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Characterization of phytoplankton exudates and carbohydrates in relation to their complexation of copper, cadmium and iron

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ABSTRACT: The goal of this study was to investigate if transparent exopolymer particles (TEP), carbohydrates, surface-active substances (SAS), reduced sulfur species (RSS), or thio/amino groups contribute significantly to the complexing capacity of phytoplankton exudates for Cu (L_{TOTCu}), Cd (L_{TOTCd}), or Fe (L_{TOTFe}). Complexing capacities and apparent stability constants (K_{app}) were determined electrochemically for Cu and Cd in cultures of the marine diatoms *Thalassiosira weissflogii* and *Skeletonema costatum*, and in a culture of the coccolithophore *Emiliana huxleyi.* Furthermore, the complexing capacity with Fe, Cu and Cd of 4 marine polysaccharides (PS) (phytagel, carrageenan, laminarin and alginic acid) was investigated. As expected, more Cu than Cd was complexed in the 3 phytoplankton cultures and in the phytagel solution. Size fractionation of the phytagel solution suggests that the binding capacity for Cu was more significant in the particulate fraction (>0.7 µm), indicating that Cu was preferably trapped within pores and channels of large hydrogels. In contrast, Cd binding sites were predominantly found in the fraction <0.7 µm, suggesting binding to the outer surfaces of gel particles to be of greater importance for larger ions. The K_{app} of the Cd complexes were higher than those of Cu, indicating stronger binding of Cd ions than of Cu ions. Solutions of carrageenan, laminarin and alginic acid did not form complexes with either Cu or Cd, and Fe-binding properties could not be detected for any of the 4 polysaccharide solutions. Thio/amino groups of sulfur-rich 'glutathione' type ligands were found in all phytoplankton cultures and were presumably responsible for the complexation of Cu. No consistent relationship was observed between TEP, carbohydrate concentration, SAS or sulfur content, or with the complexing capacity, emphasizing the high degree of heterogeneity of substance classes responsible for metal binding.

KEY WORDS: Complexation · Copper · Cadmium · Iron · Carbohydrate · TEP · Electrochemical method

INTRODUCTION

Trace metal cycling in the ocean is largely determined by organic matter that acts as metal ligands, subsequently affecting trace metal speciation (Muller et al. 2003). In surface waters, for example, >99% of Cu, Cd or Fe is complexed to organic ligands (Bruland 1992, Buck & Bruland 2005). Several substance classes, including amino acids (e.g. glutathione-like), humic substances originated from aged and microbially modified organic matter, sulfur-rich substances, and transparent exopolymer particles (TEP), have been postulated to play a central role in trace metal binding and their biochemical cycling (Leal et al. 1999, Quigley

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et al. 2001, Benner 2002, Laglera & van den Berg 2003, Muller et al. 2003, Blake et al. 2004 Bhaskar & Bhosle 2006, Laglera & van den Berg 2006, Dryden et al. 2007, Zhang et al. 2008). Phytoplankton exudates and their derivates contribute appreciably to this pool of metal ligands (Bruland et al. 1991, Aluwihare & Repeta 1999, Bhaskar & Bhosle 2006, Lorenzo et al. 2007), but metal complexing properties differ in organic matter derived from different sources. Marine metal ligands are specific for certain metals, and ligands that bind different metals differ physicochemically. As a result of this complexity, temporal and spatial variability of the speciation of any one metal in the ocean is high (Croot 2003, Wagener et al. 2008), and individual ligands are not well characterized.

Cysteine- and glutathione-like substances, which are phytoplankton derived amino acids, are known to act as trace metal ligands (Lorenzo et al. 2007). Glutathione is an important Cu-ligand responsible for the nearly complete complexation of Cu (Ross et al. 2003).

TEP, which are formed abiotically from dissolved precursors released by phytoplankton and bacteria (Passow 2002), are rich in acidic polysaccharides (PS) (especially sulfated ones), which make them extremely surface active (Mopper et al. 1995, Zhou et al. 1998). TEP and their dissolved precursors exhibit a high affinity to thorium and Fe (Honeyman & Santschi 1991, Guo et al. 2002, Quigley et al. 2002, Passow et al. 2006, Santschi et al. 2006, Zhang et al. 2008), and specifically alginic acid and carrageenans are known to bind metal ions (Gimenez et al. 1995, Kim et al. 1995). Concentrations of nickel (Ni), chromium (Cr) and Zn in the 0.2 to 0.8 µm size-range correlated well with TEP turnover rate in a lagoon off New Caledonia, suggesting that submicron TEP complex metals, but that hydrodynamically controlled reactivity affects their ability to adsorb metals (Mari et al. 2009). Consequently, TEP have also been postulated to play a central role in the cycling of trace metals (Verdugo et al. 2004, Santschi et al. 2006).

Surface-active substances (SAS), which are also derived largely from diatom exudates (Croot et al. 2007), include a variety of organic substances (proteins, PS, lipids, humic type substances) which possess hydrophobic (e.g. fatty acid chains, aromatic rings, hydrocarbons) and hydrophilic functional groups (e.g. NH_2, COOH, OH, SH) and therefore participate in electrostatic and hydrophobic interactions (Ćosović 1985, Ćosović 2005). SAS act as metal ligands and bind Cu well (Plavšić et al. 2006). Operationally defined, SAS accumulate at phase boundaries, e.g. at the seawater-atmosphere boundary or the particle surface–seawater boundary (Liss & Duce 1997).

The existing methods for these phytoplankton-derived exudation products, that appear to play such a large role in trace element cycling, are semi-quantitative. And although TEP, SAS, cysteine- and glutathione-like substances and even carbohydrates are often cited as potential ligands for trace elements, none of these substance classes is well enough characterized to determine the degree of overlap or to allow a direct comparison. Thus we attempted to look into their functional similarity, by investigating whether their respective presence was correlated with trace metal binding.

We present data on the complexing capacity of phytoplankton exudates and PS solutions used as models for TEP and precursors, to test the hypothesis that substances characterized as TEP, carbohydrates, SAS and reduced sulfur species (RSS) all act as trace metal ligands, and to determine the degree of overlap between these different substance classes. Specifically, we determined the complexing capacity for Cu and Cd (Scoullos et al. 2006) using electrochemical methods (differential pulse anodic stripping voltammetry, DPASV). Additionally we used competitive ligand exchange cathodic stripping voltammetry (CLE-CSV) (Croot & Laan 2002) to measure the Fe binding strength of the same model substances, and square wave voltammetry (SWV) scans to identify Fe^{3+} complexes with these substances. In parallel, we characterized the organic matter by using (1) constant-current chronopotentiometric stripping analysis (CPSA) to detect catalytic groups containing nitrogen (N), sulfur (S), phosphorus (P) or oxygen (O) atoms (Ciglenečki et al. 2003, Strmečki et al. 2010); (2) a voltammetric method to determine the concentration of RSS (Ciglenečki & Ćosović 1996), (3) a voltammetric method to determine the concentration and type of SAS adsorbed on the mercury electrode (Ćosović 1985) and (4 and 5) colorimetric methods to determine concentrations of TEP (Passow & Alldredge 1995) and carbohydrates, i.e. PS and monosaccharides (MS) (Myklestad et al. 1997).

MATERIALS AND METHODS

Preparations of samples. Three marine phytoplankton cultures — 2 diatoms (Bacillariophyceae), *Thalassiosira weissflogii* and *Skeletonema costatum*, as well as the coccolithophore (Prasynophyceae) *Emiliana huxleyi* — were used for experiments with exudates. Cultures were not axenic. Cultures were grown in f/2 media at 15°C, under a 16:8 h light:dark cycle at 30 to 40 µmol m^{-2} s^{-1} light. The f/2 media (Guillard & Ryther 1962) was based on filtered (0.45 µm nitrocellulose membrane, Millipore) seawater from the North Sea. Besides phosphate, nitrate, silica, and vitamins, the culture medium contained metal ions and Na_2EDTA. EDTA is added to complex the metal ions to reduce their toxicity at high concentrations. To ensure that the added EDTA or metal ions would not affect our mea-

surements, we calculated EDTA concentrations in the media according to the CHEAQS Pro (Chemical Equilibria in Aquatic Systems) program (Release P2007.1) (Verweij 2007, http:// home.tiscali.nl/cheaqs). Our calculations indicate that concentrations of free $EDTA^{4-}$ (concentration of 10^{-14} M) in the media were negligible, while other EDTA species, e.g. H_2 $(EDTA)^{2-}$, were present at even lower concentrations (10^{-15} M). The concentration of added Fe ions (as total dissolved species) was 10^{-12} M, which means that 10^{-15} M Fe was added to make the media (1 ml of trace metal mix added to 1 l of filtered seawater). Other metal ions were added at concentrations of 10^{-11} M. The final metal concentrations in the media were thus too low to seriously affect our measurements of Cu (L_{TOTCu}) or Cd (L_{TOTCd}) complexing capacity, which were about 2 orders of magnitude higher.

All cultures were harvested at cell concentrations of ~5×10^6 cells l^{-1} in their stationary phase. Stationary phase was triggered by high cell densities leading to self shading and inorganic carbon limitation, as cultures were not bubbled. Before analysis, cultures were diluted with a NaCl–Milli-Q solution (0.55 M) at a dilution factor of 1:5 by volume (10 ml culture in 50 ml total volume).

Model substances. Phytagel, alginic acid, laminarin and carrageenan (all from SIGMA) produce a clear colorless gel in seawater, and such solutions were used as model substances. Phytagel is an agar substitute produced by bacterial transformation, consisting of glucuronic acid, rhamnose and glucose and has an average molecular weight (MW) of 10^6 Da. An unfiltered solution, as well as 2 filtrates (0.2 µm Pall Corp. Acrodisc syringe filter and 0.7 µm, Whatman GF/F), of phytagel were analyzed. Laminarin is a PS consisting of glucose and manitol generated by brown algae. The PS ι-carrageenan is a sulphated PS and consists of D-galactose units (MW = 3.5 to 7.5×10^5 Da). Alginic acid consists of D-manuronic + L-glucuronic acids (MW = 1 to 60×10^4 Da). Laminarin, ι-carrageenan and alginic acid, which are all found in marine waters, are known to abiotically form gel particles similar to natural TEP (Passow & Alldredge 1995). In solutions these 4 PS exist as a size continuum from truly dissolved to particulate with gel particles several 10s to 100s of micrometers.

The solutions of laminarin, ι-carrageenan, alginic acid and phytagel were prepared for SAS, L_{TOTCu}, L_{TOTCd} and CPSA measurements by mixing the respective PS in a 25 ml (or 50 ml) flask with 2.5 ml (or 5 ml) of 5.5 M NaCl solution and filling the flask with MQ water (end concentration of 10 mg l^{-1} or 5 mg l^{-1} PS in 0.55 M NaCl solution). The PS solutions were then shaken for 4 h on a mechanical shaker table to enhance the formation of particles. An unfiltered solution, as well as 2 filtrates (0.2 µm Pall Corp. Acrodisc syringe filter and 0.7 µm, Whatman GF/F) of phytagel were analyzed.

For the SWV scans, solutions of 0.1 to 2 g l^{-1} laminarin, ι-carrageenan and alginic acid in 0.55 M NaCl were prepared and adjusted to a pH of 8, whereas for CLE-CSV measurements UV pre-treated organic-free seawater was used as a solvent.

Electrochemical instrumentation. L_{TOTCu}, L_{TOTCd}, RSS and CPSA measurements were performed with a µ-Autolab analyzer (Eco Chemie) connected to a 663 VA stand (Metrohm), with a static mercury drop electrode (SMDE) as the working electrode. The reference electrode was an Ag/AgCl (3 M KCl). A platinum electrode served as the auxiliary electrode. Identification of Fe^{3+} complexes and their stability constants was performed on a Metrohm VA 757 voltammeter (Metrohm).

Complexing capacity and stability constants of Cu and Cd. Cu complexing capacities were determined by DPASV as described previously (Plavšić et al. 1982, Scoullos et al. 2006). DPASV was applied under the following conditions: modulation time 0.04 s, interval time 0.31 s, modulation amplitude 25 mV, and step potential 5 mV. The deposition time was 60 s at –0.6 V for Cu and –0.9 V for Cd measured versus an Ag/AgCl (3 M KCl) reference electrode. We applied the direct titration method by gradually adding more metal ions to the sample. First, a solution of 0.55 M NaCl in MQ water was titrated with Cu and Cd ions, respectively, to determine the sensitivity S of the method for both metals. Second, a 0.55 M NaCl solution with phytoplankton culture or a carbohydrate added was titrated in the same way. These data can be linearized to calculate the apparent Cu complexing capacity and the corresponding apparent stability constant for each metal (Ružić 1982, van den Berg 1982).

The binding strength of metal-ligand-complex with respect to free metal is represented by its apparent stability constant K_{app}

$$[M]/[ML] = [M]/L_{TOT} + 1/K_{app}L_{TOT} \quad (1)$$

where [ML] is the amount of metal bound to the ligand, and [M] and $[L_{TOT}]$ are the uncomplexed (electrochemically labile) metal ion and ligand concentrations in the system. The total metal concentration present in the sample is $[M_{TOT}] = [M] + [ML]$, while the total ligand concentration is $[L_{TOT}] = [L] + [ML]$. [M] and [ML] are calculated from the titration data, as

$$[M] = I_p/S \quad (2)$$

and

$$[ML] = [M_{TOT}] - [M] \quad (3)$$

where I_p is the height of the DPASV metal ion peak, and S the sensitivity of the DPASV method for the respective labile metal ion. To obtain the complexing capacity and the apparent stability constant, titration

data are linearly transformed assuming a 1:1 ratio of metal to ligand in the complex (Ružić 1982, van den Berg 1982). A straight line can be fitted to the plot of [M]/[ML] versus [M] with a slope of $1/L_{TOT}$ and an intercept at $1/K_{app}$. The complexing capacity and apparent stability constant are valid only under the specific experimental conditions (Plavšić et al. 1982, Scoullos et al. 2006).

During the titrations every addition of metal ion to the sample solution was equilibrated 20 min and was measured 3 consecutive times. The reproducibility of the method for L_{TOTCu} and L_{TOTCd} is $\pm 2 \times 10^{-9}$ mol metal ion l^{-1}. Fig. 1 illustrates the determination of the complexing capacity using the example of *Skeletonema costatum*. Fig. 1A presents results of the anodic stripping voltammetric waves for Cu ion titration, where increasing amounts of Cu ions (8 to 260 nmol l^{-1} Cu) were added to a diluted culture (1:5) of *S. costatum*. The voltammetric peak of Cu ions in the culture samples appears at ca. –0.2 V (Fig. 1), which is characteristic for chloride solutions (seawater) in which oxido/reduction of Cu ions proceeds in 2 steps. First the $Cu^{\pm 0}$ is oxidized to Cu^{+0}, and this is the visible peak described above. Thereafter, the Cu^{+0} is oxidized to Cu^{2+} at a more positive potential (ca. +0.05 V) (Krznarić et al. 1992), which coincides with the oxidation wave of the Hg of the mercury drop working electrode and is observed as steep slope in current at potentials greater than 0.1 V in Fig. 1.

In Fig. 1B, the concentration of the added Cu ions is depicted on the *x*-axis versus the found (measured) Cu ion concentration for each addition of Cu to pure electrolyte (0.55 M NaCl) and to the *Skeletonema costatum* culture. Fig. 1C presents the linearization according to the Ružić–van den Berg method for the data in Fig. 1A,B.

Identification of Fe^{3+} complexes. SWV scans of laminarin, carrageenan and alginic acid solutions were made to identify the formation of Fe complexes with these model substances.

Determination of stability constants of Fe^{3+} complexes. The Fe binding strength to the model PS was investigated using CLE-CSV (Croot & Johansson 2000). All work was carried out in a class 100 clean room, and all equipment was carefully acid washed. In short, each PS was dissolved in UV pre-treated organic-free seawater and titrated with Fe standard solutions (0.5 to 5 µM), which were prepared from a 10 mM Fe^{3+} stock solution prepared from $FeCl_3 \cdot 6H_2O$ in 1% HCl (Sigma, TraceSELECT Ultra). The portion of 'free' Fe not complexed by the PS but bound to the competing ligand 2-(2-thiazolylazo)-p-cresol (TAC) was measured. TAC is a Fe ligand of known strength added to the sample in a known concentration. Fe reaches an equilibrium distribution between the PS and TAC according to the concentration of each constituent and the stability constant of each

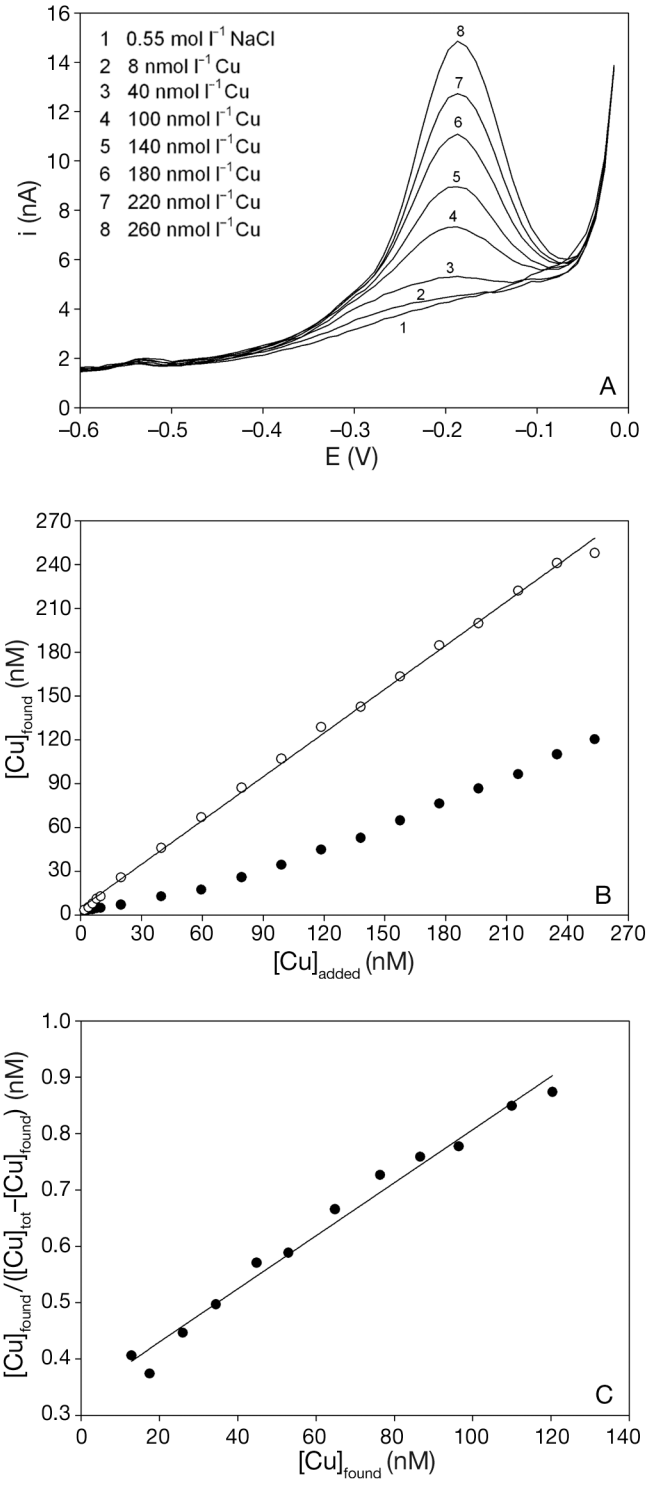

Fig. 1. *Skeletonema costatum*. Cu complexing capacity (CuCC) for *Skeletonema costatum* (1:5 diluted). (A) Voltammograms of the additions of Cu ions to 0.55 M NaCl. i (nA): oxidation current; E: electrode potential. (B) Data presented as concentrations of Cu ions added to 0.55 M Na Cl (○) or *S. costatum* culture (●) versus Cu ions found. (C) Results from B as free Cu ions found versus (Cu ions found)/(complexed Cu ions)

complex. The Fe-TAC complex can be measured electrochemically, as it induces a current when reduced on the electrode, whereas the potentially formed Fe-PS complex cannot. This method has a detection window for the conditional stability constants (K′) of competing Fe ligands depending on the concentration of added TAC (Gledhill & van den Berg 1994). TAC concentration in our experiments was 10 µM, and the respective conditional stability constant ranged from $\log K'_{FeL} = 21.4$ to 23.4.

The titration curve (i.e. added Fe versus reduction current) can be evaluated by fitting a nonlinear model of the competitive equilibrium in the solution, hence retrieving the values of the unknown parameters, i.e. the stability constant of the Fe-PS complex and the concentration of PS (Croot & Johansson 2000).

Determination of reduced sulfur species (RSS). RSS were determined by square wave cathodic stripping voltammetry (SWCSV; Ciglenečki & Ćosović 1996). Measurements were conducted directly after sample preparation and again after purging the 50 ml solution with N_2 gas to determine the fraction of RSS present as volatile RSS which are purgable.

After accumulation of RSS on the electrode surface at a deposition potential of E = −0.20 V (versus Ag/AgCl) from a stirred solution, we ran potential scans in the negative direction (up to E = −1.00 V versus Ag/AgCl), and HgS reduction peaks characteristic for RSS were recorded. Then, the solution was acidified (pH 2.0), purged with N_2 and the pH readjusted back to pH 8.0 and the RSS signal recorded again. The concentration of non-purgable sulfur species is determined after raising the pH back to pH 8.0. Samples were not readjusted to exactly the original pH, because re-adjustment was done in the electrochemical cell, so approximate concentrations of either HCl or NaOH were added. As a consequence, the signal of RSS as seen in Fig. 4A&B differed slightly in the position on potential scale.

RSS concentrations are expressed as equivalents of glutathione, determined from a calibration (from 0 to 200 nmol l^{-1}) as appropriate for the concentration range observed in the cultures. Glutathione was chosen as the standard because of its electrochemical similarity with the sulfur species observed in the 3 cultures, i.e. the appearance of the half-wave potential and the behavior upon pH changes were similar (Ciglenečki & Ćosović 1996). Furthermore, thiols/glutathione are known to be generated in phytoplankton cultures (Leal et al. 1999, Laglera & van den Berg 2003, 2006).

Constant-current chronopotentiometric stripping analysis (CPSA) for the determination of catalytic groups. CPSA produces a well-resolved catalytic peak (so called peak 'H') (Tomschik et al. 1998) that is characteristic for peptides and proteins at nanomolar concentrations (Ostatná et al. 2007). In CPSA the potential of the catalytic reaction peak (Ep) and its height $(dE/dt)^{-1}$ depend primarily on the molecular structure, i.e. atoms or a group of atoms, their position in the molecule and their adsorbability onto the surface of the working electrode. Nitrogen, sulfur, phosphorus and oxygen are the key atoms in a molecule producing a catalytic peak. Molecules with free electron pairs, which attract hydrogen ions and are easily adsorbed onto the electrode surface, lower the hydrogen overpotential, i.e. hydrogen ions need less energy for the reduction and appear at more positive potentials. Accumulation of the catalytically active compound at potential E_d = −0.20 V was achieved by stirring the solution for t_a = 60 s (accumulation time). After a quiescent period of 10 s, a constant stripping current of I_{str} = −1 µA intensity was passed through the electrolytic circuit, and CPSA curves were recorded. This method has also proved suitable for the determination of N-polymers in seawater (Strmeãki et al. 2010).

Surface-active substances (SAS). SAS were determined with phase-sensitive alternating current voltammetry (AC) (Ćosović & Vojvodić 1987, Vojvodić & Ćosović 1996). This electrochemical method measures the capacitive current (i.e. the current arising from adsorption processes, measured out-of-phase with the applied potential) separately from the faradaic current (originating from redox processes, measured in-phase with the applied potential). Out-of-phase measurements have found wide application in the study of organic substances with surface-active properties in marine and freshwater systems (Ćosović 1985, Ćosović 2005). The measurement conditions were frequency of 75 Hz, amplitude of 10 mV and phase angle of 90° so that only the capacitive component of the current was recorded versus the potential (E). In the presence of surface-active organic material, the decrease in the capacitive current below the value for the pure electrolyte solution at a chosen constant potential (E_d = −0.6 V) is a function of the amount of SAS adsorbed onto the electrode. The amount of SAS can be expressed quantitatively by an equivalent amount of a calibration substance, e.g. nonionic surfactant Triton-X-100. The nonionic surfactant Triton-X-100 proved to be a very good model substance used in many studies in seawater as well as in freshwater. This compound has been used as a model substance for SAS for more than 20 yr (Ćosović & Vojvodić 1987, Vojvodić & Ćosović 1996, Kujawinski et al. 2002, Croot et al. 2007, Gašparović et al. 2007, Plavšić et al. 2009), making our results comparable to earlier work. The potential E_d = −0.6 V is close to the potential of zero-charge of mercury in 0.55 M NaCl, i.e. the adsorption of neutral-hydrophobic molecules are favored. A 0.55 M NaCl

solution is a perfect model for investigating the adsorption of SAS because it has the ionic strength of seawater but does not contain other constituents.

TEP determinations. TEP were analyzed colorimetrically (Passow & Alldredge 1995). Six replicate samples of 50 ml each were filtered onto 0.4 µm polycarbonate filters (Poretics) and stained with Alcian blue. Gum Xanthan was used as a calibration standard, and results are expressed as Gum Xanthan equivalent per liter (GX equiv. l^{-1}).

Carbohydrate determinations. Carbohydrates (PS + MS) were determined for the whole sample (dissolved + particulate fraction) and for the dissolved fraction (<0.4 µm) (Myklestad et al. 1997). This method, which measures both neutral and charged carbohydrates, subjects the saccharides to an oxidation reaction at alkaline pH, during which Fe^{3+} is reduced to Fe^{2+}. The Fe^{2+} is then determined colorimetrically after condensation with the cromogen 2,4,6-tripyridyl-s-triazine (TPTZ) and formation of the purple color of Fe $(TPTZ)_2^{2+}$. The calibration is done with glucose, and results are expressed as glucose equivalents. Samples between 10 and 20 ml each were filtered through a 0.4 µm (PC, Poretics) filter for the determinations of dissolved carbohydrates. Total (dissolved + particulate) and dissolved MS were determined in triplicate 1 ml samples of unfiltered and 0.4 µm filtered samples, respectively. The total carbohydrate content was measured in 4 ml of filtered and unfiltered sample after hydrolysis by adding 0.4 ml 1 M HCl and heating up to 150°C for 1 h in sealed glass ampoules. The PS concentration was determined by subtracting the MS concentration from the total carbohydrate concentration.

RESULTS AND DISCUSSION

Complexing capacity and stability constant for Cu and Cd

The complexing capacity of a metal equals the total concentration of ligands that form complexes with that metal in a sample. In Table 1 the results of the complexing capacity measurements for L_{TOTCu} and L_{TOTCd} for the 3 phytoplankton cultures and the model compounds are presented. The Ružić–van den Berg method is suitable as it calculates L_{TOT} from the whole titration curve, even when full saturation of the binding sites is not achieved. All the data points on the curve have the same weight in the calculation of L_{TOT}. When the inert complex is present in the solution, it does not dissociate during the measurement (i.e. the chosen stirring rate in DPASV), so only free metal ions diffuse to the electrode surface to form an amalgam. This eliminates any problem caused by different diffusion coefficients of the metal ion and metal complex. It is possible that apparent complexing capacity can be reduced by adsorption of the organic ligand on the electrode surface. This would result from a different sensitivity for the metal ion in the 0.55 M NaCl solution with added ligand (Plavšić et al. 2006). By applying pseudopolarography, the half-wave potentials for inert organic complexes, both for model ligands and for natural samples (Croot et al. 1999, Tsang et al. 2006), can be determined. Originally developed for the calculation of stability constants for labile complexes (Branica et al. 1977), pseudopolarography has also been used in recent years (Croot et al. 1999, Tsang et al. 2006) for

Table 1. Apparent complexing capacity (CC) for copper (Cu) and cadmium (Cd) ions, and characteristics of organic matter in cultures of *Skeletonema costatum, Thalassiosira weissflogii, Emiliania huxleyi* and model polysaccharides. Cu_T, Cd_T: total concentration of copper, or cadmium; K_{app}: apparent stability constant; SAS: surface-active substances; CPSA: Constant-current chronopotentiometric stripping analysis of catalytic effect; GSSG: Glutathione; (–) not measured; blank cells: value was below detection limit. Repeated measurements in the past have given a standard deviation of 5% for these electrochemical measurements (Ciglenečki & Ćosović 1996)

Samples	Cu_T (nM)	CuCC (µM)	Log $K_{app\ Cu}$	Cd_T (nM)	CdCC (µM)	Log $K_{app\ Cd}$	SAS (mg l^{-1} equiv. Triton-X-100)	CPSA	Sulfur (as equiv. GSSG nM)
S. costatum	23.80	1.06	6.45	16.45	0.022	8.73	0.24	Yes	74
T. weissflogii	26.60	1.14	6.18	14.85	0.020	8.83	0.18	No	202
E. huxleyi	16.21	0.14	7.25	12.59	0.043	7.28	0.26	No	50
ι-carrageenan (10 mg l^{-1})		No			No		0.05	Yes	–
Alginic acid (10 mg l^{-1})			No		No		0.08	No	–
Laminarin (10 mg l^{-1})		No			No		0.32	No	–
Phytagel (5 mg l^{-1}) total	3.86	0.330	6.65	2.92	0.006	9.19	0.29	No	–
<0.7 µm	1.83	0.093	7.38	1.13	0.004	8.70	–	No	–
<0.2 µm	1.08	0.089	7.47	1.07	0.004	8.75	0.21	No	–

inert complexes. For Cu complexes pseudopolarography yielded $E'_{1/2}$ values from −0.35 to −0.82 V for model ligands and −0.66 V for *Skeletonema costatum* (Croot et al. 1999). The estimated $E'_{1/2}$ for the natural inert complexes with Cd in freshwaters range from −1.45 to −1.62 V, while for selected model substances they range from −0.7 to −1.3 V (Tsang et al. 2006). This implies that our chosen potentials for the accumulation of Cu (−0.6 V) and Cd (−0.9 V) complexes could have led to the underestimation of the complexing capacity, as possibly some complexes could have been reduced, increasing the concentration of the labile metal ions. Calculations of complexing capacities are operationally defined and only valid for the chosen conditions.

The complexing capacity for Cu ions was the highest in *Thalassiosira weissflogii* (1.14 µM) and *Skeletonema costatum* (1.06 µM) while it was an order of magnitude lower (0.14 µM) in the culture of *Emiliana huxleyi.* In *E. huxleyi* the complexing capacity for Cd ions was, however, twice that of *T. weissflogii* and *S. costatum* (0.04 vs. 0.02 µM and 0.02 µM, respectively). Appreciably lower L_{TOTCd} values, compared to L_{TOTCu} values, indicate fewer binding sites for Cd and are common (Scoullos et al. 2006). The geometrical distortion of Cu complexes due to the Jahn-Teller effect (Jahn & Teller 1937) results in an energetic stabilization of the Cu complex. The smaller radius of the Cu ion compared to Cd (0.87 Å vs. 1.09 Å) may further promote Cu-ligand binding compared to Cd-ligand binding.

Complexing capacities for both Cu and Cd were lower in the 5 mg l^{-1} phytagel solution than in those stemming from the cultures of *Skeletonema costatum* and *Thalassiosira weisflogii*. No complexing of Cu or Cd was observed in the other model substances, although carrageenans and alginic acid have been shown to bind Pb, Cd and Zn ions (Gimenez et al. 1995, Kim et al. 1995) as well as ^{234}Th (Quigley et al. 2002).

Stability constants for both Cu and Cd (Table 1) were log $K_{appCu,Cd}$ = 6 to 9. The apparent stability constants for Cd were higher than those for Cu, indicating stronger binding of Cd ions. Higher concentrations of ligands imply a lower average log K_{app}, but some fraction of the Cu may, nevertheless, be complexed more strongly than some of the Cd. It is also important to note that results of electrochemical methods are dependant on the conditions applied during measurements (e.g. stirring rate in ASV or competing ligand in CSV) (concept of the detection window) (Plavšić et al. 1982, van den Berg et al. 1990, Buck & Bruland 2005), and measured K_{app} and L_{TOT} for Cu and Cd could differ if different measurement conditions are chosen.

Some details on binding characteristics may be derived from size fractionation experiments. A L_{TOTCu} of 0.33 µM was obtained for the unfiltered phytagel solution (Table 1), whereas the L_{TOTCu} after filtration through either 0.2 µm or 0.7 µm was 73% lower at 0.09 µM, suggesting that the binding of Cu was mostly with the particulate fraction >0.7 µm of the gel solution. The surface-area-to-volume ratio of hydrogels is smaller in the larger size fraction. The much higher L_{TOTCu} in the unfiltered solution thus suggests that Cu binding sites were primarily located at the inner surface of the gels. The binding sites may be fairly nonspecific, explaining the low stability constant.

In contrast, the L_{TOTCd} was reduced by only 33% in the phytagel solution after filtration (by either 0.2 µm or 0.7 µm). The precision of the method is high enough to allow the quantification of this reduction in the L_{TOTCd}. Thus this difference indicates that Cd complexed mostly with material small enough to pass a 0.2 µm filter. The larger size of the Cd ion, compared to the Cu ion, in combination with the same charge and a stable electronic structure would make the sites on the larger surface area (after filtration) less restricted by steric factors and more available for Cd binding. Interestingly, L_{TOTCd} was positively correlated with MS concentration while the same was not true for L_{TOTCu}. MS are less likely than PS to form large gel-particles retainable by filters.

Identification of Fe^{3+} complexes with laminarin, carrageenan and alginic acid

The position of the half-wave potential of reduction is characteristic for a specific metal ion, and a peak at the potential of approximately −1.4 V is an indication of the presence of dissolved reducible Fe (presumably $Fe^{2+} \rightarrow Fe^{\pm 0}$). Calibration scans performed in artificial seawater containing 0.5 mM BisTris buffer (pH 8) revealed a peak at −1.4 V which increased linearly (Fig. 2A) with increasing Fe concentration (0 to 15 µM). The sensitivity was 1.4 nA μM^{-1}. Apart from buffering the pH, BisTris buffer also slightly complexes Fe and prevents hydrolysis and precipitation of Fe^{3+} (Taylor et al. 1994) even in its colloidal form (Schneider & Schwyn 1987).

SWV scans (Fig. 2B) of laminarin (2 g l^{-1}), alginic acid (2 g l^{-1}) and carrageenan (0.1 g l^{-1}) (in 0.55 M NaCl at pH 8) showed no peak around −1.4 V, indicating that there was no dissolved Fe species present that could be reduced. At a pH between 4 and 8, carrageenan (0.1 g l^{-1}) showed spike-like peaks at −0.5 and −0.75 V (Fig. 2B) which reflect the sulfur contained in the side chains of this PS (Plavšić & Ćosović 1998). Alginic acid also showed 2 peaks, 1 at ca. −0.2 V (unidentified) and 1 at −0.7 V (considering the spike-like shape probably caused by sulfur species). Rickard et al. (1999) report the reduction peak of Fe^{3+} at a potential of −0.2V, but as this peak is absent in the voltammograms of the carrageenan and laminarin solutions, which were also

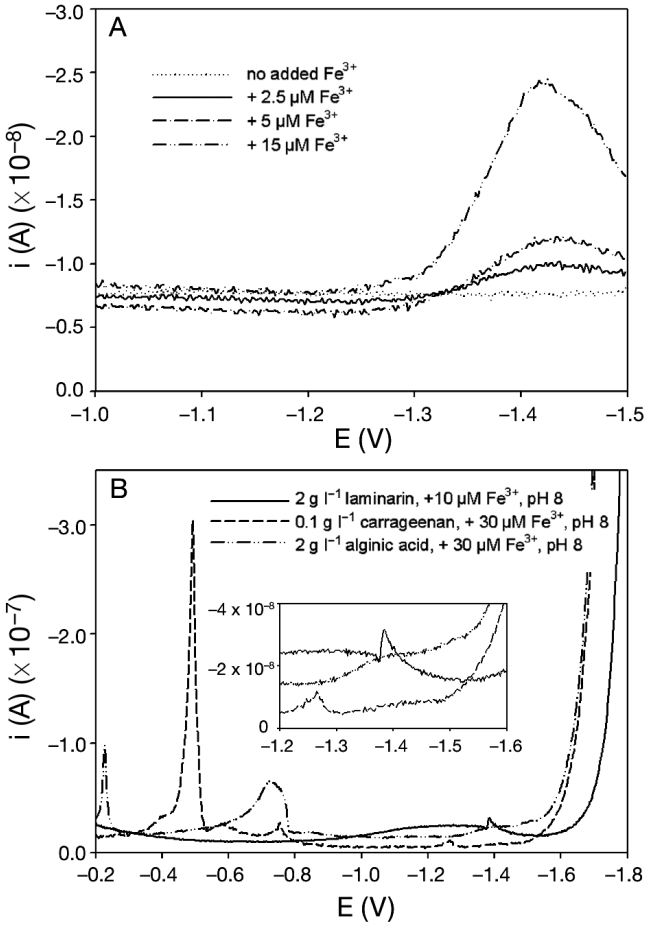

Fig. 2. Square wave voltammograms. (A) Fe^{3+} additions to a 0.5 mM BisTris solution in artificial seawater at pH 8. (B) Scans of solutions of polysaccharides in artificial seawater at pH 8, 10 to 30 µM Fe^{3+} added. Inset: detailed view of the potential range, –1.2 to –1.6 V, where peaks of Fe species are expected. i (A): oxidation curent; E: electrode potential

spiked with Fe^{3+} (30 and 10 µM respectively), it is more likely to be caused by impurities in the alginic acid. The steep increase after –1.6 to –1.7 V (Fig. 2B) is due to the hydrolytic formation of H_2 and further on the reduction of sodium ions from supporting electrolyte on the mercury drop electrode.

Determination of stability constants of Fe^{3+} complexes

The Fe titration with laminarin, carrageenan and alginic acid using CLE-CSV over a range of 0.02 to 0.5 mg l^{-1} did not show a curvature (Fig. 3) and, consequently, no stability constant could be determined. Reducing the TAC concentration by 50% to 5 µM, to lower the detection window and shift the equilibrium more towards the Fe-PS complex, gave very similar results; slopes were generally lower (Fig. 3) than those of the reference sample (UV treated organic-free seawater). Thus the titrations of PS solutions of up to 0.5 mg l^{-1} did not yield any K′ values (K′ is the conditional stability constant for FeL with respect to free Fe^{3+}), indicating that these model PS bind Fe only weakly (log K′ < 21.4) if at all. This is in accordance with the result that these PS did not show any specific Fe(III) complexation and contrasts with results from Quigley et al. (2002), who showed enhanced Fe and Cd binding in organic matter enriched in the PS fraction compared to non-enriched material. These different results may reflect differences in the composition of exudates and organic matter. Alternatively, the inability to determine K′ values for our model substances may have been due to methodological problems. Contamination of the analysed substances with Fe seems unlikely. Bound Fe should result in a FeTAC peak after initial equilibration with TAC, because TAC is a strong Fe chelator and would strip at least some Fe off the potentially contaminated PS. This was not observed. The linear increase in labile Fe makes a decreased sensitivity due to PS adsorption onto the surface of the working electrode much more plausible than an incomplete titration of the PS.

We did not attempt to measure the apparent stability constant of Fe in phytoplankton exudates because of this lack of success with the model substances.

Characterization of the organic matter

Concentrations of sulfur species

All 3 cultures contained different concentrations of sulfur (Table 1); the highest concentrations by far

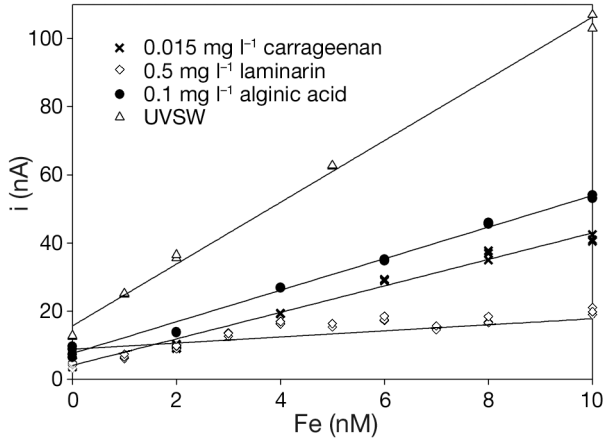

Fig. 3. Competitive ligand exchange cathodic stripping voltammetry (CLE-CSV) data of laminarin, carrageenan and alginic acid titrated with Fe^{3+}. UVSW: UV irradiated seawater, i (nA): oxidation curent

were in *Thalassiosira weissflogii*. A significant fraction of the RSS in this culture must have been present as sulfides, as indicated by the fact that an appreciable fraction of the sulfur was purged with N_2 gas. The SWCSV for *T. weissflogii* was almost halved upon acidification, purging with N_2 and readjustment of the pH (Fig. 4A). The original SWCSV for the *Skeletonema costatum* (Fig. 4B) remained the same upon acidification to pH 2, purging with N_2, and readjustment of pH back to 8.1, indicating the absence of sulfur species that could be purged by N_2 gas. *Emiliana huxleyi* (not shown) behaved in a manner similar to that of *S. costatum*, also suggesting the absence of purgable sulfur species. Assuming that about half of the sulfur in the *T. weissflogii* culture was purgable, the concentration of organic, non-purgable sulfur in *T. weissflogii* was higher than that of *S. costatum* and *E. huxleyi*. Exudation products of phytoplankton vary with species and phase (Myklestad 1974, Myklestad 1995).

The non-purgable sulfur species of all 3 cultures electrochemically resembled glutathione. Glutathione and other thiols are present in surface waters and are known to be released by phytoplankton, including *Emiliana huxleyi* (Dupont & Ahner 2005) and *Thalassiosira weissflogii* (Tang et al. 2005) when exposed to elevated concentrations of Cu or Cd. However, the presence of the more complexed ligands, i.e. phytochelatins, with the common formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ of which the tripeptide glutathione (γ-Glu-Cys-Gly) is the simplest form cannot be excluded (Mehra & Mulchandani 1995, Scarano & Morelli 1996, Cruz et al. 2005). All thiols have peaks at a potential similar to that of glutathione (Luther et al. 1985, Luther & Church 1988, Luther et al. 1990).

Presence of catalytic groups

Skeletonema costatum and the model substance ι-carrageenan both exhibited the catalytic peak 'H' (Strmečki et al. 2009, Strmečki et al. 2010) (Fig. 5, Table 1) obtained by prolonged accumulation (180 s and 300 s) and situated at the potential of ca. –1.7 V. In the case of ι-carrageenan, this catalytic peak 'H' is obtained because of catalytically active sulfate groups on PS (Strmečki et al. 2009). Protein-like complexing ligands, containing N atoms in the polymeric structure, could explain this observed peak 'H' in *S. costatum* culture (Strmečki et al. 2010). Protein-like complexing ligands are abundant during *S. costatum* blooms (Lorenzo et al. 2007).

Surface-active substances

The characteristic 'AC out of phase voltammetric curves' reveal the suppression of the capacity current in comparison to the capacity current of the pure electrolyte (0.55 M NaCl) (Fig. 6, Table 1) and are the consequence of adsorption or desorption processes (Ćosović 1985). The pronounced peak (at approx. E = –1.4 V) recorded for phytagel means that the phytagel molecules were desorbed from the electrode. Other, smaller peaks could indicate reorganization of the adsorbed molecules on the electrode surface. The highest concentrations of SAS in the phytoplankton samples were observed in *Emiliana huxleyi* (0.26 mg l^{-1} equiv. Triton-X-100) and *Skeletonema costatum* (0.24 mg l^{-1} equiv. Triton-X-100) with lower values for *Thalassiosira weissflogii* (0.18 mg l^{-1} equiv. Triton-X-100) (Table 1, Fig. 6). The SAS concentrations in *E. huxleyi* and *T.*

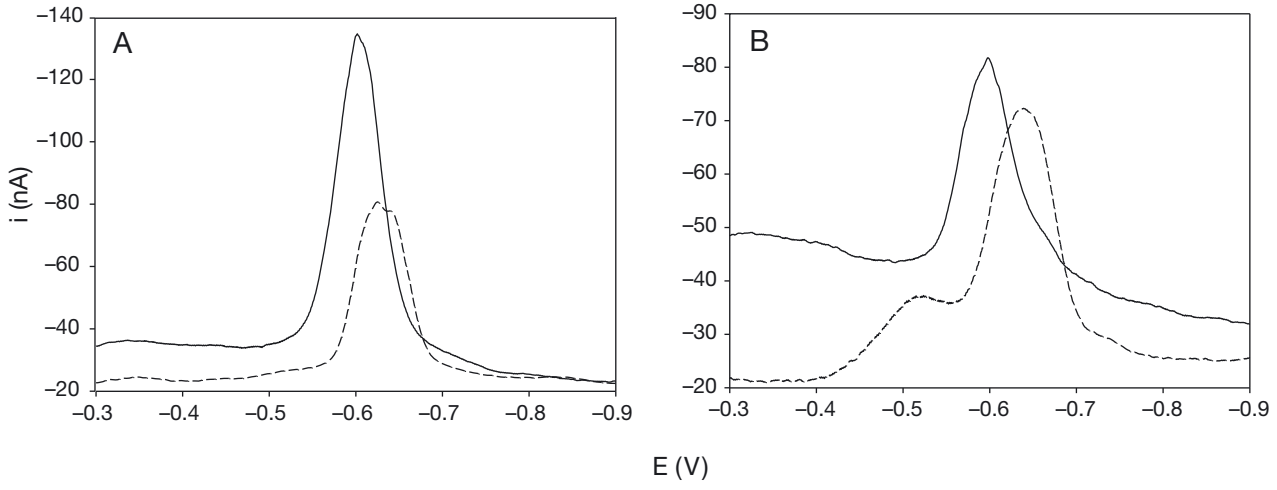

Fig. 4. Square wave voltammograms (SWV). (A) *Thalassiosira weissflogii,* (B) *Skeletonema costatum* at pH 8.1 (solid line) and after acidification, purging with N_2 and readjustment of the pH back to ~8.1 (dashed line). i (nA): oxidation current; E: electrode potential. Experimental conditions: deposition potential $E_d = -0.2$ V; deposition time $t_d = 120$ s; amplitude = 25 mV; frequency = 100 s^{-1}

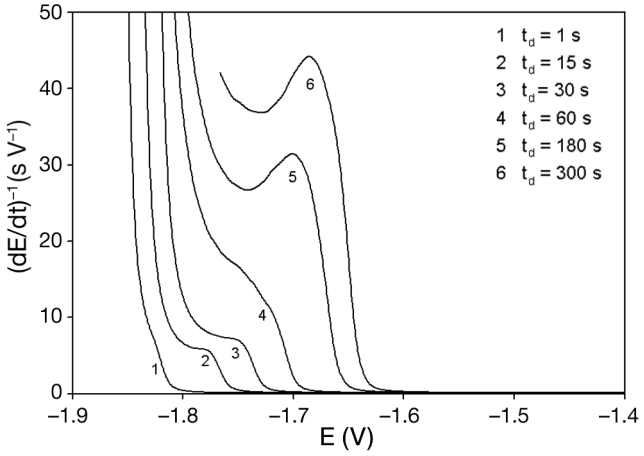

Fig. 5. Chronopotentiometric stripping analysis (CPSA) curves of *Skeletonema costatum*. E: electrode potential; t: time. Experimental conditions: deposition potential E_d = –0.2 V; stripping current I_{str} = –1 µA; maximum time of measurement = 5 s. Deposition times (t_d) are indicated in the panel

weissflogii cultures are comparable to earlier measurements (Ciglenečki & Ćosović 1996). SAS of 10 mg l^{-1} laminarin or 5 mg l^{-1} phytagel were considerably higher, whereas those of alginic acid and carrageenan were appreciably lower than those of the cultures (Table 1). In coastal regions, SAS are in the range 0.04 to 0.16 mg l^{-1} equiv. Triton-X-100 (Vojvodić & Ćosović 1996, Plavšić et al. 2009) and an order of magnitude smaller in the Southern Ocean (Croot et al. 2007). *In situ* SAS have been found elevated during the phytoplankton blooms (Croot et al. 2007) in the surface layer.

TEP and carbohydrates

The total (dissolved + particulate) carbohydrate (PS + MS) concentration ranged between 1.2 and 3.6 mg glucose equiv. l^{-1}, of which 53, 57, 79 and 98% belonged to the dissolved (<0.4 µm) pool in phytagel, *Thalassiosira weissflogii, Emiliana huxleyi* and the *Skeletonema costatum*, respectively (Fig. 7). Less than 40% of the carbohydrates were PS in *E. huxleyi* and *S. costatum*, whereas PS dominated the carbohydrate pool in *T. weissflogii* (>60%) and the phytagel solution (>80%). PS concentrations ranged between 0.5 and 2.5 mg glucose equiv. l^{-1} in the cultures and phytagel solution, with higher values in the unfiltered compared to the 0.4 µm prefiltered samples (Fig. 7). Concentrations of dissolved PS were highest in *T. weissflogii* (1.3 mg glucose equiv. l^{-1}), followed by *E. huxleyi* (0.8 mg glucose equiv. l^{-1}) and *S. costatum* (0.7 mg glucose equiv. l^{-1}) and lowest in phytagel (0.3 mg glucose equiv. l^{-1}). Particulate PS were highest in *T. weissflogii* and phytagel and almost absent in *S. costatum* (Fig. 7).

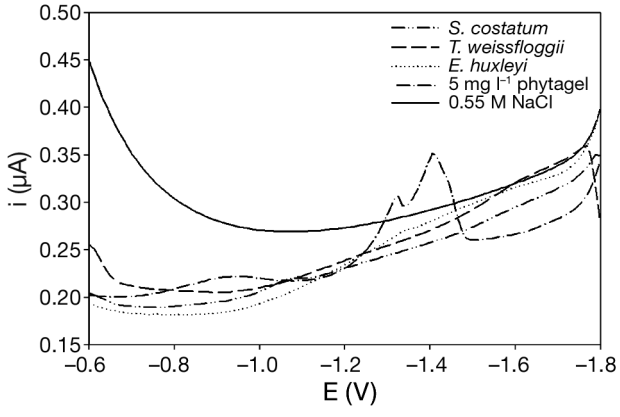

Fig. 6. Alternating current (AC)-voltammograms of phytoplankton cultures, phytagel (5 mg l^{-1}) and a 0.55 M NaCl solution. i (nA): oxidation current; E: electrode potential. Experimental conditions: deposition potential E_d = –0.6 V; deposition time t_d = 60 s; amplitude = 10 mV

TEP concentrations were highest in the phytagel solution (6.6 mg xanthan equiv. l^{-1}), followed by *Emiliana huxleyi* (2.3 mg xanthan equiv. l^{-1}) and *Thalassiosira weissflogii* (1.0 mg xanthan equiv. l^{-1}) with the lowest concentration in culture media of *Skeletonema costatum* (0.4 mg xanthan equiv. l^{-1}). TEP concentration reflected neither the pattern of particulate PS, nor that of SAS (Fig. 7).

Comparison of dissolved organic matter in different samples

TEP, SAS and RSS are substance classes that are operationally defined, and the exact chemical composition of each is unknown and variable. Most likely the compounds contributing to these classes overlap, meaning that some substances are members of more than one of these groups. As TEP consist largely of acidic PS, especially those rich in sulfur (Alldredge et al. 1993, Zhou et al. 1998), TEP most likely are SAS, and some TEP are also RSS, but many non-TEP substance classes belong to SAS and RSS. A direct comparison trying to assess the degree of overlap between these different measurements has never been made.

Our data suggest that TEP concentration was not correlated with carbohydrates, nor with SAS or sulfur concentrations. The lack of a correlation with PS confirms that the acidic PS making up TEP are a varying fraction of total PS. Additionally, the composition of TEP depends on the species generating TEP, and conversions to carbon (or glucose units) depend on TEP composition (Engel & Passow 2001).

The lack of a correlation between TEP and SAS suggests that the fraction of substances belonging to both SAS and TEP is small or variable, although TEP are known to be sticky, to promote aggregation of particles (Passow 2002) and accumulate in the surface micro-

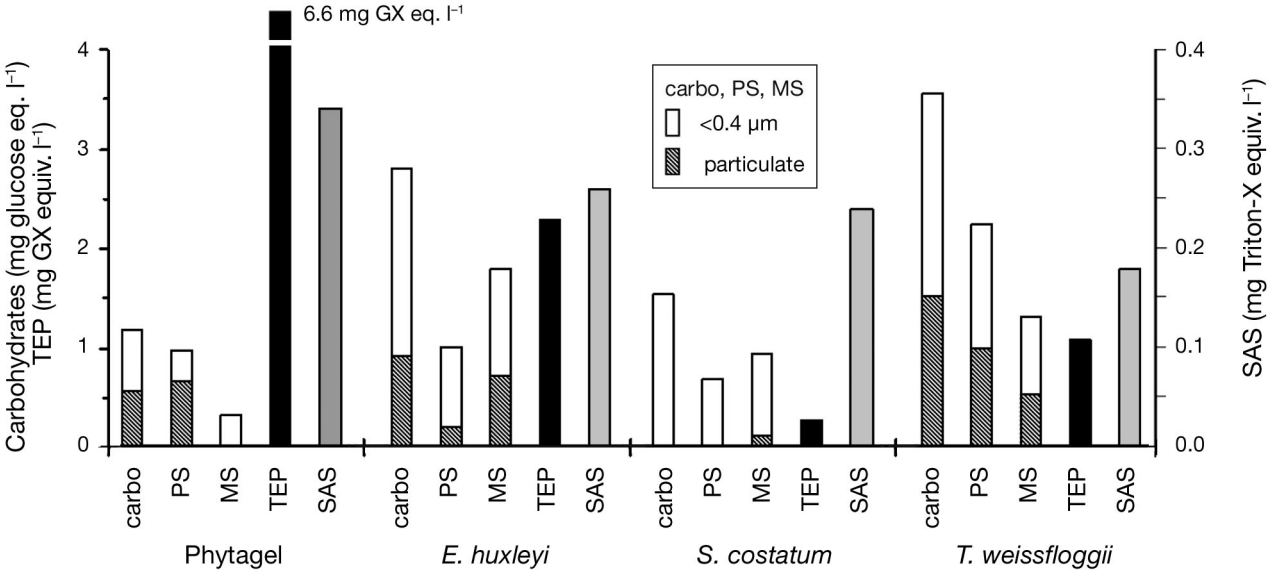

Fig. 7. Concentrations of particulate and dissolved carbohydrates (carbo = polysaccharides + monosaccharides), polysaccharides (PS), monosaccharides (MS), transparent exopolymer particles (TEP) and surface-active substances (SAS) in phytagel (5 mg l^{-1}) and in cultures of *Emiliana huxleyi*, *Skeletonema costatum* and *Thalassiosira weissflogii*

layer (Wurl & Holmes 2008). A similar lack of correlation between TEP and SAS has been observed in field samples also (unpubl. data). TEP can be rich in sulfur (Zhou et al. 1998), but our samples clearly contained other substance classes rich in sulfur as TEP and sulfur concentrations showed no correlation. The correlation between PS and sulfur (PS < 0.4 µm: $r = 0.94$, $p < 0.01$, $n = 3$, PS > 0.4 µm: $r = 0.91$, $p < 0.05$, $n = 3$) implies that a significant fraction of the total amount of sulfur was associated with PS, which did not form TEP. Our data suggest that glutathione type substances contributed appreciably to the high sulfur content in our cultures.

SAS were inversely correlated both with dissolved PS ($r = 0.96$, $p < 0.001$, $n = 4$) and sulfur content ($r = 0.99$, $p < 0.0005$, $n = 3$), suggesting that SAS in our samples consisted of sulfur-poor substances other than PS. SAS generated by phytoplankton can be rich in proteinaceous substances (Gašparović et al. 2007).

Characteristics of the phytagel model solution were not evenly distributed between the fraction retained and the fraction passing through filters, emphasizing that TEP, SAS and carbohydrates measure different substance classes. Whereas 72% of the total SAS were found in the size fraction <0.2 µm, only 53% of carbohydrates were in this size fraction, and TEP are, per definition, all retained on filters.

Characteristics of exudates of all 3 cultures differed appreciably, except for the presence of glutathione type substances in all 3 cultures. *Thalassiosira weissflogii* cultures contained large amounts of both purgable and non-purgable sulfur and high concentrations of PS (both dissolved and particulate), but relatively low TEP concentrations, whereas *Skeletonema costatum* was characterized by fairly high concentrations of non-purgable sulfur, and low concentrations of particulate carbohydrates (both PS and MS) and TEP. The presence of NH_2 groups further characterized *S. costatum* exudates. Organic matter of *Emiliana huxleyi* was characterized by high concentrations of MS and relatively low sulfur content.

Relationship between complexing capacity and dissolved organic matter characteristics

Assuming that the main respective Cu or Cd binding ligands in the phytoplankton exudates and model substances belonged to the same substance group and contributed significantly to that group, a relationship between the complexing capacity and the concentration of that substance group is expected.

Only 27% of the complexing capacity for Cu but 66% of that for Cd was retained in the 0.2 µm filtrate of phytagel. SAS, which were retained to 72% in the <0.2 µm fraction, thus seemed to have not been directly responsible for the binding of Cu, although some chemical specific component of SAS may have been. SAS may have been responsible for Cd complexing in the phytagel solution.

Characteristics of organic matter as described here varied between the different samples, and the complexing capacity of Cu or Cd showed no clear relationships to any of the types of ligands tested. TEP concentration was not correlated to the binding capacity or

the stability constants for Cu or Cd. SAS, another candidate for trace metal binding, were also not correlated to either L_{TOTCu} or L_{TOTCd}. The apparent absence of significant amounts of ligands in TEP or SAS could have several explanations. Either no ligand of importance belonged to these groups of substances, or a potential ligand belonging to TEP or SAS made up only a small fraction of TEP or SAS, which was lost in the noise of the bulk measurement. Another possibility would be that a part of Cu or Cd complexes was directly reduced due to the applied potential range (see above). Or, metal ligands generated by the different phytoplankton species belonged to different substance classes. Carbohydrates (PS or MS) could also not explain the observed binding capacities of Cu or Cd, except that the monosaccharide concentration was positively correlated to L_{TOTCu} (MS < 0.4 µm: $r = 0.95$, $p < 0.005$, $n = 4$; MS > 0.4 µm: $r = 0.85$, $p < 0.05$, $n = 4$). This could indicate that MS were primarily responsible for the complexing capacity of Cd (although correlation does not imply causation). Cd has been detected mainly complexed to organic matter in the low molecular weight fraction <1 kDa (Wells et al. 1998, Grzybowski 2000), but there is no literature to support the claim that these ligands consist of MS.

A glutathione-type ligand that has been implicated as a Cu ligand (Al-Farawati & van den Berg 1999, Leal et al. 1999, Laglera & van den Berg 2003, Tang et al. 2005, Laglera & van den Berg 2006, Marijić & Raspor 2007) was found in all 3 cultures. About 50% of natural marine Cu-complexing ligands have been found to belong to substances with a molecular weight between 1 and 10 kDa (Wells et al. 1998, Wen et al. 1999) which corresponds with molecular mass of glutathione and phytochelatins. Assuming that about half of the sulfur in the culture of *Thalassiosira weissflogii* belonged to this ligand (RSS: *T. weissflogii*: 90 glutathione equiv. nM, the other half was purgable), the concentration of organic, non-purgable sulfur was significantly ($r = 0.94$, $p < 0.01$, $n = 3$) and positively correlated to L_{TOTCu}, suggesting that glutathione type ligand could have been responsible for the binding of Cu in all 3 cultures.

SUMMARY

The 3 phytoplankton cultures and the phytagel solution complexed Cu ions and to a lesser extent Cd ions. The apparent stability constants were higher for Cd ions than for Cu. 'Glutathione type' ligands found in phytoplankton cultures are suggested to have been responsible for the binding of Cu. We found no correlations between the complexing capacity, TEP, SAS and RSS in our samples, indicating that very different substance classes are measured with these respective methods, although all appear important for trace metal binding.

No complexation for Fe^{3+} was observed for laminarin, carrageenan and alginic acid. The slopes of the titration lines with Fe in the presence of these model compounds were decreased in comparison to the UV irradiated seawater, pointing to the fact that selected model compounds are strongly adsorbed and decrease the sensitivity of the Fe determination.

Clearly new approaches are needed to classify and characterize the marine ligands that drive trace metal cycling. The combination of radioisotope work with cross-flow ultrafiltration (Schlosser & Croot 2008) is an example of a promising new approach.

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