalaR grade unless indicated otherwise. The metal standard solutions used in voltammetric determinations were prepared by dilutions of standard atomic absorption spectrometry solutions (Spectrosol grade, BDH) and acidified with sub-boiled HCL. Salicylaldoxime (SA, Sigma, 0.01 M) was prepared monthly in 0.1 M HCL. Solutions of 0.1 M dimethlglyoxime (DMG, Aldrich) were prepared in methanol (HPLC grade, Fisher Scientific) whilst 0.1 M ammonium pyrrolidinedithiocarbamate (APDC, Aldrich) was prepared in Milli-Q water. A stock solution of 0.1 M nioxime (10 μM final concentrations, Aldrich) was prepared in 0.2 M sodium hydroxide.

Sodium nitrite (5 M) solution was used as a catalyst in the cathodic stripping voltammetric (AdCSV) analyses of Co and prepared in Milli-Q water. The final concentration of sodium nitrite in the cell was 0.25 M. The sodium nitrite solution was cleaned electrochemically following the procedure described by Vega and van den Berg (1997). Cobalt was removed from this solution in a mercury pool acting as the cathode and an anode consisting of a platinum wire. The 5 M nitrite solution was placed in the electrolysis device and de-aerated for 30 min by bubbling nitrogen through the stirred solution. The electrodes were connected to the potentiostat and the bubbling rate was then reduced. A potential of -1.35 V was set to the working electrode for a period of 24 h.

Low concentrations of Ad-CSV ligands were prepared by serial dilution of the stock reagent solutions. Borate buffer was prepared by adding sodium hydroxide to the boric acid solution (1 M) (Pure grade, Fisher Scientific) to achieve pH 8.3. The buffer was cleaned by passing through a Chelex-100 (Sigma) column followed by in-line UV digestion to remove organic ligands (Achtenberg and van den Berg, 1994; Ellwood and van den Berg, 2000). 100 μL of this buffer added to 10 ml seawater (0.01 M) gave a pH of 8.3. The Chelex-100 column was cleaned beforehand by passing sub-boiled HCL (0.5 M, 100 ml) for a duration of 2-3 hours and then rinsed by Milli-Q water (200 ml), isothermally distilled ammonia NH_3 (4 M, 50 ml) and finally with Milli-Q water (a further 100 ml). The column was cleaned after each use with Milli-Q water (200 ml) and isothermally distilled ammonia NH_3 (5 M, 50 ml).

4.3.2 Cultures of *Emiliania huxleyi* in enriched natural seawater

Cultures of *E. huxleyi* were grown in natural seawater that was filter sterilised (using 0.2 µm high capacity filtration cartridges, Sartorius) using a pump (Dymax 30, Charles Austen Pump, UK) and enriched with 176 µM N and 7.26 µM P. The media was not autoclaved to avoid trace metal contamination. The starting *E. huxleyi* cell numbers were approximately 3.00 x 10^4 cells ml^{-1} for the August 2004 experiment, and 2.50 x 10^4 cells ml^{-1} for the March 2004 experiment. Dialyzed virus EhV-86 was added to the cultures on day 4 of the experiment. Full details of the experimental design, cell centrifugation and virus dialysis were given in Chapter 3, section 3.3.5. All bottles, carboys, venting caps, filters and tubes used in the biological experiments were cleaned prior to the experiments (see Chapter 3, sections 3.2.1.1 and 3.3). Samples were collected from the incubation experiments at the same time (11 am) for chemical analysis and biological numeration of cells, viruses and bacteria, as described in sections 3.2.3 and 3.3.5 (Chapter 3).

4.3.3 Determination of chlorophyll *a*, cell numbers, viruses and bacteria enumeration

Chlorophyll *a* analysis was carried out using a Turner Designs fluorometer according to the methods of Welschmeyer (1994). Full details were described in Chapter 3, section 3.2.9. *E. huxleyi* growth was monitored using an improved Neubauer haemocytometer as described by Schroeder *et al*. (2002). Virus and bacteria enumeration was carried out using a Becton Dickinson FACSort flow cytometer (Wilson *et al*., 2002 a&b). Full details of sample collection and analysis were given in sections 3.3.5 and 3.3.6.

4.3.4 Determination of nutrient and, DOC in the cultures

Nutrients were determined using a Burkard Scientific nutrient autoanalyser (model SFA-2). A detailed description of the analysis and instrumentation was given in Chapter 3, section 3.2.7. The colorimetric method used for nutrient analysis is described by Hydes (1984) and Hydes and Wright (1999).

The high temperature combustion (HTC) technique, was used for simultaneous analyses of dissolved organic carbon DOC and total dissolved nitrogen TDN. This approach provides precise and accurate results (Badr et al., 2003). The system comprised of a

Shimadzu TOC 5000A coupled to a Sievers nitrogen chemiluminescence detector (NCD) (section 3.2.8).

4.3.5 Determination of total trace metal content in seawater and algae

The total dissolved concentrations of Co, Cu Ni and Zn were determined in the seawater and the culture media after removal of biomass by filtration (using acid washed 0.4 μm polycarbonate membrane filters, Whatman). The filtration was conducted using a polysulfonic filtration unit with a hand-operated pump (Nalgene), not exceeding 0.3 bar. Microscopic examination of the algae on the filters revealed that they did not rupture or break during filtration. After filtration, aliquots of the media were acidified to pH ≈ 2 using sub-boiled HCl and UV-digested (400 W medium pressure mercury vapour lamp, Photochemical Reactors Ltd) in acid washed quartz tubes for 5 h. Metal concentrations were determined by CSV. The pH of the samples was neutralized with distilled ammonia and subsequently a 10 ml aliquot was transferred into the voltammetric cell. The voltammetric equipment used for the measurements has been described in Chapter 3, section 3.2.5. The determination of Cu, Ni and Co was carried out at pH 8.3 (0.01 M borate buffer), using the competitive ligand SA for Cu (Campos and van den Berg, 1994), DMG for Ni (Colombo and van den Berg, 1985) and Nioxime for Co (Vega and van den Berg, 1997). In order to remove all oxygen, the sample solutions were purged with oxygen free nitrogen (BOC) for 240 seconds for Co, and 300, 100 seconds for Cu and Ni respectively. All experimental conditions were provided in Table 3.2. The concentrations of total metals were quantified using the internal standard addition approach. Measurements were conducted twice and repeated when the differences between measurements were higher than 4%. The analysis of certified reference material (Table 3.3) was in good agreement with certified values. The labile dissolved metal concentration was determined as described for total dissolved metal but without previous acidification or UV digestion.

The total Cu concentrations in the algae were determined after digestion of the filters on which the biomass was collected, using 2 ml sub-boiled nitric acid. Full digestion procedures were described in Chapter 3. Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS) was used to determine total particulate Cu. For this purpose, a Perkin-Elmer 1100B AAS equipped with an HGA-700 graphite furnace and an AS-70

auto sampler were used. The total cellular Cu content (extracellular adsorption plus intracellular uptake) was calculated and metal fixed per microalgal cell was calculated from the cell count. Reference standard seaweed was used to assess the accuracy of the digestion and analysis procedures; the Cu content was found to be not significantly different from certified values.

4.3.6 The speciation of Cu in the cultures and theoretical considerations for metal speciation measurements

Copper titrations in filtered culture media were conducted to determine the concentrations of the natural Cu-complexing organic ligands (L), their conditional stability conditional constants, and free Cu^{2+} and inorganic Cu (Cu') concentrations. The competitive ligand titrations were carried out using SA according to Campos and van den Berg (1994). Eleven sample aliquots of 10 ml each were pipetted into 30 ml polystyrene cups together with SA and the borate buffer. Prior to the first titration the cups were conditioned twice with seawater containing the same range of Cu concentrations as used in the titrations. Ad-CSV was used to determine the labile Cu concentrations in the titrations, and the analysis was performed using the same conditions as described for total dissolved Cu. For Ad-CSV determinations, the pH was fixed at 8.3 (0.01 M) using borate buffer, and the SA ligand concentration was 3 μM. A previous study by Leal and van den Berg (1998) reported added Cu concentration in the range 0-50 nM for Ad-CSV Cu-complexing ligand titrations. The concentrations of detected ligands and the conditional stability constants of their Cu complexes did not vary significantly when SA concentrations were varied between 2-10 μM.

Cups were used containing sample, buffer, SA and added Cu in the range of 0-50 nM for the August 2004 experiment, and 0-200 nM for the March 2004 experiment. Two different ranges of copper concentrations were applied to check whether this could affect ligand trends (i.e. production) in the two different culture experiments. The solutions were equilibrated overnight (12-15 h) prior to analysis. The aliquots were measured sequentially, and the voltammetric cell was not rinsed between aliquots to maintain cell conditioning for the Cu and SA concentrations. The cups used were rinsed with Milli-Q water between sample titrations and the same order of cups was maintained. Triplicate measurements were performed to assess the precision of the analysis. This resulted in

relative standard deviations (RSD) of 0.53% and 0.20% for the observed ligand concentrations and the conditional stability constants, respectively.

The Ad-CSV-labile fraction of the dissolved metal is operationally determined by the analytical parameters employed during the analysis (van den Berg *et al*., 1990). The analytical competition strength of the Ad-CSV method is defined as the α-coefficient (α_{MeAL}) of the target metal with the added Ad-CSV ligand (AL), e.g. for Cu with SA:

$$\alpha_{CuSA} = 1 + K'_{CuSA+}[SA'] + \beta'_{CuSA2}[SA']^2. \quad (1)$$

Metal-organic complexes (MeL) with α-coefficients (α_{MeL}) approximately within one decade either side of the α_{MeAL} can be detected (Nimmo *et al*., 1989; Apte *et al*., 1988). The non-labile metal fraction is assumed to be complexed by natural metal binding organic ligands and is kinetically inert during the competitive ligand exchange reactions, and was calculated by subtracting the labile from the total dissolved metal concentration.

The conditional stability constants K'_{CuSA+} and β'_{CuSA2} at the appropriate salinities and pH were derived from values given in Campos and van den Berg (1994). Copper complexing ligands with low stability constants (log $K'_{CuL} \approx$ 10-12) are more abundant in estuarine and coastal waters than those with higher log K values >13 (Muller, 1998; Mofett *et al*., 1997). For this study, a relatively low α_{CuSA} value was chosen (log α_{CuSA} = 3.99) for Cu determinations using 3 μM SA. Values for the conditional stability constants of the complex formed between Ni and DMG were taken from literature (van den Berg and Nimmo, 1987) to calculate the α-coefficient for a DMG concentration of 20 μM, based on a 1:2 Ni:DMG complex log α_{NiDMG} = 8.2 to 7.6 at S = 14 to 37 and pH 8.3. For Co it was assumed that it will forms 1:2 complex with nioxime to be dominant, and experimental values (Ellwood and van den Berg, 2001) of the conditional stability constant for this complex at different pH values were used to estimate log $K'_{Co\ (nioxime)}$ = 15-16 at pH 8.3. From this, the α-coefficient for the $Co_{(nioxime)}$ complex as log $\alpha_{Co\ (nioxime)}$ = 4.74 at S = 32-35 [nioxime= 2 μM] (Ellwood and van den Berg, 2001).

The van den Berg/Ruzic data transformation was employed to compute titration data, yielding [L] and K'_{CuL}. The free Cu ion [Cu^{2+}] was calculated from this data using the thermodynamic equilibrium calculation program prepared by van den Berg. The

concentration of L not complexed by Cu was calculated using the Cu speciation model suggested by van den Berg and Donat (1992), and the α-coefficient of the complex CuL was calculated from:

$$\alpha_{CuL} = \frac{K'_{CuL}[L]}{1 + K'_{CuL}[Cu^{2+}]} \qquad (2)$$

4.4 Results and Discussion

4.4.1 Growth of Emiliania huxleyi in enriched natural seawater.

Preliminary growth experiments described in Chapter 3 section 3.3.2 were carried out to evaluate whether *E. huxleyi* would grow satisfactorily in seawater supplemented with only N and P. These experiments therefore tested the effects on growth of omitting the additions of trace metals, vitamins and EDTA to the culture media. Results presented in this chapter represent the average of two controls and three virus infected 20 L culture experiments. Figures 4.1 and 4.2 represent cell number and chlorophyll *a* concentrations observed for the August 2004 experiment. Omission of trace metals and vitamins and the absence of EDTA from the growth media had no effects on the growth rate or timing of viral lyses in these growth experiments. The results were similar to those obtained previously for the same algal species and culture medium (Leal *et al*., 1999; Vasconcelos and Leal, 2001). Growth dynamics of *E. huxleyi* in the filtered enriched seawater medium with or without virus addition were also compared in terms of the concentration of chlorophyll *a* and cell numbers. The two parameters gave a very close correlation (correlation coefficient (r) = 0.98 for infected culture and 0.94 for the control). Repeated culture experiments showed that the exponential growth phase started typically 3-4 days post incubation and was maintained for 2-3 days, after which the stationary phase set in. The maximum cell density was observed on day 6 (0.69×10^6 cells ml^{-1}), corresponding with chlorophyll *a* concentrations (25.6 μg L^{-1}). The highest cell numbers (1×10^6 cells ml^{-1}) were observed in the uninfected cultures (Figure 4.1 a). Chlorophyll *a* concentrations remained relatively stable during the early stages of stationary phase growth in the control cultures but sharply decreased on day 16 (Figure 4.2 a). Leal *et al* (1999) reported that the exponential phase started after 6-7 days of incubation. This difference could possibly be explained by the lower cell numbers used during the start of their incubations, as compared in this experiment, and/or the use of a different strain of *E. huxleyi*.

Viruses were added during the exponential growth phase (day 4). The viral induced lyses resulted in a decrease in cell numbers on day 8 compared with the uninfected cultures (Figure 4.1 b). Similarly, the infected cultures of Schroeder *et al.* (2002) and Wilson *et al.* (2002b) collapsed three days post-inoculation of viruses, while the non-infected cultures showed a typical growth curve.

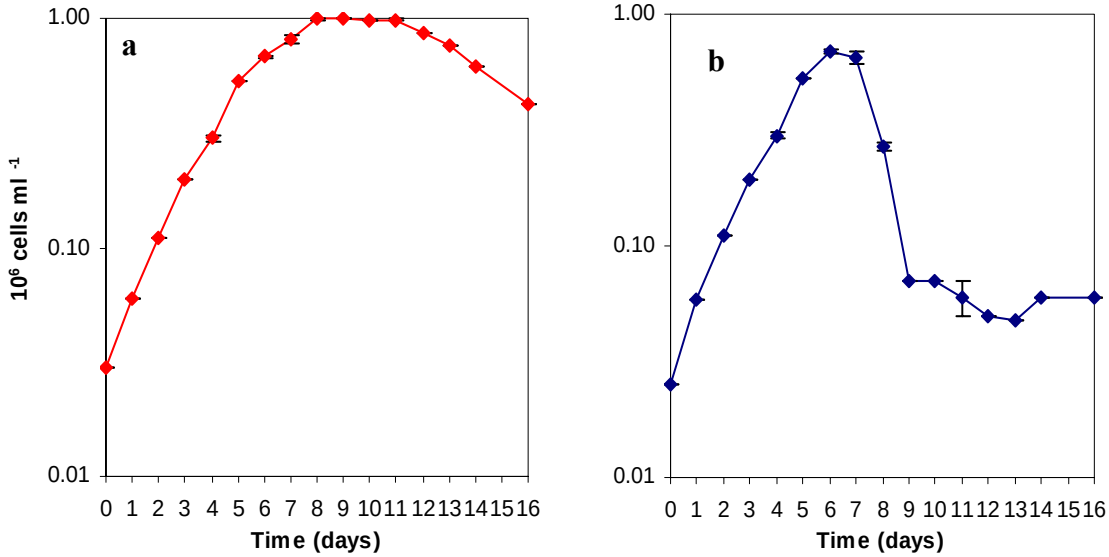

Figure 4.1: *Emiliania huxleyi* cell numbers in enriched natural seawater. (a) control culture (b) viral infected culture (viruses added on day 4).

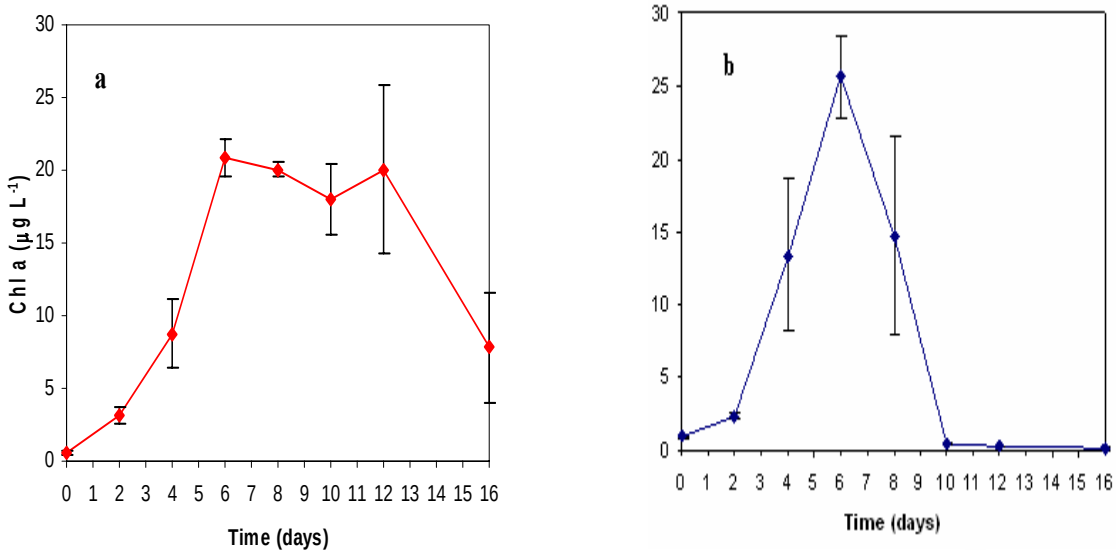

Figure 4.2: Chlorophyll *a* concentrations (a) control culture, (b) viral infected culture (viruses added day 4).

The pH of the *E. huxleyi* cultures did not vary significantly (pH 7.80) over the course of the experiments (data not shown).

Leal *et al*. (1999) showed a similar pH pattern, which remained fairly constant. This may be due to the production of organic matter and calcite coccoliths. Leal *et al*. (1999) also assessed the growth dynamics of *E. huxleyi* in cultures with and without 0.01 M HEPPS buffer. Their observations were that the growth rate was ten fold higher in the absence of buffer. This is in agreement with previous work, where Lage *et al*. (1996) used HEPPS pH buffer (pH 8) which was thought to negatively affect the algae by increasing pH toxicity. This was evaluated based on growth changes i.e. effective concentration of labile copper that reduces the growth rate by 50% of the dinoflagellate, *Amphidinium carterae*. HEPES increased toxicity (synergistic effect) as it did not significantly complex copper and this effect was possibly due to its surfactant properties (Lage *et al*., 1996). This confirms the importance of using media without added pH buffers, as was done in this research.

4.4.2 Viruses and bacteria

Virus and bacteria numbers present in the biomass-free fraction (biomass removed through centrifugation) were monitored by analyitical flow cytometry (AFC), based on their side scatter versus green fluorescence (Figure 4.3). A typical AFC signature of the *E. huxleyi*- specific virus EhV-86 was observed 1 day post virus addition in the virus addition experiment. The increase in virus numbers directly related with the decline in the *E. huxleyi* cell numbers, indicating that they crashed as a result of the virus infection (Figure 4.4). Total virus concentrations ranged from 0.029 x 10^7 particles ml^{-1} (day 5) to 9.59 x 10^7 particels ml^{-1} (day 13). The maximum EhV-86 numbers were observed on days 12 and 13, which coincided with the lowest algal cell numbers (Figures 4.1a & 4.4). Results obtained by Evans (2004) for mesocosm experiments showed a lower abundance of viruses (3.75 x 10^7 ml^{-1}) than in the present study. This is because the mesocoms experiment worked with natural occurring viruses and a lower *E. huxleyi* cell density (7 x 10^4 cells ml^{-1}). Virus concentrations observed in this study were within the range expected for a marine ecosystem (known to range from $<10^4$ to $>10^8$ ml^{-1}) (Wilson *et al*., 2002 a). Viruses and bacteria concentrations determined by AFC, throughout the viral infection of *E. huxleyi,* are presented in Figure 4.4.

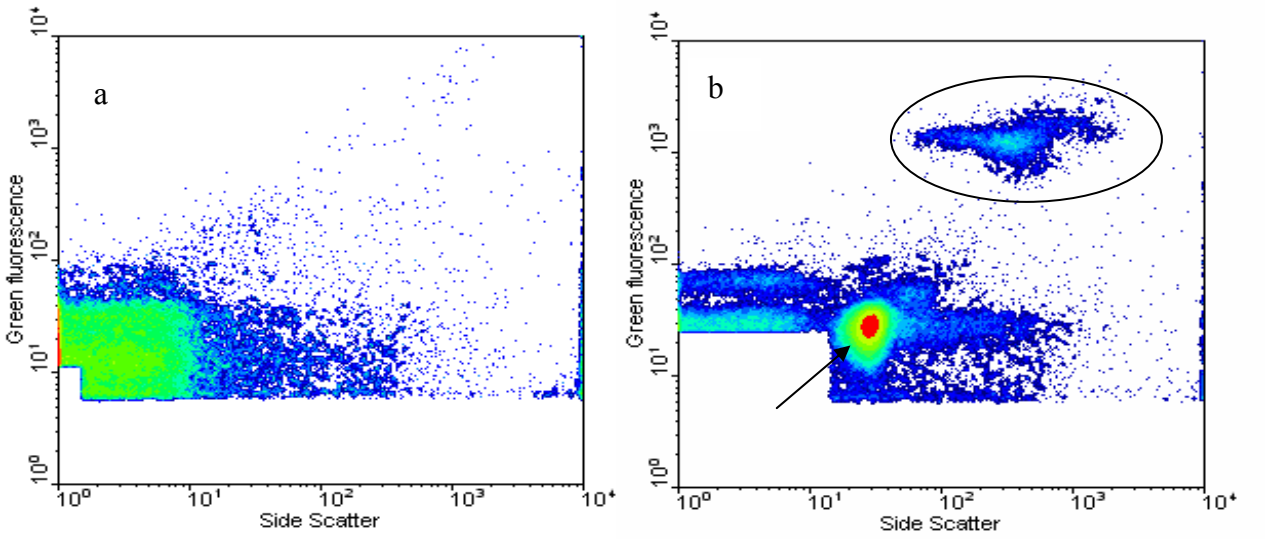

Figure 4.3: Analytical flow cytometry scatter plot of a) sample taken on day 2 before virus addition, b) sample taken on day 14 from the virus addition experiment. The arrow indicates the position of the *Emiliania huxleyi*-specific virus EhV-86, while the circle indicates the position of heterotrophic bacteria.

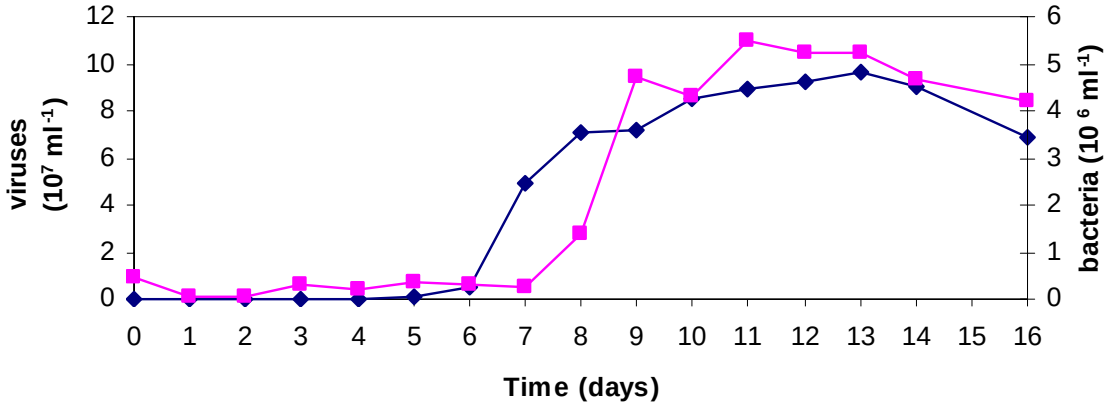

Figure 4.4: Virus and bacteria con for the August 2004 experiment (viral infected).

In the virus addition culture, bacterial numbers remained at around 0.04-0.27 x 10^6 cells ml^{-1} between days 0 and 7 (Figure 4.4). A rapid increase in bacterial numbers coincided with the start of the decline of *E. huxleyi* cell numbers and the sharp increase in free virus particles. The highest bacterial concentrations were observed between days 11 and 13 (Figure 4.4). Bacteria concentrations were lower than viruses by an order of magnitude. These observations are consistent with studies reported by Evans (2004) and Marie *et al.* (1999), who found that viral lyses stimulated bacterial production. Bacterial

concentrations in the control cultures were much lower than in the viral cultures (Figure 4.5).

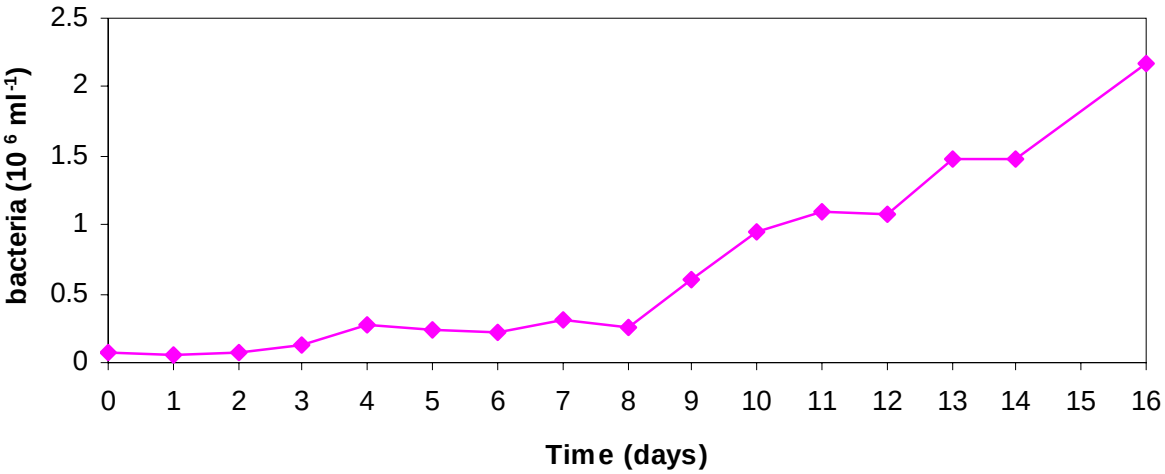

Figure 4.5: Bacteria numbers determined by AFC for the August 2004 experiment (control).

E. huxleyi cell number, viruses and bacteria concentrations, Chlorophyll *a*, nutrients and DOC concentrations for the August 2004 experiment are presented in Tables 4.1 (control cultures) and 4.2 (viral infected).

Table 4-1: Biological data (*Emiliania huxleyi*, viruses and bacteria ml^{-1}), chlorophyll *a*, nutrients and DOC in the control culture (Aug-2004).

Time (days)	*Emiliania huxleyi* 10^6 cells ml^{-1}	Bacteria 10^6 ml^{-1}	Chl *a* µg L^{-1}	NO_3 µM	PO_4 µM	DOC µM
0	0.03	0.06	0.57 ± 0.18	164.94 ± 0.36	6.66 ± 0.00	182.3 ± 18.44
1	0.06	0.05				
2	0.11	0.07	3.14 ± 0.53	162.92 ± 1.07	5.62 ± 0.17	215.3 ± 27.08
3	0.20	0.12				
4	0.30	0.27	8.76 ± 2.32	155.09 ± 0.00	4.90 ± 0.17	198.7 ± 48.29
5	0.53	0.24				
6	0.68	0.22	20.86 ± 1.33	146.00 ± 2.14	2.33 ± 0.20	141.98 ± 30.67
7	0.81	0.31				
8	0.99	0.25	20.04 ± 0.51	142.39 ± 1.61	2.45 ± 0.10	110.57 ± 10.66
9	0.99	0.60				
10	0.98	0.95	18.00 ± 2.38	141.08 ± 0.23	1.47 ± 0.17	110 .69 ± 4.37
11	0.98	1.09				
12	0.86	1.08	20.06 ± 5.74	139.13 ± 2.53	1.87 ± 0.17	136.91 ± 14.30
13	0.76	1.48				
14	0.62	1.48				
16	0.42	2.17	7.82 ± 3.76	144.5 ± 8.29	3.01 ± 0.68	122.94 ± 1.57

Table 4-2: Biological data (*Emiliania huxleyi,* viruses and bacteria ml^{-1}), chlorophyll *a*, nutrients and DOC in the viral infected culture (Aug-2004).

Time (days)	*Emiliania huxleyi* 10^6 cells ml^{-1}	Virus 10^7 ml^{-1}	Bacteria 10^6 ml^{-1}	Chl *a* µg L^{-1}	NO_3 µM	PO_4 µM	DOC µM
0	0.03	0	0.45	0.91 ± 0.06	167.04 ± 2.39	6.75 ± 0.25	165.8 ± 23.21
1	0.06	0	0.04				
2	0.11	0	0.06	2.34 ± 0.22	161.66 ± 2.20	6.34 ± 0.11	187.3 ± 5.94
3	0.19	0	0.30				
4	0.30	0.02	0.21	13.36 ± 5.26	155.10 ± 3.15	5.15 ± 0.27	184.2 ± 10.27
5	0.53	0.11	0.38				
6	0.69	0.53	0.29	25.62 ± 2.80	144.32 ± 1.91	2.57 ± 0.50	117.4 ± 23.45
7	0.65	4.96	0.27				
8	0.27	7.04	1.36	14.68 ± 6.84	151.24 ± 9.67	3.36 ± 0.37	139 ± 11.50
9	0.07	7.22	4.71				
10	0.07	8.49	4.29	0.45 ± 0.06	139.10 ± 6.20	3.91 ± 0.68	153 ± 23.23
11	0.06	8.89	5.51				
12	0.05	9.20	5.25	0.32 ± 0.01	144.50 ± 5.44	4.79 ± 0.76	155 ± 18.92
13	0.05	9.59	5.24				
14	0.06	8.98	4.68				
16	0.06	6.86	4.19	0.16 ± 0.14	138.31 ± 1.72	4.37 ± 0.71	149 ± 8.80

4.4.3 Variations in nutrient concentrations during the incubations

The nitrate and phosphate concentrations were determined in the culture media on day 0 (when the experiment started), and during the exponential and, stationary growth phases, and the decay stage for the viral infected cultures. Nutrient samples were also analysed for the control cultures. Table 4.1 and 4.2 show the nutrient concentrations in control and virus infected cultures. The concentration of phosphate decreased significantly with time in the control incubations (6.60 to 1.47 μM). The lowest phosphate concentration was observed on day 10 (1.47 μM) which coincided with the highest number of *E. huxleyi* cells (Figure 4.1). Leal *et al*. (1999) also showed that phosphate concentrations in *E. huxleyi* cultures decreased from 7.61 to 0.18 μM during their culture period. In the virus infected cultures, phosphate concentrations decreased with time during the first week, but remained fairly constant from day 8 at 4.10 μM.

Nitrate concentrations in control and virus infected cultures remained near constant throughout the experiments at around 160-148 μM. *E. huxleyi* appears to grow well under high and low nutrient concentrations or high as well as low ratios of nitrate to phosphate (Zondervan, 2007). Wal *et al*. (1994) found that different nutrient regimes in mesocosm enclosures had no influence on the gross growth rate of *E. huxleyi* populations. This was confirmed by Lessard *et al*. (2005), who showed that there is no strong relation between high N:P ratios (P more limiting than N) and the occurrence of *E. huxleyi* blooms in the field.

4.4.4 Variations in dissolved organic carbon (DOC) during the incubations

Dissolved organic carbon (DOC) ranged from 110 to 215 μM in the control cultures and from 138 to 187 μM in the viral infected culture (Tables 4.1 & 4.2). DOC decreased with time in the control culture, in parallel with the increase in *E. huxleyi* cell numbers (Figure 4.6a). A decrease in DOC was observed on day 6 in the virus infected culture, followed by a release of approximately 28 μM DOC (difference between day 6 and 10) into the media upon the viral lysis (Figure 4.6b). In a similar manner, viral lyses of *A. anophagefferens* bloom in the field resulted in a 40 μM DOC release to the water column (Gobler *et al*., 1997).

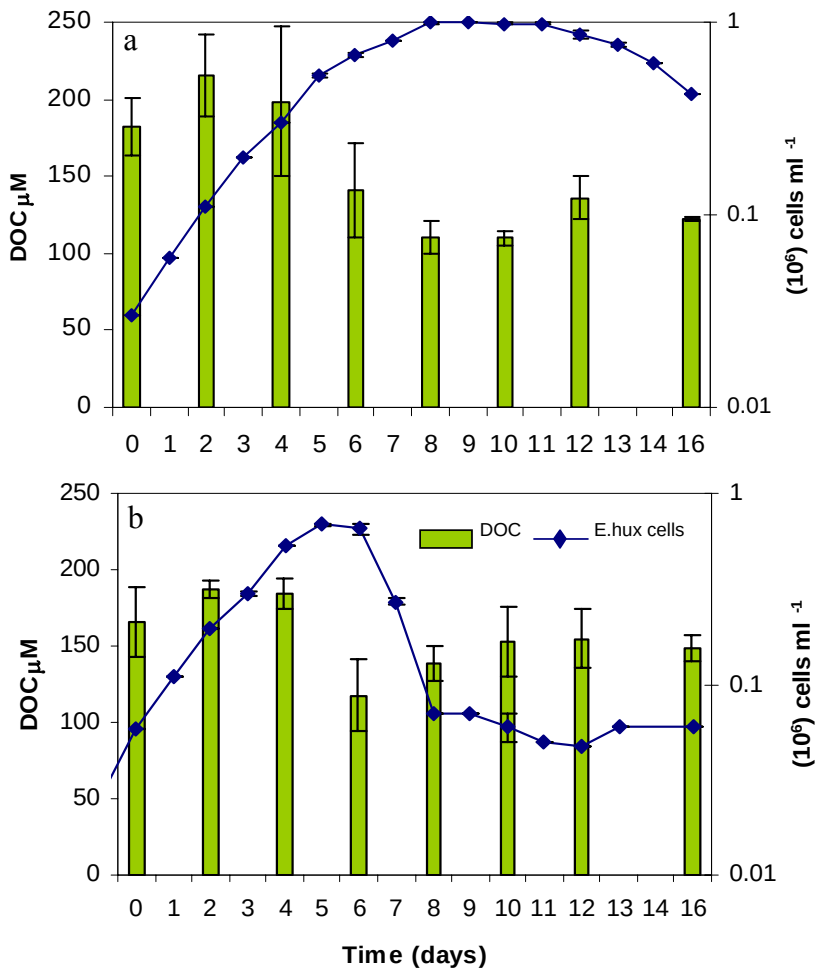

Figure 4.6: Dissolved organic carbon concentration (µM) during *E. huxleyi* culture experiment (August 2004) a) control, b) viral infected.

There was no significant correlation between *E.huxleyi* cell numbers and DOC (P> 0.05) in the virus infected culture, whereas a negative correlation was observed between cell numbers and DOC in the control culture (P<0.05 & r=0.85) (Figure 4.7). This correlation may be explained by the observed increase in heterotrophic bacteria numbers during the incubation culture period which could be responsible for removal of DOC (Bratbak *et al*., 1998). The release of DOC during cell lysis resulted in a positive correlation (P<0.05) between DOC and heterotrophic bacteria (r = 0.97) Figure 4.18. These observations indicate that the increase in DOC concentration resulted in an increased abundance of heterotrophic bacteria. The results by Middelboe *et al* (2002) similarly suggested that DOC released by the collapse of the spring phytoplankton bloom resulted in an increase in bacterial numbers and activity. No correlation found between DOC and bacteria in the control culture.

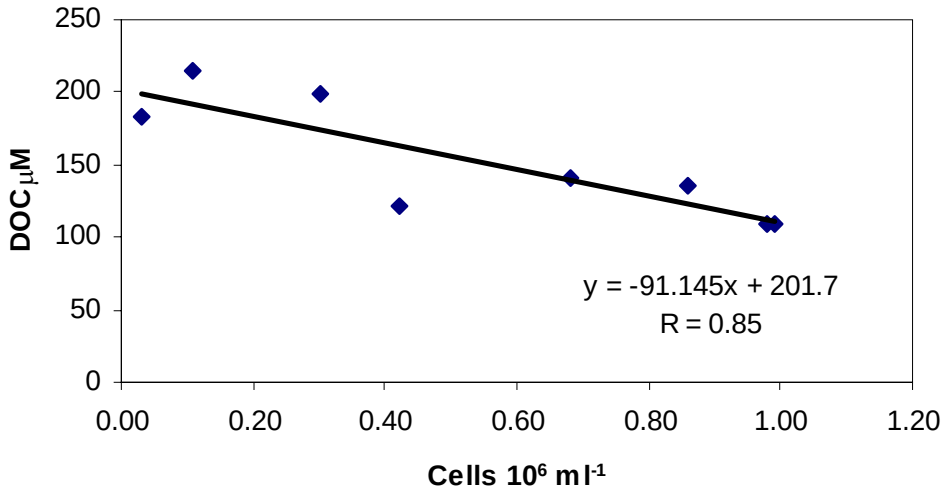

Figure 4.7: *E. huxleyi* cell numbers versus dissolved organic carbon concentrations in the control cultures.

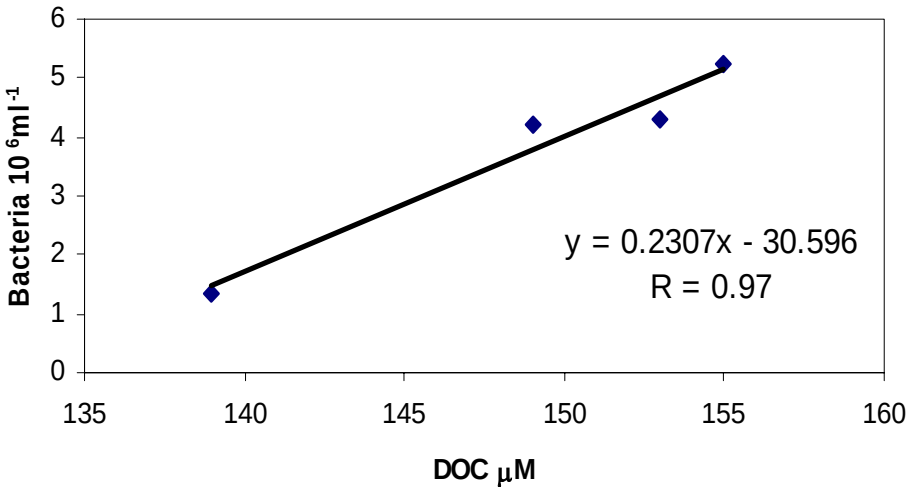

Figure 4.8: Dissolved organic carbon concentrations Versus Bacteria 10^6 ml^{-1} post viral lyses of *E. huxleyi* day 8.

4.4.5 Copper speciation during incubation experiments (August 2004 experiment).

4.4.5.1 Copper ligand titrations:

Representative Cu-ligand titration and linearization curves are shown in Figure 4.9 for virus infected cultures from the March and August 2004 experiments, and control cultures from the August 2004 experiment. The curvature at the beginning of the titration curves (Figure 4.6 a,c,e) indicates the presence of natural Cu-complexing ligands in these samples. Linear treatment of the titration data (Figure 4.6 b,d,f) produced a straight line for all samples, indicating that Cu-complexation was controlled by a single class of ligands (Campos and van den Berg, 1994).

Two sets of titrations were performed, using different added Cu concentration and a similar general trend in organic ligand concentrations was observed in the viral infected cultures. Ligand concentrations were higher (reaching a maximum of 34.32 nM, day 6) and the sensitivity of AdCSV was slightly enhanced when using the higher added Cu range (0-200 nM) compared to the lower added Cu range (0-70 nM). Measurements of Cu speciation in coastal waters by AdCSV are complicated by the presence of natural and anthropogenic surfactants (Moffett, 1995). Surfactants compete with Cu complexes for the surface of the mercury drop (Moffett *et al*., 1997). This interference becomes more pronounced at higher deposition times. Hence, for the titrations in this study the deposition times were limited to between 30 and 60 s to avoid interferences from surfactants.

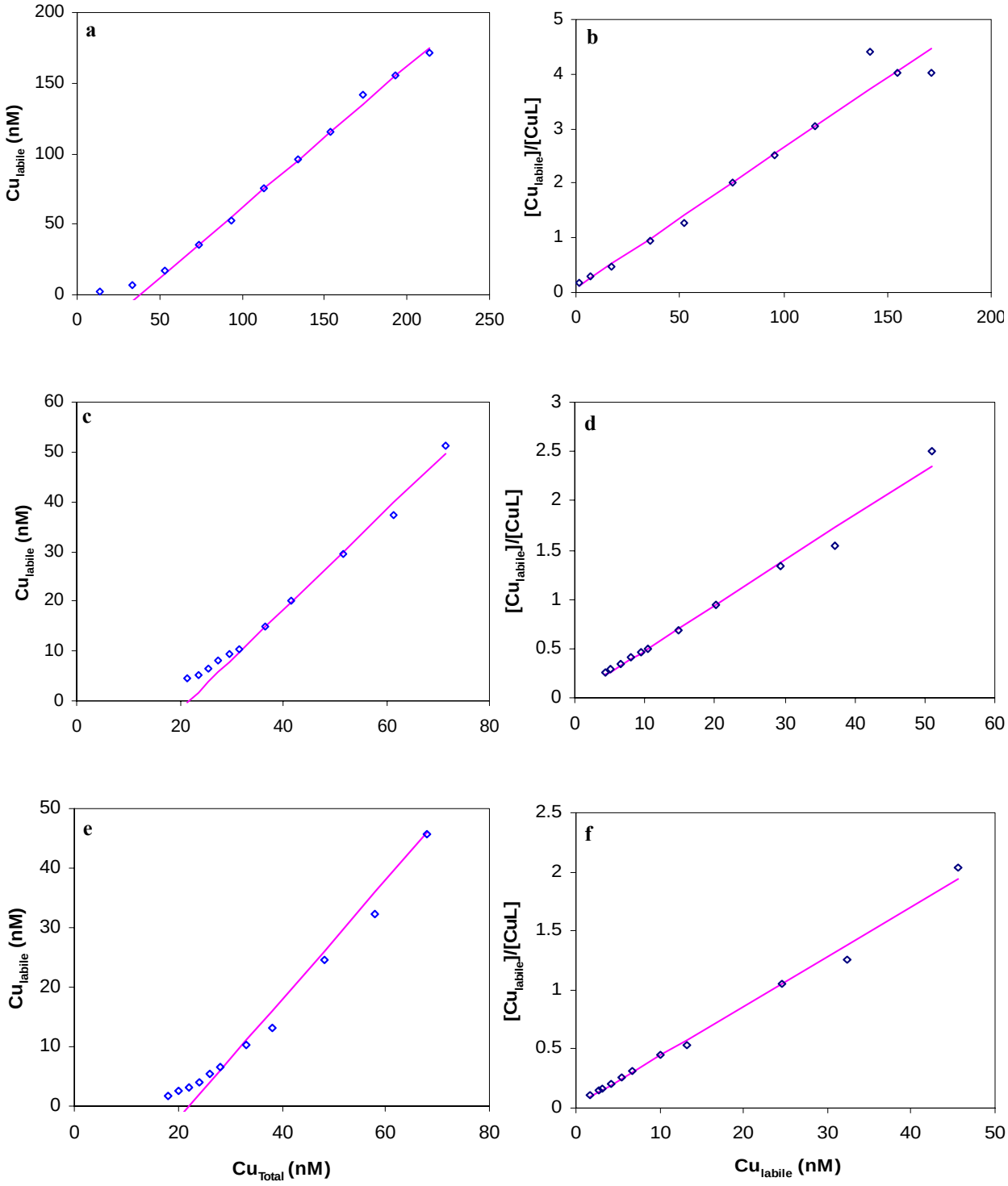

Figure 4.9: March 2004 experiment a&b, August 2004 experiment c&d (virus infected cultures); (e&f) control culture from August 2004 experiment. Titration curve for the culture samples (a, c, e), van den Berg linearization for the titration data (b, d, f). Cu_{Total} = Cu in the sample plus Cu added for the titration; CuL = Cu complexed by natural organic ligands; Cu_{labile} = Cu complexed by SA.

4.4.5.2 Total and labile dissolved Cu and organic ligands concentrations

Total dissolved Cu concentrations were near constant in the control and virus infected cultures (Tables 4.3-4.4). The concentrations ranged between ca. 17 and 20 nM. However, labile Cu concentrations decreased with incubation time in both control and virus infected cultures (Figure 4.10). In both the control and virus infected cultures, labile Cu decreased after day 6 to ca. 3 nM and then remained constant until the end of the experiment. Labile Cu concentrations in the control cultures decreased in a similar manner to those in the cultures of Leal *et al* (1999), Vasconcelos and Leal (2001). The total and labile dissolved Cu and organic ligand distributions in the control and the virus infected cultures are shown in Figure 4.10.

The total Cu concentrations observed in this experiment appeared to be optimal for *E. huxleyi* (Leal *et al.*, 1999). According to Leal *et al*. (1999) the growth of *E. huxleyi* was enhanced in the presence of 18 and 28 nM Cu, as compared with 8.4 nM. Growth of *E. huxleyi* decreased dramatically when the Cu concentration rose to 33 nM. This finding and the excellent growth achieved in our experiments confirm that total dissolved Cu concentrations were optimal for the growth of *E. huxleyi*.

In the control culture experiment, the organic Cu complexing ligand concentrations were higher than or close to total Cu concentrations. An increase in ligand concentrations was observed after day 10, when ligand concentrations reached 24.8 nM. This coincided with the lowest labile Cu concentrations. In the virus infected cultures, organic ligand concentrations were highest on day 6, and this coincided with highest cell numbers and lowest labile Cu concentrations.

The conditional stability constants (log K_{CuL}) observed during the culture experiments (control and virus infected cultures) ranged between 11.81 and 12.78. Only small differences in ligand strength were observed during the experiments, indicating that the organic ligands produced by the algae in the culture were functionally similar to those originally present in the seawater (Leal *et al.*, 1999). The conditional stability constant for the Cu-organic complexes (log $K_{CuL} < 13$) observed in all samples collected from the cultures were in the range of weak ligand class (Moffett *et* al., 1997). The conditional

stability constant observed in the current experiment was in a similar range to the values reported by Leal *et al.* (1999) and Vasconcelos *et al*. (2002). The March 2004 culture experiment results (Appendix) showed a similar range in conditional stability constants as observed in the August 2004 experiment. However, the ligand concentrations were higher (34.5 nM) in the March experiment (using a high Cu range in the titrations). Stronger ligands with conditional stability constant of Cu complexes log K_{CuL}> 13 (Moffett, 1995; van den Berg *et al*., 1987), which are designated as type 1 or class 1 ligands (L1) by Leão *et al*., 2007, were not observed in these experiments. Cyanobacteria have been considered as probably the most important source of L1 ligands in the open ocean (Croot *et al*., 2000; Gordon *et al*., 2000; Croot, 2003).

Copper in these experiments was nearly fully complexed by ligands (99.98 % CuL), yielding CuL as the major Cu species for all samples. The Cu speciation in the samples followed trends observed in other aquatic systems, where the concentration of Cu complexing ligands increased with total Cu concentration, resulting in CuL being the dominant Cu species (Kozella and Bruland, 1998; van den Berg *et al*., 1987; Moffett *et al*. 1990; Croot, 2003). Tables 4.3 and 4.4 present the concentrations of total, labile dissolved and particulate Cu, organic ligands, free Cu^{2+}, inorganic Cu', together with the conditional stability constant for the Cu-organic complexes (log K_{CuL}) and the degree of Cu-complexation (% CuL) for the control and virus infected cultures respectively.

The chemical characteristics of the Cu complexing ligands were not identified in these experiments. However, previous *E. huxleyi* culture and mescosom experiments (Leal *et al*., 1999; Muller *et al*., 2003) showed that thiols accounted for an important part of the Cu complexing ligands produced by the algae. Furthermore, Cu complexing titrations have revealed that thiol ligands could be from strong (L1) or weak (L2) organic ligands (Laglera and van den Berg, 2003). Nevertheless, the structure and exact composition of the dissolved Cu complexing ligands remains an important open question (Croot *et al*., 2000, Leão *et al*., 2007).

The most critical difference in Cu speciation between the virus infected and control cultures was in terms of organic ligand concentrations (Figure 4.10). In particular the timing of organic ligand production strongly influenced the Cu speciation. In the virus infected culture, the highest organic ligand concentrations were observed on day 6,

following the virus infection, while the highest concentration in the control cultures was observed between day 10 and 12. EhV-86 started to increase after day 6 reaching maximum on days 12 and 13 (Figure 4.4).

Cells numbers in the cultures were positively correlated ($P< 0.05$) with organic ligand concentrations in the control ($r = 0.78$) and virus infected cultures ($r = 0.80$) (Figure 4.11), which indicates that the organic ligand production was related to exudation and virus related release processes in the cultures.

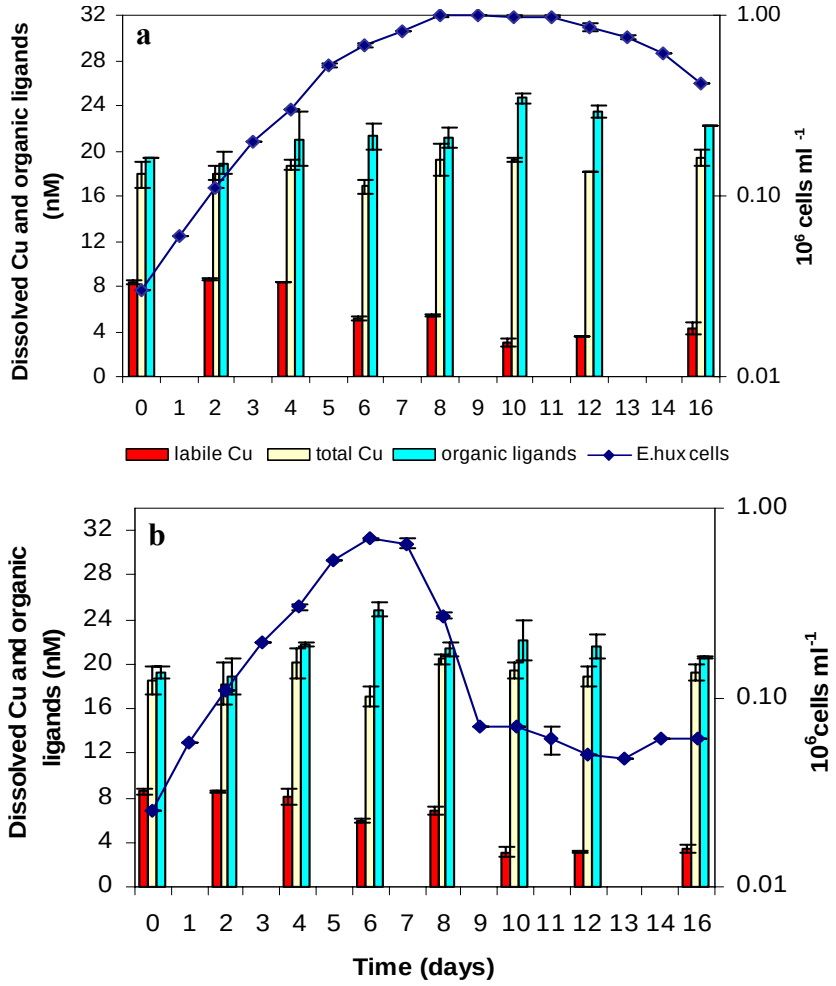

Figure 4.10: Copper speciation (labile, total dissolved Cu and organic ligands (nM) and *E. huxleyi* cell numbers in the control (a) and virus infected (b) cultures (virus added on day 4).

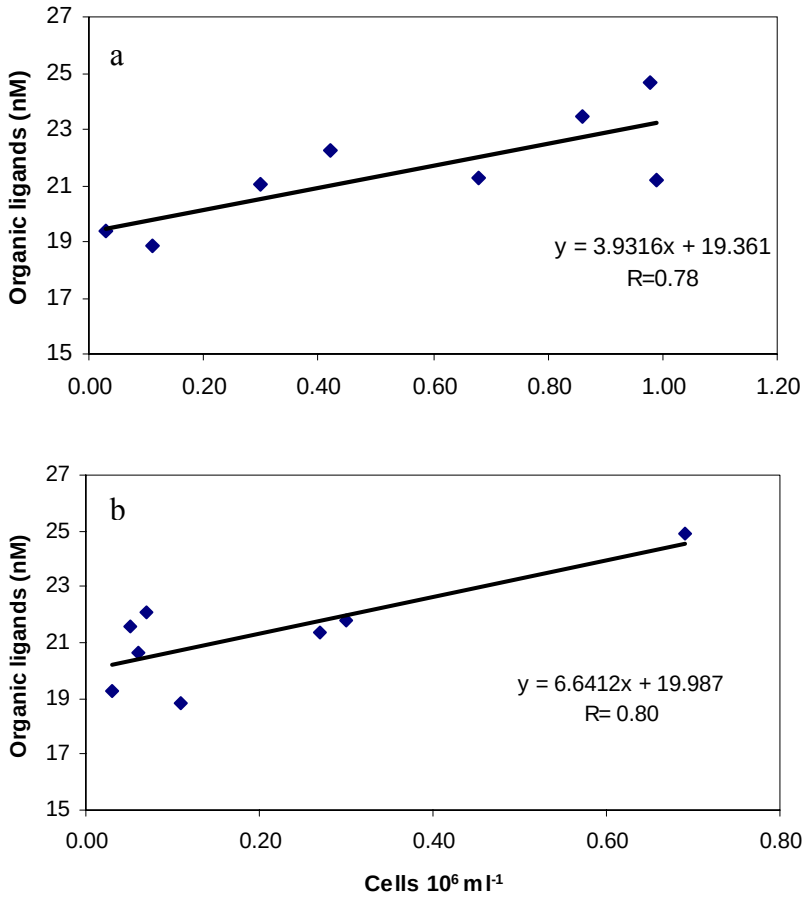

Figure 4.11: Organic Cu complexing ligand concentrations versus *E. huxleyi* cell numbers in control (a) and virus infected (b) cultures.

Table 4-3: Copper complexation in the control culture (virus free) experiment for *Emiliania huxleyi* (Aug 2004). Concentrations of total, labile dissolved and particulate Cu (Cu algae) and Cu cell, organic ligands, free Cu^{2+}, inorganic Cu', together with the conditional stability constant for the Cu-organic complexes (log K_{CuL}) and the degree of Cu-complexation (% CuL)

Time (days)	*Emiliania huxleyi* 10^6 cells ml^{-1}	Cu algae nM	Cu cell 10^{-17} mol $cell^{-1}$	Cu labile nM	Cu Total nM	L nM	Cu^{2+} M	Cu' M	log K_{CuL} M	CuL %
0	0.03	0.34 ± 0.09	1.12 ± 0.30	8.37 ± 0.20	17.87 ± 1.10	19.37 ± 0.04	2.76E-12	9.4E-11	12.86 ± 0.49	99.98
2	0.11	1.09 ± 0.36	0.98 ± 0.32	8.55 ± 0.08	17.96 ± 0.62	18.86 ± 0.98	1.03E-11	3.49E-10	12.25 ± 0.21	100.00
4	0.30	3.99 ± 1.37	1.33 ± 0.45	8.36 ± 0.05	18.73 ± 0.42	21.05 ± 2.38	1.72E-12	5.84E-11	12.76 ± 0.49	100.00
6	0.68	4.58 ± 0.78	0.67 ± 0.11	5.18 ± 0.16	16.82 ± 0.59	21.26 ± 1.19	3.25E-12	1.1E-10	12.27 ± 0.64	98.99
8	0.99	6.26 ± 1.17	0.63 ± 0.11	5.41 ± 0.05	19.17 ± 1.48	21.17± 0.87	9.6E-13	3.26E-11	12.76 ± 0.11	99.70
10	0.98	6.17 ± 1.14	0.62 ± 0.11	3.01 ± 0.35	19.26 ± 0.19	24.67 ± 0.43	6.73E-13	2.29E-11	12.73 ± 0.14	100.00
12	0.86	5.76 ± 1.55	0.67 ± 0.18	3.55 ± 0.01	18.06 ± 0.01	23.43 ± 0.54	5.15E-13	1.75E-11	12.97 ± 0.50	99.98
16	0.42	2.44 ± 0.09	0.58 ± 0.02	4.30 ± 0.49	19.43 ± 0.72	22.252 ± 0.03	2.66E-12	9.05E-11	12.51 ± 0.58	99.99

Table 4-4: Copper complexation in the virus culture experiment for *Emiliania huxleyi* (Aug 2004). Concentrations of total, labile dissolved and particulate Cu (Cu algae) and Cu cell, organic ligands, free Cu^{2+}, inorganic Cu', together with the conditional stability constant for the Cu-organic complexes (log K_{CuL}) and the degree of Cu-complexation (% CuL)

Time (days)	*Emiliania huxleyi*	Cu algae	Cu cell	Cu labile	Cu Total	L	Cu^{2+}	Cu'	log K_{CuL}	CuL
	10^6 cells ml^{-1}	(nM)	10^{-17} mol $cell^{-1}$	nM	nM	nM	M	M	M	%
0	0.03	0.42 ± 0.21	1.38 ± 0.70	8.58 ± 0.28	18.53 ± 1.31	19.25 ± 0.49	1.91E-11	6.50E-10	11.74 ± 0.12	99.99
2	0.11	1.54 ± 0.26	1.40 ± 0.23	8.60 ± 0.12	18.21 ± 1.86	18.82 ± 1.60	2.45E-11	8.33E-10	11.81 ± 0.56	98.99
4 + virus	0.3	4.32 ± 1.44	1.44 ± 0.48	8.05 ± 0.71	20.11 ± 1.33	21.77 ± 0.19	3.32E-12	1.13E-10	12.68 ± 0.55	100.00
6	0.69	6.00 ± 0.55	0.87 ± 0.07	5.93 ± 0.24	17.13 ± 0.89	24.90 ± 0.58	1.43E-12	4.86E-11	12.23 ± 0.10	99.99
8	0.27	5.19 ± 0.23	1.92 ± 0.08	6.85 ± 0.43	20.45 ± 0.48	21.35 ± 0.62	1.06E-11	3.60E-10	12.17 ± 0.16	99.99
10	0.07	3.09 ± 0.40	4.41 ± 0.57	3.13 ± 0.38	19.44 ± 0.71	22.10 ± 1.83	9.52E-12	3.24E-10	12.04 ± 0.20	100.00
12	0.05	2.39 ± 0.85	4.78 ± 1.70	3.13 ± 0.12	18.93 ± 0.88	21.57 ± 1.09	2.99E-12	1.02E-10	12.47 ± 0.36	99.98
16	0.06	2.21 ± 0.32	3.68 ± 0.53	3.33 ± 0.36	19.29 ± 0.73	20.63 ± 0.11	7.71E-12	2.62E-10	12.21 ± 0.18	100.00

4.4.5.3 Particulate Cu in phytoplankton:

The Cu associated with algal biomass, which may have been either taken up or adsorbed onto cell surface, is presented in Figure 4.12 for the control and viral infected cultures. Particulate Cu in the control cultures increased with time (0.34 nM to 6.20 nM), and could therefore be correlated with algal biomass. This finding agrees with observations by Vasconcelos and Leal (2001b) and Leal *et al*. (1999), where particulate Cu increased with time (3 nM to 19 nM). Similarly, in the virus infected culture the particulate Cu increased with time, until the cell number declined due to viral lysis. In our experiments, the intra-cellular uptake was not determined as no rinse with an EDTA or oxalate solution was conducted to remove extra-cellular Cu. Previous work by Vasconcelos and Leal (2001) has shown that 86-96% of total particulate Cu was intra-cellular, with the uptake occurring very quickly.

An important point to affirm is that the decrease in particulate Cu in the virus infected culture was associated with cell lysis as a result of virus infection on day 10. Labile Cu remained unchanged in this culture, and this issue will be discussed later in this chapter.

A strong positive correlation ($P< 0.05$) was observed between the concentration of particulate Cu and cell number in the control ($r=0.95$) similar to (Vasconcelos and Leal, 2001) findings and virus infected ($r=0.85$) cultures. These observations indicate that the two parameters were significantly correlated (Figure 4.13).

Total dissolved copper remained constant through the experiment whereas total particulate copper increased and this could be correlated with algal biomass as explained earlier. These finding agree well with those reported by Vosconcelos and Leal (2001) and Leal *et al*. (1999). However, the fact that total dissolved copper remains constant and did not increase is puzzling. Total particulate copper could be introduced through collecting and handling of samples and also through the digesting processes of the membranes.

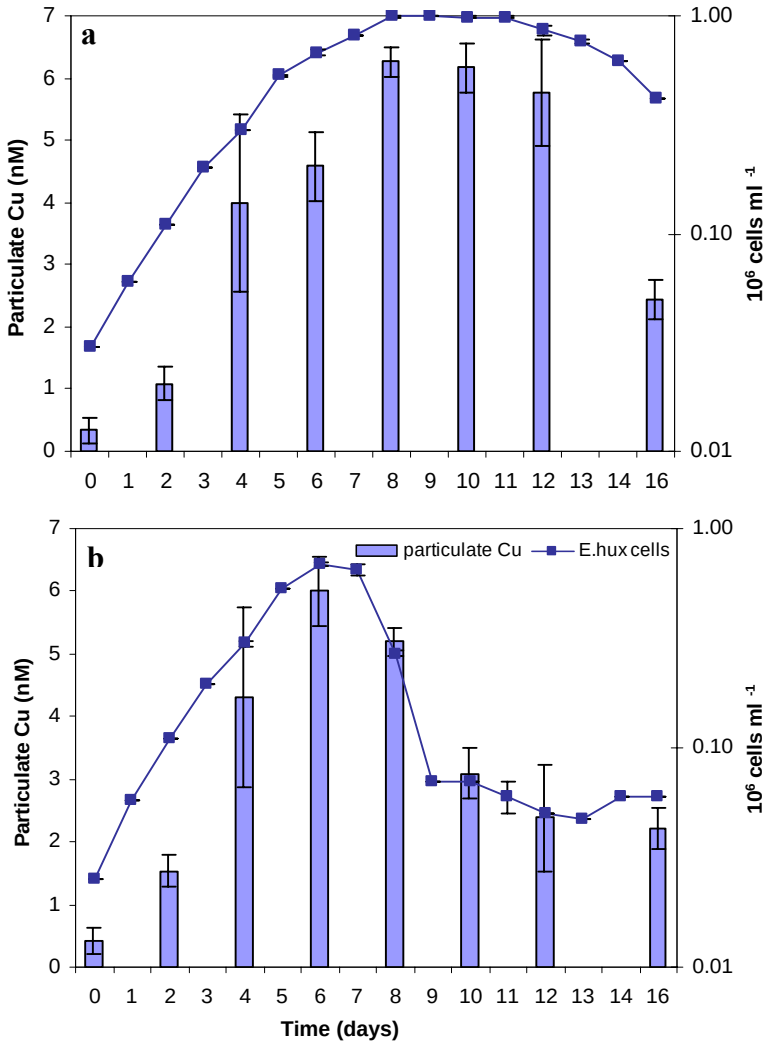

Figure 4.12: Particulate Cu concentrations and *E. huxleyi* cell numbers in control (a) and virus infected (b) cultures.

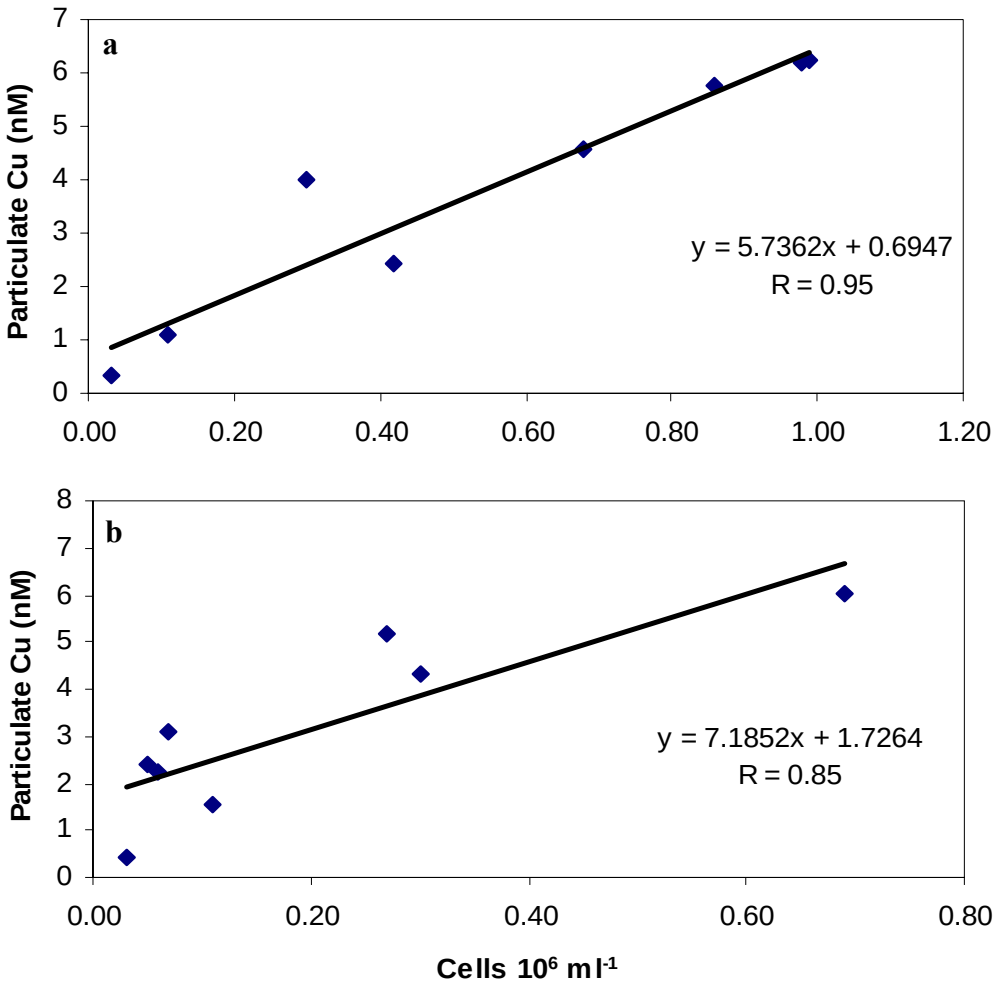

Figure 4.13: Particulate Cu concentrations versus *E. huxleyi* cell numbers in control (a) and virus infected (b) cultures.

The Cu concentrations per cell were evaluated using particulate Cu concentrations and cell densities. In the control culture the amount of Cu per cell decreased exponentially during algal growth, although the overall cellular Cu content increased. This is because of the dilution of the fixed amount of Cu over increasing numbers of cells (Leal *et al*., 1999). The rate of increase in cell numbers was greater than the rate of uptake of Cu. Copper per cell decreased when cell numbers reached a maximum in the virus infected culture (day 6). Viral lyses of *E. huxleyi* caused a sharp increase in Cu per cell, reaching a maximum of 4.78x 10^{-17} mol/cell (Figure 4.14 b). EhV-86 encodes a number of proteins that require metals for their activity, like most cellular systems that require metals for the functioning of their DNA & RNA replication enzymes. EhV-86 has for example an edonuclease (ehv018) that requires divalent metal ions for its activity (Wilson *et al*., 2005). Therefore, there is a strong possibility that the virus was using some of the free Cu during its infection cycle.

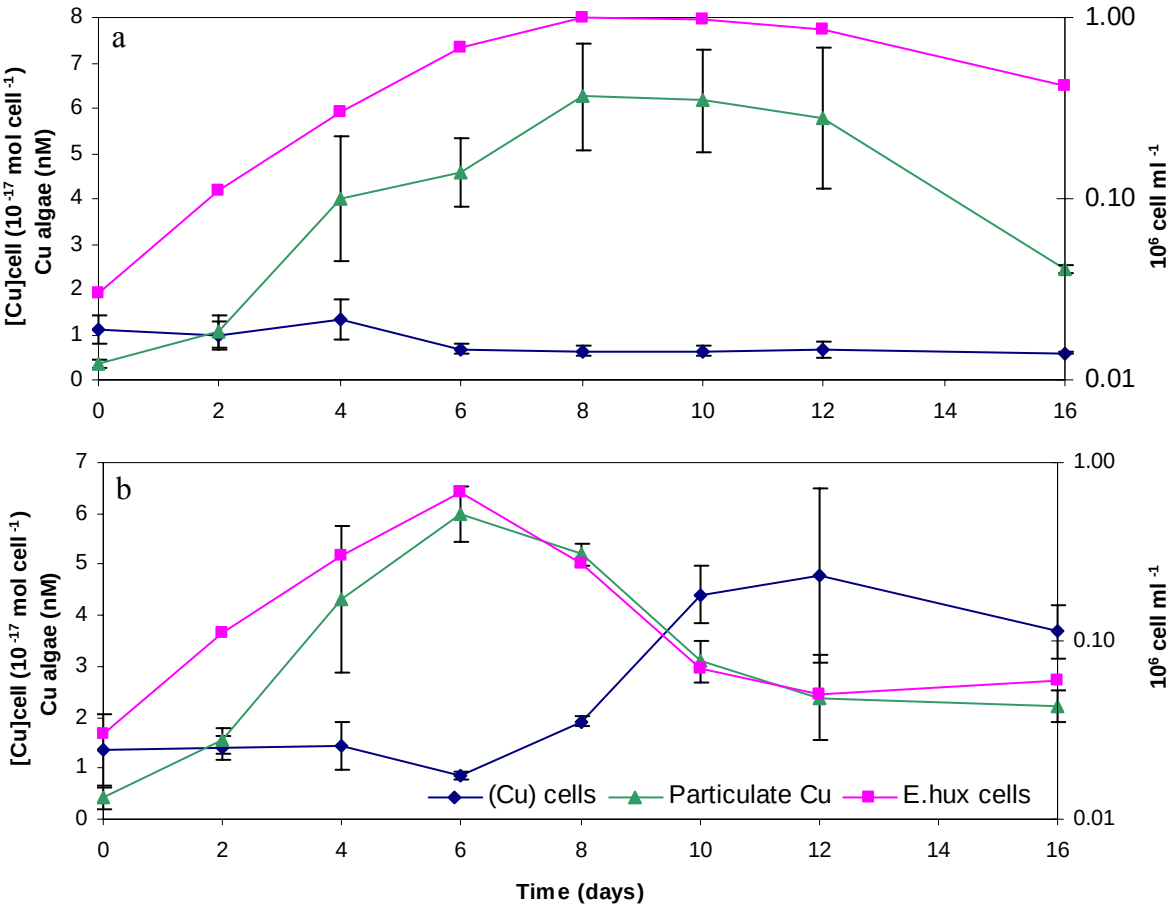

Figure 4.14: Particulate Cu concentrations and cellular Cu content and *E. huxleyi* cell numbers in control (a) and virus infected (b) cultures.

4.4.5.4 Dissolved Cu species

The chemical speciation of Cu in the culture was calculated from the determined concentrations of total dissolved Cu, organic Cu complexing ligands and their conditional stability constants. Ligand production in the control culture decreased the free aqueous Cu concentration [Cu^{2+}] with time (Figure 4.15a). The lowest [Cu^{2+}] in the control culture was between 6.73-5.15x 10^{-13} M, coinciding with the highest organic ligand concentrations. The decrease in Cu^{2+} concentrations during the control experiment was also observed by Vasconcelos and Leal (2001a), and this was related to the production of Cu complexing organic ligands. In the virus infected culture, [Cu^{2+}] decreased when enhanced organic ligand concentrations were observed (Figure 4.15b). Also production of organic ligands in both cultures decreased the concentrations of inorganic Cu'.

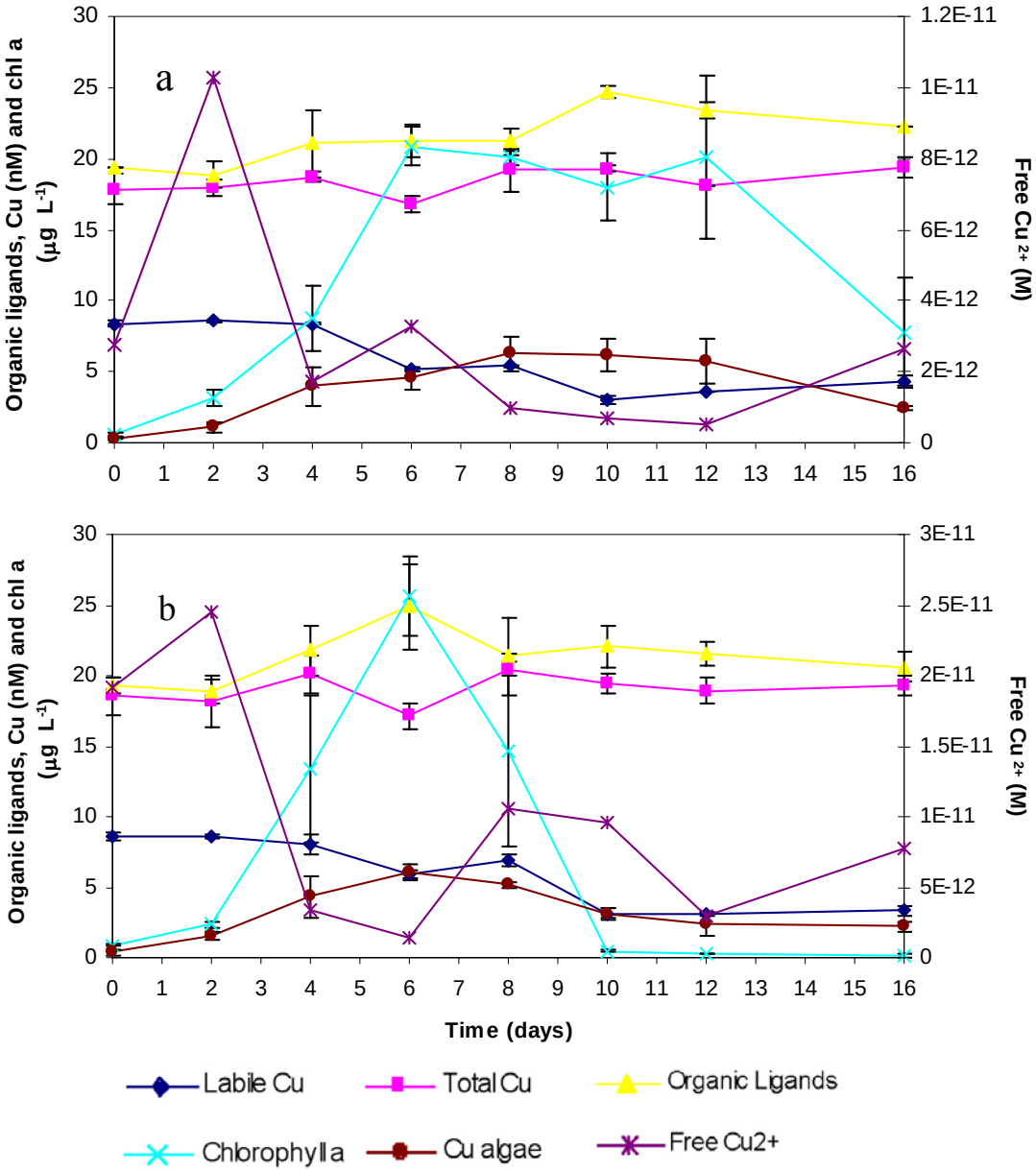

Figure 4.15: Copper speciation results and chlorophyll *a* concentrations for August 2004 experiment; control (a) and virus infected (b) cultures.

The relation between the organic ligand concentrations and log [Cu^{2+}] is plotted in Figure 4.16 for the control and virus cultures. A negative correlation between the organic ligand concentrations and log [Cu^{2+}] was observed ($P < 0.01$), with $r = 0.80$ for the control culture and $r = 0.87$ for the virus infected culture (Figure 4.16). This relationship confirms that the concentrations of Cu^{2+} in both cultures were controlled by the organic ligand concentration and they were specific ligands that bound free Cu ion.

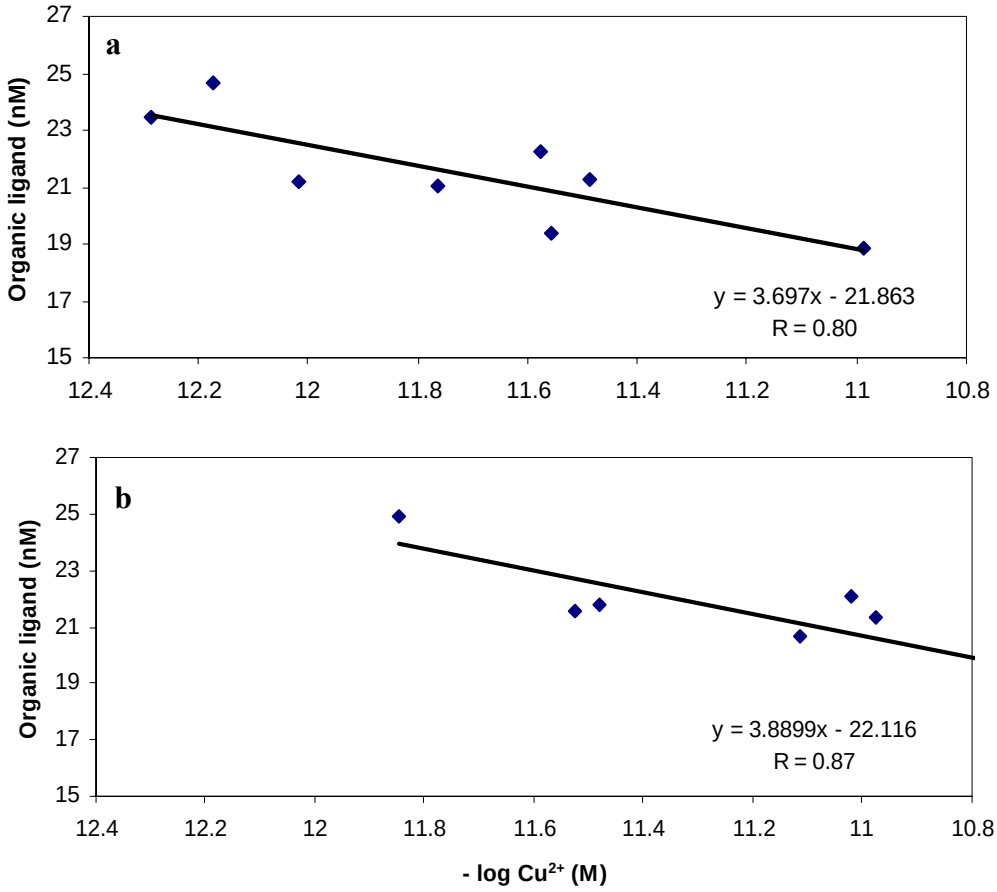

Figure 4.16: Organic ligand versus Cu^{2+} concentration in control (a) and virus infected (b) cultures.

Leal *et al*. (1999) showed that the growth of *E. huxleyi* was optimal at a concentration of 18 nM total dissolved Cu, with a 1.5 pM Cu^{2+}, and this observation is consistent with these findings. A concentration greater than 25 pM of Cu^{2+} caused organic ligand production to be simulated by *E. huxleyi* (Muller *et al*., 2003; Leal *et al*., 1999). In these current experiments (control and virus infected), lower [Cu^{2+}] were observed (Tables 4.3-4.4). This suggests that the bioavailabile Cu^{2+} fraction was negatively influencing *E. huxleyi* growth or physiology. As study by Brand *et al*. (1986) showed a high Cu tolerance of *E. huxleyi*, with a concentration threshold of 0.6 nM Cu^{2+}, which is well above the range found in the present and other studies. It is important to note that the studies by Leal *et al*. (1999) and Muller *et al*. (2003) were investigating production of organic ligands in response to added Cu and other metals, whereas no metal was added to these experiment.

4.4.5.5 Particulate to dissolved Cu ratios in the cultures

Figure 4.17 presents the ratios of particulate Cu to Cu^{2+}, particulate Cu to labile Cu, and particulate Cu to total dissolved Cu in the control and virus infected cultures for the August 2004 experiment. In the control, the ratio of particulate Cu to total dissolved Cu increased with time as cell numbers increased (Fig. 4.17a). Copper was incorporated into the particulate phase until day 12 in the control experiment, after which the ratio decreased following release of Cu from the particulate phase. The other ratios showed a similar pattern. These observations for the control cultures were in close agreement with those by Vasconcelos and Leal, (2001) as plotted in Figure 4.17 d, e.

The Cu species ratios in the virus infected cultures differed from the control experiments. It is evident that EhV-86 had an effect on the particulate and dissolved phase Cu concentrations during cell lyses. Copper became more abundant in the dissolved fraction relative to particulate Cu following day 6 (cell lyses) (Fig. 4.17). As outlined by Suttle (2005), viruses play an important role in marine biogeochemical cycles. Viral lyses of microorganisms within sinking aggregates may effectively dissolve particles and convert a fraction of them into dissolved material (Fuhrman, 1999; Wihelm and Suttle, 1999), which can subsequently be assimilated by the microbial community. The findings of the current study of a decreasing importance of the particulate fraction upon viral infection support this hypothesis. Nevertheless, Gobler *et al*. (1997) found that only 5% of Fe was released into the dissolved phase during vial lyses of *A. anpphagefferens*. This may be due to the fact that virally lysed cells release high molecular weight proteins, nucleic acids that can act as aggregating agents, promoting particle formation (Proctor and Fuhrman, 1991). In the current experiments no increase in the dissolved phase Cu concentrations were observed post viral infection. Direct comparison between current research findings and Gobler *et al*. (1997) is not possible, because of differences between metal species and viruses tested. Recent research by Poorvin *et al*. (2004) reported that the activity of naturally occurring viral populations in a high nutrient low chlorophyll (HNLC) coastal upwelling region can regenerate sufficient dissolved Fe to support the growth of phytoplankton community, indicating that viral lysis plays a key role in re-supplying microbial communities with dissolved micronutrients.

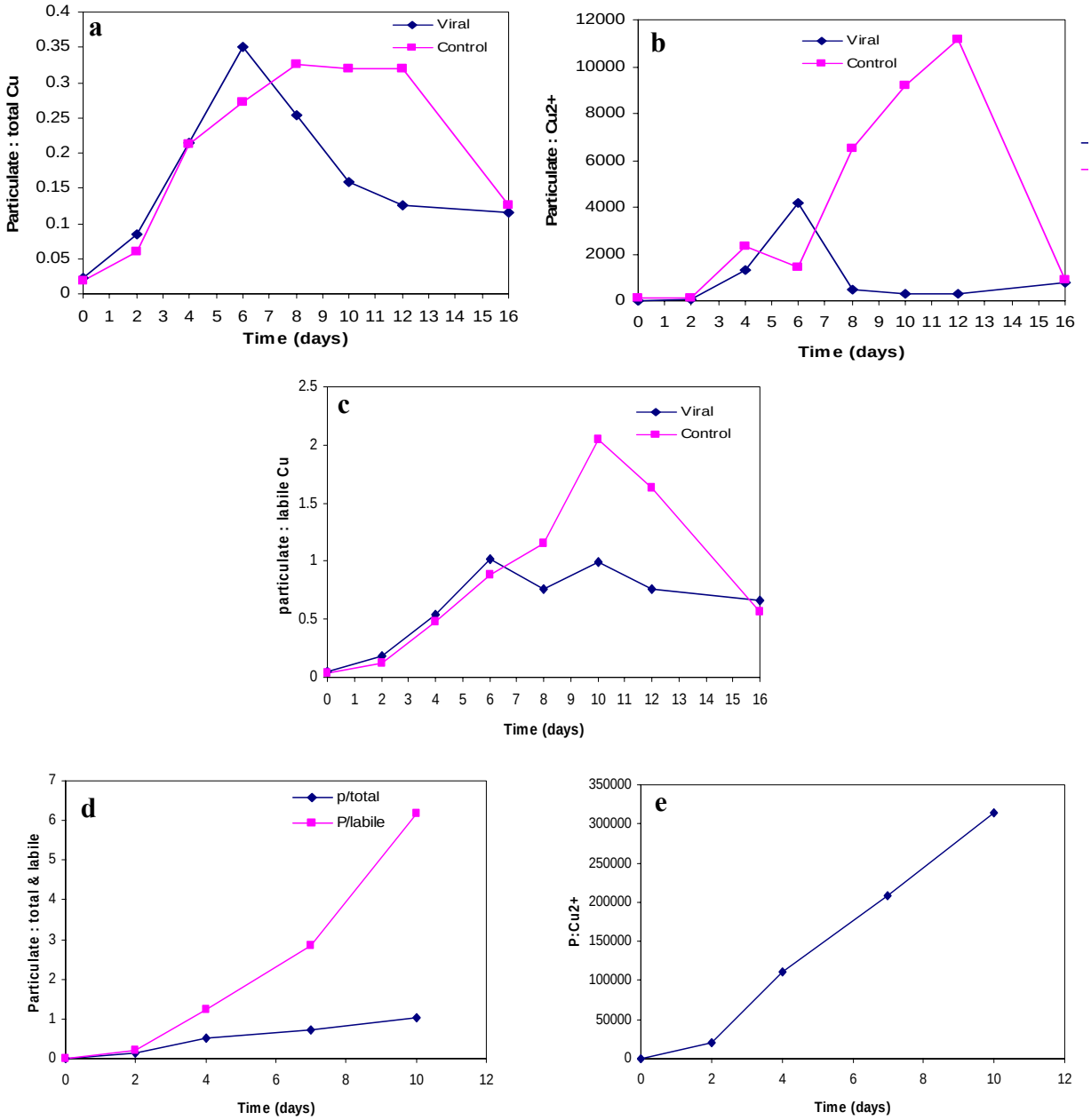

Figure 4.17: Particulate Cu to total dissolved (a), labile Cu (b) ,and Cu^{2+} (c) ratios in control and virus infected cultures for August 2004 experiment. Particulate Cu to total dissolved and labile Cu (d), and Cu^{2+} (e) ratios *E. huxleyi* cultures reported by Vasconcelos and Leal (2001).

4.4.6 Interaction between virus particles and Cu

The findings of the *in vitro* culture experiments using 20 L culture vessels (section 4.4.5) were that in virus-free cultures the amount of Cu per cell decreased exponentially during algal growth, while the overall cellular Cu content (particulate Cu) increased (Figure 4.14

and Table 4.3). Conversely, viral lysis of *E. huxleyi* caused a sharp increase in Cu per cell, reaching a maximum of 4.78 10^{-17} mol $cell^{-1}$ (Figure 4.14 and Table 4.4). Moreover, there was no significant increase in the dissolved Cu fraction in response to the decrease in the particulate Cu concentration, hence creating a the missing Cu fraction (Figures 4.10 & 4.12). It can thus be hypothesise that the virus particles played a direct role in Cu cycling, possibly by binding to the Cu directly.

This observation led to a reconsideration of the experimental design. The dissolved or labile measurements (Chapter 3 subsection 3.2.3.1) were taken from the 0.4 µm filtrate. It should be noted that virus particles pass through these filters (size range of EhV-86 is 170 to 190 nm – Schroeder et al., 2002). If this hypothesis is correct, then it is likely that the virus influenced the dissolved Cu measurements by competing with the ligands in the assay. To test this hypothesis we removed the virus fraction from the 0.4 µm filtrate by passing it through a 50 KDa filter (Viva flow 50, Sartorius - Schroeder *et al*., 2002). The permeate was reanalyzed for its Cu content. In addition, the effect of incremental EhV-86 additions on the assay was measured.

When the virus fraction was removed from samples collected on days 8 and 16 (presented in Figure 4.10), the labile Cu increased from 6.85 to 11.20 nM and from 3.33 to 7.21 nM, respectively. Titrations of these samples were conducted to measure the concentration of the natural complexing organic ligands (L), their conditional stability conditional constants and free [Cu^{2+}] in a similar manner as for the samples that were anticipated to contain viral fractions. Titration revealed that Cu organic ligands and log K_{CuL} were higher without the virus fraction. In addition, the sensitivity of the AdCSV measurements was enhanced. Ligand concentrations increased approximately by 4 nM and log K_{CuL} by 1 M. For example, on day 8 the stability constants increased from 12.17 to 13.01 M when virus fraction was removed. Consequently, the Cu^{2+} concentration decreased from 10 to 0.41 pM on day 8 and from 7.70 to 0.6 pM on day 16. This decrease can be correlated to the higher ligand concentrations observed on removal of the virus fraction. In summary, labile Cu increased upon removal of viruses from the assay, resulting in lower [Cu^{2+}]. Further experiments were conducted to investigate possible interactions between virus particles and Cu. Virus free water (Milli-Q water and 0.2 µm filtered seawater) was used to create a Cu calibration curve (0-25 nM) for CSV. The effect of EhV-86 on this standard curve was determined by adding incremental

percentages of EhV-86 (20, 50 and 100% solutions containing 1.8, 4.5, 9 x 10^7 virus particles ml^{-1}, respectively). Peaks were highest when EhV-86 was not present in the solution (Figure 4.18). The sensitivity and peak heights of the CSV were affected as the virus concentration increased, with no changes being observed in the peak height for 100% virus addition. A possible explanation is that either the virus out-competes the added ligand for Cu on the mercury drop or Cu is attached to the virus particles, which prevents Cu from complexing with the added ligand (SA).

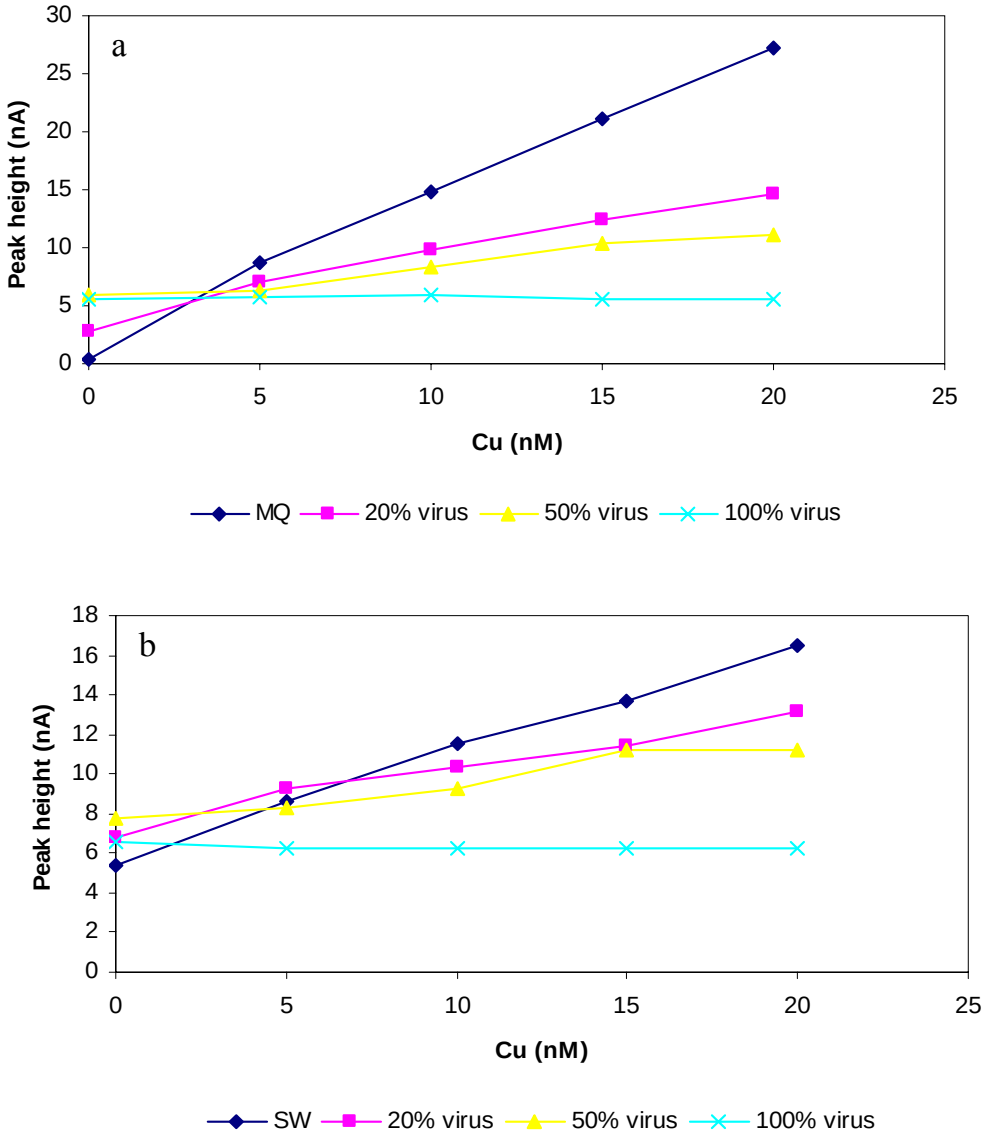

Figure 4.18: Cu and virus assay experiments using a) MQ water, b) filtered seawater.

The overarching conclusions of these experiments are that EhV-86 directly affected the Cu measurements when using the virus and Cu speciation measurements (Figure 4.18). As these speciation measurements are primarily used to quantify the presence of Cu, it

has been demonstrated here for the first time that all current measurements underestimated the Cu species concentrations when the virus fraction is not removed. This means that there was an increase in labile Cu in the current experiment as an effect of the viral lysis of *E. huxleyi*. However, the presence of viruses in the samples prevented documenting such an increase in labile Cu, but all labile Cu reported was underestimated.

Metal ions are an integral part of some viral proteins and play an important role in their survival and pathogenesis. Zinc, Mg and Cu are the commonest metal ions to bind with viral proteins (Chaturvedi and Shrivastava, 2005). EhV-86 encodes a number of proteins that require metals for their activity, like most cellular systems that require metals for the functioning of their DNA & RNA replication enzymes. EhV-86 has for example an edonuclease (ehv018) that requires divalent metal ions for its activity (Wilson *et al*., 2005). Therefore, there is strong possibility that the virus was using some of the free Cu during its infection cycle. Further work is required to confirm this observation. Moreover, Daughney *et al*. (2004) demonstrated that viruses have capsids that are reactive towards dissolved protons and iron and those virus capsids can also serve as nuclei for the growth of iron-oxide particles. Current results pertain to laboratory systems with simple chemistry and limited range of Cu-to-virus concentration ratios and so cannot be quantitatively extended to predict the role of viruses on the natural marine Cu cycle. Natural virus communities will be composed of several species, each of which might interact with Cu differently (Daughney *et al*., 2004; Suttle, 2005). Other substrates such as dissolved organic ligands might compete with the viruses for the available Cu and other dissolved elements might compete with Cu for the binding sites on the viruses.

An important question arises as to whether trace metals are complexed by viruses (10^7 ml^{-1}) in the water column, and does this apply for all viruses including bacteriophage? This has not been investigated before and these findings might serve as a base for future research focusing into the correlation between virus particles and Cu in the marine environment.

4.4.7 Nickel and Co behaviour for the August 2004 experiment

Variations of labile and total dissolved Ni and Co concentrations for the August 2004 experiment are shown in Figures 4.19-4.20. During virus infection, changes in labile and

total dissolved Ni concentrations occurred simultaneously, and the ratio of labile to total Ni concentrations was almost constant throughout the experiment (control and virus). *E. huxleyi,* it seems, did not accumulate Ni, since there was no change in the ratio of labile to total Ni in the infected and control cultures.

Labile Ni accounted for 70-80% of the total Ni concentration in the virus infected culture, and 76-91% of the total Ni concentration in the control cultures. Nickel speciation in the Mediterranean was similarly dominated by the labile fraction (> 80%) (Achterberg and van den Berg, 1997). The high labile fraction of Ni observed in these experiment indicated the limited influence of the *E. huxleyi* life cycle or EhV-86 infection on Ni speciation. Total Ni concentrations slightly decreased throughout the experiment, which might be due to adsorption onto phytoplankton cell walls. Viral infection and consequent Ni adsorption onto the virus particles may have indirectly affected the speciation of Ni, with a decrease in Ni species observed during infection as observed in copper experiments. In addition, particulate fraction of Ni could be bounded by virus particles. However, it was not possible to quatify this as particulate faction of Ni was not measured in the current study.

Nickel is an essential co-factor in the enzyme involved in urea uptake (Price and Morel, 1991). It has been reported that the diatom *Thalassiosira weissflogii* needs Ni when urea is the main source of nitrogen for growth (Price and Morel, 1991).

Tables 4.6-4.7 present labile and dissolved Ni and Co and labile to total for both metals (August 2004 experiment).

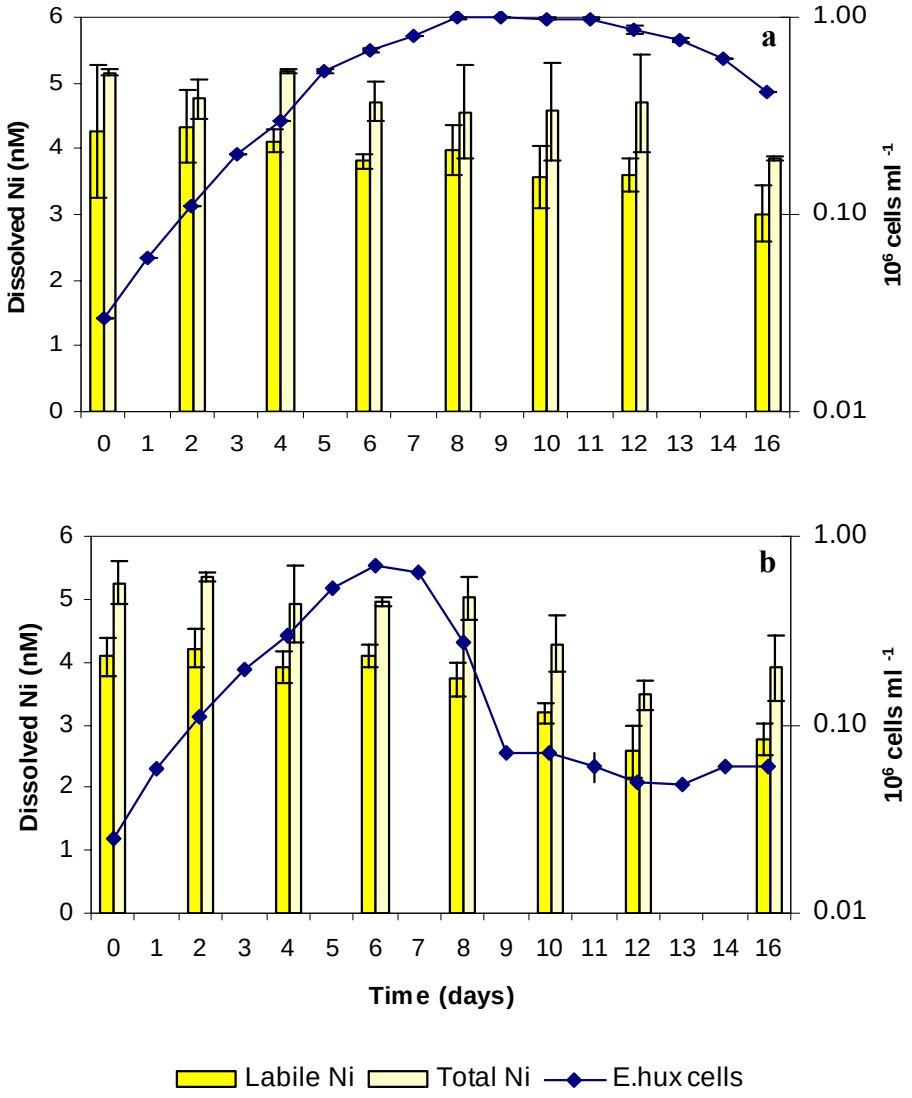

Figure 4.19: Labile and total dissolved Ni during *E. huxleyi* life cycle a) control, b) virus cultures (August 2004 experiment).

In the control culture, the ratio of labile to total Co decreased over time, which suggests accumulation by the healthy *E. huxleyi* cells, and use in for example vitamin B12. Indeed, Sunda and Huntsman's (1995) culture studies showed that *E. huxleyi* and *Synechococcus* have a strong requirement for Co. Decreases in the growth of *E. huxleyi* were observed when Co^{2+} concentrations were lowered in the culture medium (Sunda and Huntsman, 1995).

The ratio of labile to total Co in the virus infected culture decreased with time, which coincided with the high cell numbers till day 6. The ratio increased (day 8) due to virus infection, and this matched the increase in this ratio of labile to total Cu on removal of the virus fraction (section 3.3.6). This indicates that virus lyses affected the speciation of Cu and Co but with a higher influence on Cu. Adsorption onto virus, particles may explain the decrease in concentrations of the Co species in the virus infected culture.

Unlike the copper findings where total dissolved copper did not decrease with the increase in *E. huxleyi* cell number a slight decrease was observed in both Ni and Co total dissolved fraction. The decrease was most significant when viruses were highly abundant between days 10-16 which was unexpected, as it would be expected to observe Ni and Co to be released into the media. This once again highlights the role of viruses in metal speciation not only to Cu but also to Ni and Co.

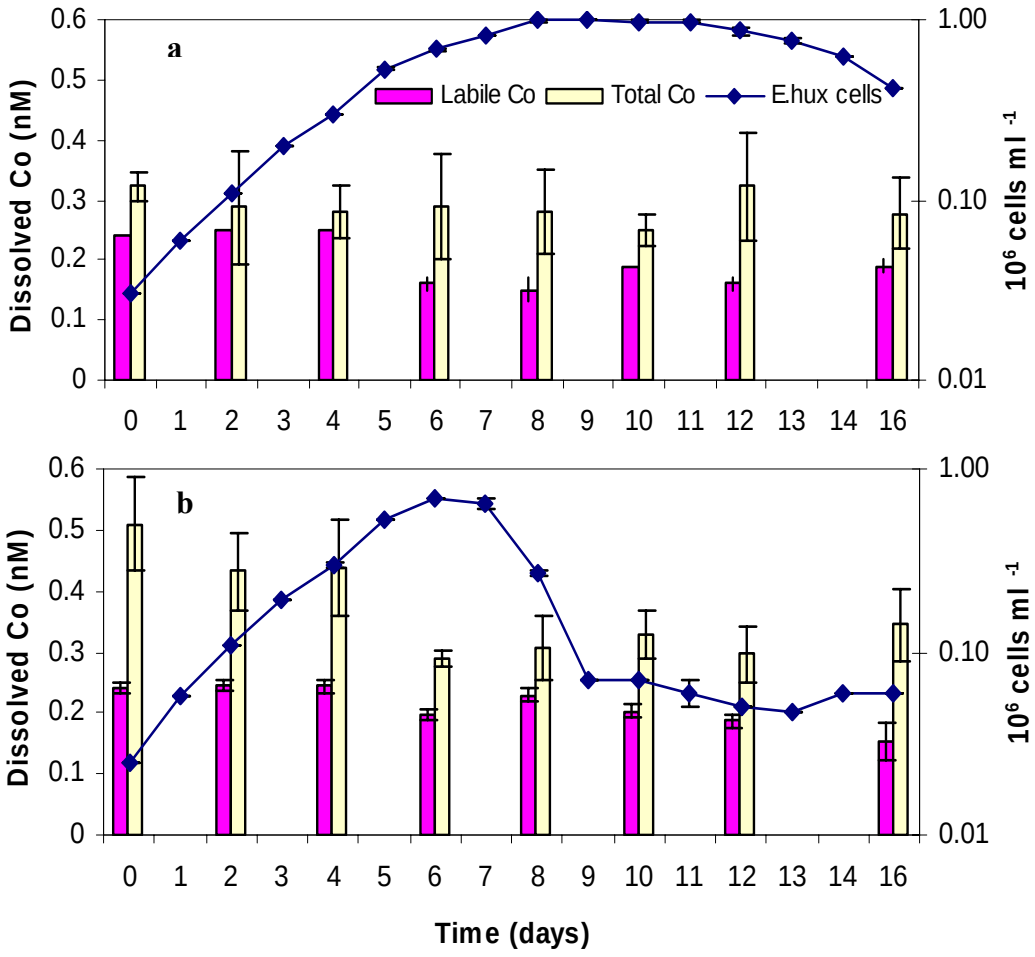

Figure 4.20: Labile and total dissolved Co during *E.huxleyi* life cycle a) control, b) virus infected cultures.

Table 4-5: Labile and total dissolved Ni, Co and labile to total % of Ni and Co in the control culture (Aug 2004).

Time (days)	Growth 10^6 cells ml^{-1}	Ni labile (nM)	Ni Total (nM)	Ni lab: total %	Co labile (nM)	Co total (nM)	Co lab: total %
0	0.03	4.26 ± 1.01	5.16 ± 0.05	82.56	0.24 ± 0	0.32 ± 0.02	74.4
2	0.11	4.34 ± 0.56	4.76 ±0.30	91.18	0.25 ± 0	0.29 ± 0.09	87.06
4	0.30	4.11 ± 0.17	5.19 ± 0.03	79.19	0.25 ± 0	0.28 ± 0.04	89.28
6	0.68	3.81 ± 0.10	4.72 ± 0.30	80.72	0.16 ± 0.01	0.29 ± 0.09	55.19
8	0.99	3.98 ± 0.37	4.56 ± 0.70	87.28	0.15 ± 0.02	0.28 ± 0.07	53.63
10	0.98	3.57 ± 0.47	4.57 ± 0.74	78.12	0.19 ± 0	0.25 ± 0.03	75.99
12	0.86	3.60 ± 0.24	4.70 ± 0.74	67.6	0.16 ± 0.01	0.32 ± 0.09	49.65
16	0.42	3.01 ± 0.42	3.85 ±0.03	78.18	0.19 ± 0.01	0.28 ± 0.06	68.48

Table 4-6: Labile and total dissolved Ni, Co and labile to total % of Ni and Co in the viral infected culture (Aug 2004).

Time (days)	Growth 10^6 cells ml^{-1}	Ni labile (nM)	Ni Total (nM)	Ni lab: total %	Co labile (nM)	Co total (nM)	Co lab: total %
0	0.03	4.08 ± 0.30	5.25 ± 0.34	77.71	0.24 ± 0.01	0.51 ± 0.08	47.24
2	0.11	4.21 ± 0.31	5.36 ± 0.08	78.54	0.24 ± 0.01	0.43 ± 0.06	56.54
4 + virus	0.30	3.92 ± 0.24	4.91 ± 0.61	79.84	0.24 ± 0.01	0.44 ± 0.08	55.73
6	0.69	4.11 ± 0.18	4.95 ± 0.08	83.03	0.20 ± 0.01	0.29 ± 0.01	68.5
8	0.27	3.73 ± 0.27	5.02 ± 0.34	74.3	0.23 ± 0.01	0.31 ± 0.05	74.71
10	0.07	3.19 ± 0.16	4.28 ± 0.45	74.53	0.20 ± 0.01	0.33 ± 0.04	61.55
12	0.05	2.58 ± 0.42	3.47 ± 0.23	74.35	0.19 ± 0.01	0.30 ± 0.05	62.89
16	0.06	2.77 ± 0.24	3.90 ± 0.52	71.03	0.154 ± 0.03	0.35 ± 0.06	44.54

4.5 Conclusions

This is the first report of the influence of viral infection of *E. huxleyi* on metal speciation. Total dissolved Cu concentrations were constant in the control and virus infected cultures. Particulate Cu concentrations in the control culture decreased with time which was related to the algal biomass. In the virus infected culture particulate Cu increased with time until the cells crashed upon infection. There was no increase in the dissolved Cu fraction in response to the decrease in the particulate Cu. Viral lyses of *E. huxleyi* caused a sharp increase in Cu per cell.

Experiments conducted to investigate possible interactions between virus particles and Cu revealed important findings. The overarching conclusions of these experiments were that EhV-86 directly affected the Cu measurements when using the virus and Cu speciation measurements. It has been demonstrated for the first time that all current measurements underestimated the concentrations of Cu species when the virus fraction was not removed. This means that there was an increase in labile Cu in the current experiment as an effect of the viral lysis of *E. huxleyi*. However, the presence of viruses in the samples prevented documenting such an increase in labile Cu, but all labile Cu reported was underestimated.

Important question arises as to whether trace metals are complexed by viruses (10^7 ml^{-1}) in the water column, and whether this applies to all viruses. This has not been investigated before and these findings might serve as a base for future research focusing into the correlation between virus particles and Cu in the marine environment.

Changes in labile and total dissolved Ni concentrations corresponded, with the ratio of labile to total Ni being almost constant throughout the experiment (control and virus). *E. huxleyi,* it seems, did not accumulate Ni, since there was no change in the ratio of labile to total Ni in both cultures. In the control culture, the ratio of labile to total Co concentrations decreased over time, which indicated accumulation by the healthy *E. huxleyi* cells, and which might be used for vitamin B12 production. Viral lysis affected the speciation of Cu and Co, but the effects were stronger for Cu.

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Chapter 5

5. The speciation of trace metals in the English Channel during *E. huxleyi* bloom 2005-2006

5.1 Abstract

The aim of this part of the study was to determine the trace metal speciation in samples collected during or post a natural *E. huxleyi* bloom event in the Western English Channel. Samples were collected during the summers of 2005 and 2006. Virus and plankton abundances, the concentrations of trace metals (Cu, Co, Ni, and Zn) and other relevant parameters were measured in depth profiles. In the 2005 survey, chlorophyll *a* concentrations ranged from 0.03 to 3.45 μg L^{-1} and higher concentration were observed during the 2006 survey, reaching a maximum of 3.70 μg L^{-1}. None of the viruses detected using AFC exhibited a signature typical of *E. huxleyi* viruses (EhV-86). Only able smaller bacteriophage/cynophage-like viruses were detected. The concentration of viruses and bacteria were within the range expected for the marine ecosystem. Generally viruses were highest in the near surface waters and decreased with depth. Molecular evidence has confirmed the presence of EhVs in these blooms. However, it is highly likely that *E. huxleyi* viruses were present at concentration below the limit of AFC detection. The *Synechococcus* density in the current study coincided with the highest chlorophyll *a* and the highest concentrations of *E. huxleyi*. *Synechococcus* dominated the phytoplankton community during both surveys. Hence, at the time of sampling, the *E. huxleyi* bloom was being succeeded by *Synechoccocus* in both years.

Total dissolved Cu concentrations ranged between 1.87-3.73 nM in 2005, and between 2.11- 4.43 nM in 2006. All results indicated that ligand concentrations (3.62-5.98 nM in 2005, 6.10-9.76 nM in 2006) exceeded total dissolved Cu concentration. Copper organic ligand in both surveys presented high conditional stability constants (Log K_{CuL} 12.20-13.77 M), which is characteristic of the strong Cu-binding L_1 ligand class. The Cu^{2+} concentration range was higher in 2005 (0.14-1.69 pM) than in 2006 (0.01-0.73 pM), when a slightly higher ligand concentrations was observed. The synchronicity of the appearance of L_1 and *Synechoccocus* abundance points strongly to these cyanobacteria as a strong ligand source. A comparison between Cu speciation in the culture experiments and findings of coastal surveys is difficult as the survey studies looked at different 'snap shots' of bloom dynamics. However, it is assumed that EhV-86 viruses were present in the coastal waters and equivalent to 5% virus solution used in chapter 4 (which is below AFC detection limit); this virus concentration will still potentially interfere with the Cu measurements.

The average surface Ni values in the surveys were 3.99 nM for 2005 and 3.23 nM for 2006. The labile fraction of Ni was relatively high, in particular during 2005 survey. Total dissolved Zn concentrations ranged between 5.78-8.67 nM in 2005, and higher concentrations were observed in 2006. Total dissolved Co ranged between 0.12-0.22 nM in 2005, and between 0.16-0.45 nM in 2006. In both years, the non labile fraction Co was dominant which indicates that Co was strongly complexed by exudates from the phytoplankton community.

5.2 Introduction

The English Channel is a marginal coastal sea located between the south coast of England and the northern coast of France with a maximum depth range of 40 m in the east to 100 m in the west (Tappin and Reid, 2000). During the summer months the western Channel current speeds are relatively low and so the waters become thermally stratified (Qurban *et al*., 2004). This seasonal stratification and the stability of the water column are ideal for the development of phytoplankton blooms (Tyrell and Taylor, 1996; Tappin and Reid, 2000).

The concentrations and distribution of trace metals in seawater are controlled by a combination of processes. Trace metals are delivered to coastal seas by rivers, wet and dry deposition derived from industrial sources and arid and semi-arid continental regions, and from sedimentary inputs (Tappin *et al.,* 1993; Statham *et al.,* 1999; Bruland and Lohan, 2004; Morel *et al.,* 2004). Processes removing trace metals from seawater include active biological uptake or passive scavenging on either living or nonliving particulate material with subsequent sedimentation (Bruland and Lohan, 2004; Tappin *et al.,* 1993). Availability of trace metals in seawater is determined by their chemical speciation (van den Berg and Donat, 1992; Leal and van den Berg, 1998). Several metals are known to be complexed by natural organic ligands of as yet unknown composition (Leal and van den Berg, 1998; Sunda and Huntsman, 1998; Bruland *et al.,* 1991).

Emiliania huxleyi regularly forms extensive and intensive blooms in many coastal and oceanic regions (Brown and Yoder, 1994; Wilson *et al*., 2002b). The high reflectance of *E. huxleyi* blooms allows them to be captured by satellite imagery (Holligan *et al*., 1993; Tyrrell and Merico, 2004) which is caused by backscattering of light by the coccoliths detached from *E. huxleyi* cells (Gordon and Du, 2001). Coccoliths are shed continuously, however, when the cells die or lyse, large amounts of coccoliths become detached and the colour of the surrounding water turns milky white. It is in this period of the bloom that it can easily be captured by satellite imagery. High reflectance areas of blooms are likely to comprise of dead or dying *E. huxleyi* cells. One of the main mechanisms for the termination of *E. huxleyi* blooms is thought to be viral lysis (Wilson *et al*., 2002b, Schroeder *et al*., 2002).

Viruses are the most abundant biological constituents of the oceans (Bergh *et al.,* 1989) and are known pathogens of both heterotrophic and autotrophic marine organisms (Paul *et al.,* 1993; Furhman, 1999; Wilson *et al*., 2002 a). Viruses exert a strong influence over the diversity and abundance of phytoplankton communities by maintaining populations at non-blooming levels (Larsen *et al.,* 2001), and by causing the rapid demise of phytoplankton blooms (Wilson *et al.,* 2002 a,b). The majority of reports of virus-induced algal mortality in seawater have been for the coccolithophore *E. huxleyi* (Bratbak *et al.,* 1996; Wilson *et al.,* 2002a). Viruses were responsible for the demise of a reported *E. huxleyi* bloom in the English Channel in 1999 (Wilson *et al*., 2002b). Two virus strains, EhV-84 and EhV-86, that lyse cultures of the *E. huxleyi* host strain 1516, were isolated from this bloom in the English Channel. Several studies have shown that virus numbers increased following the demise of *E. huxleyi* bloom (Brussaard *et al*., 1996; Castberg *et al*., 2001; Jacquet *et al*., 2002, Schroeder *et al*., 2003, Martinez-Martinez *et al*., 2007). Interest in marine viruses stems not only from their ability to shape planktonic communities, but also from their impact on the biogeochemistry of the oceans (Furhman, 1999; Suttle, 2005).

As indicated above, the English Channel is an important transition zone between the continent and the open ocean. *E. huxleyi* blooms occur frequently in these regions in summer periods and it is thought that viruses are responsible for the demise of these blooms (Schroeder, pers. comm.). There are no known studies on trace metal speciation in a natural *E. huxleyi*-virus bloom dynamic cycle, and therefore this study is unique. The aim was to determine the effects of the *E. huxleyi* bloom termination on the speciation of trace metals in coastal water.

The aim of this study is to investigate trace metal speciation dynamics in a phytoplankton bloom in coastal waters. Previous work in Western English Channel (Tappin *et al*., 1993), the North Sea (Tappin *et al*., 1995) and in the Central English Channel (Statham *et al*., 1999) did not observe any depletion of metals concurrent with the depletion in nutrients. In addition, the aim was to determine whether correlations existed between findings from laboratory based culture experiments (Chapter 4) and those in the field.
This chapter presents results from surveys in the English Channel undertaken during *E. huxleyi* blooms in the summers of 2005 and 2006. First, the research site will be

introduced, followed by a brief description of the sampling regime, the parameters measured and method of analysis. The results and discussion section will describe and analyse the physical parameter profiles, and the distribution of chlorophyll *a, E. huxleyi*, *Synechococcus*, and viruses. Trace metal (Cu, Co, Ni and Zn) depth profiles during the sampling period will be interpreted, and the speciation of Cu will be discussed in detail.

5.3 Study site

The main study was conducted on the *RV Squilla*, the vessel from Plymouth Marine Laboratory (PML), on the 5th of August 2005 and 26th of July 2006 during *E. huxleyi* blooms in the Western English Channel. Sampling locations were based on satellite observations (Figure 5.1), courtesy of Dr Peter Miller (Remote Sensing Group, PML). Three stations were sampled during each survey. The survey in 2005 went from 49° 21.26′N 5°15.20′W (station 1, high satellite reflectance area) via 49° 30.17′N 5°08.08′W (station 2, edge of high satellite reflectance area) to 49° 40.01′N 5°05.54′W (station 3 outside the area of high reflectance). The 2006 survey went from 50° 11.58′N 4°19.98′W (station 1 outside the area of high reflectance) via 49° 54.72′N 4°39.66′W (station 2, edge of high satellite reflectance area) to 49° 32.22′N 5°14.46′W (station 3, high satellite reflectance area). Prior to each sample collection, a CTD cast was carried out to provide a depth profile of the temperature and salinity. In 2005, seawater was collected at 10 m intervals down to 60 m, and in 2006, seawater was collected at 5 m intervals down to 40 m depth.

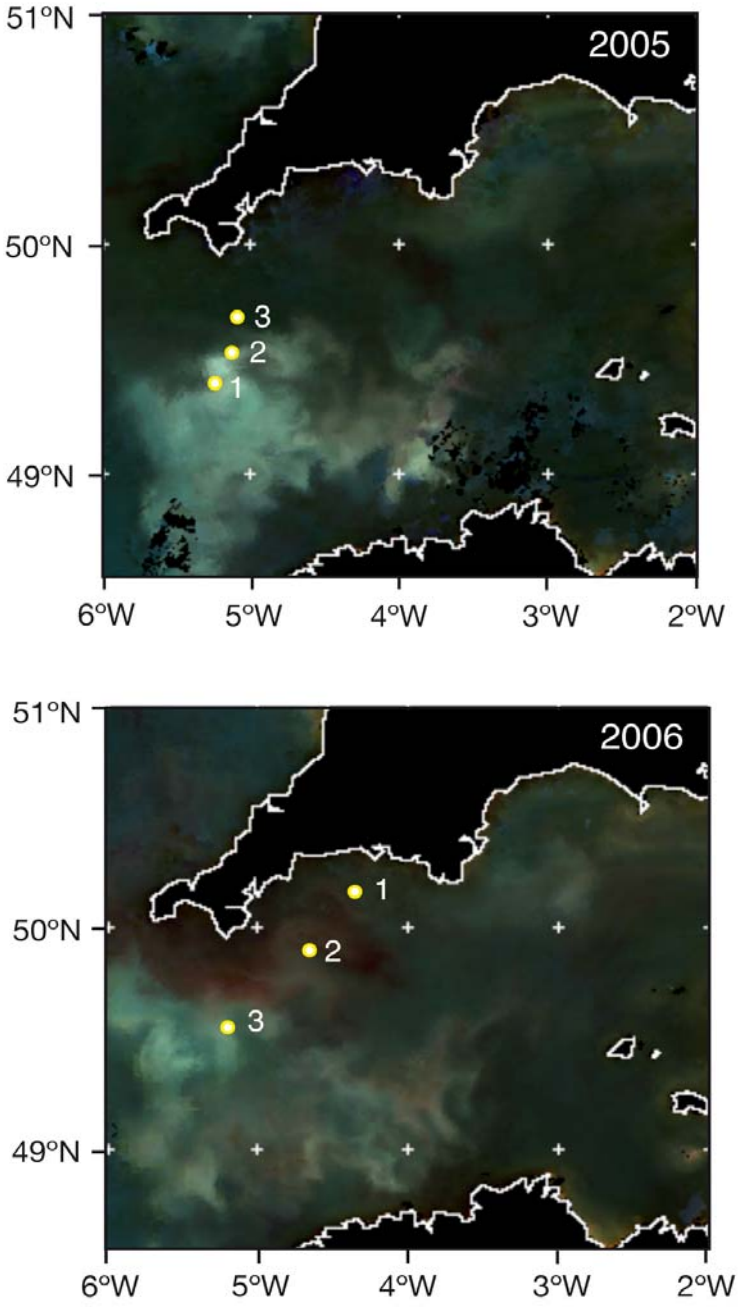

Figure 5.1: True colour satellite images of the high reflectance *E. huxleyi* blooms (2005-2006) south of Plymouth, UK. Sammpling track with approximate positions of stations: (a) 5th of August (2005) 49° 21.26´N 5°15.20´W (station 1, in high reflectance area); 49° 30.17´N 5°08.08´W (station 2, edge of high reflectance area); 49° 40.01´N 5°05.54´W (station 3 outside), (b) 26th of July 2006, 50° 11.58´N 4°19.98´W (station 1 outside); 49° 54.72´N 4°39.66´W (station 2, edge of high reflectance area); 49° 32.22´N 5°14.46´W (station 3, in high reflectance area). Satellite images courtesy of Plymouth Marine laboratory, UK, Remote Sensing Group.

5.4 Sampling and Analysis

5.4.1 Samples for chemical analyses:

Seawater samples were collected using 10 L Teflon-coated GoFlo bottles (General Oceanics) attached to a Kevlar Line. These GoFlo bottles are specifically used for trace-metal clean sampling of seawater at different depths (Statham *et al.,* 1999). The GoFlo samplers (supplied by National Marine Facilities Division in the National Oceanography Centre) were cleaned one week prior to the sampling in the laboratory using HCl (2% v/v, 24 h) rinsed 3 times by Milli-Q water and then covered with polyethylene sheets until used. Precautions against contamination were applied throughout the sampling and filtration processes. Details of sampling were given in section 3.2.3.3.

Samples for trace metal analysis were collected using acid cleaned low density polyethylene bottles (Nalgene). Each sample storage bottle was rinsed several times with the seawater sample before filling, and the collected samples were stored in re-sealable plastic bags. All trace metal sample handling and preparation was carried out in a laminar flow hood.

Samples were filtered using acid clean 0.4 µm polycarbonate filters (Whatman). Samples for the determination of total dissolved metals were acidified with quartz-distilled HCl to pH 2 and stored until analysis. Samples for labile dissolved metals were stored (4ºC) until analysis. Filters for quantification of total particulate metal concentrations in algae were stored in a Petri dish (-20^{o}C). Samples for nutrients and dissolved organic carbon measurements were collected using cleaned Pyrex glass bottles and filtered using glass fibre filters of 0.7 µm (Whatman).

5.4.2 Biological Samples

Samples for bacterial and viral enumeration were collected as described in Wilson *et al*. (2002 a&b). For each depth at each station, a 1.5 ml aliquot of seawater was collected and fixed with 0.5% glutaraldehyde (final concentration) and kept in a cool box at 4^{o}C. Upon return to the laboratory (within 24 h), the samples were stored frozen at – 80^{o}C until analysis (within two weeks). Samples for *E. huxleyi* cell and coccoliths counts, were collected in sterile Blue Max^{TM} 50 ml polypropylene conical centrifuge tubes (Becton

Dickinson Labware, USA) and maintained in the dark at ambient seawater temperature in covered flow-through tanks (Wilson *et al.,*2002 b). These samples were immediately analysed according to the methods described by Marie *et al.* (1999) and Wilson *et al.* (2002 a,b). Analyses were carried out courtesy of Dr Claire Evans, PML, using a Becton Dickinson FACSort flow cytometer. Full details of measurements were given in section 3.3.6.

5.4.3 Determination of chlorophyll *a*, nutrients, and DOC in the English Channel

Chlorophyll *a* analysis was carried out using a Turner Designs fluorometer (Welschmeyer, 1994). A detailed description of the analysis is provided in section 3.2.9. Nutrients were determined using a Burkard Scientific nutrient autoanalyser (model SFA-2). The colorimetric method used for nutrient analysis is described by Hydes (1984) and Hydes and Wright (1999). A more detailed description of the analysis and instrumentation is given in section 3.2.7. For the 2005 survey, ammonia measurements were undertaken using the spectrophotometric indephenol blue method (data courtesy of Malcolm Woodward (PML)).

The high temperature combustion (HTC) technique was used for DOC analyses (Badr *et al*., 2003). The system comprises a Shimadzu TOC 5000A, coupled to a Sievers nitrogen chemiluminescence detector (NCD) (section 3.2.8).

5.4.4 Determination of total particulate and dissolved total and labile trace metals in seawater

The determination of total and labile dissolved metals (Cu, Ni, Zn, Co) in seawater was performed using adsorptive cathodic stripping voltammetry (AdCSV). The voltammetric equipment used for the measurements has been described in section 3.2.5.4. The reagents and standards, together with the full details of AdCSV measurements, have been provided in sections 4.3.1 & 4.3.5. The analyses of certified reference material (CASS-4) for all metals were in good agreement with certified values (Table 3.3). Digestion of the filters and measurement of total particulate Cu using Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS) have been described in section 3.2.6.

5.4.5 Natural organic ligands and copper species in the English Channel

Concentrations of free aqueous Cu ions (Cu^{2+}) and natural organic ligands (L), together with the conditional stability constants (log K_{CuL}) of the ligands, in the filtered samples collected from Western English Channel were obtained using competitive ligand titrations with SA as added ligand (Campos and van den Berg, 1994), as described in section 4.3.6. The limit of detection for Cu by AdCSV using adsorptive collection of complexes with SA (0.1 nM) is lower than using catechol 0.2 nM (van den Berg, 1984), tropolone 0.4 nM (van den Berg and Donat, 1992) or oxine 0.2 nM (van den Berg, 1986). The high sensitivity of SA as a competitive ligand enhances the reliability of results obtained using the titrations.

In brief, eleven sample aliquots of 10 ml each were pipetted into polystyrene cups, pH buffer (0.01 M borate) and ligand (2 μM SA) were added. Copper was added in a range between 0-20 nM to the 2005 samples, and in a range between 0-30 nM to the 2006 samples. The solutions were equilibrated for at least 16 h at room temperature. The scanning parameters were as those reported for the determination of total dissolved Cu.
The sensitivity was calibrated using Cu additions (two or three depending on the relative peak heights) to the voltammetric cell containing the aliquot with the highest Cu concentration, where the ligands were saturated with Cu. The voltammetric measurement was conducted one minute later, thus allowing for equilibration of added Cu with SA. Triplicate titrations resulted in RSD of 1.48 nM and 1.50 for the ligand concentration and $LogK_{CuL}$, respectively.

5.5 Results and Discussion

5.5.1 Physical parameters

E. huxleyi blooms typically occur in stratified water (Nanninga and Tyrell, 1996) and under conditions of high irradiance (Tyrell and Merico, 2004). Indeed, during the *E. huxleyi* blooms, there was a well-developed thermocline at all stations during both surveys (Figures 5.2 & 5.3). The stratified region was characterised by warmer water overlying colder water of higher salinity (Figures 5.2 & 5.3, Tables 5.1 & 5.2). Qurban *et al*. (2004) noted that during the summer months, the Western Channel current speeds are relatively low and consequently the waters become thermally stratified. The seasonal

stratification and the stability of the water column influence the growth of phytoplankton (Tyrell and Taylor, 1996; Nanninga and Tyrell, 1996). In 2005 all stations had a thermocline at approximately 15 m depth and salinity did not vary significantly throughout the vertical profile resulting in weak haloclines.

Station 2 sampled during the 2006 survey had a deeper summer thermocline (25 m) compared to other stations and the stations sampled in 2005. The salinity profile for station 1 in 2006, was near constant, whereas a halocline was observed at stations 2 and 3. Note that the surface water temperature was approximately 2^{o}C warmer in the 2006 survey compared to 2005. Tables 5.1 & 5.2 show the physical data, nutrients and DOC in the 2005 and 2006 surveys.

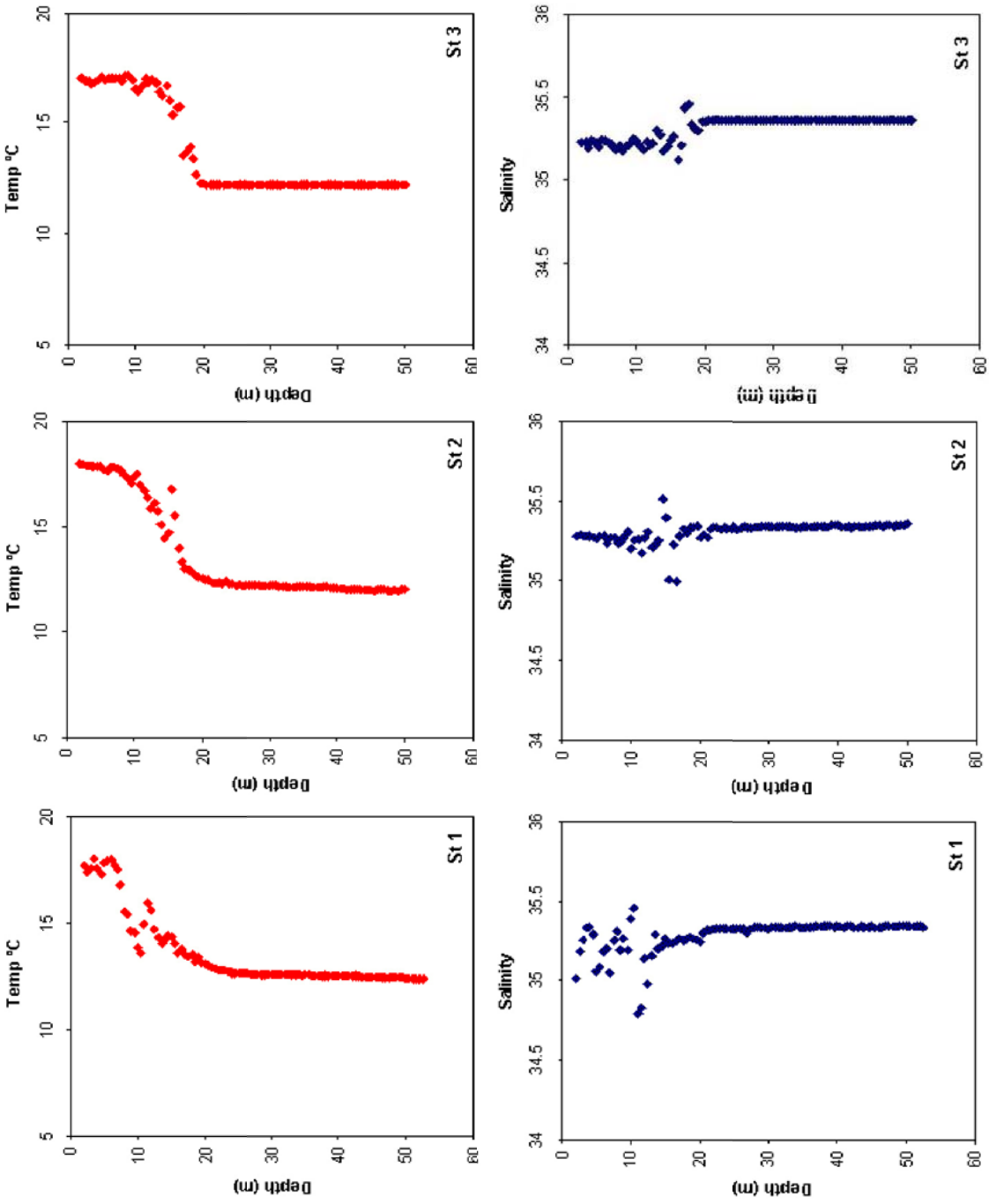

Figure 5.2: Temperature and salinity depth profiles in the Western English Channel, 2005 survey.

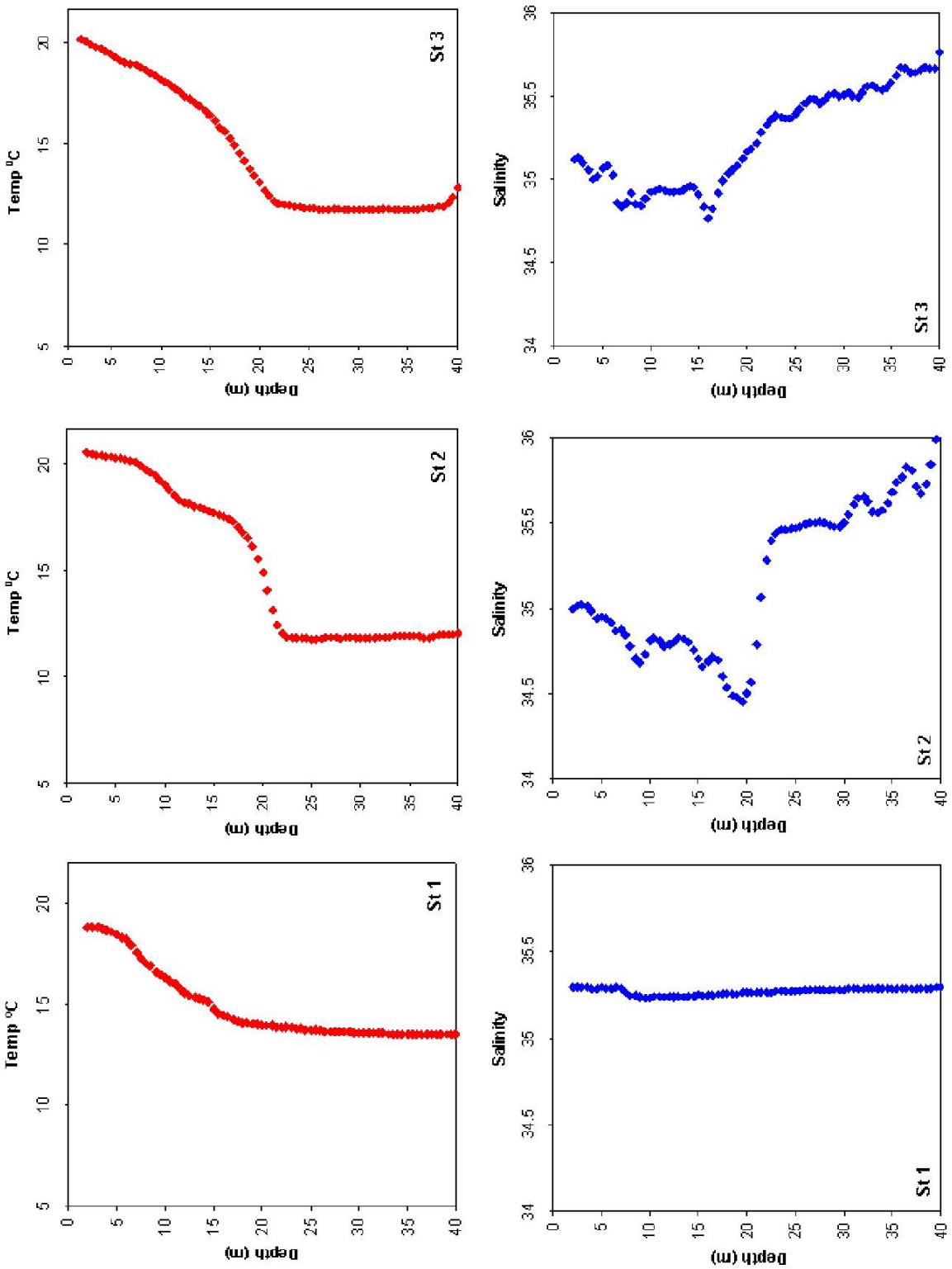

Figure 5.3: Temperature and salinity depth profiles in the Western English Channel, 2006 survey.

Table 5-1: Physical data (temperature and salinity) chlorophyll *a*, DOC and nutrients (NO_3, PO_4, NH_4) for depth profiles of 2005 survey.

station	Depth m	Temp °C	Salinity	Chlorophyll *a* µg L^{-1}	DOC µM	NO_3 µM	PO_4 µM	NH_4 µM
1	5	17.84	35.05	0.37	206.13	<0.02	0.08	0.29
	10	13.82	35.38	0.61	123.50	0.2	<0.01	0.08
	20	13.01	35.24	1.61	95.23	<0.02	<0.01	0.14
	40	12.51	35.33	0.38	94.55	2.5	0.1	0.27
	60	12.38	35.33	0.11	81.76	2.4	0.1	<0.05
2	5	17.85	35.26	0.52	132.14	<0.02	<0.01	<0.05
	10	17.36	35.2	0.77	199.71	<0.02	<0.01	0.05
	40	12.07	35.34	3.45	129.25	<0.02	<0.01	<0.05
	60	12	35.35	0.09	132.67	1.7	0.04	<0.05
3	5	17.05	35.25	0.51	136.05	<0.02	<0.01	<0.05
	10	16.48	35.23	0.4	171.32	<0.02	<0.01	<0.05
	20	12.23	35.35	0.26	113.56	1.5	0.03	0.2
	40	12.17	35.35	0.03	107.64	1.5	0.08	0.2
	60	12.16	35.35	0.25	114.32	2.2	0.09	0.3

Table 5-2: Physical data (temperature and salinity) chlorophyll *a*, DOC and nutrients (PO_4,) for depth profiles of 2006 survey.

station	Depth m	Temperature °C	Salinity	Chlorophyll*a* µg L^{-1}	DOC µM	Phosphate µM
1	1	18.84	35.39	0.30	91.37	0.1
	5	18.43	35.29	0.48	89.16	0.03
	10	16.27	35.23	0.67	197.28	<0.03
	15	14.77	35.23	0.95	110.73	<0.03
	25	13.72	35.27	0.73	98.46	<0.03
	40	13.47	35.29	0.80	78.04	<0.03
2	1	20.48	35	3.02	109	0.1
	5	20.27	34.94	3.70	224.93	0.12
	10	18.98	34.81	3.02	102.9	0.25
	15	17.65	34.7	1.87	98.99	0.27
	25	11.77	35.47	1.70	75.53	0.36
	40	12.03	36.12	0.34	242.43	0.41
3	1	20.18	35.11	0.63	107.58	0.08
	5	19.42	35.06	0.52	85.93	0.07
	10	18.2	34.91	0.59	116.63	<0.03
	15	16.4	34.91	3.41	134.96	<0.03
	25	11.8	35.4	2.91	132.88	0.18
	40	12.77	35.76	0.54	85.56	0.31

5.5.2 Results for Biological measurements

5.5.2.1 Chlorophyll a

The concentrations of chlorophyll *a* measured in the summer of 2005 and 2006 are shown in Figure 5.4. Chlorophyll *a* concentrations were lowest at the bottom of the profiles as a result of light limitation of the phytoplankton. In the 2005 survey, chlorophyll *a* ranged from 0.03 μg L^{-1} to a maximum of 3.45 μg L^{-1} at 40 m (station 2, edge of the high reflectance area). The chlorophyll *a* levels at each station in 2005 showed different profiles (Figure 5.4), with the highest concentrations at the edge of the high reflectance area. High reflectance areas caused by backscattering of light by the detached coccoliths represent regions of inactive cells (Wilson *et al*., 2002 b). Higher chlorophyll *a* concentrations are expected to be observed at station 2 as more active cells were present (this will be discussed later in the chapter).

Chlorophyll *a* concentrations were higher during the survey in 2006 compared with 2005. The highest concentrations of chlorophyll *a* concentrations were observed at station 2, reaching a maximum of 3.70 μg L^{-1} at 5 m depth. High concentrations of chlorophyll *a* were also recorded at 15 m at station 3 (3.41 μg L^{-1}; station situated within the high reflectance area,). The lowest chlorophyll *a* concentration was observed at station 1, which was outside the high reflectance area.

A recent investigation in the Western English Channel (Qurban, 2008) observed that summer sea surface chlorophyll *a* ranged around 4-6 μg L^{-1}, which is in good agreement with the highest chlorophyll *a* observed in the current study. These values agree well with values reported in the literature (Wilson *et al*., 2002 b) for samples collected during a demise of an *E. huxleyi* bloom in summer 1999 (at station 1 in high reflectance area).

Tappin and Reid (2000) found that, during the summer months, a chlorophyll *a* maximum is located at the pycnocline separating surface nutrient poor waters from nutrient rich deep waters. Exceptionally, large blooms of algae can occur in the Western channel when conditions are favourable (Tappin and Reid, 2000), including low winds and strong stratification. This matches the situation in the current study, where on both occasions the chlorophyll *a* maxima occurred at in the upper 40 m.

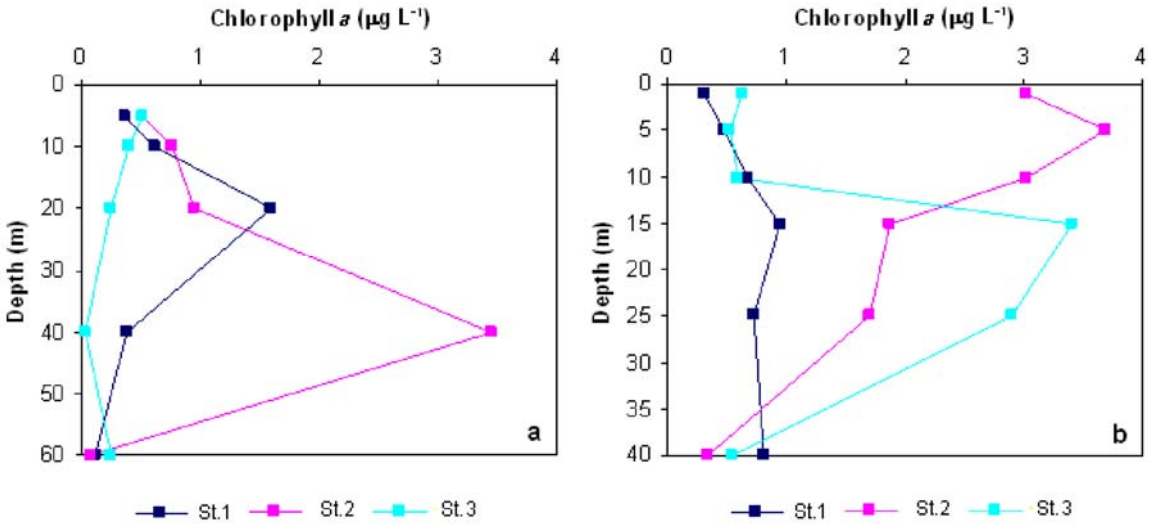

Figure 5.4: Chlorophyll *a* (µg L^{-1}) depth profiles in the 2005 (a) and 2006 (b) surveys.

5.5.2.2 Viruses and Bacteria

None of the viruses detected using AFC exhibited a signature typical of the *E. huxleyi* viruses (section 4.4.2). Similarly, AFC analysis of viruses detected by Evans (2004) from samples collected from the English Chanel summer 2001 did not show a signature of *E. huxleyi* viruses. We were only able to detect smaller bacteriophage -like viruses (Figure 5.5a). Circle indicates heterotrophic bacteria detected from samples collected during 2005-2006 survey's (Figure 5.5b)

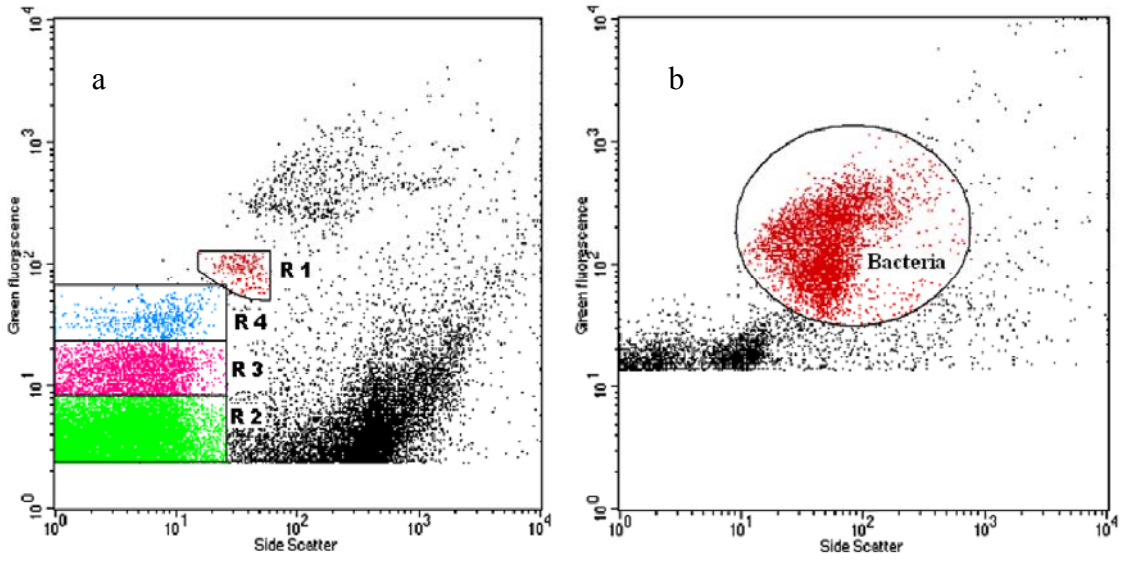

Figure 5.5: Examples of samples collected from the Western English Channel and analysed by flow cytometry: (a) detected viruses groups (R2,R3,R4) are bacteriophage viruses and R1 is unidentified group; (b) heterotrophic bacteria circled.

The distribution of viruses and bacteria during the summer of 2005 and 2006 can be found in Figures 5.6 and 5.7, respectively. The concentration of viruses detected during

summer 2005 ranged from 2.69 x 10^7 ml^{-1} to 6.51 x 10^7 ml^{-1} and was generally highest near the surface and decreased with depth. All stations exhibited an approximately similar viral abundance. A comparable distribution was observed for bacteria and the concentrations were one order of magnitude lower than those of viruses.

For the summer 2006 survey, virus particle concentrations ranged from 4.4 x 10^6 ml^{-1} to 2.6 x 10^8 ml^{-1} and were also high in the surface waters and decreased with depth. Maximum virus concentrations were observed at station 2 (edge of the *E. huxleyi* reflectance area), followed by station 3 (inside the *E. huxleyi* reflectance area), and the lowest virus concentrations were found at station 1 (outside the *E. huxleyi* reflectance area). Bacteria did not correlate well with the distribution of viruses, but the highest concentrations were observed at station 2.

In general, the virus concentrations observed in this study were within the range expected for a marine ecosystem (Wilson *et al.,* 2002 a; Suttle, 2005). Wilson *et al.* (2002 b), reporting on large viruses, and Evans (2004), reporting on small viruses, observed total virus concentrations in the English Channel during *E. huxleyi* bloom similar to the concentrations observed in this study. However, virus concentrations were one order of magnitude higher in the current study at station 2 (edge of the *E. huxleyi* high reflectance area, 2006) compared to the other stations. No significant statistical relationship was observed between viruses and chlorophyll *a* in 2005. However, a positive relationship (R^2= 0.44) was observed in 2006 survey. Viruses and biological findings for the 2005 and 2006 surveys are shown in Tables 5.3 & 5.4.

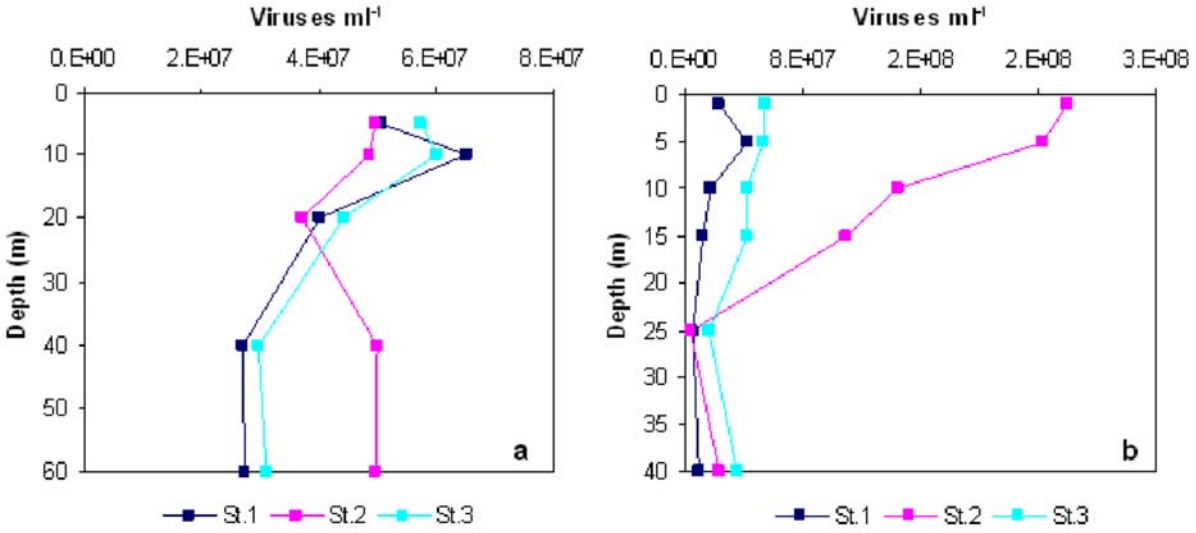

Figure 5.6: Depth profiles of viruses (ml^{-1}) for 2005 (a) and 2006 (b) surveys.

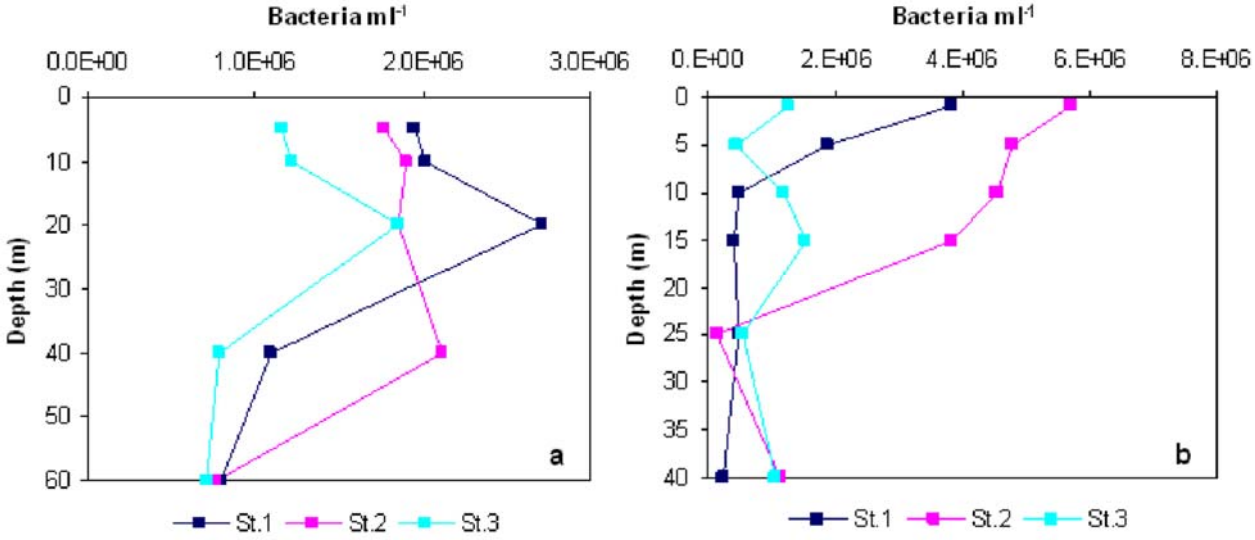

Figure 5.7: Depth profiles of bacteria (ml^{-1}) for 2005 (a) and 2006 (b) surveys.

5.5.2.3 E. huxleyi and Synechococcus Distribution

Figure 5.8 show *E. huxleyi* profile in the 2005 and 2006 surveys. *E. huxleyi* concentrations were highest at station 2 (edge of reflectance area) in 2005, ranging from less than 100 to a maximum of 1010 cells ml^{-1} at 40 m. The highest concentrations of *E. huxleyi* were correlated with the observed highest chlorophyll *a* at 40 m for the three stations. *E. huxleyi* cell numbers dropped at station 3. Concentrations at station 1 were between 61 to 494 cells ml^{-1}, and decreased with depth at all stations. Detached coccolith concentrations were low outside the high reflectance areas at stations 2 and 3, ranging between 1.21 x 10^4 and 6.90 x 10^4 coccoliths ml^{-1}. Inside the high reflectance areas, the concentrations were one order of magnitude higher (1.21 x 10^5 coccoliths ml^{-1}). These findings agree with those of Wilson *et al.* (2002 b) regarding *E. huxleyi* cell concentrations and coccoliths during an *E. huxleyi* bloom in the Western English Channel in 1999.

The summer 2006 survey showed higher *E. huxleyi* cell concentrations compared to the 2005 survey. The highest cell numbers were observed at station 3 (inside the high reflectance area), ranging from 1200 to 2250 cells ml^{-1}, and coinciding with the highest chlorophyll *a* concentrations recorded at 15 and 25 m. In the surface waters at station 2, *E. huxleyi* cell numbers were highest (2450 cells ml^{-1}) for all stations sampled in 2006, and that also coincided with a high chlorophyll *a* concentration. Coccoliths data for the 2006 surveys are unavailable. A significant positive trend was observed between *E. huxleyi* cell numbers and chlorophyll *a* in 2005 (R^2= 0.66) whereas a weaker relationship (R^2 = 0.20) was recorded in 2006.

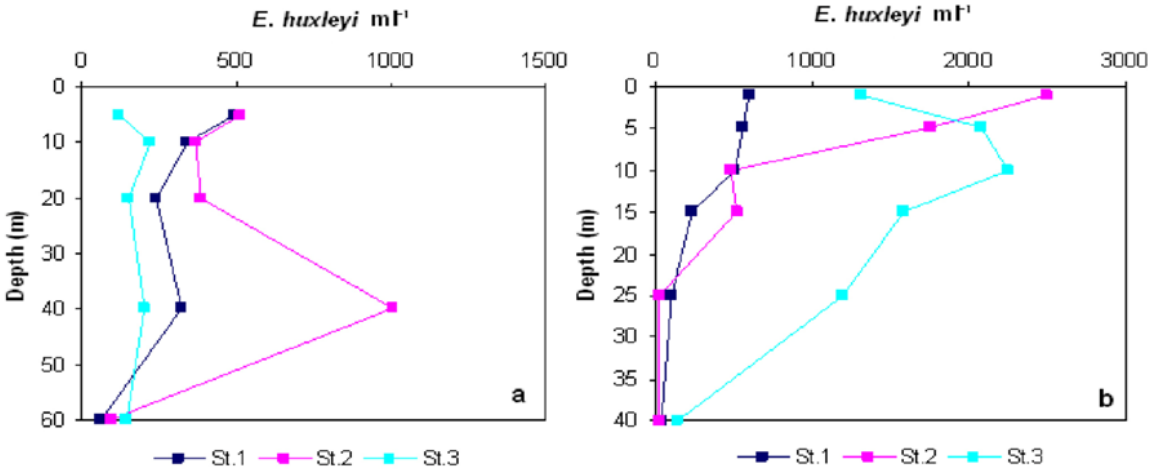

Figure 5.8: Depth profiles of *E. huxleyi* cells (ml^{-1}) concentrations determined by AFC: (a) 2005, (b) 2006.

The cyanobacterium *Synechococcus* dominated the phytoplankton community (with respect to cell numbers) during both surveys (Figure 5.9). The highest concentrations of *Synechococcus* were observed at station 1, 2005, reaching 1.17 x 10^5 cells ml^{-1} and were one order of magnitude lower at other stations. Concentrations decreased with depth. The highest concentrations of *Synechococcus* during 2006 were for example found at 25 m depth at station 3 (1.17 x 10^5 cells ml^{-1}) and at depths of 1, 5 and 10 m at station 2 (5.52-6.89 x 10^4 cells ml^{-1}). Synechococcus numbers were somewhat lower during the 2006 survey. Muhling *et al* (2005) reported *Synechococcus* abundance in the Gulf of Aqaba, Read Sea over an annual cycle with a maximum cell density of 3.4 x 10^4 ml^{-1}.

The *Synechococcus* density in the current study coincided with the highest chlorophyll *a* and the highest concentrations of *E. huxleyi* (Tables 5.3 & 5.4). This was confirmed by the positive trend observed between *Synechococcus* density and chlorophyll *a* in 2006 (R^2= 0.44).

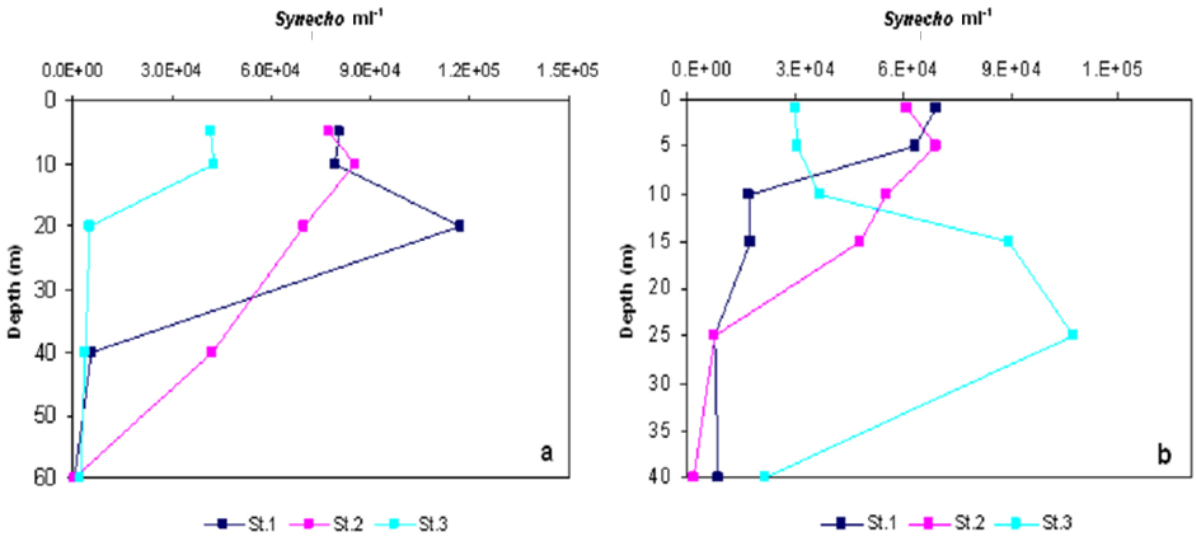

Figure 5.9: Depth profiles of *Synechococcus* (particles ml^{-1}) concentrations through the water column determined by AFC: (a) 2005, (b) 2006.

Table 5-3: Biological data (*Synechococcus*, *E. huxleyi*, Coccoliths, virus, and bacteria ml^{-1}) determined by AFC for 2005 survey.

Station	Depth m	*Synechococcus* cells ml^{-1}	*E. huxleyi* cells ml^{-1}	Coccoiths ml^{-1}	Virus ml^{-1}	Bacteria ml^{-1}
1	5	8.06E+04	494	1.22E+05	5.07E+07	1.96E+06
	10	7.92E+04	344	1.05E+05	6.51E+07	2.01E+06
	20	1.17E+05	239	4.97E+04	4.03E+07	2.71E+06
	40	5.66E+03	320	1.92E+04	2.69E+07	1.10E+06
	60	4.17E+02	61	9.59E+03	2.72E+07	7.97E+05
2	5	7.71E+04	514	6.53E+04	4.95E+07	1.77E+06
	10	8.52E+04	364	7.12E+04	4.86E+07	1.90E+06
	40	4.24E+04	1008	7.21E+04	5.00E+07	2.11E+06
	60	5.47E+02	97	1.44E+04	4.96E+07	7.70E+05
3	5	4.19E+04	121	1.61E+04	5.74E+07	1.15E+06
	10	4.25E+04	219	1.46E+04	6.03E+07	1.22E+06
	20	5.14E+03	150	1.28E+04	4.41E+07	1.85E+06
	40	3.57E+03	202	7.20E+03	2.96E+07	7.81E+05
	60	2.12E+03	146	9.95E+03	3.12E+07	7.13E+05

Table 5-4: Biological data (*Synechococcus*, *E. huxleyi*, virus, and bacteria ml^{-1}) determined by AFC for 2006 survey.

Station	Depth	*Synechococcus* cells ml^{-1}	*E. huxleyi* cells ml^{-1}	Virus ml^{-1}	Bacteria ml^{-1}
1	1	6.90E+04	603	2.39E+07	3.81E+06
	5	6.33E+04	557	4.19E+07	1.88E+06
	10	1.68E+04	503	1.74E+07	4.62E+05
	15	1.74E+04	233	1.29E+07	4.24E+05
	25	7.81E+03	110	5.04E+06	4.86E+05
	40	8.65E+03	40	9.20E+06	2.34E+05
2	1	6.11E+04	2497	2.60E+08	5.72E+06
	5	6.89E+04	1750	2.43E+08	4.79E+06
	10	5.52E+04	487	1.45E+08	4.55E+06
	15	4.78E+04	523	1.09E+08	3.82E+06
	25	7.84E+03	27	4.43E+06	1.48E+05
	40	1.63E+03	20	2.36E+07	1.12E+06
3	1	2.98E+04	1310	5.42E+07	1.27E+06
	5	3.05E+04	2076	5.38E+07	4.58E+05
	10	3.70E+04	2250	4.27E+07	1.19E+06
	15	8.95E+04	1580	4.30E+07	1.55E+06
	25	1.07E+05	1196	1.68E+07	5.58E+05
	40	2.17E+04	140	3.59E+07	1.05E+06

Other phytoplankton determined by AFC in samples collected during the 2005 survey included dinoflagellates, nanophytoplankton, cryptophytes and picoeukaryotes. The concentrations of these organisms increased from stations 2 to 3, inside the high reflectance area (data not shown). Dinoflagellates were most numerous at station 1 (20 m, 2963 particles ml^{-1}) and station 2 (40 m, 1935 particles ml^{-1}), which correlated with the highest observed chlorophyll *a* concentration (3.45 µg L^{-1}) and *E. huxleyi* cell numbers. For the 2006 survey, picoeukaryotes and nanoflagellates were most abundant at the edge of the high reflectance area (station 2). High cell numbers at depths of 1, 5 and 10 m coincided with the enhanced chlorophyll *a* concentrations and high *E. huxleyi* cell numbers.

In summary of this section, maximum chlorophyll *a* concentrations at each station in both years were observed at different depths in the Western English Channel. Chlorophyll *a* concentrations and *E. huxleyi* cells concentrations were correlated and higher in 2006 compared to 2005. *Synechococcus* dominated the phytoplankton community in both years. It can be concluded that at the time of sampling, the *E. huxleyi* bloom was being succeeded by *Synechoccocus* in both years.

None of the viruses detected exhibited an AFC signature typical of *E. huxleyi* viruses (see section 4.4.2; Figure 4.3). As the *E. huxleyi* bloom was being succeeded by *Synechoccocus,* this observation that the virus community was dominated by cynophage and not *E. huxleyi* viruses is not to surprising. Consequently, its highly likely that the *E. huxleyi* viruses were present at concentrations below the limit of AFC detection. Wilson *et al.* (2002 b) The findings of (Wilson *et al*., 2002 b) during demise of *E. huxleyi* bloom observed in Western English Channel showed strong evidence for the role of viruses in bloom decimation in these waters. It was reported that at stations inside the bloom area, concentrations of viruses, bacteria and free coccoliths were higher, whereas cell counts were lower compared to other stations outside the high reflectance area. This indicates that viruses induced lysis of *E. huxleyi* occurs because of large concentrations of free coccoliths detaching from cells. This was also supported by the isolation of two viruses, EhV-84 and EhV-86 (used in cultures, chapter 4), from the high reflectance area, which lysed cultures of the *E. huxleyi* host strain CCMP1516 (Schroeder *et al*., 2002 & 2003 Wilson *et al*., 2002 b). Indeed, as was described earlier, detached coccolith counts were highest at station 1 in 2005. Molecular evidence has confirmed the presence of EhVs in

these blooms (Schroeder, pers. comm.). The low level of EhVs indicates that the biology had progressed on and another virus-host population had been established.

During an *E. huxleyi* bloom in the North Sea (Wilson *et al.,* 2002a), it was reported that viruses played a minor role in *E. huxleyi* mortality and that microzooplankton out-competed viruses for algal prey/hosts. However, new molecular based evidence suggests that viruses did in fact play an important role in terminating this bloom (Schroeder, pers. comm.). Moreover, high levels of visibly infected *E. huxleyi* at the same location were observed by Brussaard *et al.* (1996), which indicated that viruses may be linked to the decline of *E. huxleyi* blooms. As discussed earlier, studies have reported that viruses were the cause for the decline of *E. huxleyi* populations despite previous reports linking microzooplankton grazing as more important in the demise of blooms than viruses (Wilson *et al.,* 2002a).

Indeed, in both surveys, *E. huxleyi* concentrations were relatively low (i.e. high reflectance are where large *E. huxleyi* bloom occurred at some stage), with maximum concentrations of only just over 2000 cells ml^{-1} in 2006 at stations 2 and 3, and over 1000 cells ml^{-1} in 2005 at station 2. Therefore, this represents in both years a completely senescent bloom [corresponding to a situation many days after the end of the lab experiment (Chapter 4)] and not a bloom in the initial phases of senescence (equivalent to the start of the crash in the lab experiment).

Much higher *E. huxleyi* concentrations of up to 6000 cells ml^{-1} were observed by Head *et al.* (1998) in the North Sea. Furthermore, *E. huxleyi* concentrations up to two orders of magnitude greater than this were observed in coastal mesocosm studies (Jacquet *et al*., 2002) and in open water sites (Holligan *et al*., 1993). Wilson *et al.* (2002 a) argued that viruses would play a much more important role towards the end of a bloom when virus concentrations would reach the threshold level required to cause termination of that bloom. Indeed mesocosm experiments (e.g. Jacquet *et al*., 2002) have revealed that high concentrations of *E. huxleyi* virus-like particles have been observed immediately after the demise of *E. huxleyi* populations.

It should also be noted that AFC is routinely used to detect 'free' viruses in the water column. Hence, viruses in infected cells are not included in total counts. Sampling of the

same area prior to the succession of *E. huxleyi* by *Synechococcus* might have revealed an increase in those viruses (EhV-86). Therefore, different results for virus numbers may have been achieved if sampling had been conducted throughout different stages of the bloom. This is always difficult to assess, as it is not often logistically possible to be on a ship throughout the progression of a bloom. Evans (2004) suggested that it is possible that *E. huxleyi* in addition to being infected by members of the family *Phycodnaviridae* (e.g. EhV-86*)*, which are easily identified by AFC, can also be infected by other viruses which have different characteristics. In summary, during the current study in the English Channel, it was highly probable that viral infection had a major role in the decline of *E. huxleyi* bloom but it was not possible to distinguish the causative viral agent from the overall virus community or it may be that sampling was conducted too late and the typical coccolithovirus had dropped below the limits of AFC detection.

5.5.3 Results of Chemical analyses

5.5.3.1 DOC in the Western English Channel

Dissolved organic carbon (DOC) and nutrient concentrations for the 2005 and 2006 surveys are shown in Tables 5.1 and 5.2. DOC concentrations ranged between 82-206 μM in 2005 (Figure 5.10). A general trend of a decrease in DOC concentrations with depth can be identified, the highest concentrations were found at all stations in the near surface waters. Maximum DOC concentrations in the 2005 surveys (206 μM) were observed at station 1 (in the high reflectance area) and so coincided with enhanced phytoplankton productivity. This was also coincided with the highest liths (1.22×10^5 ml^{-1}) observed in the first 10 m of the water column. Minimum DOC concentrations were typically obtained at depth at all stations sampled in 2005. DOC concentrations in 2006 fell into a similar range as for 2005 (Table 5.1). Concentrations ranged between 75-225 μM and the highest concentrations (225 μM) were observed at station 2 (edge of the high reflectance area, Figure 5.10). This coincided with the highest chlorophyll *a* concentration and the highest *E. huxleyi* cell numbers (Table 5.4) which were observed at depths between 5 and 15 m. Similar observations were made at depths 15 and 25 m at station 3 (in the high reflectance area). No significant statistical relationship was observed between DOC and biological parameters. The high levels of DOC observed in this study can be attributed to phytoplankton production dominating DOC dynamics through the sampling period. In terms of DOC concentrations, Aminot and Kerouel

(2004) reported concentrations of between 61 and 62 µM in the surface waters of the North East Atlantic (Bay of Biscay), which are lower than the concentrations observed in the present study. However, higher concentrations (81 to 83 µM) for the North West Atlantic were reported in bloom conditions (Chen *et al.,*1996). Pan *et al.* (2005) reported DOC concentrations between about 65 and 88 µM in samples collected from the North Sea.

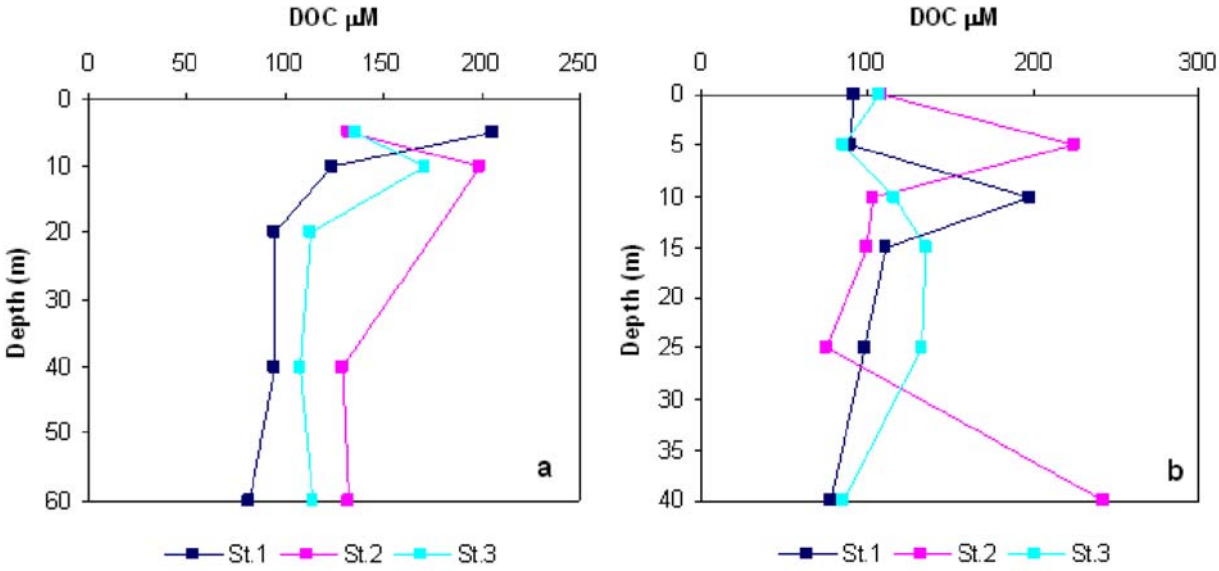

Figure 5.10: Depth profile of DOC (µM) through the water column a) 2005, b) 2006.

It has been reported that a DOC peak can be associated with a chlorophyll *a* maximum (Kepkay and Wells, 1992). This was observed in the 2006 surveys, where the highest DOC correlated with the chlorophyll maximum at station 2. An increase in DOC also accompanied an increase in chlorophyll *a* and highest *E. huxleyi* cell number at station 3. However this was not observed in the 2005 surveys as the chlorophyll maximum occurred at greater depths in comparison to DOC.

Figure 5.11 does show a weak positive correlation between DOC and chlorophyll *a* in both years (stronger in 2006). Kepkay and Wells (1992) stated that there is no simple relationship between DOC and chlorophyll *a*. The highest DOC concentrations observed at different depths is most likely due to enhanced primary productivity at these stations. Kirchman *et al.* (1991) reported that the breakdown of DOC is undertaken by bacteria.

Packard *et al.* (2000) demonstrated that bacterial remineralisation dominated DOC dynamics after a phytoplankton bloom had occurred, and that DOC generation by zooplankton feeding could serve to offset bacterial degradation at night.

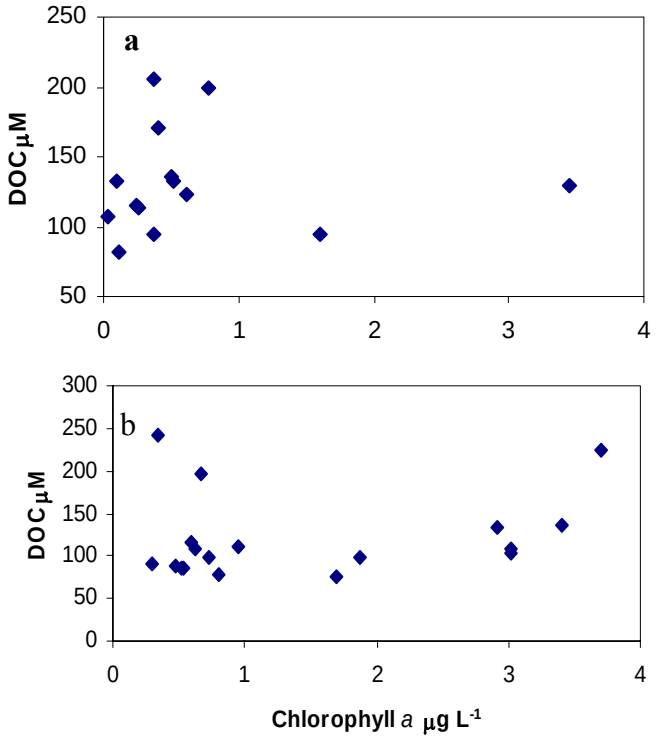

Figure 5.11: Correlation between chlorophyll *a* and DOC, a) 2005 and b) 2006.

5.5.3.2 Nutrients

The nutrients observed in the English Channel exhibited important variations in concentrations, which can be attributed to the variation in productivity in the water column. All nutrients increased down the water column, due to phytoplankton uptake in the surface waters, and light limited productivity combined with remineralisation of sinking organic debris at depth.

Phosphate concentrations in the Western English Channel during the 2005 survey were between below detection limit, <0.01 µM and 0.1 µM (Table 5.1). Higher phosphate concentrations were observed at deeper depths at all stations. Concentrations were generally low and agreed well with the sea surface values reported by Tappin *et al.* (1993). Qurban (2008) reported low surface phosphate concentrations (< 0.05 µM) in the Western English Channel in summers 2003-2004, which are close to the values reported in the present study. However, higher concentrations (0.39 to 0.91 µM) were reported in

the central English Channel (Bentley *et al*. 1999). This was thought to be caused by freshwater inputs.

E. huxleyi has been shown to grow efficiently at low phosphate concentrations (Egge and Heimdal, 1994). Tyrell and Taylor (1996) reported phosphate values between 0.18 and 0.2 µM in the North Atlantic. Lower phosphate (0.04 to 0.1) was observed in the English Channel (Garcia-Soto *et al*., 1995) during late stages of an *E. huxleyi* bloom.

Phosphate concentrations in 2006 varied between below detection limit and 0.41 µM (Table 5.2). Concentrations increased with depth at stations 2 and 3. Low surface concentrations coincided with the highest chlorophyll *a* and highest *E. huxleyi* observed at similar depths. The nitrate vs phosphate (2005) plot (Figure 5.12) shows a strong positive correlation.

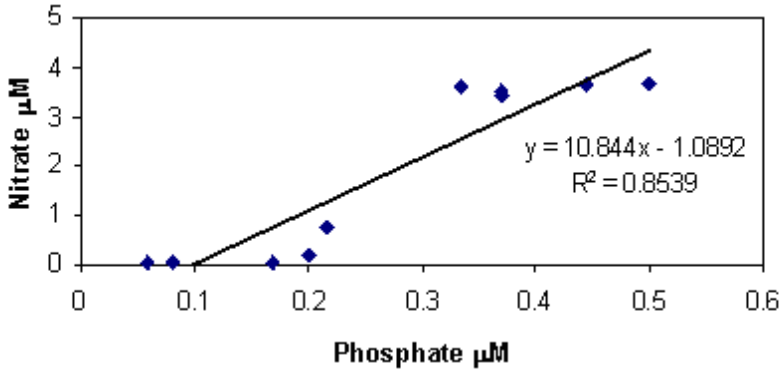

Figure 5.12: Relationship between phosphate and nitrate for 2005 survey.

Nitrate concentrations varied between below the detection limit to 0.20-2.50 µM (Table 5.1). Bentley *et al.* (1999) reported nitrate concentrations in the Central English Channel between 8 and 14 µM, which is higher than the concentrations observed in this study. The lower maximum observed in this study, is likely due to the shallower sampling depths compared with the Bentley (1998) study. However, nitrate remained between 2 and 5 µM during an oceanic North Atlantic *E. huxleyi* bloom (Tyrell and Taylor, 1996), which is in close agreement with the values reported in the current study. Tappin *et al.* (1993) sampled a station in the English Channel at a similar position to that in the current study; they found low nitrate concentrations in summer, in agreement with current observations. Stations sampled in winter in the English Channel by Tappin *et al*. (1993) showed higher nutrients concentrations compared with summer, which is an indication of

higher primary productivity and consequent nutrient uptake in the summer months. A recent survey in the Western English Channel by Qurban (2008) observed highest surface nitrate concentrations in early spring, with a maximum of 9 μM in March 2003, and a minimum of 0.1 μM in summer. Nitrate concentrations increased in winter (December) to 2.84 μM due to winter mixing (Qurban, 2008).

E. huxleyi has two alkaline phosphatase systems and the highest observed affinity for inorganic phosphate for a phytoplankton species. Consequently it is able to out-compete other algal groups for P at levels down to the nM range (Riegman *et al.,* 2000). This was documented in this study where phosphate concentrations were low, and actually below detection limit at many depths. Phosphate concentrations were low in the current study, compared to reported literature values. In addition nitrate concentrations were relatively low in surface waters and increased with depth. The nitrate: phosphate ratio was not calculated, as many concentrations either for phosphate or nitrate were below the detection limit.

Ammonium (NH_4^+) concentrations for the 2005 survey ranged from below detection limit to 0.05-0.29 μM (Table 5.1). Bentley *et al.* (1999) reported higher values (0.5 to 0.8 μM) for Central Atlantic Ocean surface waters than the concentrations observed in this study. Ammonium is the preferential source of nitrogen for most species of phytoplankton and inhibits the utilization of nitrate (Varela and Harrison, 1999). Skoog *et al.* (2001) reported that the highest NH_4^+ concentrations were found within bloom waters and that this is indicative of the recycling of organic nitrogen from the degradation of dissolved or particulate organic matter fractions.

Nutrient limited *E. huxleyi* cells can acquire extra layers of coccoliths, possibly allowing them to sink to greater depths to access nutrient-richer waters (Paasche, 2001). This was demonstrated at station 2 (40 m) for the 2005 survey where the chlorophyll maximum was found at greater depths in the water column, with high nutrient concentrations and *E. huxleyi* cell numbers. Similarly, the highest chlorophyll observed in 2006 coincided with the highest *E. huxleyi* cell numbers. A lack of nitrate data for the 2006 survey makes it difficult to link cell numbers to the nutrient status of the waters. However, phosphate concentrations were relatively high at deeper depths, where high chlorophyll *a* and *E. huxleyi* cell numbers were also observed.

Blooms of *E. huxleyi* almost always occur in areas where nitrate and/or phosphate concentrations are very low (Holligan *et al*., 1993; Buitenhuis *et al*., 1996; Tyrrell and Merico, 2004). From these findings it has been argued that *E. huxleyi* is able to build up its massive blooms by out competing other phytoplankton species at levels of inorganic P down to the nM range (Riegman *et al*., 2000; Egge and Heimdal, 1994).

The ability to grow at low nutrient concentrations was confirmed by Lessard *et al*. (2005), who showed that there is no strong link between high N:P ratios and the occurrence of *E. huxleyi* blooms. They suggested that most *E. huxleyi* blooms occurred in N limiting waters, which is enabled by the ability of *E. huxleyi* to use non-nitrate forms of N (amino acids, amides and urea) (Palenik and Henson, 1997), and under low P conditions due to its exceptional P acquisition capacity (Riegman *et al*., 2000).
However, other studies using mesocosms have reported that *E. huxleyi* is able to grow well under both high and low nutrient concentrations. Wal *et al*. (1994) found that different nutrient regimes in mesocosm enclosures had no influence on the gross growth rate of *E. huxleyi* populations. Furthermore, blooms in the Eastern Bering Sea under enhanced P concentrations (Olson and Storm, 2002) offer evidence that phosphate limitation is not a prerequisite for *E. huxleyi* bloom formation. Lessard *et al*. (2005) believe that ecosystem modelling related to *E. huxleyi* should not contain a dependency on a high N:P ratio as a trigger for blooms of this species.

5.5.4 Trace metals in the Western English Channel

Adsorptive cathodic stripping voltammetric methods have been applied to studies in seawater of the speciation of Cu (van den Berg, 1984; Moffett *et al*., 1990), Zn (Ellwood and van den Berg, 2000; Lohan *et al*., 2002) and Co (Ellwood and van den Berg, 2001). Typically more than 99% of Cu in marine waters exists as organic complexes (Coal and Bruland, 1998). Approximately 98% of dissolved Zn in surface waters is complexed with organic ligands (Ellwood and van den Berg, 2000; Lohan *et al*., 2002). More than 90% of Co is complexed to strong cobalt-binding organic ligands (Ellwood and van den Berg, 2001). The number of studies looking at the speciation of trace metals during phytoplankton blooms is limited because both of the difficulty of capturing blooms in the field and attributing any observed changes in the metal concentrations to bloom processes (Luoma *et al.,* 1998).

5.5.4.1 Copper

Organic ligands play a key role in the speciation of dissolved Cu in seawater, with a large fraction (80->99%) being complexed by natural ligands (Buck and Bruland 2005; Coal and Bruland 1998; van den Berg and Donat 1992; Moffett *et al.,* 1990). Ligands produced by cyanobacteria are thought to contribute to the complexation of Cu (Moffett and Brand, 1996). Luoma *et al.* (1998) found that dissolved Cu was not depleted during a diatom bloom, indicating that the requirement for Cu by these organisms did not outstrip supply. Buck and Bruland (2005) found that Cu concentrations and associated speciation remained relatively constant during a phytoplankton bloom. They suggested that the phytoplankton community did not influence the presence of Cu binding organic ligands, total dissolved and Cu^{2+} in San Francisco Bay. These observations are consistent with previous studies, which have shown that diatom blooms do not substantially impact Cu concentrations or speciation (Beck *et al*., 2002). Similarly, Luengen *et al*. (2007) reaffirmed that diatom blooms did not significantly affect ambient dissolved Cu concentrations. These observations are consistent with our laboratory based experiments as total dissolved Cu did not vary through the *E. huxleyi* growth cycle (Chapter 4). Therefore, we would expect that total dissolved Cu would not be affected strongly as a result of the bloom.

Total dissolved Cu in the 2005 survey ranged between 1.87 to 3.73 nM, and between 2.11 to 4.43 nM during the post bloom of *E. huxleyi* in 2006 (Figures 5.13 & 5.14). These values agree well with those reported by Tappin *et al.* (1993) (3.2 nM in the Western English Channel) and by Statham *et al.* (1999) (4.1 nM in Atlantic waters, Table 4 in Statham *et al*., 1999). Campos and van den Berg (1994) also reported Cu concentrations in NE Atlantic at around 1.3 to 3.1 nM. Although the sampling stations in both years are considered as shelf sea stations, total Cu concentrations were in a close agreement with values reported in oceanic waters.

Comparison between average Cu (4.1±1.59 nM) in the central English Channel (Statham *et al.,* 1999) and values reported for the Atlantic (Statham *et al.,* 1999, in Table 4 and Campos and van den Berg, 1994) revealed that values are higher in the Channel, particularly closer to the coasts. This reflects inputs of land and sediment derived natural and anthropogenic metals. All stations sampled in our study were away from land and freshwater input, which indicates that those sources are less likely to have influenced the distribution of metals in this part of the English Channel.

Labile Cu concentrations in 2005 and 2006 ranged between 0.22 & 1.30 nM and 0.57 & 2.17 nM, respectively. The highest concentration of particulate Cu in the 2005 surveys was at station 1 (0.33 nM) whereas the lowest observed was at station 3 (40 m) (0.022 nM). The concentrations of particulate Cu (0.29 to 0.93 nM) were higher in 2006 compared to the 2005, which reflects the higher chlorophyll *a* measured in 2006 and higher biological species abundances in that year.

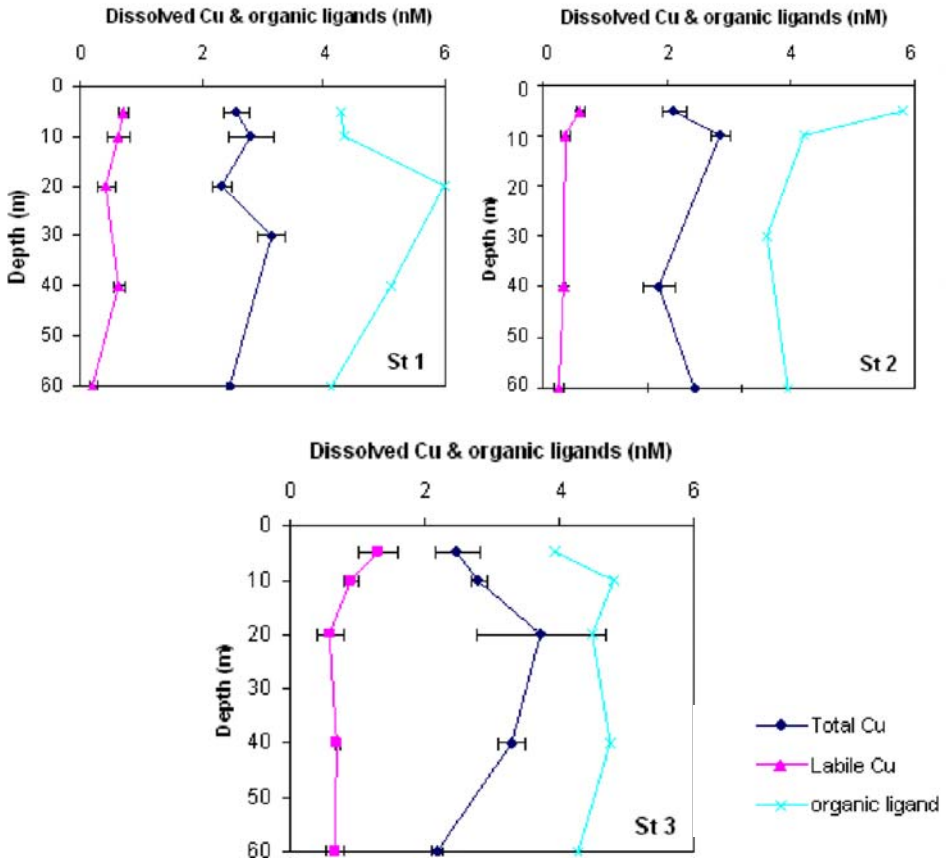

Figure 5.13: Depth profiles of concentrations of total and labile dissolved copper and organic ligands (nM), 2005 survey.

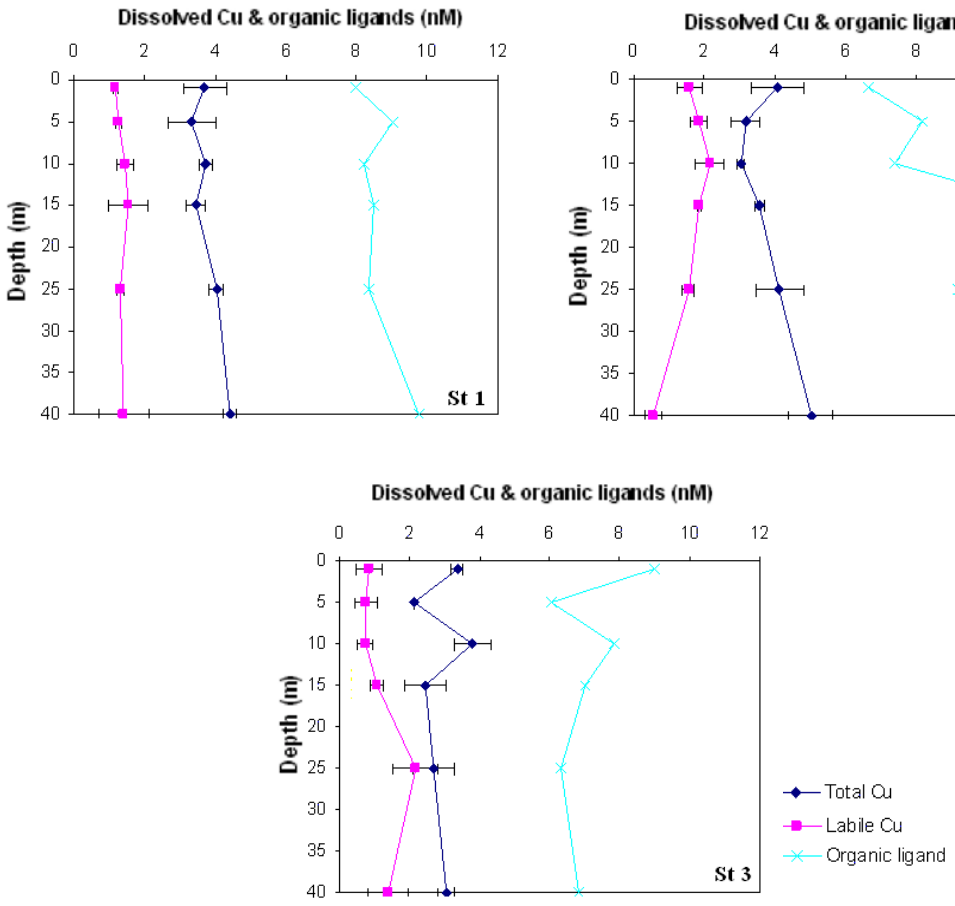

Figure 5.14: Depth profiles of concentrations of total and labile dissolved copper and organic ligands (nM), 2006 survey.

5.5.4.2 Speciation of Cu in the Western English Channel

Titrations were carried out in order to determine Cu speciation with respect to the conditional stability constant and the concentration of the natural organic Cu-binding ligands. Figure 5.15 shows representative Cu-ligand titration of English Channel samples (2005 & 2006). The titration curves for both samples showed a clear curvature (Figure 5.15 a) at low Cu concentrations (beginning of the titration curve), which indicates the presence of natural Cu-complexing ligands (i.e. not all natural ligands were saturated by the Cu initially present in the sample) (Achterberg and van den Berg, 1994). At higher Cu additions, the CSV response was found to increase linearly with the added Cu concentration, indicating that the natural ligands were saturated. Plots of $[Cu_{labile}]/[CuL]$ vs. $[Cu_{labile}]$ were linear for all samples (Figure 5.15 b), indicating that the complexation was controlled by a single class of competing ligands (Campos and van den Berg, 1994), as was the case for all samples determined. The parameters obtained from the titration

and linearization together with total, labile dissolved and particulate copper for both transects are presented in tables 5.5-5.6.

Copper organic complexes in both surveys presented high conditional stability constants (Log K_{CuL} 12.2-13.77), in the range for strong ligands, at the detection window (log α_{CuSA} = 3.99) used. The concentrations of these ligands were 3.62-5.98 nM in 2005 and 6.10-11.51 nM in 2006 (Figures 5.13-5.14). Campos and van den Berg (1994) reported similar ligand concentrations in samples collected in NE Atlantic, varying between 3 and 8 nM (Log K_{CuL} 13.1), which is in good agreement with the values reported in the current study. Note that similar detection windows (utilising 2 μM SA) were used in both studies. The stability of the complexes in Campos and van den Berg (1994) and the present study were high, with values for the log conditional stability constants of ca. 13. These results confirm Campos and van den Berg's (1994) findings that ligand competition using SA was a suitable approach to investigating the complexation of Cu in seawater. Achterberg and van den Berg (1994) reported ligand concentrations of ca 7 nM, also in Atlantic surface waters, a maximum of ca 12 nM at 200 m and conditional stability constants ranging between 12 and 13 (log values), using tropolone as a competitive AdCSV ligand (log α_{CuTrop} = 3.29). Surface organic ligand values (Achterberg and van den Berg, 1994) were in agreement with the values observed in the 2006 survey.

The ligand concentrations observed in current study were always greater than the Cu concentrations. The Cu^{2+} concentrations ranged between 0.14-1.69 pM in 2005, with lower concentrations in 2006 (0.01-0.73 pM) because of the higher ligand concentrations (Figures 5.16-5.17). The inorganic Cu´concentrations ranged between 3.16-13.40 pM in 2005 and similarly to Cu^{2+} lower range was observed in 2006 (Tables 5.5-5.6).

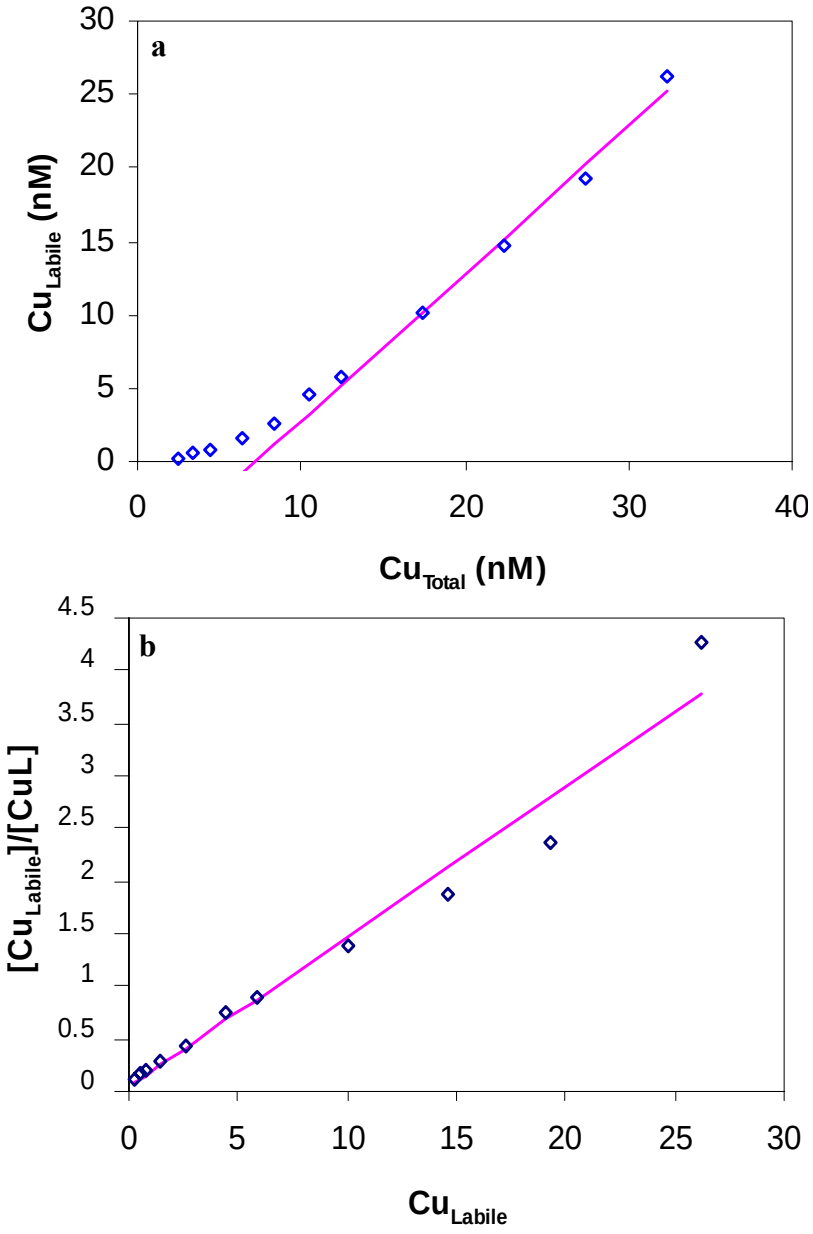

Figure 5.15: a) Typical titration curve for the Western English Channel samples (Station 3 depth 15m, 2006 survey); b) van den Berg linearization for the titration data. Cu $_{Total}$ = Cu in the sample plus Cu added for the titration; CuL = Cu complexed by natural organic ligands; Cu $_{labile}$ = Cu complexed by SA.

Table 5-5: Copper specation in the Western English Channel (5th of August 2005). Concentration of dissolved labile and total and particulate Cu, and total organic ligands (nM), Cu^{2+}, Cu', together with the conditional stability constants of the Cu organic complexes.

Station	Depth	Labile dissolved Cu	Total dissolved Cu	Cu lab:total	particulate Cu	Ligand	log K_{CuL}	[Cu']	[Cu^{2+}]
	M	nM	nM	%	nM	nM	M	pM	pM
1	5	0.73 ± 0.08	2.56 ± 0.21	28.27	0.32 ± 0.02	4.29	12.84	4.89	0.21
	10	0.64 ± 0.18	2.81 ± 0.37	22.78	0.22 ± 0.07	4.34	13.15	2.99	0.13
	20	0.44 ± 0.13	2.33 ± 0.16	18.67	0.32 ± 0.09	5.98	12.30	7.32	0.31
	40	0.64 ± 0.09	3.13 ± 0.23	20.26	0.34 ± 0.01	5.10	12.84	5.26	0.23
	60	0.22 ± 0.07	2.44 ± 0.05	9.00	0.26 ± 0.10	4.13	13.02	3.16	0.14
2	5	0.61 ± 0.06	2.11 ± 0.20	28.67	0.19 ± 0.02	5.80	12.55	5.76	0.25
	10	0.36 ± 0.08	2.85 ± 0.15	12.43	0.15 ± 0.01	4.21	12.51	14.60	0.64
	40	0.35 ± 0.01	1.87 ± 0.27	18.72	0.24 ± 0.03	3.62	12.34	11.20	0.49
	60	0.26 ± 0.08	2.44 ± 0.76	10.43	0.22 ± 0.01	3.95	12.74	6.67	0.29
3	5	1.30 ± 0.28	2.48 ± 0.33	52.42	0.20 ± 0.07	3.93	11.99	39.00	1.69
	10	0.90 ± 0.10	2.81 ± 0.11	32.14	0.11 ± 0.01	4.80	12.59	8.33	0.36
	20	0.59 ± 0.20	3.73 ± 0.96	15.82	0.07 ± 0.08	4.49	12.92	13.40	0.58
	40	0.70 ± 0.04	3.29 ± 0.20	21.12	0.02 ± 0.01	4.74	12.68	10.90	0.47
	60	0.66 ± 0.14	2.17 ± 0.08	30.41	0.42 ±0.06	4.27	12.87	3.22	0.14

Table 5-6: Copper specation in the Western English Channel (27th of July 2006). Concentrations of dissolved labile and total and particulate Cu, and total organic ligands (nM), Cu^{2+}, $Cu^{'}$, together with the conditional stability constants of the Cu organic complexes.

Station	Depth	Labile dissolved Cu	Total dissolved Cu	Cu lab:total	Particulate Cu	Ligand	$logK_{CuI}$	$[Cu^{'}]$	$[Cu^{2+}]$
	m	nM	nM	%	nM	nM	M	pM	pM
1	1	1.21 ± 0.08	3.71 ± 0.62	32.48	0.21 ± 0.01	7.99	13.51	0.62	0.03
	5	1.28 ± 0.09	3.35 ± 0.66	38.23	0.29 ± 0.02	9.02	13.25	0.77	0.03
	10	1.46 ± 0.24	3.73 ± 0.18	39.14	0.74 ± 0.10	8.24	13.77	0.32	0.01
	15	1.55 ± 0.56	3.46 ± 0.28	44.65	0.09 ± 0.02	8.47	13.06	1.38	0.06
	25	1.31 ± 0.10	4.05 ± 0.21	32.35	0.12 ± 0.01	8.33	13.49	0.70	0.03
	40	1.42 ± 0.71	4.43 ± 0.19	32.05	0.25 ± 0.02	9.76	13.13	1.42	0.06
2	1	1.59 ± 0.34	4.05 ± 0.74	39.21	0.93 ± 0.07	6.65	12.10	16.83	0.73
	5	1.85 ± 0.22	3.17 ± 0.40	58.11	0.68 ± 0.20	8.18	12.20	7.72	0.33
	10	2.16 ± 0.40	3.03 ± 0.13	71.29	0.29 ± 0.02	7.37	11.92	11.44	0.49
	15	1.85 ± 0.06	3.45 ± 0.15	52.05	1.22 ± 0.23	11.51	12.92	1.18	0.05
	25	1.56 ± 0.16	4.21 ± 0.67	37.70	0.35 ± 0.02	9.20	12.68	4.21	0.18
	40	0.57 ± 0.23	5.02 ± 0.62	11.35	0.17 ± 0.04	9.76	13.68	0.35	0.01
3	1	0.85 ± 0.37	3.35 ± 0.17	25.37	0.34 ± 0.05	8.99	12.68	2.84	0.12
	5	0.73 ± 0.32	2.11 ± 0.01	34.36	0.12 ± 0.03	6.06	13.44	0.45	0.02
	10	0.72 ± 0.20	3.80 ± 0.52	18.95	0.27 ± 0.01	7.85	13.43	0.80	0.03
	15	1.06 ± 0.19	2.44 ± 0.61	43.24	0.27 ± 0.01	7.01	13.40	0.49	0.02
	25	2.17 ± 0.65	2.67 ± 0.60	81.12	0.40 ± 0.07	6.30	13.31	0.83	0.04
	40	1.37 ± 0.58	3.04 ± 0.22	44.99	0.43 ±0.01	6.84	12.83	3.33	0.15

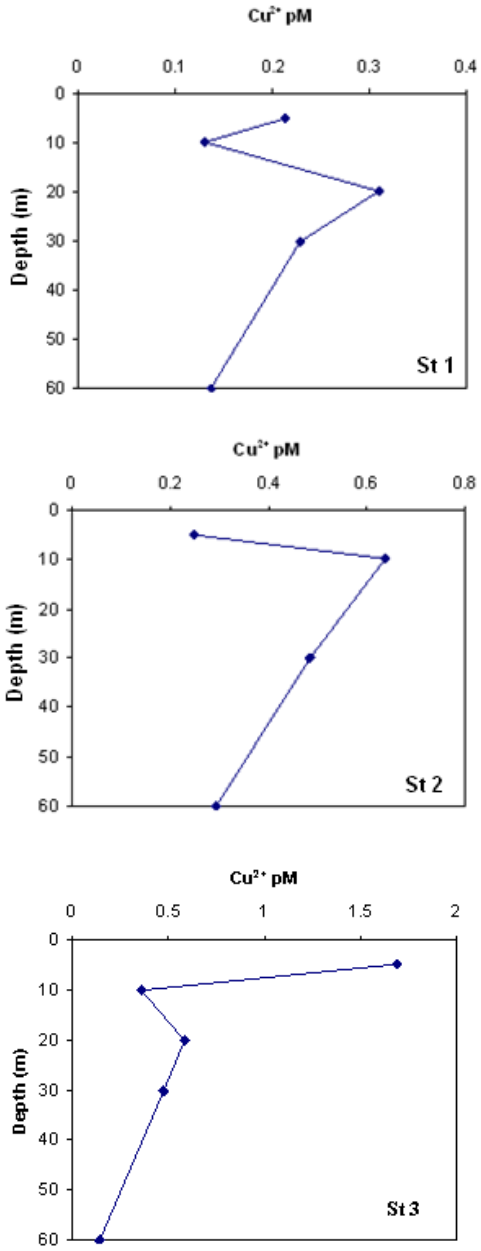

Figure 5.16: Depth profiles of Cu^{2+} (pM) for 2005 survey

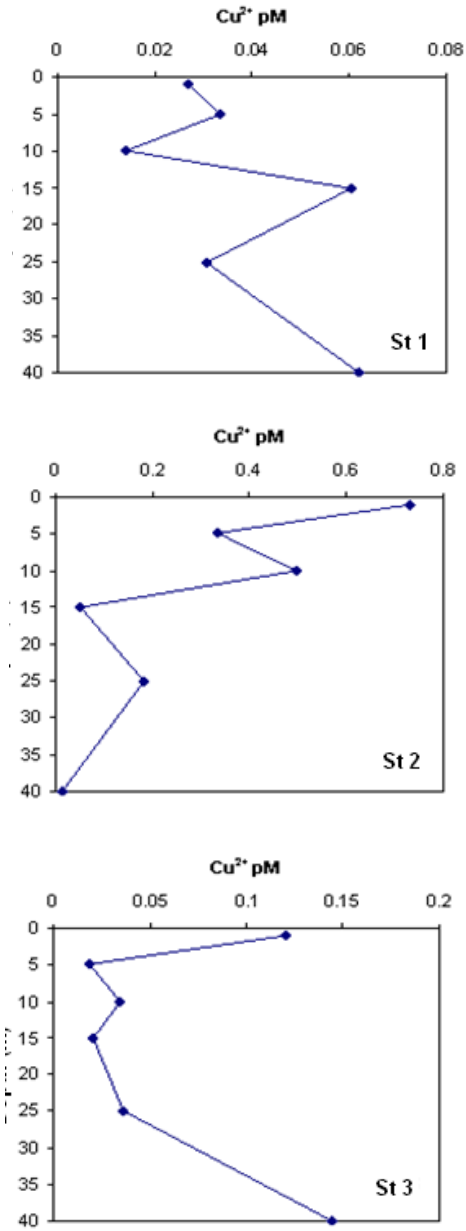

Figure 5.17: Depth profiles of Cu^{2+} (pM) for 2006 survey

Earlier studies on Cu speciation in open ocean waters have found low concentrations of L_1 ligands in an almost 1:1 relationship with ambient dissolved Cu (van den Berg *et al*., 1987; Moffett *et al*., 1990; Moffett, 1995; Croot, 2003). However, the ratio in the present study (1.7:1 in 2005, 2:1 in 2006) was slightly higher than the published ratios. Tang *et al.* (2001) found a 5:1 molar ratio between ligand and total dissolved Cu. This ratio is higher than that reported in the current study. Tang *et al*. (2001) suggested that excess of ligand might indicate that not all Cu complexing ligands are actually bound by Cu in Galveston Bay waters, which reflects the possibility that organic ligands are not specific

to Cu but can be complexed by other metals (Ni and Zn) in the same waters (Tang, 2000). It is unclear whether the conclusions reached by Tang (2000) for Cu speciation in the river influenced Galveston Bay, with high Cu and ligand concentrations and a different experimental set-up, are transferrable to the Western Channel study, where lower oceanic levels of Cu and ligands were observed.

The observed Cu speciation results in this study agree well with the values reported by Buck and Bruland (2005), who reported ligand concentrations exceeding total dissolved copper concentrations, and copper being strongly complexed by L_1 ligands. The strong organic complexation of copper resulted in very low Cu^{2+}, which did not exceed pM levels and was suitably below the toxicity limit for aquatic micro-organisms (Buck and Bruland, 2005).

5.5.4.3 Possible source and sinks for Cu complexing ligands:

Fieldwork in the Sargasso Sea has shown a strong link between the presence of strong Cu complexing ligands (L_1) and the cynobacterium *Synechoccocus* (Moffett, 1995). Laboratory cultures of *Synechoccocus* isolated from the Sargasso Sea have also been found to produce strong Cu binding ligands when under Cu Stress (Moffett and Brand, 1996). *Synechoccocus* growth will be significantly inhibited if there is no organic complexation of Cu in ambient open ocean seawater (1-5 nM Cu) (Croot, 2003 and Croot *et al*., 2000). Thus, *Synechoccocus* benefits from the production of (L_1) ligands in the presence of elevated Cu concentrations. Also the production of these ligands creates conditions more favourable for its growth as it maintains Cu^{2+} concentrations low.

In the present work, the synchronicity of the appearance of enhanced L_1 concentrations and *Synechoccocus* abundance points strongly to these cyanobacteria as a possible strong ligand source, as *Synechoccocus* dominated the phytoplankton community in both survey years. Interestingly, there was no correlation between bulk chlorophyll and ligands or Cu^{2+} in either year, indicating that only a small part of the phytoplankton community might be responsible for ligand (L_1) production. There are no statistically significant relationships ($P>0.05$) found between *Synechoccocus* and complexing ligands for both years.

The data presented here are consistent with the hypothesis that *Synechococcus* is a strong candidate for L_1 production (Moffett *et al*., 1990; Moffett and Brand, 1996; Croot, 2003; Croot *et al*., 2000; Muller *et al*., 2005). The strong ligand L_1 was only found in Gullmar Fjord, Sweden (Croot, 2003) in regions of, and times of, significant (>1 x 10^7 cells L^{-1}) *Synechococcus* abundance. Similarly, in the current surveys in the English Channel, *Synechococcus* average abundance was 4.20 x 10^7 cells L^{-1}. Currently only *Synechococcus* has been reported to produce L_1 type ligands (Croot, 2003; Croot *et al*., 2000; Muller *et al*., 2005). In this study, a 1:1 correlation between L_1 concentration and *Synechococcus* abundance was not found and this was not in agreement with data from Croot (2003) and Croot *et al.* (2002). This might indicate that those ligands are present in the water column throughout the year but only in a higher concentration during the periods of high *Synechococcus* abundance. Sampling the same location at different times throughout the year would be very useful to document any changes in organic ligand concentrations and speciation of copper in the English Channel.

However, other organisms present in the water column could be responsible for L_1 production- in particular, heterotrophic bacteria. Presently, there is no evidence for production of strong ligands (L_1) by any species of heterotrophic bacteria (Croot, 2003; Muller *et al.,* 2005), although it cannot be discounted. Of the eukaryotic species present in samples where L_1 was observed, there was no strong candidate for L_1 production. As discussed in Chapter 4 and reported by Leal *et al*. (1999) and Vasconcelos and Leal (2001), *E. huxleyi* is reported to produce weak ligands (L_2) and has a high Cu tolerance (Brand *et al*. 1986). According to Croot (2003) many of the algae (e.g. *Ceratium* sp, *Prorocentrum* sp) are reasonably Cu tolerant and known to produce weaker ligands. For example, dinoflagellates (*Amphidinium carterae*) produce weak Cu complexing ligands (Log K = 12) when under Cu stress (Croot *et al*., 2000). Diatoms also produce Cu complexing ligands during different stages of growth (Zhou and Wangersky, 1989; Croot *et al*., 2000), with one species of the common coastal diatom *S. costatum* found to produce Cu chelating ligands with log K ≈ 12 (Croot, 2003).

Weak dissolved copper complexing ligands, not detected by the established analytical conditions, should be at concentrations higher than strong ligands (Moffett *et al*., 1997; Croot, 2003; Tang *et al*., 2001) and could also be important for the total metal buffering capacity of the system, despite their weaker binding strengths.

This study therefore indicates that the phytoplankton presented in stations sampled affected the speciation of Cu on the study area, with the production of strong ligands resulting in low Cu^{2+} concentrations.

5.5.4.4 Possible role of viruses in copper speciation

As mentioned earlier, the viruses detected in the 2005 and 2006 surveys where bacteriophage viruses only; the flow cytometry signature of EhV-86 was not detected. Therefore, a comparison between Cu speciation from the culture experiments (Chapter 4) and the Western English Chanel data would be difficult as the studies are looking at different 'snap shots' of the bloom dynamics. Large viruses (e.g. EhV-86) were not identified, as they were more than likely at levels below AFC detection (6.9×10^5 ml^{-1}, Schroeder, pers. comm.). It was shown in Chapter 4 (section 4.4.6) that a 20% virus solution (which represents a late *E. huxleyi* senescent bloom) did interfere with the AdCSV measurements. Therefore we assume that EhV-86 were equivalent to 5% solution which is below AFC detection limit, but it will still potentially interfere with the measurements.

As viruses pass through the membrane used for filtration, we assume that there is less free Cu^{2+} available. This is because Cu^{2+} may been bound by viruses or used by them, as they require metals for the functioning of their DNA and RNA replication enzymes (Wilson *et al*., 2005). This is only a speculation, and no data is yet available to prove it. Results by Daughney *et al*. (2004) demonstrated the potential of marine bacteriophage to serve as nuclei for iron adsorption and precipitation. Therefore, viruses detected in the current study (cyanophage), and the undetected viruses responsible for the demise of the *E. huxleyi* bloom i.e. EhV-86 may affect metals speciation either by metal adsorption onto the virus particles or by using free metal ions in their proteins structure.

Total dissolved Cu concentrations observed in this study were similar to those reported in the literature. Since there were no pronounced differences between Cu concentrations in this study and previous published work, it is difficult to determine whether the virus particles were chelating metals to a great extend. This could be documented if the virus fraction was removed, similar as for the culture samples (Chapter 4), in particular for dissolved labile copper. Consequently, the effects of viruses in the water column must

not be ignored as they potentially can affect the speciation of Cu in the water column. More studies are required here.

5.5.4.5 Nickel, Zinc and Cobalt in the Western English Channel

Both Zn and Ni have a nutrient-type oceanic distributions characterized by a depletion in surface ocean waters as a result of phytoplankton uptake (Bruland and Lohan, 2004). Luengen *et al.* (2007) hypothesised that those metals would be depleted during a phytoplankton bloom. It would be expected, as Luengen *et al.* (2007) hypothesised, that those metals are to be assimilated by phytoplankton during the blooms observed in our study, unless they were strongly complexed to organic ligands. In open ocean waters cobalt has a unique profile, with generally low concentrations in surface waters and an increase in the thermocline (Knauer *et al.,* 1982; Martin *et al*., 1993). The current study was undertaken in shelf sea waters, with more complex water circulations compared with open ocan environments.

Ni

In vertical oceanic profiles of dissolved Ni, this element exhibits a surface nutrient depletion, with surface values typically in the 1-5 nM range (Bruland *et al*., 1994; Tappin *et al*., 1993). Nonetheless, laboratory data demonstrate that Ni is necessary for the assimilation of urea. For example, the diatom *Thalassiosira weissflogii* requires Ni when urea is its main source of nitrogen (Price and Morel, 1991).

Total dissolved Ni concentrations in the 2005 survey ranged from 2.95 to a maximum of 5.50 nM at 5 m at station 2 (edge of high reflectance area). The vertical profiles showed a decrease in total and labile Ni with depth at stations 1 and 2 (Figure 5.18). Labile Ni concentrations ranged from 1.24 to 2.34 nM. The enhanced Ni concentrations in the surface waters indicate atmospheric inputs. The highest total Ni observed in 2006 was at 40 m at station 2 (3.53 nM). There were no significant concentration variations in the depth profiles in 2006 (Figure 5.19). The average surface Ni values in the current study (3.99 nM for 2005 and 3.23 nM for 2006) agree well with values reported in the literature for dissolved Ni in the English Channel (3.8 nM Tappin *et al.,* 1993). A higher concentration is observed for dissolved Ni (Statham *et al.,*1999) close to the Solent (7.14 nM), which is affected by riverine inputs, relative to lower concentration in the Central

Channel (typically 2-2.3 nM), of principally Atlantic origin. This is close to values observed in the current study.

In oceanic waters and in the Mediterranean, Ni speciation is dominated by the labile fraction (>80 %) (Achterberg and van den Berg, 1997). The labile fraction was high in the current study in the depth profiles, and in particular in both transects (40-70%). These percentages lie within the range (40-80%) reported for estuaries and coastal waters (Nimmo *et al*., 1989; van den Berg and Nimmo, 1987; Braungardt *et al*., 2007).
Labile and total dissolved Ni concentrations in both years are shown in Figures 5.18-5.19. Tables 5.7-5.8 show labile and total dissolved Ni, Zn and Co concentrations together with the labile to total % for the 2005 and 2006 surveys.

Despite their need for Ni in the enzyme urease, which assists the assimilation of urea, in the current study marine phytoplankton did not deplete Ni concentration in the surface seawaters to values below a few nanomolars. The plentiful supply of Ni in the system, combined with a relatively low requirement resulted in a lack of obvious biological Ni depletion in depth profiles (Morel *et al.* ,2004).

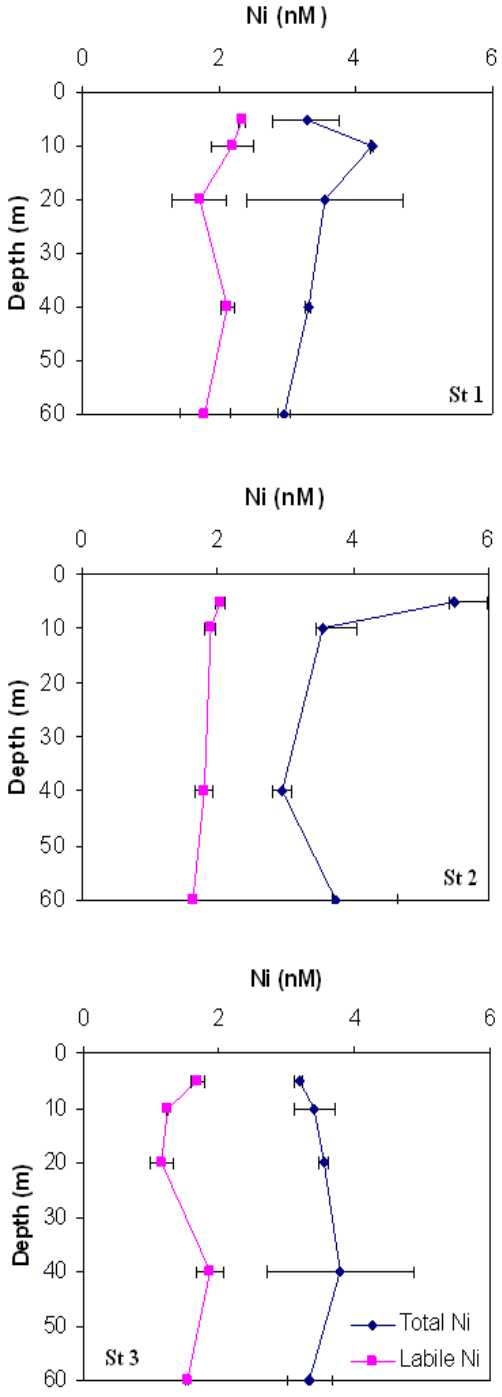

Figure 5.18: Depth profiles of labile and total dissolved Ni (nM) for 2005 survey.

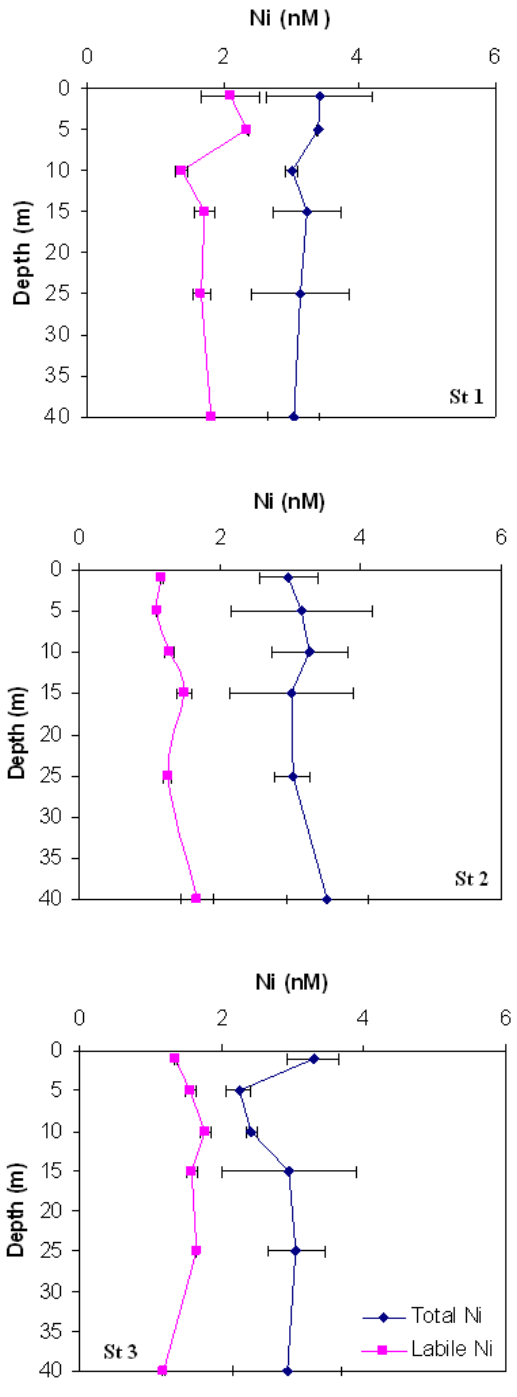

Figure 5.19: Depth profiles of labile and total dissolved Ni (nM) for 2006 survey

Table 5-7: Labile and total dissolved metal (Ni, Zn, Co) concentrations (nM) and ratio of labile to total of Ni, Zn and Co (%) for 2005 survey.

station	Depth	labile Ni	Total Ni	Ni Lab:Tot	Labile Zn	Total Zn	Zn Lab:Tot	Labile Co	Total Co	Co Lab:Tot
	M	nM	nM	%	nM	nM	%	nM	nM	%
1	5	2.34 ± 0.04	3.29 ± 0.49	71.23	2.96 ± 0.26	7.82 ± 0.76	37.79	0.11 ± 0.03	0.22 ± 0	51.13
	10	2.19 ± 0.30	4.25 ± 0.02	51.47	2.30 ± 0.01	5.78 ± 0.53	39.74	0.06 ± 0	0.18 ± 0.01	31.66
	20	1.71 ± 0.39	3.55 ± 1.15	48.03	2.55 ± 0.34	8.60 ± 0.54	29.65	0.07 ± 0	0.19 ±0	37.83
	40	2.12 ± 0.09	3.30 ± 0.04	64.09	1.70 ± 0.11	7.50 ± 1.31	22.62	0.07 ± 0.01	0.23 ±0	31.77
	60	1.80 ± 0.36	2.95 ± 0.09	60.95	2.11 ± 0.44	6.82 ± 1.08	30.96	0.11 ± 0.03	0.13 ±0.03	84.71
2	5	2.05 ± 0.07	5.50 ± 0.49	37.31	1.71 ± 0.37	7.55 ± 0.27	22.65	0.06 ± 0.01	0.17 ± 0.00	36.53
	10	1.89 ± 0.09	3.55 ± 0.50	53.17	1.63 ± 0.37	6.93 ± 0.62	23.47	0.06 ± 0.03	0.13 ± 0.07	43.03
	40	1.79 ± 0.14	2.95 ± 0.15	60.78	2.64 ± 0.44	6.37 ± 0.81	41.44	0.07 ± 0.01	0.16 ± 0.04	43.13
	60	1.63 ± 0.04	3.74 ± 0.92	43.45	1.91 ± 0.51	7.00 ± 0.26	27.31	0.05 ± 0.01	0.10 ± 0.00	45.45
3	5	1.69 ± 0.10	3.18 ± 0.06	53.14	1.70 ± 0.42	8.67 ± 1.74	19.61	0.07 ± 0.02	0.12 ± 0.04	53.47
	10	1.24 ± 0.01	3.42 ± 0.30	36.31	1.57 ± 0.06	7.01 ± 1.23	22.33	0.07 ± 0	0.19 ± 0.02	37.71
	20	1.16 ± 0.17	3.56 ± 0.06	32.63	1.28 ± 0.33	6.47 ± 0.21	19.78	0.07 ± 0	0.18 ± 0.05	39.24
	40	1.87 ± 0.20	3.79 ± 1.08	49.41	1.60 ± 0.36	9.88 ± 1.20	16.15	0.08 ± 0	0.22 ± 0	37.19
	60	1.54 ± 0.02	3.34 ± 0.33	45.96	1.86 ± 0.18	8.27 ± 0.90	22.44	0.06 ± 0.02	0.24 ±0.06	26.62

Table 5-8 Labile and total dissolved metal (Ni, Zn,Co) concentrations (nM) and ratio of labile to total of Ni, Zn and Co (%) for 2006 survey.

Station	Depth	labile Ni	Total Ni	Ni Lab:Tot	Labile Zn	Total Zn	Zn Lab:Tot	Labile Co	Total Co	Co Lab:Tot
	m	nM	nM	%	nM	nM	%	nM	nM	%
1	1	2.10 ± 0.43	3.42 ± 0.78	61.35	4.25 ± 0.15	8.79 ± 1.68	48.29	0.03 ± 0	0.16 ± 0.06	19.05
	5	2.35 ± 0.01	3.39 ± 0.02	69.28	3.68 ± 0.60	10.84 ±1.3	33.90	0.07 ± 0	0.45 ± 0.04	15.92
	10	1.39 ± 0.09	3.00 ± 0.08	46.17	4.37 ± 0.73	7.80 ± 0.15	56.06	0.08 ± 0	0.34 ± 0.09	23.76
	15	1.72 ± 0.14	3.23 ± 1.00	53.33	4.21 ± 0.08	6.75 ± 1.41	62.42	0.14 ± 0.06	0.26 ± 0.05	52.70
	25	1.68 ± 0.13	3.13 ± 0.71	53.51	3.02 ± 0.11	7.43 ± 0.71	40.65	0.09 ± 0.01	0.18 ± 0	49.45
	40	1.83 ± 0.05	3.04 ± 0.39	60.13	5.20 ± 0.84	7.43 ± 1.77	69.99	0.11 ± 0.01	0.18 ± 0.02	59.65
2	1	1.17 ± 0.02	2.98 ± 0.41	39.26	3.98 ± 0.39	8.12 ± 0.76	49.01	0.07 ± 0.01	0.26 ± 0.02	26.51
	5	1.10 ± 0	3.17 ± 1	34.70	5.44 ± 0.74	7.82 ± 0.69	69.57	0.06 ± 0.01	0.15 ± 0.06	38.75
	10	1.29 ± 0.06	3.29 ± 0.54	39.27	4.14 ± 0.63	6.25 ± 0.21	66.24	0.06 ±0	0.26 ± 0.07	23.72
	15	1.50 ± 0.11	3.03 ± 0.88	49.59	2.48 ± 0.37	7.22 ± 1.88	34.28	0.05 ± 0	0.37 ± 0.01	12.88
	25	1.26 ± 0.05	3.04 ± 0.25	41.52	3.92 ± 1.01	8.50 ± 0.62	46.12	0.07 ± 0	0.28 ± 0.05	24.37
	40	1.68 ± 0.23	3.53 ± 0.57	47.59	2.71 ± 0.98	5.74 ± 0.22	47.17	0.05 ± 0	0.50 ± 0.08	10.57
3	1	1.36 ± 0.02	3.30 ± 0.36	41.27	7.53 ± 0.62	8.49 ± 0.26	88.69	0.05 ± 0.01	0.26 ± 0.04	18.86
	5	1.56 ± 0.08	2.25 ± 0.18	69.49	7.02 ± 0.74	8.32 ± 0.89	84.38	0.04 ± 0.01	0.34 ± 0	12.09
	10	1.77 ± 0.08	2.43 ± 0.08	72.99	6.90 ± 0.37	8.67 ± 1.77	79.58	0.05 ± 0.01	0.35 ± 0.04	14.00
	15	1.59 ± 0.09	2.95 ± 0.95	53.86	4.97 ± 0.33	6.45 ± 1.71	76.98	0.04 ± 0	0.29 ± 0.01	14.82
	25	1.65 ± 0	3.05 ± 0.41	54.10	7.83 ± 1.53	9.81 ± 0	79.77	0.05 ± 0.02	0.16 ± 0.02	33.77
	40	1.19 ± 0.02	2.93 ± 0.76	40.44	6.71 ± 0.71	9.40 ± 0.28	71.33	0.06 ± 0.01	0.16 ± 0.01	35.16

Zn

Zn had the highest concentration observed compared to other metals investigated in the current study. The Zn profiles in both years are presented in Figures 5.20-5.21. Dissolved Zn concentrations ranged from 5.78 to 8.67 nM in 2005. The highest total dissolved Zn concentration was observed in 2005 at 40 m at station 3 (9.88 nM). An important observation of Zn concentrations in the 2005 survey was that the non-labile fraction was higher than the labile fraction (labile to total Zn ranged between 16-38%, Table 5.7) at all stations which indicates that Zn was highly complexed by the phytoplankton community. Compared with 2005, higher total dissolved Zn concentrations were observed in 2006, reaching 10.84 nM at station 1 (depth 5m) (Table 5.8). The Zn concentrations were in good agreement with previously observed Zn concentration profiles for the study region (7.6 nM by Tappin *et al.,* 1993 and 5.3 ± 1.98 nM by Statham *et al.,* 1999). However, Ellwood and van den Berg, (2000) reported lower Zn concentrations nearer the English coast (1.5 nM). Non-labile Zn concentrations in 2006 were also higher compared with labile concentrations, with the exception of station 3 (Table 5.8). It is unclear why Zn was complexed less strongly at station 3 compared with other stations in 2006 or 2005. This may be due to the higher total dissolved Zn observed at station 3. Bruland (1989) and Ellwood and van den Berg (2000) indicated that ≈ 98% of dissolved Zn was highly complexed by organic ligands in open ocean waters. However, a comparison between the current findings and studies mentioned above is not possible due to the lack of speciation data and higher Zn concentrations observed.

Determination of Zn speciation in marine systems has always been a challenge, due to the difficulty in collecting uncontaminated samples for total dissolved Zn and Zn-complexing ligand concentrations. Zinc is present in nearly 300 enzymes that perform many different metabolic functions in organisms (Ellwood and van den Berg, 2000; Lohan *et al.,* 2002). Unlike Cu, Zn is not toxic to phytoplankton at the concentration observed in the open ocean. There appears to be no immediate advantage to the phytoplankton community in reducing [Zn^{2+}] through organic complexation (Bruland and Lohan, 2004). Ambient Zn speciation measurements in the North Atlantic were compared to phytoplankton incubation experiments (Brand *et al*., 1983; Sunda and Huntsman, 1992), and indicated that Zn does not limit phytoplankton growth (Ellwood and van den Berg, 2000). This was furthermore enforced by Crawford *et al*. (2003) and Lohan *et al*. (2005) who did not observed immediate Zn limitation in oceanic waters using ship-board bio-assay experiments. The addition of Zn to the incubations showed no

major chlorophyll *a* biomass change in comparison to the Fe enriched treatments where chlorophyll *a* increased 20-fold above initial concentrations (Lohan *et al.*, 2005). Total dissolved Zn concentrations observed in the current survey indicates that Zn was not a limiting factor for phytoplankton at stations sampled in 2005 and 2006.

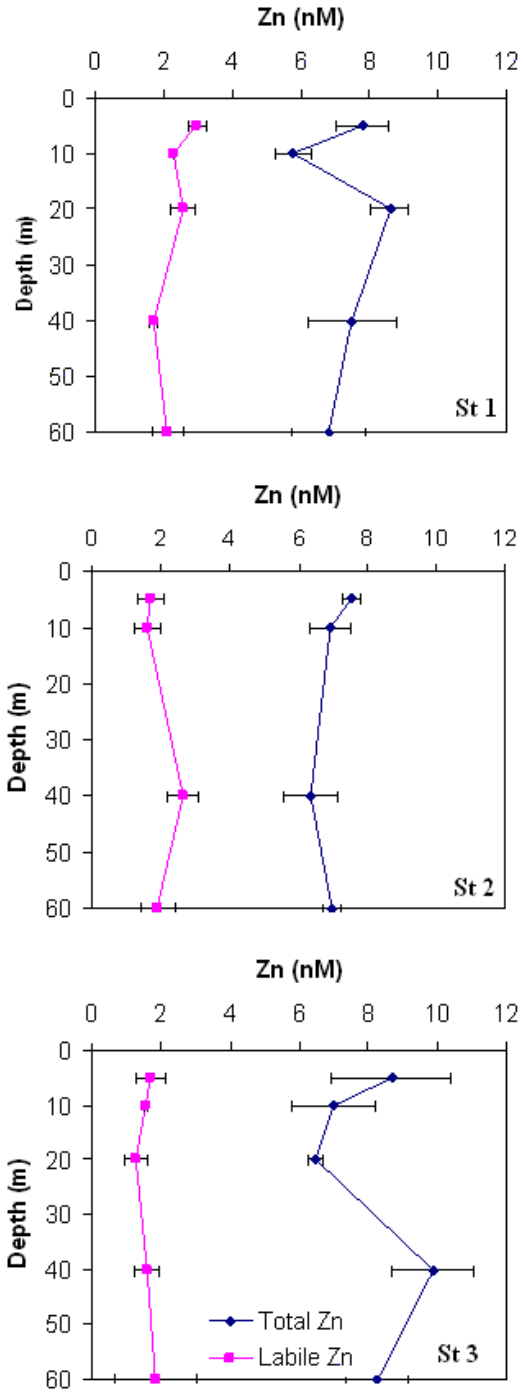

Figure 5.18: Depth profiles of labile and total dissolved Zn (nM) for 2005 survey.

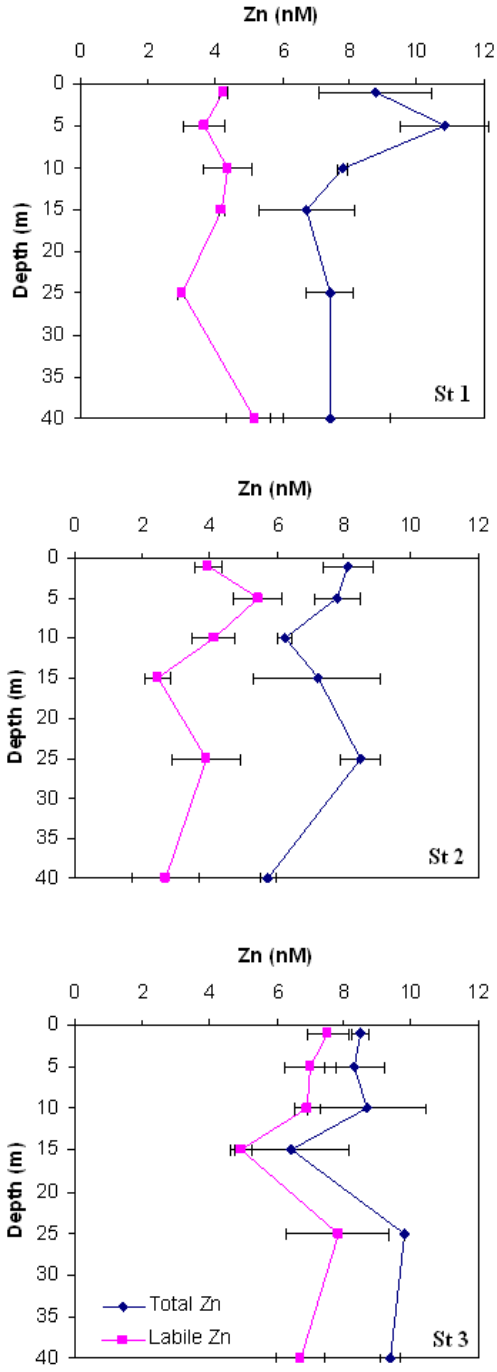

Figure 5.19: Depth profiles of labile and total dissolved Zn (nM) for 2006 survey.

The lack of a measurable decline in dissolved Zn concentrations in this study (2005-2006 surveys) were different from observations in previous field studies (Luoma *et al.,*1998) and in mesocosm experiments (Wang *et al.,*2005). In the field study of 1994, Luoma *et al.* (1998) found that dissolved Zn was depleted in a coastal phytoplankton bloom in the South Bay. This was confirmed in mesocosm studies (Riedel and Sanders, 2003). Wang *et al*. (2005) showed that phytoplankton accumulated Zn in Hong Kong coastal waters. However, a bloom of diatoms (Luengen *et al.,* 2007) did not show any depletion of Zn.
The contrasting results in the current study could be because sampling started too late to capture a clear Zn drawdown, or Zn was rapidly repartitioned from the particulate phase (Luengen *et al.,* 2007). Organic complexation may limit Zn bioavailability. Indeed, the non-labile fraction of Zn in the present study was dominant in the majority of samples, which may indicate strong ligand complexation of Zn.
Although Cu and Zn have nutrient-like behaviour in the ocean (Bruland, 1983), there was no observable depletion of these metals concurrent with the depletion of nutrients. Other studies (Tappin *et al*., 1993; Tappin *et al*., 1995; Statham *et al*., 1999) have demonstrated depletion in micronutrients without any decrease in dissolved trace metals such as Cu, Zn and Ni. The enhanced metal supply in shelf waters, combined with small requirements of micro-organisms for these metals, may explain these findings.

Co

Total dissolved Co concentrations ranged between 0.12 and 0.22 nM in 2005 and between 0.15 and 0.45 nM in 2006. In both years, similarly to Zn (except station 3, 2006), the non-labile fraction was dominant compared to the labile fraction (15-38% in 2006, Table 5.8). This indicates that Co was strongly complexed by the phytoplankton community. Zhang *et al*. (1990) and Ellwood and van den Berg (2001) showed that Co was highly complexed by organic ligands. However, a recent study by Braungardt *et al*. (2007) in Huelva Estuary (Southwest Spain) reported high labile fraction of Co >88% of total dissolved Co. The concentrations of Co in profiles observed in this study were in good agreement with the values reported by Statham *et al.* (1999) (0.30 nM), and Tappin *et al*. (1993) (0.08-0.56 nM). Total dissolved and labile Co concentrations in the Western English Channel are displayed in figure 5.22-5.23.

Cobalt plays a particularly important role in the growth of cyanobacteria. Cobalt is required in vitamin B 12. Both *Prochlorococcus* and *Synechococcus* show an absolute

Co requirement that Zn cannot substitute for (Saito *et al*., 2002; Sunda and Huntsman, 1995).

The importance of cobalt in the physiology and ecology of cyanobacteria is underscored by evidence showing that they produce strong specific cobalt chelators. Production of such organic complexes has been observed during a *Synechococcus* bloom in the equatorial Pacific (Saito and Moffett, 2001) and uptake of organically complexed cobalt has been demonstrated in *Prochlorococcus* cultures (Saito *et al*., 2002). These results have led to the hypothesis that cobalt ligands in surface seawater are produced by cyanobacteria and that they are 'cobalophores' whose function in cobalt chelation and uptake is analogous to that of siderophores for iron. As *Synechococcus* dominated the phytoplankton population in our surveys, if speciation of Co had been performed in the current study, it would have been possible to document the production of Co ligands more likely produced by these cyanobacteria.

As with the other metals referred to earlier, it was possible that a decrease in dissolved Co could not be observed because of the timing of the sampling in 2005 and 2006. Furthermore, the supply of Co in the shelf seawaters may have been higher than the demand by the microbial community, and hence no clear Co depletions could be observed in the depth profiles related to a relatively enhanced cell numbers.

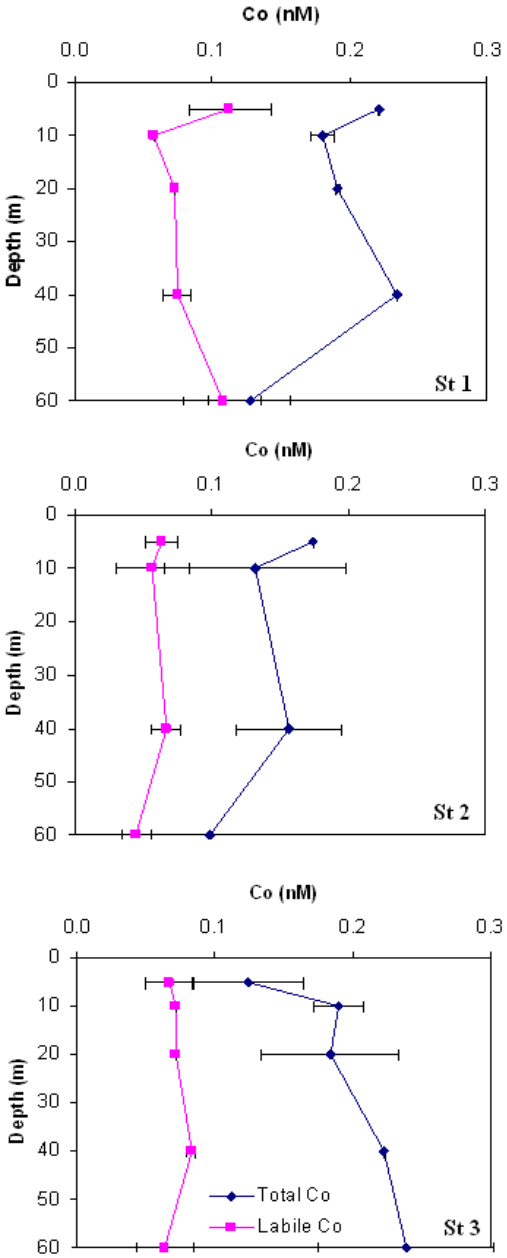

Figure 5.20: Depth profiles of labile and total dissolved cobalt (nM) for 2005 survey.

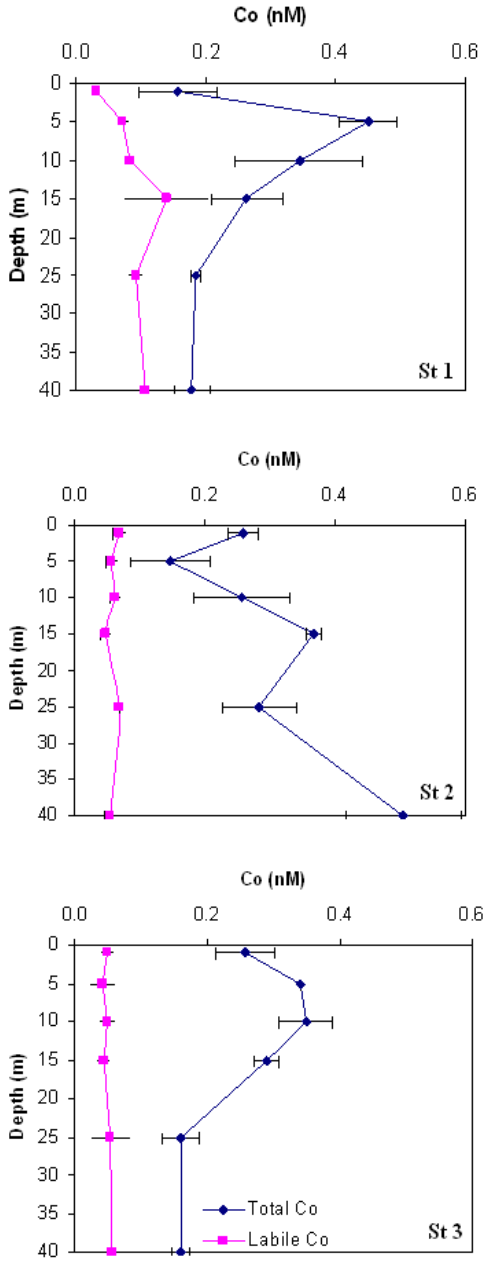

Figure 5.21: Depth profiles of labile and total dissolved cobalt (nM) for 2006 survey.

5.5.5 Correlation of trace metals with salinity and chlorophyll *a*

Figures 5.24-5.25 display the correlation between dissolved metals and salinity for the 2005 and 2006 surveys. A narrow salinity range was observed during the surveys and no clear correlations between metals and salinity was observed. In case of a wider salinity range, it would have been expected to see enhanced metal concentrations in low salinity samples collected close to the coast and freshwater input, reflecting the terrestrial inputs of metals in these waters. Tappin *et al.* (1993) observed wider salinity ranges in their study in the English Channel and found that mixing of Atlantic Ocean surface water with freshwater containing higher concentrations of dissolved trace metals was a key factor determining the distribution of trace metals. These workers (Tappin *et al*., 1993) observed a significant inverse linear correlation of metals with salinity. Nevertheless, in summer surveys in the Western Channel, Cd, Cu, lead and Zn showed no linear relationship with salinity (Tappin *et al*., 1993). This observation was attributed to biological removal of metals during the phytoplankton growth season. Similarly, it is likely that in the current study, conducted during the summer months, biological processes may have contributed to the lack of correlation between metals and salinity.
Overall, the variability observed in the metal-salinity relationship was probably due to the interaction of a number of factors, including biological processes, aeolian inputs, exchange across the benthic boundary and metal partitioning between dissolved and particulate phases (Tappin *et al*., 1993; Tappin *et al*., 1995; Statham *et al*., 1999).

As time-series sampling was not carried out at the same stations in this study, it was hence difficult to assess the relationship between metals and the biology in the water column at the stations sampled in 2005 and 2006. Nevertheless, a comparison of chlorophyll a profiles and metal profiles indicated that there were no clear links between chlorophyll and metal species. These observations indicate that in the dynamic shelf waters of the study, the supply of the metals was sufficient to remove any indications of metal depletions by biological uptake.

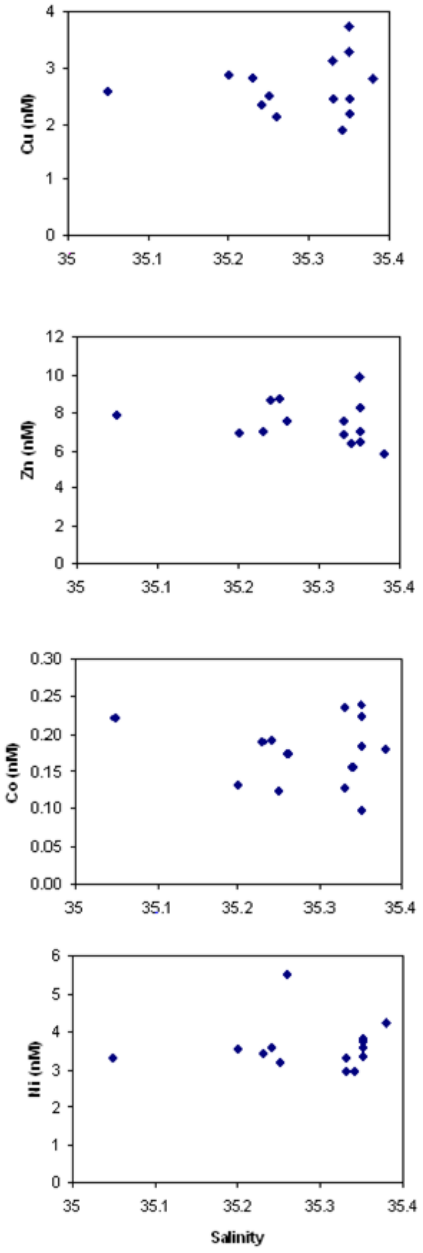

Figure 5.20: Salinity vs trace metals metals for 2005 survey

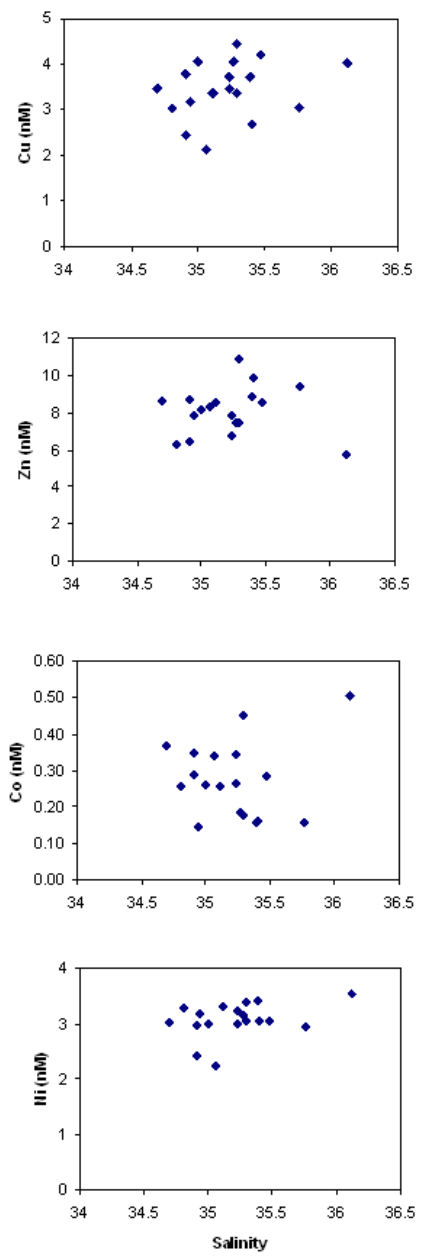

Figure 5.21: Salinity vs trace metals for 2006 survey.

5.6 Conclusions

This study is the first to report on the speciation of Cu, Co, Ni, and Zn in the Western English Channel during and post a natural *E. huxleyi* bloom. The *Synechococcus* density in the current study coincided with the highest chlorophyll *a* and the highest concentrations of *E. huxleyi*. *Synechococcus* dominated the phytoplankton community during both surveys. Hence, at the time of sampling, the *E. huxleyi* bloom was being succeeded by *Synechoccocus* in both years. None of virus detected using AFC exhibited a signature of *E. huxleyi* viruses (EhV-86). Molecular evidence has confirmed the presence of these viruses in both surveys. *E. huxleyi* viruses were present at concentrations below the limit of AFC detection. The detected viruses were likely bacteiophage-like viruses. A general trend of decrease in DOC concentrations with depth was observed, the highest concentrations being found at all stations in the near surface waters. All nutrients increased in concentration with depth in the water column. Total dissolved metals observed in our study agreed well with values reported in the English Channel and the Atlantic Ocean. There was no observable depletion of these metals concurrent with the depletion of nutrients.

Copper speciation study revealed that organic ligand exceeded total dissolved Cu concentrations, and the ligands had strong binding strengths for Cu. The Cu^{2+} concentrations ranged between 0.14-1.69 pM (2005), with lower concentrations in 2006 (0.01-0.73 pM), associated with higher ligand concentrations. The synchronicity of the appearance of L_1 and *Synechoccocus* abundance points at these cyanobacteria as a strong ligand source.

A comparison between Cu speciation from the culture experiments (Chapter 4) and the Western English Chanel data was difficult as the studies looked at different parts of the bloom dynamics. Since there were no pronounced differences between Cu concentrations in this study and previous published work, it is difficult to determine whether the virus particles were chelating metals. This could be documented if the virus fraction was removed as observed in the culture samples (Chapter 4), in particular with respect to dissolved labile Cu.

Viruses detected in current study (cyanophage) and the viruses that were responsible for the demise of *E. huxleyi* bloom, but not detected, i.e. EhV-86, may affect metal

speciation either by metal adsorption into viruses or by using free metal ions in their protein structure.

The labile fraction of Ni was high during the surveys which indicated the absence of strong organic complexation. In both years, for Co and Zn, the non-labile fraction was dominant compared to the labile fraction. This indicates that these elements were strongly complexed by organic ligands produced by the phytoplankton community. In the case of Co, cyanobacteria (e.g. *Synechoccocus*) have been suggested to play a key role in Co biogeochemistry. *Synechoccocus* was dominating the phytoplankton community in this study. No clear correlations between total dissolved metals and salinity or chlorophyll *a* were observed.

The real picture is more complicated than this as many species are present. In addition the effect of viruses cannot be eliminated. Further study is needed on the chemical speciation of metals in the Western English Channel. Collecting samples from the same stations during different seasons is essential to be able to clarify the whole picture of the area on a wider scale.

5.7 References

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Chapter 6

6. Conclusions and Future Work

The main objective of this study was to examine the effects of phytoplankton growth, senescence and decay on dissolved trace metal concentrations and their physico-chemical speciation. This objective was fulfilled by establishing a baseline for Cu, Ni, Zn & Co speciation and distribution following *E. huxleyi* blooming events in the Western English Channel in 2005 and 2006. Moreover, this study revealed for the first time the importance of coccolithoviruses in regulating trace metal speciation during and follow *E. huxleyi* bloom development. This is especially important as our current understanding of the potential role of viruses in metal speciation was limited to comparatively only a few studies (Gobler *et al.*, 1997; Poorvin *et al*., 2004; Daughney *et al*., 2004).

The voltammetric technique, AdCSV, is a cost effective, rapid and sensitive analytical method to quantify organically complexed, inorganic and free metal forms in the dissolved phase. Extensive analyses of Certified Reference estuarine water (SLEW-2, 1999) and nearshore seawater (CASS-4, 2002) were in close agreement with certified values, thus providing confidence in the methods and instruments used. This study used the AdCSV technique to determine Cu, Ni, Zn & Co speciation in both *in vitro* and *in situ* environments. In batch *E. huxleyi* culture based experiments, total dissolved Cu, Ni & Co concentrations were found to be stable irrespective of the means of phytoplankton decay. This is in agreement with observations reported previously (Vasconcelos and Leal, 2001; Leal *et al*., 1999). However, speciation analyses revealed that the labile Cu fraction increased when viruses were responsible for the phytoplankton decay. This observation was only made when the virus fraction was removed from the samples. Removal of the virus fraction resulted in an overall increase in labile Cu (day 8) from 3.33 nM to 7.21 nM (ca. 4-4.5 nM). The labile Co and to a lesser extent labile Ni measurements were not directly affected by the coccolithovirus but unfortunately due to time constraints, it was not possible to resolve this issue in this study. In addition, Cu was incorporated into the particulate phase during active growth after which the ratio of

particulate to total dissolved decreased following release of Cu from the particulate phase. The ratios of particulate to Cu^{2+} in the virus infected cultures also differed from the control experiments. It is therefore evident that EhV-86 had an effect on the particulate and dissolved fractions of Cu during cell lysis, with Cu^{2+} becoming more abundant in the dissolved fraction relative to particulate Cu following cell lysis. This supports the hypothesis that viral lysis of microorganisms converts material from particulate to dissolved phases (Fuhrman, 1999; Wihlem and Suttle, 1999). Recently, Brussaard *et al* (2008) discussed how grazing and virus mediated cell mortality both regenerate nutrients but in a different forms. Grazing results in the release of for example faecal pellets (Brussaard *et al*., 2008), whereas viruses regenerate dissolved elements in organic forms (Wihlem and Suttle, 1999). Poorvin *et al*. (2004) suggested that viruses regenerate Fe in dissolved organic species that are more biological available to phytoplankton (Hutchins *et al*., 1999).

In the virus-free cultures the amount of Cu per cell decreased exponentially during algal growth while the overall cellular Cu content (particulate Cu) increased. Viral lysis of *E. huxleyi* caused a sharp increase in Cu per cell. There is therefore a strong possibility that EhV-86 is using some of the free Cu during its infection cycle. The EhV-86 genome encodes a number of proteins that requires divalent metal ions for its activity (Wilson *et al*., 2005). Future investigations are needed to confirm that this is in fact the case.

When determining the effect of incremental EhV-86 additions on the Cu assay, the highest CSV peaks where observed when viruses were not present in solution. No changes were observed in the peak height for 100% virus addition. Titrations without viruses showed higher organic ligand concentrations (4 nM) and $LogK_{CuL}$ by 1. All this showed that viruses potentially interfere with the measurements of Cu. The overarching conclusion of the experiments is that EhV-86 directly affected the Cu measurements. As these speciation measurements are primarily used to quantify the presence of Cu, it was demonstrated for the first time that all current measurements underestimated the Cu species concentration. As automated technologies using an auto sampler with Ad-CSV capability is being developed to routinely monitor in real-time *in situ* and *in vitro* samples, caution needs to be heeded as these samples are likely to be influenced by viruses such as EhV-86.

Results from the culture experiments pertain to laboratory systems with simple chemistry and a limited range of Cu-to-virus concentration ratios and can not be extended to predict the role of viruses in natural marine Cu cycle. Dissolved organic ligands may compete with the viruses for the available Cu and other dissolved trace metals may compete with Cu for the binding sites on the viruses (Daughney *et al*., 2004). Although present results cannot be directly correlated to natural seawaters, they demonstrate the need of further research on the potential role of viruses in oceanic waters. Daughney *et al*. (2004) showed the potential of marine bacteriophage to serve as nuclei for iron adsorption and precipitation. Important questions arise to whether trace metals are complexed by viruses in the water column and whether this applies for all viruses? In addition, would metals adsorbed to or precipitated onto virus particles be available for assimilation by phytoplankton or bacteria? This has not been investigated before and this study demonstrates that future research is warranted to relate viruses and trace metals in the marine environment. For example, recent work by a colleague at NOCS, Dr Aurelie Devez, revealed that *E. huxleyi* survived toxic levels of Cu (10 μM) when EhV-86 was added to the media. In the current study, total dissolved Cu concentrations were one order of magnitude lower, however, the fact the higher concentration metals can affect the effectiveness of viruses and *vice versa,* this interaction cannot be ignored and needs to be investigated further.

Little is known on trace metal speciation in natural *E. huxleyi*-virus bloom assemblages, and therefore samples were collected during the latter stages of two *E. huxleyi* blooms in the Western English Channel. It was hoped to correlate findings from laboratory based culture experiments with those in the field. Unfortunately, at the time of sampling and analyzing samples for both *E. huxleyi* blooms, the interference by coccolithovirus on Cu measurements was not realised, and potentially these measurements were influenced by the presence of viruses. Nevertheless, no discernable coccoliuthovirus AFC signature could be detected in the collected samples. As the *E. huxleyi* bloom was being rapidly succeeded by a *Synechoccocus* bloom (on both occasions), it became clear that the coccolithoviruses were less than 5% of the virus stock solution used in the laboratory virus-Cu assays. Molecular analyses on the samples confirmed the presence of coccolithoviruses in these blooms (Schroeder, pers. comm). Therefore, the measurements taken were at the very least minimally affected by the presence of the coccolithoviruses

but it cannot be excluded the potential inhibitory effects by other viruses, notably bacteriophage.

Total dissolved metal concentrations observed in the current study agreed well with values reported by other workers for the English Channel and the Atlantic Ocean. Copper speciation measurments revealed that organic ligand concentrations exceeded total dissolved Cu concentrations and the ligands showed high conditional stability constants and can be characterised as part of the strong copper binding L_1 ligand class. *Synechoccocus* was dominating the phytoplankton community in the region. The synchronicity of the appearance of L_1 and *Synechoccocus* abundance points strongly to these cyanobacteria as the strong ligand source. A direct comparison between Cu speciation from the culture experiments and the Western English Channel data was difficult as the studies looked at different parts of the bloom dynamics. There was no pronounced difference between Cu concentrations in current study and previous published work; hence it is difficult to determine whether the virus particles were chelating metals. In both years, for Co and Zn, the non-labile fraction was dominant compared to the labile fraction. This indicates that these elements were strongly complexed by organic ligands produced by the phytoplankton community. In the case of Co, cyanobacteria (e.g. *Synechoccocus*) have been suggested to play a key role in Co biogeochemistry. No clear correlations between total dissolved metals and salinity or chlorophyll *a* were observed in the study. Further studies are needed on the chemical speciation of metals in the Western English Channel. Collection of samples from the same stations during different seasons is essential to be able to clarify the whole picture of the area on a wider scale.

Finally, are we potentially underestimating trace metal concentrations (for example Cu) in the oceans and coastal waters when coccolithoviruses such as EhV-86 and/or other viruses are present in the water column? In this case, strong evidence suggested that any inhibitory effect did not come from the coccolithoviruses but the potential effects of the cyanophage fraction could not be excluded. To resolve this question, further work is required to look at viruses and metals interactions.

Summary of proposed future work:

1. Further investigations are required into the Cu-virus particle interactions. The particles appear to act as a adsorption medium for Cu. Whether other metals also interact with virus particles also should be a focus of research, and the physical metal-virus interactions may influence the biogeochemical cycles of the metals.
2. Further work is required on the biological/biochemical interactions between Cu and viruses, as this metal appears to reduce the effectiveness of the viral action
3. This study is one of the first to study metal speciation during life cycles of phytoplankton. More such studies will be required in the laboratory and field using different phytoplankton species to investigate whether these observations are 'typical' for phytoplankton communities
4. Viral lysis has been used in this study as a means to induce cell death and study metal speciation changes. The influence of zooplankton grazing of phytoplankton on metal speciation will form a very interesting study as well.
5. Copper has received a great deal of attention in this study. Investigating zooplankton and viral lysis effects on phytoplankton communities with respect to iron speciation will be an important research area.

6.1 References

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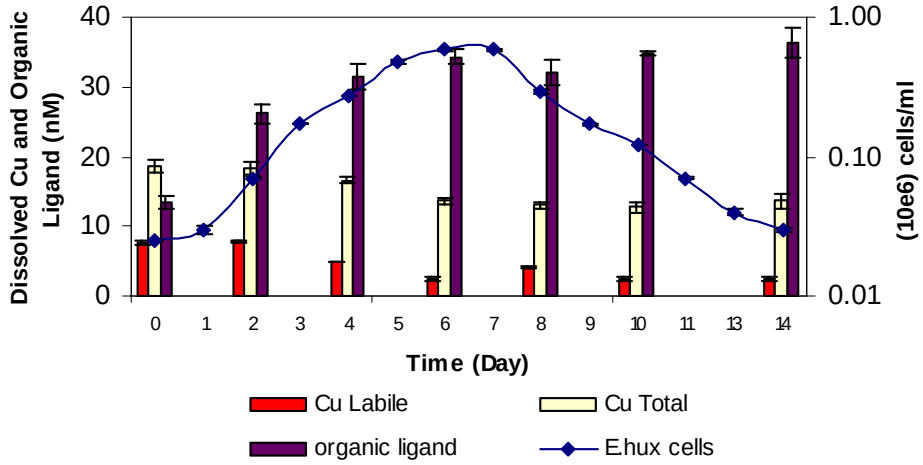
March 2004 culture experiment results (Copper speciation)

Time (days)	Growth 10^6 cells ml^{-1}	Cu algae (nM)	Cu cell mol $cell^{-1}$	Cu labile (nM)	Cu Total (nM)	L (nM)	Cu^{2+} M	Cu' M	Log K_{CuL} M	CuL %
0	0.025	0.35	1.39E-17	7.70	18.68	13.48	2.79E-10	4.46E-09	11.38	100
2	0.07	0.56	8.04E-18	7.81	18.30	26.11	3.31E-12	5.3E-11	12.33	99.8
4	0.27	1.11	4.13E-18	4.85	16.58	31.58	4.68E-12	7.49E-11	11.38	99.7
6	0.58	2.04	3.52E-18	2.48	13.65	34.32	9.46E-13	1.51E-11	11.89	100
8	0.29	3.76	1.3E-17	4.09	13.06	25.72	1.17E-12	1.87E-11	12.02	100
10	0.12	2.06	1.72E-17	2.41	12.69	34.82	9.02E-14	1.44E-12	12.84	99.9
14	0.03	0.54	1.79E-17	2.58	13.68	36.38	1.7E-13	2.73E-12	12.58	100



+ Virus (day 4) March 2004 exp