

**BINDING OF THORIUM (IV) TO CARBOXYLATE, PHOSPHATE AND SULFATE  
FUNCTIONAL GROUPS FROM MARINE EXOPOLYMERIC SUBSTANCES (EPS)**

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## ABSTRACT

Th(IV) isotopes are important proxies in oceanographic investigations, and are used as tracers of particle dynamics and particulate organic matter (POC) fluxes out of the euphotic zone through the use of  $^{234}\text{Th}/\text{POC}$  ratios. These approaches rely on empirically determined and variable POC to  $^{234}\text{Th}$  ratios, which might be controlled, in parts, by the abundance of exopolymeric substances (EPS). EPS contain acidic polysaccharides (APS) and are excreted by both phytoplankton and bacteria. To this end, radiotracer experiments with EPS from microbial cultures were conducted to determine the binding environment of  $^{234}\text{Th}(\text{IV})$ -binding ligands in colloids and suspended particles in marine systems. In these experiments, the  $^{234}\text{Th}$  distribution during isoelectric focusing (IEF) and polyacrylamide gel electrophoresis (PAGE) was related to the functional group composition of EPS and of colloidal organic matter (COM) isolated from the Gulf of Mexico (GOM) using cross-flow ultrafiltration. EPS was extracted from phytoplankton (*Emiliania huxleyi* and *Synechococcus elongatus*) and bacteria (*Sagittula stellata* and *Roseobacter gallaeciensis*) cultures by repeated alcohol precipitation. Phosphate and sulfate concentrations were determined using ion chromatography (IC). IEF profiles indicated that 49% to 65% of the  $^{234}\text{Th}$  labeled EPS from plankton and bacteria as well as COM samples from the GOM was found concentrated below pH of 4, near an isoelectric point,  $\text{pH}_{\text{IEF}}$ , of about 2. The carboxylic acid maxima for extracted EPS and COM samples appeared close to the  $\text{pH}_{\text{IEF}}$  of  $^{234}\text{Th}(\text{IV})$ . The phosphate maximum appeared at the same  $\text{pH}_{\text{IEF}}$  as  $^{234}\text{Th}(\text{IV})$  for EPS from *Roseobacter gallaeciensis* and *Synechococcus elongatus*. The sulfate maximum was found at the same  $\text{pH}_{\text{IEF}}$  as  $^{234}\text{Th}(\text{IV})$  for EPS from *S. elongatus* and COM. The molecular weight (MW) of the strongly Th(IV) binding ligand varied from 1-14 kDa, depending on the species, but was about 10 kDa in COM. Thus, depending on the species of plankton or bacteria, the MW and specific functional group composition of the strongly  $^{234}\text{Th}(\text{IV})$ -binding amphiphilic biomolecule can vary. Therefore, different acidic functional groups can, at times, contribute to the binding of Th(IV) to the EPS chelating ligand, which can also have different MWs. This implies that the binding environment for Th(IV), which is present at total concentrations at least a million times lower than the acid functional groups, consists of strong polydentate chelate complexes in clustered structures of carboxylate, sulfates and/or phosphates. The combination of strongly chelating groups and amphiphilicity gives this biomolecule the unique properties of a “sticky” ligand.

## Introduction

Air-sea exchange of CO<sub>2</sub>, coupled to downwelling and/or the “biological pump”, are the main mechanisms of the ocean to counteract increasing anthropogenic CO<sub>2</sub> concentrations in the atmosphere. The “biological pump”, i.e., new production or carbon export flux, consists of the fraction of the primary production of organic matter resulting from CO<sub>2</sub> fixation by phytoplankton that is exported as particulate organic matter (POM) to the deep ocean, thus escaping internal recycling. Carbon export fluxes can be assessed by measuring biogenic particle fluxes caught in sediment traps (Buesseler *et al.*, 1992, 1995), as well as by the product of measured <sup>234</sup>Th flux times measured ratios of particulate organic carbon (POC) to <sup>234</sup>Th(IV) in either sinking (caught in sediment traps) or large (filterable) particles (Moran *et al.*, 2003, and references therein). Organic carbon to <sup>234</sup>Th(IV) ratios in suspended matter, used to determine new production rates, decrease with increasing depth and particle size, and are often different for settling particles caught in sediment traps than for large (>53 μm) suspended particles (Buesseler *et al.*, 1995, 1998, 2005; Murray *et al.*, 1996; Bacon *et al.*, 1996; Moran *et al.*, 1997; Santschi *et al.*, 2003, 2005; Hung *et al.*, 2004).

Thorium-234 (<sup>234</sup>Th(IV), t<sub>1/2</sub>=24.1 days) is a naturally occurring highly particle-reactive radionuclide continuously produced from alpha decay of Uranium-238 (t<sub>1/2</sub>=4.47 x10<sup>9</sup> years) in seawater. Uranium in seawater exists as the soluble carbonate species [UO<sub>2</sub>(CO<sub>3</sub>)<sub>3</sub>]<sup>4-</sup> that is relatively un-reactive to particles (less than 0.1% of uranium is particulate in seawater). Thus, uranium exhibits a rather conservative distribution in the open ocean (Ku *et al.*, 1977; Edwards *et al.*, 1987), with an average U-concentration of 1.4 x10<sup>-8</sup> M at a salinity of 35, thus providing a constant source of its daughter product, <sup>234</sup>Th. Long-lived <sup>232</sup>Th(IV) occurs in seawater at concentrations of about 4.5 x 10<sup>-12</sup> M (Choppin and Wong, 1998) and short-lived <sup>234</sup>Th(IV) at about 10<sup>-18</sup> M. This can be compared to natural seawater concentrations of major and minor cations, which are in the millimolar to micromolar range. The Th(IV) concentration is thus well below the solubility limit of ThO<sub>2</sub> of about 10<sup>-9</sup> M (Fanghänel and Neck, 2002). While Th(IV) might form a humic acid complex in seawater (Nash and Choppin, 1980), it was also found strongly associated with an acid polysaccharidic (APS) rich compound in both laboratory and field experiments (Quigley *et al.*, 2002; Guo *et al.*, 2002, Santschi *et al.*, 2003). Acidic polysaccharide fibrils have been identified as important biopolymers in different aquatic systems (Allredge *et al.*, 1993; Leppard, 1995, Santschi *et al.*, 1998, 2005), and are likely derived from

exopolymeric substances (EPS) excreted by micro-organisms. EPS compounds are amphiphilic or amphiphatic (Buffle, 1990; Leppard, 1995, 1997) and act as biosurfactants (Ron and Rosenberg, 2001). Because of their surface activity, EPS in the ocean form alcian-blue stainable transparent exopolymeric particles (TEP, Alldredge et al., 1993). APS compounds, even though generally only a minor fraction (~10%) of the polysaccharide pool (Santschi *et al.*, 2003; Hung et al., 2003a), are present in both particulate and colloidal material, and play a critical role in the formation of marine snow flocs, mucilaginous aggregates, and the removal of trace elements and radionuclides from the water.

Using radiolabeled glucose, Stoderegger and Herndl (1998) determined the incorporation of glucose into intracellular and capsular pools (i.e., EPS) to acquire production estimates. Their results indicated that 55% of labeled glucose was incorporated intra-cellularly and 45% to capsular material. Release rates of the capsular material represented about 25% of bacterial respiration suggesting that a significant portion of the DOC pool is composed of bacterially derived semi-labile EPS (Stoderegger and Herndl, 1998).

It was recently reported that the strongly Th(IV)-binding APS compound has a low  $pH_{IEP}$  and  $pK_a$  of  $\leq 3$  (Quigley et al., 2002), and a high sticky coefficient in seawater of 0.9 (Quigley et al., 2001). Such a “sticky” macromolecular ligand is likely instrumental in removing Th(IV) from the ocean. The exact functional group composition of this APS compound is, however, not well known, even though Santschi et al. (2003) reported that it might also contain phosphate.

In order to determine the functional group composition, isoelectric focusing experiments were conducted with samples of EPS taken from cultured phytoplankton and bacteria, as well as of colloidal macromolecular organic matter from the Gulf of Mexico.

## 2. MATERIALS AND METHODS

### 2.1. Sampling

Colloidal organic matter (COM) water samples were taken from the chlorophyll *a* maximum layer inside a Cold Core Ring (CCR, Station 4, 27°38'N, 94°59'W) at a depth of 72 m along a N-S transect in the Gulf of Mexico (GOM) aboard the R/V Gyre during May 17-25, 2001 (Santschi et al., 2003; Hung et al., 2003a,b). Briefly, cross-flow ultrafiltration (CF-UF) was used to extract the colloidal fraction from large volumes of seawater (Benner, 1991; Guo *et al.*, 1994,

1996, 2000a,b). An Amicon DC10L ultrafiltration system was used with a 1kDa spiral-wound polysulfone cartridge (Amicon, S10N1). About 200 liters of seawater were processed, and the concentrated colloidal fraction was reduced to ~ 2 liters. All ultra-filtration experiments were carried out aboard ship within 6-10 h of seawater collection (Guo *et al.*, 1994). The concentrated colloidal sample was further desalted through diafiltration using nanopure water and subsequently freeze dried for purification as well as <sup>234</sup>Th labeling experiments.

## 2.2. Exopolymeric substances (EPS) from bacteria and phytoplankton

Phytoplankton and bacteria cultures were used to obtain polysaccharide- and APS-enriched EPS isolates from particle phases using established procedures (Staats *et al.*, 1999). Illumination for the cultures was provided by white fluorescent tubes and photo flux density was 12.5  $\mu\text{E m}^{-2} \text{s}^{-1}$ , measured by a LI-COR (model LI-250 Light Meter) quantum Radiometer/Photometer equipped with a quantum sensor (LI-Q323926). The species used to extract EPS included the marine bacteria *Roseobacter gallaeciensis* (Strain ATCC 700781) and *Sagittula Stellata* (Strain ATCC 700073) and the phytoplankton *Emiliana huxleyi* (Strain CCMP 374) and *Synechococcus elongatus* species (Strain CCMP 1379). These bacteria and phytoplankton species are very common in the Gulf of Mexico and the open ocean. *B. gallaeciensis* and *Sagittula stellata* (obtained from the American Type Culture Collection, ATCC 700073) were cultured in marine broth 2216 (Difco Laboratories) media at 27-29 °C. After 2 to 4 days of incubation, the cultures were sampled for harvesting EPS (see below).

The two phytoplankton species, *Emiliana huxleyi* (CCMP 374) and *Synechococcus elongatus*, obtained from the Bigelow Center for the Culture of Marine Phytoplankton, were cultured in f/2-Si medium at 20 °C under a 12:12 day/night irradiance cycle. From these cultures, EPS was harvested (see below) after 10-14 days of incubation. Both bacteria and phytoplankton cultures were used for radioisotopic incubation experiments.

### *Procedure for harvesting, i.e., extraction and purification of EPS:*

EPS extraction and precipitation was performed as described in Staats *et al.* (1999), which minimizes cell lysis and maximizes extraction efficiency through the use of tap water rather than nanopure water ([Figure 1](#)).

After the bacterial and phytoplankton incubations, 35 ml of media solution was placed into centrifuge tubes. The bacteria or phytoplankton cells were centrifuged (Sorvall RC-5B

Refrigerated Superspeed Centrifuge) at 10,000 rpm for a period of 30 minutes at 20° C, in order to separate phytoplankton or bacteria from seawater and marine broth media. The supernatant seawater or marine broth solutions were then decanted. The bacterial and phytoplankton pellet on the bottom of the container was washed with tap water to extract surficial EPS (Staats *et al.*, 1999). The aqueous supernatant was separated by centrifugation and four volumes of 95% ethanol and 5% methanol were slowly added to the aqueous supernatant resulting in a alcohol:water ratio of 4:1. The resulting solution was stored in -20° C overnight to promote precipitation of EPS. After that, the ethanol:water mixture was ultracentrifuged for 30 minutes at a temperature of -20°C. This separation and purification procedure was performed a total of five times, after which the EPS was lyophilized. The EPS material thus isolated was then further separated through isoelectric focusing and 2D SDS-PAGE to determine the spectrum of <sup>14</sup>C, <sup>32</sup>P, <sup>35</sup>S, <sup>234</sup>Th(IV), as well as phosphate (PO<sub>4</sub><sup>3-</sup>), and sulfate (SO<sub>4</sub><sup>2-</sup>). *S. stellata* and *S. elongatus* yielded about 2 mg (2% yield) and 1 mg (1% yield) of EPS material, respectively, after IEF separation.

#### *Radioisotopic Incubation [<sup>32</sup>P, <sup>35</sup>S and <sup>234</sup>Th(IV)]:*

In order to verify mass balance and extraction efficiency, incubations were conducted in parallel but on smaller scale (30 ml) with all species. Incubation conditions and duration for the radioisotopic incubations were identical to experiments without radioisotopes i.e., EPS material was harvested after 10-14 days of incubation. Triplicate cultures of each species were incubated with small amounts of <sup>32</sup>P (in the form of H<sub>3</sub>PO<sub>4</sub> in 0.02N HCl), <sup>35</sup>S (in the form of H<sub>2</sub>SO<sub>4</sub> in water) and <sup>234</sup>Th(IV) (in nanopure H<sub>2</sub>O, after purification according to Quigley *et al.*, 2001, 2002). Small amounts of radiolabeled material were then extracted as described above, including micro-organism cells by centrifugation, EPS by alcohol precipitation as described above, and 0.1 N HCl wall leachates. Activity levels used were high enough for mass balance evaluations but generally not for IEF experiments.

### **2.3. Laboratory Analyses**

#### *Determination of the Relative Concentration of Carboxylic Acid:*

Carboxylic acid functional groups of samples of humic acid (as a test sample), EPS harvested from cultures, and COM from the Gulf of Mexico were radiolabeled with <sup>14</sup>C-

methylamine according to published procedures (Warwick et al., 1993, as modified by Alain Reinhardt, CABE, University of Geneva, Switzerland, personal communication). 20.0 mg of Suwannee River Humic Acid Standard (Int. Humic Substances Society), or 5.0 mg of microbial (bacteria and phytoplankton) EPS were added to 10.0 ml 0.05M NaCl adjusted to pH of 7.0 and filtered through a 0.22mm white GSWP 47mm filter. Under nitrogen atmosphere,  $0.322 \times 10^{-4}$  Moles 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Fluka-Chemika) was added to the filtered sample.  $6.95 \times 10^{-7}$  Moles  $^{14}\text{C}$ -methylamine (ICN), an amount sufficient to react with about 1% of the number of carboxylic acid groups present in solution, was added to a humic acid standard and/or the microbial samples, respectively (assuming identical fraction of carboxylic acid groups as in the Suwannee River Humic Acid Standard). The basis for labelling only about 1% was so that the chemical behaviour of the macromolecular compound would not be significantly altered. The pH of the solution was adjusted to 7.0, and the reaction vessel was stirred overnight. The pH of the solution was then lowered to 2 using concentrated nitric acid. The acidic solution was subsequently placed in a dialysis bag (Spectrum CE molecular weight cut-off, MWCO 500) and inserted in 900ml of 0.05 M NaCl at pH 2 for 3 days. The solution was recovered from the dialysis bag and neutralized to a pH of 7.0, then dialysed again with nanopure  $\text{H}_2\text{O}$  to remove unwanted salts prior to isoelectric focusing. The sample was then freeze-dried and resuspended in nanopure  $\text{H}_2\text{O}$  prior to carrying out isoelectric focusing and 2-D SDS-PAGE (Quigley et al., 2002). Finally, individual parts of the gel strips were sectioned and subsequently analyzed on a liquid scintillation counter.

#### *Isoelectric focusing and 2-D SDS-PAGE:*

Isoelectric Focusing Electrophoresis (IEF) IEF is an electrophoretic method that separates molecules according to their isoelectric point (pI or  $\text{pH}_{\text{IEF}}$ ). Isoelectric focusing (IEF) experiments were conducted as a means of primarily determining the  $\text{pH}_{\text{IEF}}$  of  $^{234}\text{Th}(\text{IV})$  binding ligands. Two types of IEF were obtained and presented, IEF obtained from a one-dimensional IEF strip, and IEF obtained from a 2D SDS-PAGE, where the sum of the molecular weight (MW) subfractions at each pH were combined to result in a one dimensional profile (or spectrum). IEF is performed under denaturing conditions to provide the highest resolution and the cleanest results. This is undertaken by mixing urea, carrier ampholytes, and detergent (Triton X) to ensure that each sample (polymer) is only present in one configuration and aggregation and

intermolecular interactions are minimized. Each sample (ca. 2 mg of microbial EPS) was loaded into each IPG strip (Amersham Biosciences, Immobiline Dry Strip, pH 3-10, 11cm, Lot No: 300135), in addition to 100  $\mu$ l of rehydration solution. The IPG strips were placed into the reswelling tray for at least 12 hours. Consequently the IPG strips were then placed in the Electrophoresis apparatus (Amersham Biosciences, Multiphor II Electrophoresis System) and run for 17.5 hours with a current profile of 19,701 Volt-hours (Vh), and the pH values calibrated separately. 2D SDS-PAGE separates molecules according to two physical properties, i.e., net surface charge (pH) and molecular size. The charged molecules migrate during isoelectric focusing through the gel toward one of the electrophoresis electrodes until protonation or deprotonation within the pH gradient results in a net neutral charge for the molecule ( $\text{pH}_{\text{IEF}}$ ) (Trubetskoj *et al.*, 1992). The second dimension run, which was carried out on the same sample, was a standard polyacrylamide homogeneous gel (T=15% Amersham Pharmacia Biotech). The Multiphor II system (Amersham Pharmacia Biotech) was used for sample preparation and electrophoresis, according to the manufacturer's recommended procedures. The pH along the length of the isoelectric focusing gel was monitored, and the molecular weight (MW) gradient or homogeneous gel was calibrated by use of rainbow colored MW marker standards (Amersham Pharmacia Biotech).

*Liquid Scintillation Counting (LSC) -  $^{14}\text{C}$ ,  $^{234}\text{Th(IV)}$ :*

$^{14}\text{C}$  and  $^{234}\text{Th(IV)}$  in the samples were counted for 10 minutes by a Beckman Model 8100 Liquid Scintillation Counter. The conventional energy windows used were 18 keV to 0.156 MeV for  $^{14}\text{C}$  and 0.199 MeV for  $^{234}\text{Th(IV)}$ , respectively. The detection limits for  $^{14}\text{C}$  and  $^{234}\text{Th}$  analyses were 6.4, and 6.3 dpm, respectively. Measurement errors were  $\pm 10\%$  for a 2-sigma error (Quigley *et al.*, 2001).

*Determination of organic phosphate and sulfate, carbohydrates, proteins, monosaccharides and uronic acids:*

The organic phosphorus and sulfate were extracted from the IEF gel sections with Triton X-100, and analyzed according Grotjan *et al.*, (1986) as modified by Silvestri *et al.*, (1982). The method utilizes acid hydrolysis of organic material with subsequent lyophilization followed by pyrolysis with ultimate injection into ion chromatograph (IC). The EPS samples were dissolved

in nanopure water in glass tubes and the solution was pyrolyzed. The dry residue was then dissolved in 2 ml of nanopure water for the analysis by ion chromatography. Total carbohydrates in EPS were measured by a spectrophotometric method (Myklestad et al., 1997), as modified by Hung and Santschi (2001). The concentration of protein in EPS was measured by the bicinchoninic acid method and colorimetric detection (Smith et al., 1985). Bovin serum albumin was used as a standard to quantitatively calculate the protein content. The concentration of uronic acids in EPS was analyzed according to Filisetti Cozzi and Carpita (1991), as modified by Hung and Santschi (2001). The concentrations of monosaccharides and acidic polysaccharides were measured, after methanolysis, by the method of Doco et al. (2001) using alditol as a standard. Briefly, EPS were suspended in methanol/HCl solution and kept at 80 °C for 16 hr in a heating block. After that, the solution was cooled down and concentrated to dryness at 40 °C under a stream of nitrogen gas. 0.3 ml of TriSil reagent was added to the solutions at 80 °C for 20 mins and the extra reagents were removed by a stream of nitrogen gas. The residue was extracted by hexane. Hexane was then concentrated to 20 µL. Finally, 2 µL of hexane was used for measurement by GC-EI-MS (Polaris-Q GC/MS) with a J&W Scientific column (DB 1701, 0.25 mm ID, 30 m). The temperature of the GC program was set up as follows: 120 °C to 145 °C at 1 °C /min, and 145 to 180 °C at 0.9 °C /min, and 180-230 at 50 °C /min. Peaks of neutral monosaccharides and acidic polysaccharides in the EPS were identified by comparison of retention time and mass spectral fragmentation patterns of monosaccharide and acidic polysaccharide standards.

*Hypothesis testing:*

Acidic polysaccharides (those containing anionic acidic functional groups, i.e., carboxylate, phosphate or sulfate) are mainly responsible for surface complexation of  $^{234}\text{Th(IV)}$  compared to neutral polysaccharides. If there is no observed correlation between any of the anions in the acidic region and  $^{234}\text{Th(IV)}$ , then the null ( $H_0$ ) will have to be accepted.  $H_0$ :  $^{234}\text{Th(IV)}$  will have no correlation with any of the individual acidic functional groups for all samples.

### 3. RESULTS AND DISCUSSION

#### 3.1. Radioisotope Labeling Incubation Experiment - $^{14}\text{C}$ and $^{234}\text{Th(IV)}$

The purpose for conducting this radionuclide experiment was to verify mass balance and extraction efficiency. IEF gel electrophoresis was performed on radiolabeled EPS in order to study and compare the isoelectric (pH) profile of  $^{14}\text{C}$  incubated material with that non-incubated  $^{234}\text{Th}$  labeled EPS.

##### *Bacterial Incubations:*

The mass balance for the EPS extraction method was obtained for all species and each individual step of the method. Total recoveries for  $^{234}\text{Th(IV)}$  were 60-82% for the marine bacteria *R. gallaeciensis*, 93-97% for *S. stellata*, 66-122%, for *E. huxleyi*, and 93-99% for *S. elongatus*. Less than 4% of the total activity of  $^{234}\text{Th(IV)}$  remained in the marine broth after incubation. The majority, i.e., 56-75% and 79-89% of the total  $^{234}\text{Th(IV)}$  activity was associated with the microbial pellet for *R. gallaeciensis* and *S. stellata*, respectively. The water extraction of the microbial pellet resulted in 1% and 8% extraction efficiency for *R. gallaeciensis* and *S. stellata*, respectively. Very little of the  $^{234}\text{Th(IV)}$  label remained on the centrifuge tubes (ranging from 1-7% of total  $^{234}\text{Th}$  activity). Eventually, between 0.5% and 2% of the  $^{234}\text{Th}$  was extracted as Th(IV)-reactive EPS produced by *R. gallaeciensis* and *S. stellata* from the cell surface, respectively. Thus a large portion of the  $^{234}\text{Th(IV)}$ , as was the case with  $^{32}\text{PO}_4$  (not shown), remained on the cell surface, indicating that the extraction procedure only removed a small fraction of the thorium-binding biomolecule.

##### *Phytoplankton Incubations:*

At the end of the incubation period, less than 8% of total activity of  $^{234}\text{Th}$  remained in the marine broth. 41-61% and 32-76% of the total  $^{234}\text{Th(IV)}$  activity remained associated with the microbial pellet for *E. huxleyi* and *S. elongatus*, respectively. The water extraction of the microbial pellet resulted in 33% and 17% extraction efficiency for *E. huxleyi* and *S. elongatus*, respectively. Very little of the  $^{234}\text{Th(IV)}$  label remained on the centrifuge tubes (ranging from 2-3% of total  $^{234}\text{Th(IV)}$  activity). Only about 10% of  $^{234}\text{Th(IV)}$  associated with EPS material was extracted from the microbial cells (9% and 11% for *E. huxleyi* and *S. elongatus*, respectively).



49 % and 55 % of the total activity of  $^{234}\text{Th(IV)}$  were found near pH of 2 for *R. gallaeciensis*, *S. stellata*, *E. huxleyi*, *S. elongatus* and GOM COM, respectively.

IEF and 2D SDS-PAGE for  $^{234}\text{Th(IV)}$ -labeled EPS were conducted in parallel to corroborate the  $^{234}\text{Th(IV)}$  affinity to the acidic region, and showed similar distribution patterns for all species examined. The IEF spectra obtained from 2D SDS-PAGE of *R. gallaeciensis* and *S. stellata* exhibited similar profiles from that of the simple IEF, while IEF spectra obtained from 2D SDS-PAGE for *E. huxleyi* and *S. elongatus* exhibited nearly identical profiles to those of the simple IEF (not shown). Although there were slight variations, statistically speaking (see section 3.4) there was no significant difference.

IEF of carboxylic acid labeled EPS from microbial species with dimethylamine ( $[\text{C}^{14}\text{CH}_3]_2\text{NH}$ ) generally resulted in  $^{14}\text{C}$ -maxima at low pH, e.g., 30 % at pH between 3.14 and 2.31 for *R. gallaeciensis*, 56 % at pH between 1.8-2.3 for *S. stellata*, 46 % at pH between 1.7 – 3.9 for *E. huxleyi* (Figure 2). *S. elongatus* exhibited two major  $\text{pH}_{\text{IEF}}$  peaks at either electrode, with 31 % below pH of 4. The general trend was an increase in activity at lower pH.

There were not only  $\text{PO}_4^{3-}$  peaks in the IEF spectrum that were coinciding with the  $^{234}\text{Th}$  and  $^{14}\text{C}$  maxima at low pH, but also peaks at higher pH values, as phosphate would also be found in other biomolecules such as DNA, RNA, or phospholipids. For example, there were phosphate peaks at  $\text{pH} < 5$  for *R. gallaeciensis*, three major peaks (pH 2.3, 4.5 and 9.65) for *S. stellata*, three peaks (pH 2.7, 4.75 and 11.4) for *E. huxleyi* (Figure 2) and two distinct peaks (pH 3.71 and 8.92) for *S. elongatus*. The IEF spectra of  $\text{SO}_4^{2-}$  also had peaks at low pH, e.g., for *R. gallaeciensis* at pH 3.1, two major peaks (pH 1.8 and 9.6) for *S. stellata*, two regions of elevated activity (pH 1.7, 7.9-11.4) for *E. huxleyi* and two distinct peaks (pH 2.0 and 8.9-11.2) for *S. elongatus*.

The IEF and 2D SDS-PAGE spectra of COM from the Gulf of Mexico showed nearly identical profiles (not shown), with no significant differences between the two methods. The  $^{14}\text{C}$ -labeled carboxylic acid profile had one significant peak located at the anode pH 2.2, phosphate showed three, and sulfate showed two major peaks (Figure 3). The phosphate peaks were at pH 2.2, 4.1 to 5.00 and 6.1. Sulfate peaks were at pH 2.2 and 9.25 to 10.3. Sulfate in the neutral and basic region could originally have been present as sulfur species different from sulfate esters that were hydrolyzed and/or oxidized through our procedures.

In summary,  $^{234}\text{Th}$ , sulfate and phosphate from the colloid and the EPS samples all had coincident peaks at low pH near 2, while P and S also had peaks at higher pH values.

### 3.3. 2D SDS-PAGE (IEF+1D SDS-PAGE) of Thorium (IV) bound to EPS

Two dimensional (2D) sodium dodecyl sulfate (SDS) polyacrylamide gel (PAGE) electrophoresis using a 15% homogeneous gel (2D SDS-PAGE 15%) was performed on all samples to determine and isolate the  $^{234}\text{Th(IV)}$  binding ligand and associate it with a specific functional group. In the marine bacteria sample of *R. gallaeciensis*, a combined 24% of  $^{234}\text{Th(IV)}$  activity was associated with the extracted EPS that had a MW of <1 kDa at pH 2.0 and 2.3 (Figure 4). This was similar to the IEF results, although the IEF plot had a higher amount (i.e., 38% total) for same pH regions. Upon further inspection, the sum of the  $^{234}\text{Th(IV)}$  activity for the pH 2.0 and 2.3 (from 2D SDS-PAGE) was 42%. At an approximate pH of 2, 24% of the total  $^{234}\text{Th(IV)}$  activity was associated with the organic matter in the MW category less than 1 kDa in size range. Clearly, for *R. gallaeciensis*, the  $^{234}\text{Th}$ -labeled EPS material was mostly in the truly dissolved fraction.

For the marine bacteria sample of *S. stellata*, a combined 37% of total  $^{234}\text{Th(IV)}$  activity was associated EPS around 3.5 kDa at pH 1.9 and 2.3 (Figure 5). This was corroborated from the IEF plot, although the IEF plot had a combined total of 47% for the same pH regions. The sum of the  $^{234}\text{Th}$  activity for the pH 2.0 and 2.3 (from 2D SDS-PAGE) was 50%. Evidently, for *S. stellata*, at an approximate pH of 2, 37% of the total  $^{234}\text{Th(IV)}$  activity was associated with the organic matter in the MW category of 3.5 kDa.

For *E. huxleyi*, a combined 27% of total  $^{234}\text{Th(IV)}$  activity was associated with EPS with a molecular weight of 14.3 kDa and a  $\text{pH}_{\text{IEP}}$  between 1.7 and 4.7 (Figure 6). This was corroborated from the IEF plot, although the IEF plot had a combined total of 50% for the same pH range. The sum of the  $^{234}\text{Th(IV)}$  activity for the pH 2.0 and 2.3 (from 2D SDS-PAGE) was 50%. Interestingly, Magaletti et al. (2004) recently isolated, using Gel Permeation Chromatography, a 12 kDa fragmentation product of EPS from different diatom species, *Cyndrotheca fusiformis* commonly found in mucilage aggregates of the Adriatic, besides a 20kDa and 700 kDa product.

For the *S. elongatus*, a combined 17% of total  $^{234}\text{Th(IV)}$  activity was associated with EPS with a MW ranging from 1 to 6.5 kDa (average of 3.7 kDa) for pH 1.85 (Figure 7). This was corroborated from the IEF plot, although the IEF plot had a combined total of 32 % for same pH range. The sum of the  $^{234}\text{Th(IV)}$  activity for the pH 1.85 (from 2D SDS-PAGE) was 46%. *S. elongatus*, at approximately pH of 1.85, 17% of the total  $^{234}\text{Th(IV)}$  activity was associated with

the EPS in the MW category less than 3 kDa. In this case, there was another peak above 45kDa size accounting for ~14% total  $^{234}\text{Th(IV)}$  activity. When this material was run again on a gradient (8-18%) SDS-PAGE instead of typical 15% homogenous SDS-PAGE, a large portion of the activity was associated with material above 160 kDa. This indicated that the  $^{234}\text{Th(IV)}$ -binding ligand can be present in different MW fractions, which all might have similar chelating functional group domains for strongly binding Th(IV); however, all have low  $\text{pH}_{\text{IEP}}$ . One way to explain this observation is that the low molecular weight ligands might be breakdown products of larger macromolecular ligands.

#### *Carboxylic Acid Functional Group - $^{14}\text{C}$ :*

2D SDS-PAGE (15%) was performed on all samples to map out the activity associated with the  $^{14}\text{C}$ -labeled carboxylic acid functional group of *R. gallaeciensis* (example in Figure 8). The main reason for conducting 2D SDS-PAGE on carboxylic acid functional group for  $^{14}\text{C}$ -dimethylated amine was to correct for the dimethylamine peak ( $\text{pK}_a = 10.9$ , Mol. Weight = 33 Da). Even though every attempt was made to dialyze unwanted salts and unreacted dimethyl amine from organic material, dimethylamine was still present, indicated by an anomaly in the initial IEF profiles, where a significant amount of the  $^{14}\text{C}$  activity was found in the basic region. When the Suwannee River humic acid test standard was run, there was no activity in the high pH region, likely due to a more favorable ratio of carboxyl groups to dimethylamine. Even though the amount of the marine sample was scaled down 4 fold, there might still have been excess reactant if the carboxylate concentration was lower in EPS. Regardless, these results allowed to correct for this low MW  $^{14}\text{C}$  peak in the IEF profile by using the results from the 2D SDS-PAGE experiment.

### **3.4. Statistical Analysis**

Statistical analysis was conducted using SPSS software (SPSS Inc., Chicago, IL 60606) 2-tailed, bivariate correlation ( $\alpha = 0.05$ ) after testing for normality using Kolmogorov-Smirnov test. All data resulted in being parametric indicating that any correlations having a p-value at or below 0.05 were selected to be significant.

Our null ( $H_0$ ) hypothesis was stipulated for the analysis of the IEF spectra (section 3.2) as follows: A p-value indicates the probability of the hypothesis being tested (usually  $H_0$ ) is true (a

p-value of 0.01 indicates there is a 1% chance that the hypothesis being tested is true). With a critical level for rejection ( $\alpha$ ) of a hypothesis set at 0.05, the null hypothesis ( $H_0$ ) is rejected with any p-value smaller than 0.05. Thus, the smaller the p-value the more confident one can be to reject the null hypothesis and to accept the alternative hypothesis (Dytham, 1999).

#### *Comparison of IEF Results:*

Bivariate correlation analysis was conducted for each species, using the criteria stated above. All samples (*R. gallaeciensis*, *S. stellata*, *E. huxleyi*, *S. elongatus*, and GOM COM) displayed significant correlations between the IEF spectrum of  $^{234}\text{Th(IV)}$  over the whole pH range for the 1D-IEF ( $^{234}\text{Th(IV)}$  IEF) and the IEF obtained from the sum of the parts from 2D SDS-PAGE, which allowed us to use both IEF and IEF from 2D SDS-PAGE separation techniques for statistical tests. Selected examples are given in Figures 2 and 3.

In short, IEF spectra of EPS from *R. gallaeciensis*, *E. huxleyi* and *S. elongatus* showed significant correlations between  $^{234}\text{Th}$  and  $^{14}\text{C}$ -carboxyl,  $^{31}\text{P}$ -labeled and stable phosphate, and  $^{35}\text{S}$ -labeled and stable sulfate in the acidic range ( $\text{pH} < 7$ ), while *S. stellata* showed no significant correlations because of slight differences of  $\leq 0.5$  pH units in their respective peaks. Most importantly, COM from the Gulf of Mexico showed significant correlations between all parameters over the whole pH range. This difference is likely due to the fact that labile DNA, RNA and other biomolecules (with binding capacity for  $^{234}\text{Th}$ ), which are expected to have  $\text{pH}_{\text{IEP}}$  in the neutral to basic range, are present in the extracted EPS at considerably higher concentrations than in marine colloids.

### **3.5. Chemical Composition of EPS**

The content of hydrophilic polysaccharides from the EPS samples from *S. stellata* and *S. elongatus* isolated by IEF from the pH 1.9 region was 14 % and 8 % of the total carbon content, while the carbon content of more hydrophobic proteins was 2.6 % for *S. stellata* (Table 1). Thus, the Th(IV)-binding ligand is amphiphilic (or amphiphatic). Approximately 15 and 23 % of the total polysaccharides from *S. stellata* and *S. elongatus* were composed of hydrophilic uronic acids, respectively. Overall, the sum of polysaccharides and proteins was about 17 % of the organic carbon for *S. stellata* (Table 1). The polysaccharide and protein data agree with the analysis of bulk EPS, which showed 6% TCHO and 5% proteins for the unattached, dissolved

fraction, and 12% TCHO and 5% proteins for the attached, particulate fraction of EPS. The remainder of EPS mass might have been lipids, refractory sugars or other unknown compounds. Moreover, water could be another important fraction of the isolated EPS, which is hygroscopic, similar to humic acids (Buffle, 1990; Leppard, 1997).

Two important anions are also found in the EPS after IEF separation, phosphate and sulfate. The molar ratios for the EPS, in terms of total carbon: SO<sub>4</sub>, were 72:1 and 30:1 for *S. stellata* and *S. elongatus*, respectively, which are similar to the molar ratios of C:S in marine particles, which range from 50 to 70 (Cutter, 1982; Chen et al., 1996). In other words, if all the carbon would be composed of hexoses, the sugar: SO<sub>4</sub> ratios would be 12:1 and 5:1 for *S. stellata* and *S. elongatus*, respectively. The sulfate results are similar to those recently published for bulk polysaccharide-rich mucilage by Mecozzi et al. (2005), who reported a molar ratio of total carbon to total sulfur ranging from 18-66, with an average value of 30. The molar ratios of carbon to phosphate in the EPS isolate, after IEF separation, was 243 and 200 for *S. stellata* and *S. elongatus*, respectively. The C:P ratio of 200 in the EPS is about twice that of the Redfield ratio. This ratio is very similar to the C:P ratio in labile dissolved organic matter of 154 to 245 (average of 199), reported by Hopkinson and Vallino (2005). Therefore, the ratios of C:S or C:P in our purified EPS samples are similar to elemental ratios in bulk to elemental ratios in bulk dissolved organic matter.

Given total organic carbon concentrations of 10<sup>-4</sup> M and a carboxylate concentration of 1 meq/g-C (Santschi et al., 1995) in seawater, organic phosphate, sulfate or carboxylate concentrations of the order of 10<sup>-6</sup> M, and Th(IV) concentrations of the order of 10<sup>-12</sup> M (determined by <sup>232</sup>Th), there would be at least ~ 10<sup>6</sup> potential acidic binding sites for one <sup>234</sup>Th(IV) ion. The vast majority of such sites would be occupied by other metals in the ocean, such as calcium. This would imply that rarer but clustered acidic binding sites of variable composition are likely important as chelating sites. Thus, the steric environment and not necessarily the exact functional group might actually be responsible for thorium-234 complexation to macromolecular organic matter.

*Carbohydrate Composition:* The gas chromatography (GC) mass spectrometry (MS) provided secondary characterization of the organic material that complexed with <sup>234</sup>Th(IV) and was extracted from bacterial (Figure 9a) and phytoplankton (Figure 9b) cultures after alcohol

precipitation and IEF separation at pH of 2. The total acid polysaccharide (APS) content was calculated by the sum of individually identified monosaccharide peaks from the GC spectra (Table 2). The determination of simple sugars was accomplished by comparing a library of mass spectra to that of sample mass spectra at a specific retention time (Figures 9a and 9b).

The majority of the organic material extracted from both *S. stellata* and *S. elongatus* isolated EPS samples showed allose, glucose, galactose, mannose and xylose as the main simple sugar monomers in the methanolized polysaccharide fraction (Table 2). The acidic polysaccharides in both *S. stellata* and *S. elongatus* were galacturonic acid, mannuronic acid and glucuronic acid (Table 2). However, there were many unknown peaks, which could not be identified in these isolated EPS samples, but could nonetheless be quantified by comparison to the standard peak. Overall, the normalized URA to total sugars measured by the spectrometric method (15 and 23% of TCHO) and GC-MS (19 and 26%) are in agreement for both *S. stellata* and *S. elongatus*, respectively. Bergamaschi et al. (1999) reported that uronic acids, URA, contain galacturonic, glucuronic and mannuronic acids, with a range of 2 to 6% of total carbohydrates. Aluwihare et al (2002) also found that high molecular weight dissolved organic matter contains some URA, such as glucuronic acid and galacturonic acid. In comparison, our URA concentrations are higher than these previous reports because 1) an EPS sample extracted from pure phytoplankton or bacteria cultures should contain more high molecular weight polysaccharides and less monosaccharides using repeated alcohol precipitations and 2) the samples chosen in this study were isolated at specific pH and molecular weight values rather than from the bulk material.

#### 4. SUMMARY AND CONCLUSIONS

The primary objective of our experiments was to test how the  $^{234}\text{Th(IV)}$  is bound to oceanic particles, which could be important to our understanding of the variability of  $\text{POC}/^{234}\text{Th}$  ratios in the ocean. This was accomplished through controlled laboratory experiments with exopolymers responsible for  $^{234}\text{Th(IV)}$  complexation and extracted from pure cultures of phytoplankton (*S. elongatus*, *E. huxleyi*), bacteria (*R. gallaeciensis*, *S. stellata*) as well as colloidal organic matter (COM) from the Gulf of Mexico. The microbial incubation experimental results indicate that most of the  $^{234}\text{Th(IV)}$  is taken up at the cell surface and is not easily removed

from it, with  $^{234}\text{Th}$  found at ratios similar to the mass of cells to removable EPS in solution. Thus, while potential binding sites for  $^{234}\text{Th(IV)}$  near the cell surface were not assessed, an apparently small but representative Th-tagged EPS sample can still be extracted from the cells for further separation and characterization.

The experiments described in this paper are representative for fresh phytoplankton bloom conditions that can produce rapidly sinking aggregates controlled by the excretion of EPS (Allredge *et al.* 1993; Verdugo *et al.*, 2004, and references therein). While it is possible that the isolation, purification and drying of isolated materials could have affected the subsequent Th binding experiments, there are valid reasons for believing that the results described in this paper are relevant to oceanic conditions. These reasons include: 1) Similarity between the results of Th(IV) binding experiments at ambient pH of Quigley *et al.* (2002) who used similar procedures, with those of Hirose (1996, 2004) and Hirose and Tanoue (1994, 1998, 2001), who conducted Th(IV) binding experiments to suspended particles, colloids, phytoplankton and bacteria at pH of 1 (discussed in more detail in Santschi *et al.*, 2005), suggests that the Th(IV) binding ligand groups are robust to different conditions and non-labile. 2) The fact that the Th(IV) binding ligand group can have different MWs and slightly different  $\text{pH}_{\text{IEP}}$  values suggests that this ligand group can be found in different macromolecules of different preparation histories and conditions.

For the primary characterization, results indicate that in all EPS and COM samples, the majority of  $^{234}\text{Th(IV)}$  (50-60 %) was concentrated near a pH of 2. As an A-type metal, Th(IV) prefers O-containing ligands, i.e., could preferentially bind to carboxyl, phosphate and sulfate functional groups of EPS. Based on bivariate correlation analyses, the carboxylic acid functional group was significantly associated with  $^{234}\text{Th(IV)}$  for *R. gallaeciensis*, *E. huxleyi*, *S. elongatus* and Gulf of Mexico COM, the phosphate functional group was associated with  $^{234}\text{Th(IV)}$  for *R. gallaeciensis*, and *S. elongatus* sample, and the sulfate functional group was most closely related to  $^{234}\text{Th(IV)}$  for *S. elongatus* and the COM from the Gulf of Mexico.

Hence,  $^{234}\text{Th(IV)}$ -binding molecules co-occurred with carboxylic acid, phosphate and/or sulfate functional groups in the isoelectric focusing profiles of the different bacterial and phytoplankton EPS as well as COM samples. The exact functional group responsible for  $^{234}\text{Th(IV)}$  binding may be variable and species dependent. Thus, the fact that different EPS with different functional group composition all strongly bind  $^{234}\text{Th(IV)}$  may support intramolecular

chelation through a clustered acid functional group environment. The steric enhancement effect appears not to depend on the exact acid functional group that is associated with  $^{234}\text{Th(IV)}$ .

Marine bacteria *R. gallaeciensis* and *S. stellata* had Th-complexed EPS with a MW of 1 kDa and 3.5 kDa, respectively. Phytoplankton *E. huxleyi* and *S. elongatus* had EPS of MWs of 14.3 kDa and 1-6.5 kDa, respectively, much lower than the typical EPS MW of  $10^5$  Da or higher (Decho, 1990; Leppard, 1995, 1997). These MW estimates compare to the 12 kDa MW of marine colloidal Th-binding ligands of Quigley *et al.*, (2002), and the 12 kDa MW carbohydrate-rich macromolecule, as one of the 3 main fractions of EPS isolated from the marine diatom *Cylindrotheca fusiformis* (Magaletti *et al.*, 2004).

Approximately 8-14 % of the total carbon content of EPS from *S. Stellata* and *S. elongatus*, extracted with IEF at a pH close to 2, was composed of hydrophilic carbohydrates, and 26 % of hydrophobic proteins, while 15 and 32% of the total polysaccharide content was composed of hydrophilic uronic acids for *S. stellata* and *S. elongatus*, respectively. Thus, our Th(IV)-binding biomolecules are amphiphilic (or amphiphatic), which gives them surfactant properties. From the analysis of the organic carbon, sulfate and phosphate concentrations that were determined in the IEF experiments, EPS from *S. stellata* and *S. elongatus* had a carbon to phosphate-P ratio of 243 and 200, and carbon to sulfate-S ratio of 72 and 30, respectively.

The presence of Th-binding amphiphilic ligands of MWs of 1-15 kDa in the extracted EPS of much higher MW agrees with the observations of Santschi *et al.* (1998), which showed that polysaccharide-rich fibrils were usually covered with much smaller and more spherical molecules, like pearls on a necklace. Since coagulation and flocculation are controlled by charge neutralization as well as hydrophobic forces (Wilkinson and Reinhardt, 2004; Reinhardt, 2004), bridging flocculation and attachment to surfaces might be regulated by the presence of hydrophobic moieties of the EPS (Sternström, 1989; Ahimou *et al.*, 2001). Thus, it is likely that these relatively small Th-binding ligand biomolecules are tightly attached to EPS, but were liberated by the solvents and detergents used in IEF and PAGE.

Based on our results, different acidic functional groups might, at times, contribute to the binding of Th(IV) to the surface-active EPS ligand. This implies that the binding environment for Th(IV) likely consists of strong polydentate chelate complexes involving acid groups, i.e., requiring the presence of clustered structures of acid functional groups of carboxylate, sulfates and/or phosphates/phosphonates in macromolecules of MWs between 1-10 kDa. Thus, the steric

environment, and not necessarily a single functional group, is most important for thorium-234 complexation to “sticky” macromolecular organic matter compounds. The physical and chemical properties of these biomolecular chelate compounds (type and quantity of acid functional groups present) found in EPS might therefore affect the extent of  $^{234}\text{Th(IV)}$  particle sorption, and thus, the  $\text{POC}/^{234}\text{Th(IV)}$  ratio.

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## REFERENCES

- Ahimou, F., Paquot, M., Jacques, P., Thonart, P., and Rouxhet, P.G. 2001. Influence of electrical properties on the evaluation of the surface hydrophobicity of *Bacillus subtilis*. *J. Microbiol. Methods*, 45, 119-126.
- Allredge, A.M. Passow, U., and Logan, B., 1993. The abundance and significance of a class of large, transparent organic particles in the ocean. *Deep-Sea Res. I*, 40: 1131-1140.
- Aluwihare, L.I., Daniel, J.R., and Chen, R.F., 2002. Chemical composition and cycling of dissolved organic matter in the mid-Atlantic bight. *Deep-Sea Res. II*, 49, 4421-4437.
- Bacon, M. P., Cochran, J.K., Hirschberg, D., Hammar, T.R. and Fler, A.P., 1996. Export flux of carbon at the equator during the EqPac time-series cruises estimated from <sup>234</sup>Th measurements. *Deep-Sea Res. II*, 43 (4-6): 1133-1153.
- Benner, R., 1991. Ultra-filtration for the concentration of bacteria, viruses, and dissolved organic matter. In: D.C. Hurd and D.W. Spencer (Editors), *Marine Particles: Analysis and Characterization*. Am. Geophys. Union, Washington, DC, pp. 181-185.
- Bergamaschi, B.A., Walters, J.S., and Hedges, J.I., 1999. Distributions of uronic acids and O-methyl sugars in sinking and sedimentary particles in two coastal marine environments. *Geochim. Cosmochim. Acta*, 63, 413-425.
- Buesseler, K.O., Andrews, J. A., Hartman, M. C., Belostock, R., and Chai, F., 1995. Regional estimates of the export flux of particulate organic carbon derived from thorium-234 during the JGOFS EqPac program. *Deep-Sea Research II*, 42 (2-3), 777-804.
- Buesseler, K. O., Bacon M. P., Cochran J. K., and Livingston, H. D., 1992. Carbon and nitrogen export during the JGOFS North Atlantic bloom experiment estimated from <sup>234</sup>Th:<sup>238</sup>U disequilibria. *Deep-Sea Research*, 39 (7/8): 1115–1137.

- Buesseler, K.O., Ball, L., Andrews, J.A., Benitez-Nelson, C., Belostock, R., Chai, F., and Chao, Y., 1998. Upper ocean export of particulate organic carbon in the Arabian Sea derived from thorium-234. *Deep-Sea Res. II*, 45 (10-11): 2461-2487.
- Buesseler, K.O., Benitez-Nelson, C.R., Moran, S.B., Burd, A., Charette, M., Cochran, J.K., Coppola, L., Fisher, N.S., Fowler, S.W., Gardner, W.D., Guo, L.D., Gustafsson, O., Lamborg, C., Masque, P., Miquel, J.C., Passow, U., Santschi, P.H., Savoye, N., Stewart, G., and Trull, T. 2005. An assessment of particulate organic carbon to thorium-234 ratios in the ocean and their impact on the application of  $^{234}\text{Th}$  as a POC flux proxy. This volume.
- Buffle, J. *Complexation Reactions in Aquatic Systems: An Analytical Approach*; Ellis Horwood: New York, 1990.
- Chen, C.T.A., Lin, C.-M., Huang, B.-T. and Chang, L.F., 1996. Stoichiometry of carbon, hydrogen, nitrogen, sulfur and oxygen in the particulate matter of the western North Pacific marginal seas. *Mar. Chem.*, 54: 179-190
- Choppin, G.R. and Wong, P.J., 1998. The chemistry of actinide behavior in marine systems. *Aquat. Geochem.*, 4: 77-101.
- Cutter, C.A., 1982. Processes affecting the distribution and speciation of selenium in seawater. In: *Ph.D. Thesis*, University of California, Santa Cruz, CA (1982), p. 168.
- Decho, A.W., 1990. Microbial exopolymer secretions in the ocean environments their role(s) in food webs and marine processes. *Oceanogr. Mar. Biol. Ann. Rev.*, 28: 73-153.
- Doco, T., O'Neill, M.A., Pellerin, P., 2001. Determination of neutral and acidic glycosyl-residue compositions of plant polysaccharides by GC-EI-MS analysis of the trimethylsilyl methyl glycoside derivatives. *Carbohydrate Polymers*, 46: 249-259.

- Dytham, C., 1999. *Choosing and Using Statistics: A Biologists Guide*. Blackwell Science, Inc, Malden, MA, 218pp.
- Edwards, R.L., Chen, J.H., Ku, T.L., and Wasserburg, G.J., 1987. Precise timing of the last interglacial period from mass spectrometric determination of thorium-230 in corals. *Science*, 236: 1547-1553.
- El Shafei, G.M.S., 1996. The polarizing power of metal cations in (Hydr)oxides. *J. Colloid Interface Sci.*, 182: 249-253.
- Fanghänel, T., and Neck, V. 2002. Aquatic chemistry and solubility phenomena of actinide oxides/hydroxides. *Pure Appl. Chem.*, 72, 1895-1907.
- Filisetti-Cozzi, T.M., and Carpita, N.C., 1991. Measurement of uronic acids without interference from neutral sugars. *Analyt. Biochem.*, 197 (1):157-162.
- Grotjan, Jr., H.E., Padrnos-Hicks, P.A., and Keel, B.A., 1986. Ion chromatographic method for the analysis of sulfate in complex carbohydrates. *J. Chromatogr.*, 367: 367-375.
- Guo, L.-D., Coleman, C.H., Santschi, P.H., 1994. The distribution of colloidal and dissolved organic carbon in the Gulf of México. *Mar. Chem.*, 45: 105-119.
- Guo, L.-D., Hung, C.-C., Santschi, P.H. and Walsh, I.D., 2002. <sup>234</sup>Th scavenging and its relationship to acid polysaccharide abundance in the Gulf of México. *Mar. Chem.*, 78 (2-3): 103-119.
- Guo, L.D., Santschi, P.H., Cifuentes, L., Trumbore, S.E., and Southon, J., 1996. Cycling of high-molecular-weight dissolved organic matter in the Middle Atlantic Bight as revealed by carbon isotopic (<sup>13</sup>C and <sup>14</sup>C) signatures. *Limnol. Oceanogr.*, 41(6): 1242-1252.

- Guo, L.D., Santschi, P.H., and Warnken, K.W., 2000a. Trace metal composition of colloidal material in estuarine and marine environments. *Mar. Chem.*, 70: 257-275.
- Guo, L., Wen, L.-S., Tang, D., and Santschi, P.H., 2000b. Re-examination of cross-flow ultrafiltration for sampling aquatic colloids: evidence from molecular probes. *Mar. Chem.*, 69: 75-90.
- Hopkinson, C.S., and Vallino, J.J. 2005. Efficient export of carbon to the deep ocean through dissolved organic matter. *Nature*, 433: 142-145.
- Hirose, K., 1996. Determination of a strong organic ligand dissolved in seawater: Thorium-complexing capacity of oceanic dissolved organic matter. *J. Radioanalyt. Nucl. Chem., Articles*, 204: 193-204.
- Hirose, K., 2004. Chemical Speciation of Thorium in Marine Biogenic Particulate Matter. *The Scientific World Journal*, 4: 67-76.
- Hirose, K., Tanoue, E., 1994. Thorium-particulate matter interaction. Thorium complexing capacity of oceanic particulate matter: Theory. *Geochim. Cosmochim. Acta* 58: 1-7.
- Hirose, K., Tanoue, E., 1998. The vertical distribution of the strong ligand in particulate organic matter in the North Pacific. *Mar. Chem.* 59: 235-252.
- Hirose, K., Tanoue, E., 2001. Strong ligands for thorium complexation in marine bacteria. *Mar. Environ. Res.* 51: 95-112.
- Hung, C.-C. and Santschi, P. H., 2001. Spectrophotometric determination of total uronic acids in seawater using cation-exchange separation and pre-concentration by lyophilization. *Analyt. Chim. Acta*, 42 (7): 111-117.

- Hung, C.-C., Guo, L., Santschi, P.H., Alvarado Quiroz, N.G., and Haye, J.M., 2003a. Distribution of carbohydrate species in the Gulf of México. *Mar. Chem.*, 81: 119-135.
- Hung, C.-C., Guo, L., Schultz, G., Pinckney, J.L., and Santschi, P.H., 2003b. Production and fluxes of carbohydrate species in the Gulf of México. *Global Biogeochemical Cycles* 17(2): 1055, doi10.1029/2002GB001988.
- Hung, C.-C., Guo, L., Roberts, K.A., Santschi, P.H., 2004. Upper ocean carbon flux determined by the  $^{234}\text{Th}$  approach and sediment traps using size-fractionated POC and  $^{234}\text{Th}$  data from the Gulf of Mexico. *Geochem. J.*, 38, 601-611.
- Hung, C.-C., Santschi, P.H., and Gillow, J.B. 2005. Isolation and characterization of extracellular polysaccharides produced by *Pseudomonas fluorescens* Biovar II. *Carbohydrate Polymers*, in press.
- Ku, T. L., Knauss, K.G., Matthieu, G.G., 1977. Uranium in the open ocean: concentration and isotopic composition. *Deep-Sea Res.*, 24: 1005-1017.
- Leppard, G.G., 1995. The characterization of algal and microbial mucilage and their aggregates in aquatic ecosystems. *Sci. Tot. Environ.*, 165: 103-131.
- Leppard, G.G., 1997. Colloidal organic fibrils of acid polysaccharides in surface waters: electron-optical characteristics, activities and chemical estimates of abundance. *Colloids Surf. A: Physicochem. Engin. Aspects*, 120: 1-15.
- Magaletti, E., Urbani, R., Sist, P., Ferrari, C.R., and Cicero, A.M., 2004. Abundance and chemical characterization of extracellular carbohydrates released by the marine diatom *Cylindrotheca fusiformis* under N- and P-limitation. *Eur. J. Phycol.*, 39, in press.

- Mecozzi, M., Pietrantonio, E., Di Noto, V., and Papai, Z. 2005. The humin structure of mucilage aggregates in the Adriatic and Tyrrhenian seas: hypothesis about the reasonable causes of mucilage formation. *Mar. Chem.*, 95: 255-269.
- Moran, S.B., Charette, M.A., Hoff, J. A., Edwards, R.L., and Landing, W.M., 1997. Distribution of Th-230 in the Labrador Sea and its relation to ventilation. *Earth Planet. Sci. Lett.*, 150 (1-2): 151-160.
- Moran, S.B., Weinstein, S.E., Edmonds, H.N., Smith, J. N., Kelly, R. P., Pilson, M.E.Q., and Harrison, W.G., 2003. Does  $^{234}\text{Th}/^{238}\text{U}$  disequilibrium provide an accurate record of the export flux of particulate organic carbon from the upper ocean. *Limnol. Oceanogr.*, 48 (3): 1018-1029.
- Murray, J.W., Young, J., Newton, J., Dunne, J., Chappin, T., Paul, B., and McCarthy, J., 1996. Export flux of particulate organic carbon from the central equatorial Pacific determined using a combined drifting trap- $^{234}\text{Th}$  approach. *Deep-Sea Res. II*, 43 (4-6): 1095-1132.
- Myklestad, S.M., Skånøy, E., and Hestmann, S., 1997. A sensitive and rapid method for analysis of dissolved mono- and polysaccharides in seawater. *Mar. Chem.*, 56 (3-4): 279-286.
- Nash, K.L., and Choppin, G.R., 1980. Interaction of humic and fulvic acids with Th(IV). *J. Inorg. Nucl. Chem.*, 42: 1045-1050.
- Quigley, M.S., 2000. Tracing colloid-colloid and colloid-particle interactions using thorium. Ph.D. Dissertation, Texas A&M University; College Station.
- Quigley, M.S., Honeyman, B.D., and Santschi, P.H., 1996. Thorium sorption in the marine environment: equilibrium partitioning at the hematite/water interface, sorption/desorption kinetics and particle tracing. *Aquat. Geochem.*, 1: 277-301.

- Quigley, M.S., Santschi, P.H., Guo, L.-D. and Honeyman, B.D., 2001. Sorption irreversibility and coagulation behavior of  $^{234}\text{Th}$  with marine NOM. *Marine Chemistry*, 76: 27-45.
- Quigley, M.S., Santschi, P.H., Hung, C.-C., Guo, L., and Honeyman, B.D., 2002. Importance of acid polysaccharides for  $^{234}\text{Th}$  complexation to marine organic matter. *Limnol. Oceanogr.*, 47: 367-377.
- Reinhardt, A., 2004. Contrasting roles of natural organic matter on colloidal stabilization and flocculation in freshwaters. Ph.D. dissertation, Department of Inorganic, Analytical and Applied Chemistry, CABB, University of Geneva, Sciences II, Geneva 4, CH1211, Switzerland.
- Ron, E.Z., and Rosenberg, E., 2001. Natural roles of biosurfactants. *Env. Microbiol.*, 3: 229-236.
- Santschi, P.H., Guo, L., Baskaran, M., Trumbore, S., Southon, J., Bianchi, T.S., Honeyman, B.D., and Cifuentes, L., 1995. Isotopic evidence for the contemporary origin of high-molecular weight organic matter in oceanic environments, *Geochim. Cosmochim. Acta*, 59(3): 625-631.
- Santschi, P.H., Balnois, E., Wilkinson, K. J., Zhang J., Buffle, J., and Guo, L., 1998. Fibrillar polysaccharides in marine macromolecular organic matter as imaged by atomic force microscopy and transmission electron microscopy. *Limnol. Oceanogr.*, 43 (5): 896-908.
- Santschi, P.H., Hung, C.-C., Schultz, G., Alvarado-Quiroz, N., Guo, L., Pinckney, J., Walsh, I., 2003. Control of acid polysaccharide production and  $^{234}\text{Th}$  and POC export fluxes by marine organisms. *Geophysical Research Letters*, 30, doi, 10.1029/2002GL016046.
- Santschi, P.H., Murray, J.W., Baskaran, M., Benitez-Nelson, C.R., Guo, L., Hung, C.-C., Lamborg, C., Moran, S.B., Passow, U., and Roy-Barman, M. 2005. Thorium speciation in seawater. *Mar. Chem.*, this volume.

- Silvestri, L. J., Hurst, R. E., Simpson, L., and Settine, J. M., 1982. Analysis of sulfate in complex carbohydrates. *Analytical Biochemistry*, 123: 303-309.
- Smith, P.K., Krohn R.I., Hermanson, G.T., Mallia A. K., Gartner F.H., Provenzano M.D., Fujimoto E.K., Goeke N.M., Olson B.J., and Klenk D.C., 1985. Measurement of protein using Bicinchoninic acid. *Analytical Biochemistry*, 150 (1): 76-85.
- Staats, N., De Winder, B., Stal, L. J., and Mur, L.R., 1999. Isolation and characterization of extracellular polysaccharides from the epipelagic diatoms *Cylindrotheca closterium* and *Navicula salinarum*. *European Journal of Phycology*, 34: 161-169.
- Stenstöröm, T.A., 1989. Bacterial hydrophobicity, an overall parameter for the measurement of adhesion potential to soil particles, *Appl. Environ. Microbiol.*, 55: 142-147.
- Stoderegger, K., and Herndl, G. J., 1998. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. *Limnol. Oceanogr.*, 43(5): 877-884.
- Trubetskoj, O.A., Trubetskaya, O.E., and Khomutova, T.E., 1992. Isolation, purification and some physico-chemical properties of soil humic substances fractions obtained by polyacrylamide gel electrophoresis. *Soil Biol. Biochem.*, 24: 893-896.
- Verdugo, P., Alldredge, A.L., Azam, F., Kirchman, D.L., Passow, U., Santschi, P.H., 2004. The oceanic gel phase: a bridge in the DOM-POM continuum. *Mar. Chem.* 92: 67-85.
- Warwick, P., Carlsen, L., Randall, A., Yhao, R., and Lassen, P., 1993. <sup>14</sup>C and <sup>125</sup>I labelling of humic material for use in environmental studies. *Chem. And Ecol.*, 8: 65-80.
- Wilkinson, K.J., and Reinhardt, A. 2004. Contrasting roles of natural organic matter on colloidal stabilization and flocculation in freshwaters. In: *Flocculation in Natural and Engineered*

Environmental Systems, Droppo, I.G., Leppard, G.G., Liss, S.N., and Milligan, T.G., eds.,  
CRC Press, Boca Raton, FL., ch. 7, pp. 143-170.

Table 1. Composition of exopolymeric substances (EPS) produced by *S. stellata* and *S. elongatus* in IEF fraction at an isoelectric point ( $\text{pH}_{\text{IEP}}$ ) of about 2.

(A)

Type	$\text{pH}_{\text{IEP}}$	OC	TCHO	Protein*	URA	$\text{SO}_4^{-2}$	$\text{PO}_4^{-3}$
		(mmole-C/g)	(mmole-C/g)	(mmole-C/g)	(mmole-C/g)	(mmole/g)	(mmole/g)
<i>S. stellata</i>	1.90	15.4	2.2	0.4	0.34	0.213	0.064
<i>S. elongatus</i>	1.85	15.2	1.2	n.d.	0.28	0.508	0.076

(B)

Type	$\text{pH}_{\text{IEP}}$	TCHO/OC	Protein/OC	URA/OC	$\text{C}/\text{SO}_4^{-2}$	$\text{C}/\text{PO}_4^{-3}$
		(%)	(%)	(%)	mole ratio	mole ratio
<i>S. stellata</i>	1.90	14.3	2.6	2.2	72	243
<i>S. elongatus</i>	1.85	7.9	n.d.	1.8	30	200

Table 2. Neutral monosaccharide and acid polysaccharide content from GC-MS of isolated EPS after methanolysis (Table 1)

<i>Type</i>	<i>S. stellata</i>	<i>S. elongatus</i>
	% of total	% of total
Allose	6.7	6.1
Glucose	14.9	13.4
Galactose	6.0	4.1
Mannose	3.9	5.6
Galacturonic acid and Mannuronic acid	18.8	24.8
Glucuronic acid	0.2	1.5
Unknown sugars	51.7	55.6
Total	100	100

Fig. 1. Extraction procedure of microbial cultures and natural organic matter samples

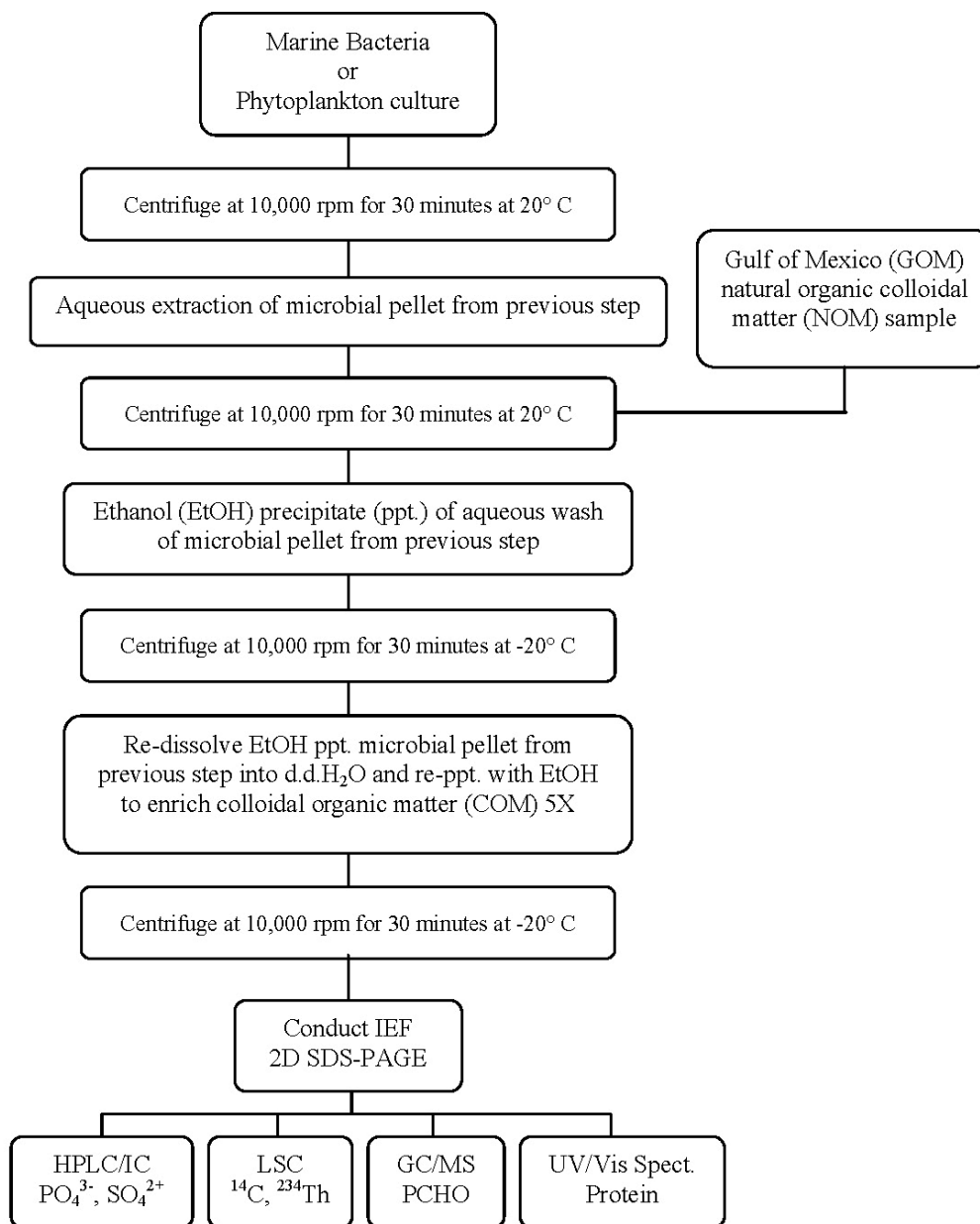


Fig. 2. Example of isoelectric focusing profile of a)  $^{234}\text{Th(IV)}$ -labeled EPS from *Emiliana huxleyi*, as compared to that of b)  $^{14}\text{C}$ -labeled carboxylic acid, c) phosphate ion and d) sulfate ion.

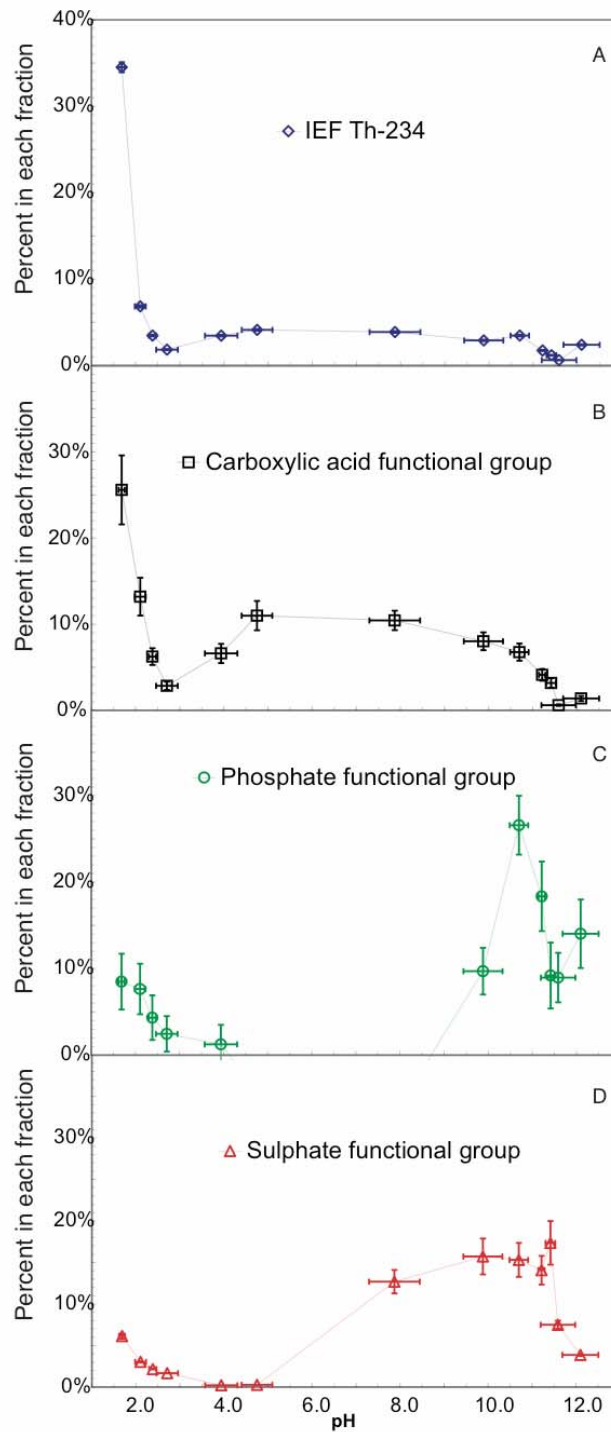


Fig.3. Isoelectric focusing profile of a)  $^{234}\text{Th(IV)}$ -labeled COM from the Gulf of México (Station 4, 72m depth, at chlorophyll A maximum; Hung et al., 2003a,b), as compared to b)  $^{14}\text{C}$ -labeled carboxylic acid, c) phosphate ion and d) sulfate ion.

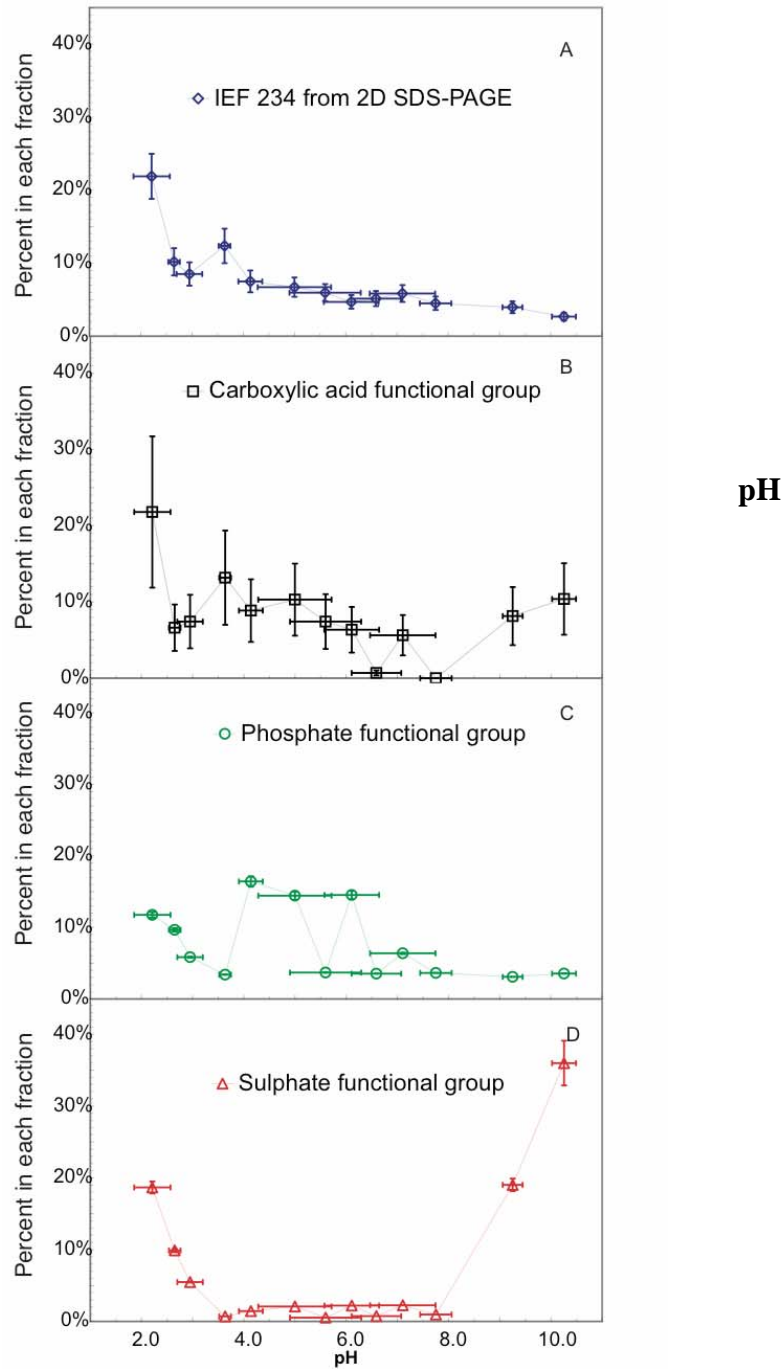


Fig. 4. Plot of 15% 2D SDS-PAGE of  $^{234}\text{Th(IV)}$  for polysaccharide enriched fraction extracted from marine bacteria *R. gallaeciensis*.

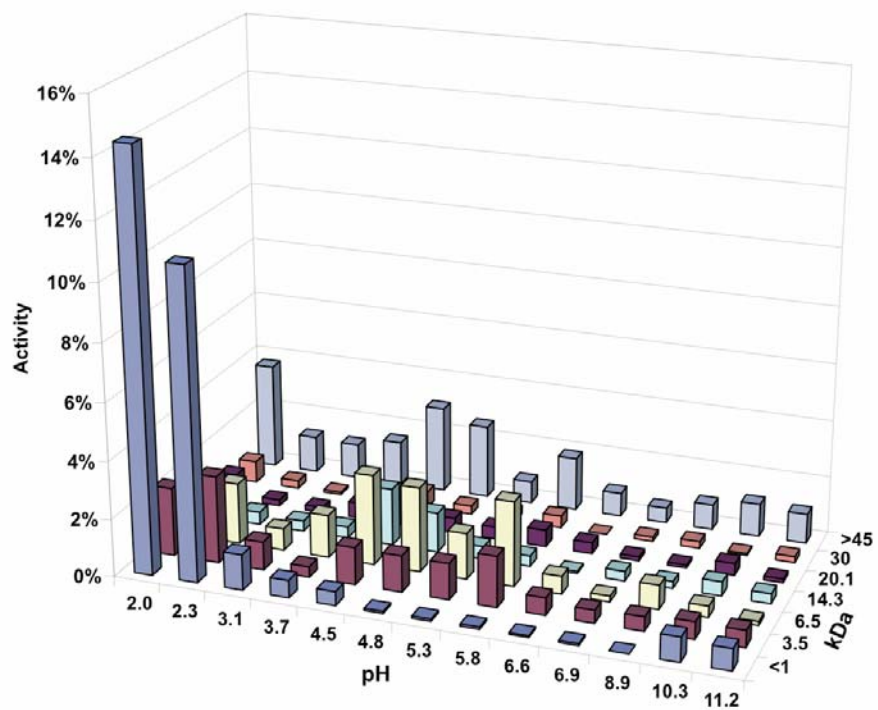


Fig. 5. Plot of 15% 2D SDS-PAGE of  $^{234}\text{Th(IV)}$  for polysaccharide enriched fraction extracted from marine bacteria *S. stellata*.

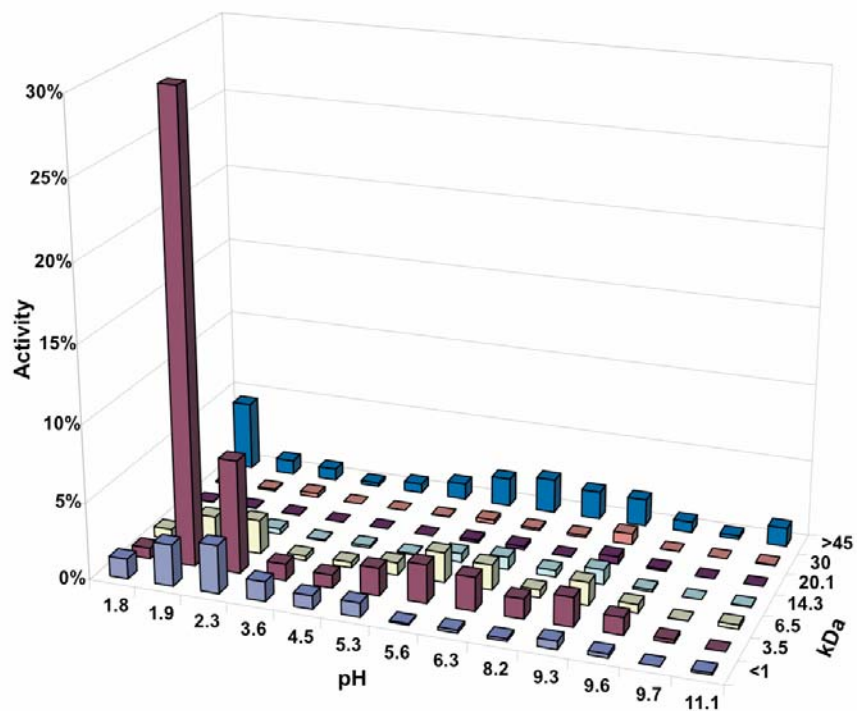


Fig. 6. Plot of 15% 2D SDS-PAGE of  $^{234}\text{Th(IV)}$  for polysaccharide enriched fraction extracted from phytoplankton *E. huxleyi*.

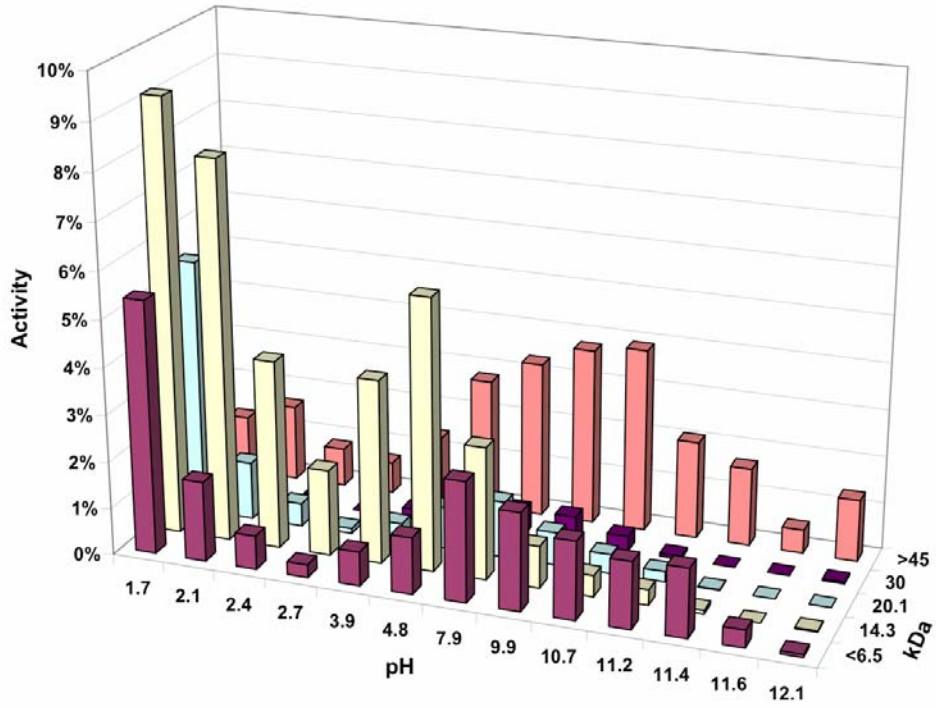


Fig. 7. Plot of 15% 2D SDS-PAGE of  $^{234}\text{Th(IV)}$  for polysaccharide enriched fraction extracted from phytoplankton *S. elongatus*.

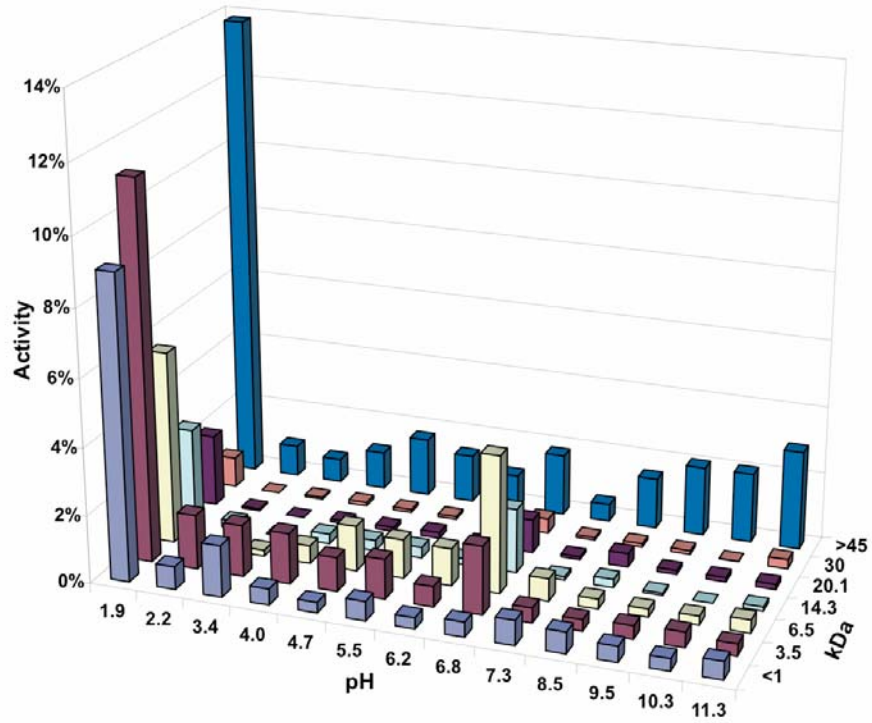


Fig. 8. Plot of 15% 2D SDS-PAGE of  $^{14}\text{C}$ -methylamine labeling of COOH functional groups of the polysaccharide enriched fraction extracted from marine bacteria *R. gallaeciensis*. a) with the unreacted  $^{14}\text{CH}_3\text{NH}_2$  peak and b) without the  $^{14}\text{CH}_3\text{NH}_2$  peak.

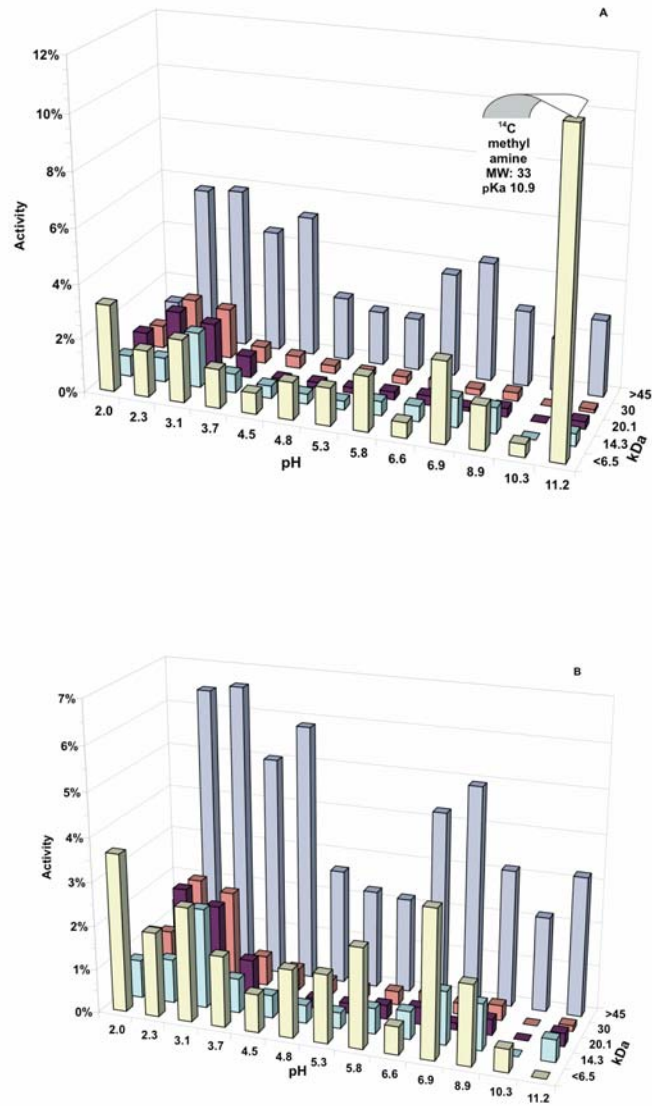


Fig. 9. Gas Chromatogram of derivatized EPS from *S. stellata* (a) and *S. elongatus* (b) with the following peaks: alditol (1), allose (2), glucose (8), mannose (10), galactose (11, 14), galacturonic/mannuronic acids (12), glucuronic acid (13) and other sugars (3, 4, 5, 6, 7, 9 and other peaks without labels).

