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### Abstract

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# Influence of the Composition of Natural Organic Matter on Pb Bioavailability to Microalgae

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The current work examines the effects of model allochthonous (humic substances) and autochthonous (microbial polysaccharides) natural organic matter (NOM) on Pb speciation and bioaccumulation. The results demonstrated that polysaccharides, in particular alginic acid, had complexing properties and effects on Pb bioaccumulation by the green alga *Chlorella kesslerii* that were similar to equivalent complexing capacity of humic substances. Pb uptake decreased in the presence of humic, alginic, and polygalacturonic acids with respect to noncomplexed Pb, but accumulated Pb was higher than predicted from measured Pb<sup>2+</sup> concentrations or from previous results obtained in the presence of simple synthetic ligands. An improved fit between experimental observations and Pb speciation was obtained by taking into account the formation of a ternary complex at the algal surface. The contribution of the ternary complexes to Pb bioaccumulation was dependent on the relative binding constants of the Pb to the NOM and to the binding sites on the biological surface. In the presence of the humic acid, a decreased surface charge and increased membrane permeability were considered to be of secondary importance to explain the observation of increased Pb uptake with respect to that predicted on the basis of [Pb<sup>2+</sup>]. The environmental implications of the results are discussed with respect to the development of site-specific water quality criteria.

## Introduction

Natural organic matter, NOM, plays an important role in aquatic ecosystems by binding trace metals and thus influencing their speciation, bioavailability, and toxic effects (1, 2). In thermodynamic models such as the Free ion activity model, FIAM (3), or its derivative the Biotic ligand model, BLM (4), a reduction of the equilibrium concentration of free ions due to binding by NOM is generally expected to reduce metal biouptake and bioavailability. Nonetheless, a fundamental understanding of the relationship between trace metal speciation, biological uptake and effects is still lacking for many environmental systems, although considerable

progress has occurred over the past few years (reviewed in ref 5). Of the papers that have compared trace metal bioaccumulation or toxicity with speciation in the presence of NOM, no consensus has emerged as to whether data conform to the FIAM. Examples of enhanced toxicity and enhanced protection have been documented (6). For example, while Cd uptake by the common carp *Cyprinus carpio* was directly proportional to [Cd<sup>2+</sup>] (7) in the presence of the humic acid, Cd uptake and toxicity to the blue mussel *Mytilus edulis* were higher than predicted by [Cd<sup>2+</sup>] (8). Pb biouptake by the alga *Chlorella kesslerii* was enhanced in the presence of the Suwannee River fulvic acid, SRFA, with respect to the expected reduction based upon [Pb<sup>2+</sup>] (9). Similarly, in the presence of riverine organic matter, Pb uptake by the alga *Chlorella vulgaris* and by the diatom *Stephanodiscus hantzschii* was higher than predicted by the FIAM, while for the same organisms, Cd and Cu uptake followed FIAM predictions (10). Note that all of the above studies considered the role of the allochthonous, humic- like fraction of natural organic matter. Other components of organic matter such as polysaccharides have been practically ignored in the literature, even though they can play a quantitatively important role in many conditions (2, 11). For example, autochthonous organic matter including polysaccharides may comprise up to 25% of NOM in freshwaters (12), especially during algal blooms (2, 13), and up to 80% of organic carbon in seawaters (14, 15); it is the primary component of flocs and biofilms (16, 17).

The aim of the present work was thus to gain additional insight into the role of the composition of NOM on metal complexation and bioavailability in natural waters. More specifically, the emphasis was on the quantification of Pb complexation by different components of natural organic matter, such as polysaccharides and humic substances, and the determination of the influences (both direct and indirect) of NOM on Pb bioaccumulation by the green algae *Chlorella kesslerii*. Since biological effects due to metals are always initiated by metal bioaccumulation, a more thorough understanding of the fundamental processes underlying bioaccumulation will lead to an improved capacity to evaluate the impact of metals on aquatic organisms. By *simultaneously* examining metal speciation (i.e., Pb<sup>2+</sup> measurements) and biological uptake in the presence of NOM, this work should provide novel information on the role of NOM composition on metal uptake, especially with respect to the applicability and limitations of the existing equilibrium models (i.e., FIAM, BLM) under environmentally relevant conditions.

## Experimental Section

**Choice of Natural Organic Matter.** A model humic acid and several microbially produced polysaccharides were selected for the study since these components represent the majority of recalcitrant organic matter in natural aquatic systems. A standard humic acid isolated from the Suwannee River, SRHA, Georgia, was obtained from the International Humic Substances Society (Colorado School of Mines, Golden, CO) (18). Several model polysaccharides were purchased from Sigma: an alginic acid (AA) isolated from *Macrocystis pyrifera*; a polygalacturonic acid (PGA) isolated from citrus fruit, and xanthan (XAN) from *Xanthomonas campestris*. A polysaccharide isolated from the gram negative bacteria, *Rhizobium meliloti*, was separated and purified following a procedure adapted from ref 19. This exopolysaccharide has been shown to correspond primarily to the anionic polysaccharide succinoglycan, SUC (20). Stock solutions of each type of NOM with a concentration of 1 g L<sup>-1</sup> were prepared in Milli-Q

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water, filtered through 0.22- $\mu\text{m}$  filters (Millipore), and stored at 4 °C for a maximum of 15 days. Experimental solutions containing between 2.5 and 100 mg C L<sup>-1</sup> NOM were prepared by dilution of the stock solutions. Total organic carbon was measured using a Shimadzu TOC 500/5000A following acidification to pH = 2.0 with concentrated HCl (Baker suprapur, 1  $\mu\text{L}/\text{mL}$ ).

**Determination of Pb Speciation.** (i) *Pb<sup>2+</sup> Selective Electrode Measurements.* A low-detection-limit Pb<sup>2+</sup>-selective electrode (Pb<sup>2+</sup>-SE) with an optimized membrane composition based on the ionophore 4-*tert*-butylcalix[4]arenetetrakis thioacetic acid dimethylamide (2I) was employed to measure Pb<sup>2+</sup> activities. To minimize zero-current ion fluxes, the inner electrolyte composition was designed to match that of the sample being measured, as detailed previously (9). For example, a mixture of 10<sup>-6</sup> M Pb(NO<sub>3</sub>)<sub>2</sub> and 10 mg C L<sup>-1</sup> SRHA in MES (2-[*N*-morpholino]ethanesulfonic acid, Sigma) buffered to pH 6.0 was used as the internal filling solution for the measurement of Pb<sup>2+</sup> in the presence of 10 mg C L<sup>-1</sup> SRHA. Potentials were measured with a 16-channel electrode monitor (Lawson Labs Inc., Malvern) at room temperature in stirred solutions, by simultaneously recording data from at least 3 electrodes. Electrodes were preconditioned for at least 12 h in mixtures corresponding to the composition of the internal solution prior to calibration with a series of Pb solutions in the Pb<sup>2+</sup> concentration range of 10<sup>-7</sup> to 5  $\times$  10<sup>-5</sup> M. The slope of the response curve was 29.0  $\pm$  1.5 mV, very close to the theoretically predicted values of 29.6 mV. Given that the measurements were performed at constant ionic strength, *I*, data are presented below as concentrations rather than activities.

(ii) *Pb<sup>2+</sup> Determination Using Centricon Devices.* In parallel to Pb<sup>2+</sup>-SE measurements, Pb bound to the polysaccharides was quantified using a centricon ultrafiltration device (Millipore) with membrane cutoff of 30 kDa. Because the membrane cutoff was much lower than the average molar masses of each of the polysaccharides (AA: ca. 3  $\times$  10<sup>5</sup> g mol<sup>-1</sup>; PGA: ca. 8  $\times$  10<sup>5</sup> g mol<sup>-1</sup>; XAN: ca. 5  $\times$  10<sup>6</sup> g mol<sup>-1</sup>; SUC: ca. >10<sup>6</sup> g mol<sup>-1</sup>), Pb bound to the biopolymers was assumed to be entirely retained on the membranes, whereas free metal ions and small complexes were likely to pass completely into the filtrate. Prior to use, the centricon devices were washed 3 times with 0.1 M NaOH and rinsed 3 times with MilliQ water in order to remove organic surfactants from the membranes, as recommended by the manufacturer. Prior to measurements, the centricon devices were conditioned with experimental medium by performing at least 5 consecutive runs of 1 mL each of the experimental medium. The filtrate was removed, the device was turned over, and a further centrifugation was performed in order to prevent NOM from clogging the membrane. This treatment was found to significantly decrease both Pb losses from the centricon devices and the clogging of the membrane by NOM. Filtrates were collected for Pb measurements following an additional 3 consecutive runs with the experimental medium. Controls with solutions containing Pb in the absence of NOM were run in each experiment to estimate potential Pb adsorptive losses to the devices. Typically, each experiment with 1 mL of experimental medium required a 20-min centrifugation at 4500 rpm (2500g). Given the low average molar mass of the SRHA with respect to the centricon membrane cutoff, this technique was not applied to Pb speciation determinations using the SRHA.

(iii) *Thermodynamic Calculations.* Speciation calculations for Pb in the centricon filtrates were performed with the speciation program MINEQL+ (version 3.01a, Procter and Gamble, OH) and MINTEQA2 for windows (Allison Geoscience Consultants Inc. and Hydrogeologic Inc., USA). In the presence of SRHA, Pb<sup>2+</sup> concentrations were also estimated using the NICA–Donnan approach incorporated

into a Visual MINTEQA2 model based upon a density of carboxylic sites of  $Q_{1\text{max}} = 0.00315 \text{ mol g}^{-1}$ ; a distribution width (heterogeneity),  $m_{\text{H1}} = 0.62$ ; a phenolic site density of  $Q_{2\text{max}} = 0.00255 \text{ mol g}^{-1}$  with  $m_{\text{H2}} = 0.41$ ; and a parameter  $b = -0.49$  for determination of the SRHA Donnan volume (22).

(iv) *Determination of Pb–NOM Stability Constants.* Average conditional stability constants ( $K_{\text{PbL}}$ ) were estimated by assuming that the complexing site concentration was in large excess with respect to the metal concentration and that a 1:1 Pb–ligand complex was formed (1). Constants were determined from slopes of the plots of the degree of complexation,  $[\text{Pb}]_{\text{tot}}/[\text{Pb}^{2+}]$ , as a function of the concentration of the complexing sites, expressed as the concentration of carboxylic groups per gram of polysaccharide,  $[\text{COOH}]$  according to the equation

$$\frac{[\text{Pb}]_{\text{tot}}}{[\text{Pb}^{2+}]} = 1 + K_{\text{PbL}}[\text{COOH}] \quad (1)$$

The total concentration of binding sites,  $[\text{COOH}]_{\text{tot}}$ , was determined from an acid–base titration at constant ionic strength (5  $\times$  10<sup>-3</sup> M) (23).

**Algal Determinations.** (i) *Determination of Pb Uptake.* Bioaccumulation experiments were carried out with green alga, *Chlorella kesslerii* (University of Toronto culture collection, UTCC 266). The algae were cultured in OECD medium (24) at 20 °C, with rotary shaking (100 rpm) and a 12:12-h light/dark regime. Cells in their mid-exponential growth phase were harvested by gentle filtration and washed (3  $\times$  5 mL) with a metal-free medium, then resuspended in an experimental medium containing known quantities of NOM and Pb in MES at pH 6.0. Studies were performed with total Pb concentrations ( $[\text{Pb}]_{\text{tot}}$ ) of 10<sup>-6</sup> M which are near to the half saturation concentration for Pb ( $K_{\text{M}} = 3.3 \times 10^{-6}$  M Pb corresponding to the Pb concentration where the uptake is 50% of maximal flux (25)) and within the range of reported EC<sub>50</sub> (50% effect concentration, i.e., growth inhibition) for this species. Aliquots (40-mL) of the algal suspensions were filtered (3.0- $\mu\text{m}$  nitrocellulose filters, Millipore) at given times after resuspension in the experimental medium. Algae were then rinsed three times with 5 mL of a 5  $\times$  10<sup>-3</sup> M MES solution (pH = 6.0). The rinse was followed by a 1-min wash with 10<sup>-2</sup> M EDTA in order to distinguish surface-bound from internalized Pb (26). Internalized Pb was determined following digestion of the filtered, EDTA-washed algae with 0.5 mL of concentrated, ultrapure HNO<sub>3</sub> (Baker Instra-Analyzed Reagents). Dissolved, adsorbed, and internalized Pb fractions were measured by inductively coupled plasma mass spectrometry (Hewlett-Packard 4500, Agilent Technologies, Palo Alto, CA). For each experimental run, cell densities, sizes, and surface distributions were determined using a Coulter Multisizer II (50- $\mu\text{m}$  orifice) or a Coulter Multisizer III (20- $\mu\text{m}$  orifice) particle counter. Uptake was examined in short-term experiments (maximum 60 min) in order to reduce the effect of efflux, exudate production, and variable cell sizes and numbers. Experiments were performed with algae having a mean cellular diameter of 3.6  $\mu\text{m}$  and a cell density of 1–5  $\times$  10<sup>6</sup> cell mL<sup>-1</sup>. Mass balances were performed by comparing initial Pb concentrations to the sum of dissolved, adsorbed, and internalized Pb. Data were not used if the mass balance exceeded a 20% variation with respect to initial added Pb.

(ii) *Membrane Permeability Determinations.* Sorbitol is a lipophilic molecule that is accumulated by passive diffusion for this algae. The effect of NOM on lipidic membrane permeability (9, 26, 27) was determined by incubating the algae with a small spike of <sup>14</sup>C-D-sorbitol (10 GBq mmol<sup>-1</sup>, Anawa, Switzerland). Similar to the uptake experiments, algae were collected in their mid-exponential growth phase, filtered,

rinsed with NOM-free experimental medium and resuspended in a medium containing 10 mg L<sup>-1</sup> of NOM and [<sup>14</sup>C]-D-sorbitol in MES (pH = 6.0). Twenty milliliter samples were filtered after a contact time of 30 min in a manner similar to the uptake experiments. A solution of 10<sup>-2</sup> M nonradiolabeled D-sorbitol was used to rinse the algae on the filters. <sup>14</sup>C activities were quantified on a Beckmann LS 6500 scintillation counter following digestion in a scintillation cocktail (Packard Biosciences Filter count).

(iii) *Estimation of the Direct Interaction of the NOM with the Algal Cells.* Adsorption of the NOM to the surface of *C. kesslerii* was estimated by electrophoretic mobility (EPM) measurements using laser Doppler velocimetry (Malvern Zetasizer 2000). The cells' EPM were measured following a 30-min exposure to each model NOM, using concentrations that corresponded to the bioaccumulation experiments. In parallel, SRHA adsorbed to the algal surface was determined from the difference between bulk SRHA concentrations before and after a 30-min contact with algae (9). In this case, SRHA concentrations were quantified using UV-vis absorbance measurements at 280 and 326 nm (Lambda 4 UV-Vis Spectrophotometer, Perkin-Elmer). The concentration of the SRHA was varied from 1 to 15 mg C L<sup>-1</sup>.

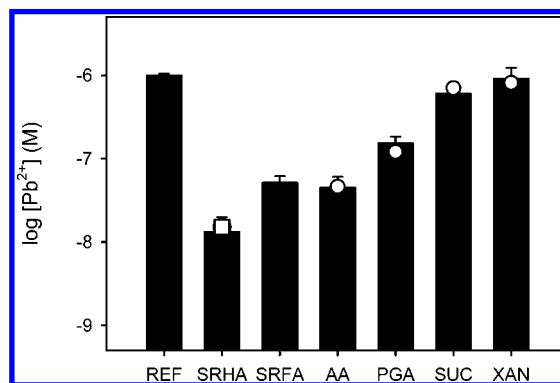
A similar methodology was used to determine the adsorption of Pb-SRHA complexes on the algal surface. Pb-SRHA concentrations were increased by increasing [Pb]<sub>tot</sub> from 5 × 10<sup>-7</sup> M Pb to 10<sup>-4</sup> M Pb in the presence of a constant [SRHA] of 10 mg C L<sup>-1</sup>. Such a setup allowed SRHA and Pb-SRHA surface complexes to be distinguished. Two types of controls were run for each experiment. The first control contained SRHA and/or Pb in the absence of algae and was used to estimate potential adsorptive losses to the filters. The second set of controls, containing algae and Pb, was used to determine the possible influence of extracellular products on the absorbance signal.

## Results and Discussion

**Pb Speciation in the Presence of NOM.** Successful prediction of the impact of NOM on Pb accumulation requires that the metal binding characteristics of the environmental ligands (e.g., the values of conditional stability constants and binding site concentrations) are known. In contrast to humic substances where the large data sets are available (e.g., ref 28), such information is scarce or unavailable for metal-polysaccharide complexes (with the exception of a limited number of studies using radionuclides, e.g., ref 29). Therefore, the first step in this study was to quantify Pb<sup>2+</sup> concentrations over a large range of polysaccharide concentrations in order to determine conditional stability constants for several Pb-polysaccharide complexes.

(i) *Determination of Free Lead Concentrations.* Pb<sup>2+</sup> concentrations decreased significantly in the presence of 10 mg C L<sup>-1</sup> SRHA, SRFA, AA, or PGA in the following order: [Pb<sup>2+</sup>]<sub>SRHA</sub> (1.3 × 10<sup>-8</sup> M) << [Pb<sup>2+</sup>]<sub>SRFA</sub> (5.1 × 10<sup>-8</sup> M) ≈ [Pb<sup>2+</sup>]<sub>AA</sub> (4.6 × 10<sup>-8</sup> M) < [Pb<sup>2+</sup>]<sub>PGA</sub> (1.5 × 10<sup>-7</sup> M) as determined by the Pb<sup>2+</sup>-SE. In the presence of similar concentrations of succinoglycan or xanthan, less than 40% or 9% of the total Pb was complexed, corresponding to [Pb<sup>2+</sup>] of 6.0 × 10<sup>-7</sup> and 9.1 × 10<sup>-7</sup> M, respectively (Figure 1). On the basis of Figure 1 and the relative concentrations of the various NOM components (11), organic Pb speciation will be dominated by complex formation with humic-like substances in the natural aquatic environment.

Good agreement was observed between [Pb<sup>2+</sup>] measured by the Pb<sup>2+</sup>-SE (Figure 1, bars) and those evaluated in the centricron filtrates for each of the model polysaccharides (Figure 1, points). Speciation calculations (MINTEQA2) suggested that, under the studied conditions (pH = 6.0, I = 5 × 10<sup>-3</sup> M), more than 97% of the lead in the centricron filtrates solutions was present as Pb<sup>2+</sup>, with only small



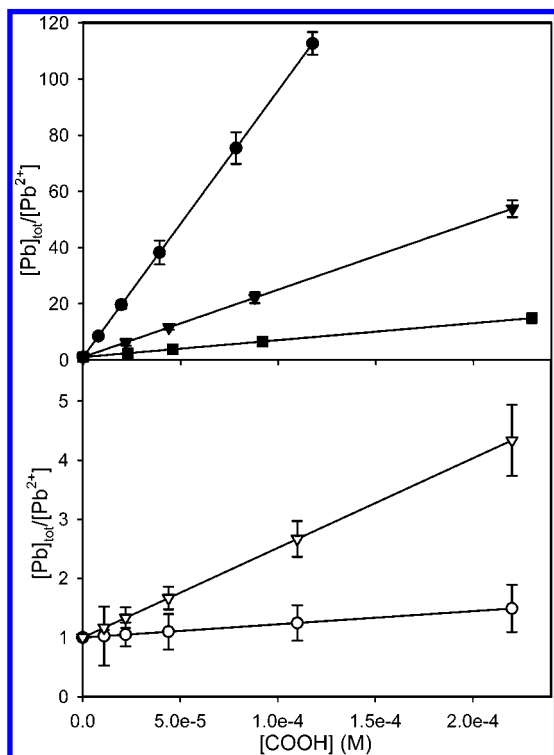
**FIGURE 1.** Pb<sup>2+</sup> measured using a Pb<sup>2+</sup>-selective electrode in the absence (REF) or presence of 10 mg C L<sup>-1</sup> humic acid (SRHA), fulvic acid (SRFA), alginate acid (AA), polygalacturonic acid (PGA), succinoglycan (SUC), and xanthan (XAN). ([Pb]<sub>tot</sub> = 10<sup>-6</sup> M, I = 5 × 10<sup>-3</sup> M, pH = 6.0). Error bars represent standard deviations (N = 3).

contributions of the carbonate or hydroxo species. Centricron experiments were not possible for the SRHA solutions. In that case, Pb<sup>2+</sup>-SE measurements gave good agreement with free metal ion concentrations calculated by the NICA-Donnan approach (square in Figure 1).

Complexation properties of the NOM, including conditional stability constants for the Pb complexes, were also determined in a series of experiments using increasing concentrations of NOM quantity. The proportion of complexed lead increased with increasing NOM. For example, by increasing the concentration of AA or PGA from 2.5 to 50 mg C L<sup>-1</sup>, [Pb<sup>2+</sup>]/[Pb]<sub>tot</sub> decreased from 0.16 to 0.01 (AA) or from 0.42 to 0.035 (PGA). For xanthan, the [Pb<sup>2+</sup>]/[Pb]<sub>tot</sub> ratio decreased from 0.98 to 0.68; for succinoglycan, it decreased from 0.86 to 0.14 when the NOM concentration was increased from 10 to 100 mg C L<sup>-1</sup>. For the humic substances, an increase from 1 to 15 mg C L<sup>-1</sup> lead to a decrease in [Pb<sup>2+</sup>]/[Pb]<sub>tot</sub> from 0.11 to 0.0089 (SRHA) or from 0.35 to 0.019 (SRFA). These results implied that for natural conditions where the polysaccharides predominate (e.g., inside flocs or biofilms (30)), they could play an important role in decreasing free metal concentrations.

(ii) *Determination of Average Conditional Stability Constants of Pb-NOM Complexes.* Concentrations of carboxylic acid sites, [COOH]<sub>tot</sub>, were determined by acid-base titration at constant I followed by data treatment using the method of Katchalsky (31). Estimates of 4.4 × 10<sup>-3</sup> mol g<sup>-1</sup> for alginate acid, 2.7 × 10<sup>-3</sup> mol g<sup>-1</sup> for xanthan, 4.6 × 10<sup>-3</sup> mol g<sup>-1</sup> for PGA, and 2.2 × 10<sup>-3</sup> mol g<sup>-1</sup> for succinoglycan were obtained. For the SRHA, a binding site concentration of 4.9 × 10<sup>-3</sup> mol g<sup>-1</sup> was taken from the literature (18). On the basis of the titration curves, >95% of the polysaccharide binding sites (COOH) were deprotonated under the studied conditions (pH = 6.0, I = 5 × 10<sup>-3</sup> M), while for SRHA this proportion was about 80% (32).

Average conditional stability constants (in M<sup>-1</sup>) were determined from the slope of the plot of the degree of complexation, [Pb]<sub>tot</sub>/[Pb<sup>2+</sup>], as a function of the concentration of carboxylic groups (Figure 2): log K<sub>PbSRHA</sub> = 6.0, log K<sub>PbAA</sub> = 5.5, log K<sub>PbSUC</sub> = 4.3, log K<sub>PbPGA</sub> = 4.5, and log K<sub>PbXAN</sub> = 3.4. It is very difficult to compare the obtained stability constants with literature data, since complexation data in the literature are very scarce and, where available, have been obtained under variable experimental conditions of pH, I, and Pb/NOM ratio. Nonetheless, the values appear consistent with those obtained previously for SRHA (e.g., ref 13) and AA (log K<sub>PbAA</sub> = 5.79, [COOH]<sub>tot</sub> = 4.39 × 10<sup>-3</sup> mol g<sup>-1</sup> at I = 0.1 M, pH = 3.0 (33)).



**FIGURE 2.** Ratio between total and free lead concentrations as a function of the binding site concentration expressed as carboxylic groups, SRHA (●), AA (▼), PGA (■), SUC (▽), and XAN (○). Error bars represent standard deviations ( $N = 3$ ).

It must be stressed that the log  $K$  values are means that are only valid for a given Pb/NOM ratio, pH, and ionic strength. Although more sophisticated determinations of intrinsic stability constants or statistical functions that represent a distribution of constants are available (reviewed in ref 34), they were considered to be outside the scope of this paper. Instead,  $[Pb^{2+}]$  was determined precisely under the exact conditions of the Pb bioaccumulation studies presented below.

**Lead Biouptake in the Presence of NOM.** Within the conceptual framework of the existing equilibrium models, including the FIAM and the BLM, sensitive binding sites on the organism are considered to be in equilibrium with the external medium and thus in competition for available metal with all other ligands (35, 36). Furthermore, Pb internalization and the subsequent biological effects are assumed to be

proportional to the concentration of filled sites ( $\{Pb-R_S\}$ , basis of the BLM) (4, 6), which can be directly related to the free metal concentration in solution ( $[Pb^{2+}]$ , basis of the FIAM). In Table 1, some of the main chemical equilibria leading to biological uptake are summarized with equations derived for two principal cases: reaction of  $Pb^{2+}$  with the cell surface and the reaction of both  $Pb^{2+}$  and  $Pb$ -NOM with the cell surface.

(i) *Pb<sup>2+</sup> Interacts with Sensitive Sites on the Algal Surface.* In most natural systems, metal concentrations are below those corresponding to the half saturation constant for which 50% of the sensitive sites on the algae are filled with metal (i.e.,  $K_S[Pb]_{tot} < 1$ ). For example, for the interaction of Pb with *C. kesslerii*,  $K_M = 1/K_S = 10^{-5.52}$  M (25). Complexation of Pb by NOM is predicted to decrease internalized Pb according to eq 4 (Table 1). In the present work,  $K_S[Pb]_{tot}$  corresponded to 0.31, implying that the metal internalization should decrease by a factor of  $(1 + K_{PbL}[L])$  with respect to the system containing no ligand. For example, on the basis of the stability constants determined above, for 10 mg C L<sup>-1</sup> of NOM, Pb uptake was predicted to decrease by a factor of ca. 80 for SRHA, 23 for AA, 4 for PGA, and 1.7 for succinoglycan. Although complexation by SRHA, AA, and PGA reduced Pb biouptake, agreement was qualitative only: uptake was superior to that predicted by the measured reduction in  $[Pb^{2+}]$  (Figure 3, circles). For example, internalized Pb was 25-fold higher than predicted in the presence of 10 mg C L<sup>-1</sup> SRHA and 8-fold higher than predicted for the same concentration of AA (Figure 3, bars). In contrast, for xanthan and succinoglycan,  $K_{PbL}[L] < 1$  such that no significant effect on uptake was expected nor observed for the concentrations that were employed (eq 4).

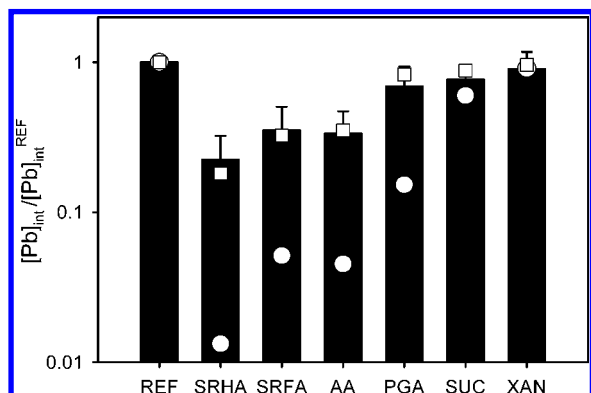
For the SRHA and to a lesser extent for the AA and the PGA, the observed deviation of the experimentally determined internalized Pb (Figure 4, points) from FIAM predictions (Figure 4, dotted line) increased with increasing NOM concentrations. Clearly, predictions of Pb uptake on the basis of free lead concentrations significantly *overestimated* the protective effect of the humic, alginic, and polygalacturonic acids, in agreement with previous observations for the SRFA (9). To explain this higher-than-expected Pb bioaccumulation in the presence of NOM, several “direct” effects of the NOM on the algae were examined further.

(ii) *Effects of the NOM on Membrane Permeability.* The role of the NOM on the overall (passive) algal membrane permeability was probed using the neutral marker, sorbitol. In the presence of 10 mg C L<sup>-1</sup> of SRHA, membrane permeability increased about 20%, while for AA, PGA, xanthan, and succinoglycan no significant differences in

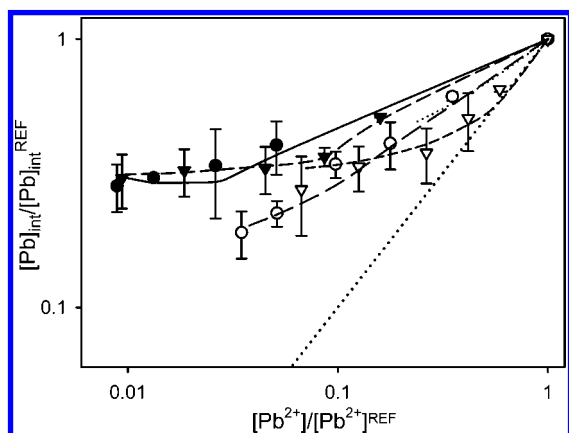
**TABLE 1.** Simplified Equations Used To Predict the Effect of NOM on Internalized Pb<sup>a</sup>

case	$Pb^{2+}$ reacts with sensitive sites	$Pb^{2+}$ and PbL react with sensitive sites
Reaction	$Pb^{2+} + R_S \xrightleftharpoons{K_S} Pb-R_S$ (2)	$Pb^{2+} + R_S \xrightleftharpoons{K_S} Pb-R_S$
Pb bound to sensitive sites	$\{Pb-R_S\} = \{R_S\}_{max} K_S [Pb^{2+}]$ (3)	$PbL + R_S \xrightleftharpoons{K_{SPbL}} LPb-R_S$ $\{Pb-R_S\} + \{LPb-R_S\} = \{R_S\}_{max} [Pb^{2+}](K_S + K_{SPbL}K_{PbL}[L])$ (6)
Response relative to the reference system	$\frac{[Pb]_{int}}{[Pb]_{int}^{REF}} = \frac{1}{1 + K_{PbL}[L]}$ (4)	$\frac{[Pb]_{int}}{[Pb]_{int}^{REF}} = \frac{K_S + K_{SPbL}K_{PbL}[L]}{K_S(1 + K_{PbL}[L])}$ (7)

<sup>a</sup> Equations 3 and 6 have been obtained from the “extended” FIAM (45) under the condition that metal concentration is lower than that corresponding to the half saturation constant ( $K_M = 10^{-5.52}$  M). Two cases are considered: (i) only  $Pb^{2+}$  reacts with sensitive sites on algal surface, or (ii) both  $Pb^{2+}$  and PbL react with the sensitive sites.  $[\ ]$  and  $\{ \}$  are the bulk and surface concentrations, respectively. L (i.e., ligand) corresponds to the NOM in solution,  $R_S$  is the free concentration of a sensitive site on the surface of the organism (e.g., transporter, carrier, ion channel), while  $K_{PbL}$ ,  $K_S$ , and  $K_{SPbL}$  are the stability constants defining the equilibrium reactions. PbL corresponds to a Pb complex with the humic substances or polysaccharides in solution;  $Pb-R_S$  is a surface complex resulting from the interaction of  $Pb^{2+}$  with sensitive sites at the biological surface; and  $LPb-R_S$  is a ternary complex on the cell surface. The superscript REF refers to a reference system containing the same total Pb concentration in the absence of ligand.



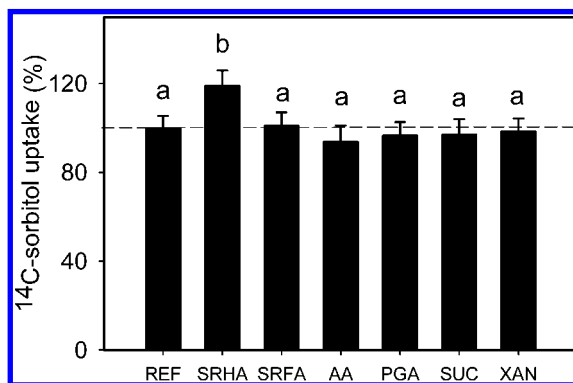
**FIGURE 3.** Logarithmic representation of internalized Pb concentrations normalized to the reference system in the presence of 10 mg C L<sup>-1</sup> of humic acid (SRHA), fulvic acid (SRFA), alginic acid (AA), polygalacturonic acid (PGA), succinoglycan (SUC), and xanthan (XAN). The reference system,  $[Pb]_{int}^{REF}$ , corresponds to the same total Pb concentration in the absence of NOM (pH = 6.0,  $I = 5 \times 10^{-3}$  M,  $[Pb]_{tot} = 1.2 \times 10^{-6}$  M). Experimentally determined values (bars) were compared to model predictions based upon the FIAM (eq 4, circles) and extended FIAM (eq 7, squares). Error bars represent standard deviations ( $N = 3$ ). The value for the SRFA (9) is given for comparison.



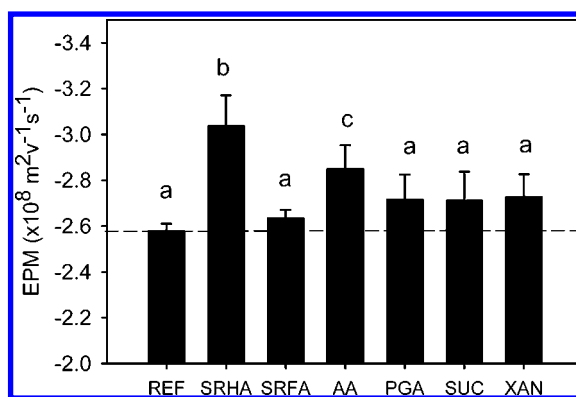
**FIGURE 4.** Logarithmic representation of internalized Pb ( $[Pb]_{int}$ ) normalized to the reference system in the absence of NOM ( $[Pb]_{int}^{REF}$ ) as a function of the measured  $[Pb^{2+}]$  normalized to Pb in the absence of NOM ( $[Pb^{2+}]^{REF}$ ) at pH = 6.0,  $I = 5 \times 10^{-3}$  M. Ligand concentrations increase from right to left on the figure, that is, [SRHA] (●) and [SRFA] (○): 1 to 15 mg C L<sup>-1</sup>; [AA] (▼) and [PGA] (▽): 2.5 to 50 mg C L<sup>-1</sup>. The dotted line represents the 1:1 line that corresponds to the FIAM (eq 4). Other lines represent the model prediction according to "extended" FIAM (eq 7). Error bars represent standard deviations ( $N = 3$ ). Values for SRFA (9) are given for comparison.

sorbitol incorporation were observed with respect to the reference system ( $p < 0.05$ , Student-Neuman-Keuls test) (Figure 5).

These observations are in agreement with previous results using the lipophilic Cd diethyldithiocarbamate as a marker that demonstrated that the addition of SRHA increased the membrane permeability of *Pseudokirchneriella subcapitata* (37). It was suggested that, due to its surfactant-like properties, humic acid may intercalate among membrane phospholipids, resulting in a decrease in lipid-lipid interactions and an overall increase in membrane permeability (38). In such a case, an effect is likely to be observed for the SRHA, while no influence would be expected for the polysaccharides. These expectations were confirmed by results in Figure 5. Although it is likely that changes in the overall membrane permeability could affect sensitive sites involved in Pb internalization and thus affect Pb uptake, the absence of



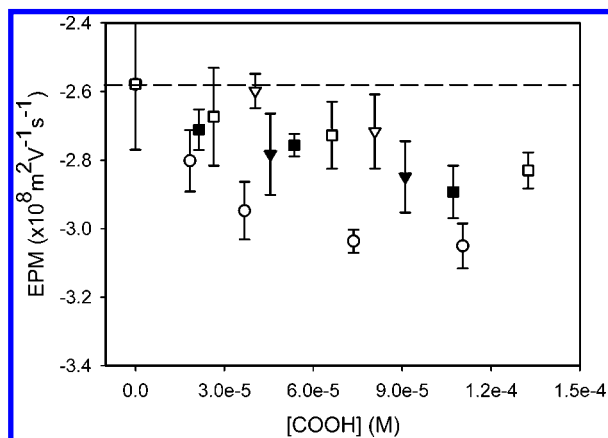
**FIGURE 5.** Cellular <sup>14</sup>C-D-sorbitol content after a 30-min contact time with 10 mg C L<sup>-1</sup> of humic acid (SRHA), fulvic acid (SRFA), alginic acid (AA), polygalacturonic acid (PGA), succinoglycan (SUC), and xanthan (XAN) (pH = 6.0,  $I = 5 \times 10^{-3}$  M). Dashed line indicates the control value in the absence of NOM. Error bars represent standard deviations ( $N = 3$ ). Different letters indicate significant differences between the means ( $p < 0.05$ , Student-Neuman-Keuls test).



**FIGURE 6.** Variation of the electrophoretic mobility (EPM) of *C. kesslerii* in the presence of 10 mg C L<sup>-1</sup> of humic acid (SRHA), alginic acid (AA), polygalacturonic acid (PGA), succinoglycan (SUC), and xanthan (XAN) (pH = 6.0,  $I = 5 \times 10^{-3}$  M, contact time = 30 min.). Error bars represent standard deviations ( $N = 6$ ). Different letters indicate significant differences among means ( $p < 0.05$ , Student-Neuman-Keuls test).

differences in sorbitol uptake in the presence of the polysaccharides, despite large increases in Pb uptake, suggested that the NOM had an additional effect on the algae.

(iii) *Adsorption of NOM on the Algal Surface.* The adsorption of NOM to the algal surface was probed using electrophoretic mobility measurements. Although a quantitative interpretation of mobility measurements is beyond the scope of this paper, this technique has the advantage of being much more sensitive than classical adsorption measurements, i.e., variations of algal electrophoretic mobilities, EPM are observed, even at low adsorbed NOM concentrations. Due to its high charge density, the adsorption of NOM to the algal surface leads to a decreased (i.e., more negative) EPM (cf. refs 9, 40). Indeed, the exposure of the algae to both humic and polysaccharide solutions generally resulted in a decreased algal EPM (pH = 6.0 and  $I = 5 \times 10^{-3}$  M; Figures 6 and 7). Indeed, for solutions containing 10 mg C L<sup>-1</sup> of NOM, a more negative EPM was observed for the SRHA and AA, while for the SRFA, succinoglycan, PGA, and xanthan, the effect was not statistically different from control values ( $p < 0.05$ , Student-Neuman-Keuls test; Figure 6). EPM values became more negative with increasing NOM in the external medium (Figure 7), in agreement with surface charge densities measured in the presence of polysaccharides for four kinds of heterotrophic bacteria (39) and for EPM and



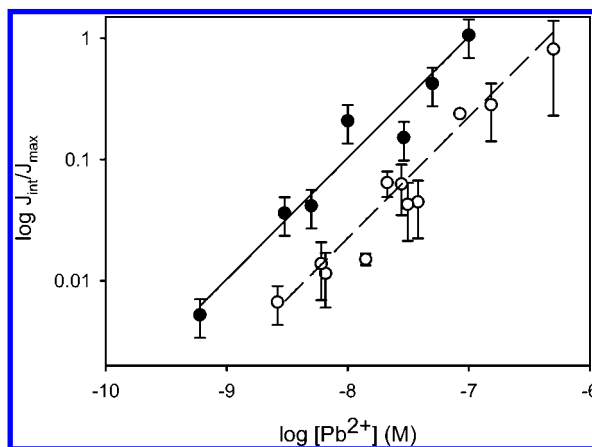
**FIGURE 7.** Variations of the electrophoretic mobility (EPM) of *C. kesslerii* in the presence of increasing quantities of SRHA (○), AA (■), PGA (□), SUC (■), and XAN (▽) (pH = 6.0,  $I = 5 \times 10^{-3}$  M, contact time = 30 min.). Ligand concentrations are expressed as carboxylic groups in mol L<sup>-1</sup>. Standard deviations are given when larger than the symbol size ( $N = 6$ ).

adsorption data quantifying the interaction of humic substances to the surfaces of biological cells including green algae (9, 40, 41). The adsorption of the SRHA to *C. kesslerii* was also confirmed in an independent set of experiments using differences in the SRHA absorbance (280 nm) in solution prior to and following contact with algae (data not shown).

The other advantage of using EPM measurements is that surface potentials can eventually be estimated. Biological “interphases”, including algal surfaces, contain numerous charges that generate a potential at the surface relative to the bulk phase that will affect the concentration of the metal near the surface (42). This implies that each substance that modifies the algal surface potential may also affect metal concentrations at the water–biological interface, which will modify the apparent metal-binding properties. Previous results have shown that the surface potential of *C. kesslerii* is dependent on the pH (43), ionic strength, and presence of divalent cations (e.g., Ca<sup>2+</sup>) (44). In addition to indicating that NOM is being sorbed to the algal surface, an increase of the negative electrophoretic mobilities, as observed here, is strong evidence for an increase in the negative surface potential of the algal cells. Such an increase could result in an increased concentration of Pb<sup>2+</sup> in the reactive layer at the cell–water interface, although the increase is unlikely to account for the very large increase in biouptake (Figures 3 and 4) (9).

(iv) *Formation of Ternary Pb–NOM Complexes on the Algal Surface.* The formation of a ternary complex and its contribution to the biological response is a reasonable possibility that could explain enhanced Pb internalization observed in the presence of humic and alginic acids. As observed above (Figure 4), deviations between the experimentally obtained and the predicted values of internalized Pb increased with increased ligand concentrations. A similar tendency was observed for Pb adsorbed to the cell wall (i.e., EDTA-extractable) and that bound to the biological membrane (data not shown). An increased adsorption of both NOM and Pb at the cell surface in parallel to an increased Pb internalization suggested that ternary surface complexes, {LPb–R<sub>s</sub>}, may have been formed, preceding Pb uptake.

The formation of ternary complexes has already been considered within the conceptual framework of the “extended FIAM” (45, 46). In such a case, the amount of “bioavailable” Pb is considered to be proportional to the sum of {Pb–R<sub>s</sub>} and {LPb–R<sub>s</sub>}, corresponding to the interaction of free Pb or PbL with the sensitive sites (Table 1, eq 6). To predict the



**FIGURE 8.** Experimentally determined uptake fluxes,  $J_{int}$ , normalized to the maximal uptake flux  $J_{max}$  as a function of the concentration of free lead. Experiments were performed in the presence of 10 mg C L<sup>-1</sup> SRHA (●) or SRFA (○). The solid and long dashed lines represent fits according to eq 8.

contribution of mixed complexes on Pb bioavailability, two limiting cases can be considered. First, if the free metal has much higher binding affinity to membrane sensitive sites than the complex, PbL, i.e.,  $K_S \gg K_{SPbL}K_{PbL}[L]$ , then the contribution of the ternary complex to metal bioavailability will be negligible, and a good correlation is expected between the measured uptake flux and model prediction using the “classical FIAM”. On the other hand, for  $K_S \ll K_{SPbL}K_{PbL}[L]$ , the ternary complex should contribute to the bioavailability in a manner that depends on the ratio  $K_S + K_{SPbL}K_{PbL}[L]/K_S(1 + K_{PbL}[L])$  (Table 1, eq 7).

The application of the above approach requires that the stability constants for all complexes, including those of the ternary complexes, are known. Therefore, average conditional stability constants of the LPb–R<sub>s</sub> complex,  $K_{SPbL}$ , were estimated from slope of experimentally determined, normalized (to  $J_{max}$ ) uptake fluxes versus the measured free metal ion concentration (Figure 8), according to eq 8 which is valid for low metal concentrations where  $\{R_s\} = \{R_s\}_{max}$ :

$$\frac{J_{int}}{J_{max}} = (K_S + K_{SPbL}K_{PbL}[L])[Pb^{2+}] \quad (8)$$

For this purpose, to avoid “direct” effects of the humics on the algae,  $J_{int}$  (and  $[Pb]_{int}$ , which is equivalent to an integrated  $J_{int}$  over time) were measured in a separate set of experiments where  $[Pb^{2+}]$  was varied by decreasing the total metal concentration while keeping [NOM] constant.

Given that the stability constant,  $K_S$ , for the interaction of Pb<sup>2+</sup> with *C. kesslerii* has been determined to be 10<sup>5.5</sup> (25) and  $K_{PbSRHA} = 10^6$ , an average value of 10<sup>4.9</sup> could be estimated for the formation of the ternary complex,  $K_{SPbL}$ , according to eq 8. Note that solely on the basis of these results, it is not possible to distinguish between (i) a ternary complex where Pb acts as bridge between NOM and the algal surface (LPb–R<sub>s</sub>) and (ii) adsorption of the Pb–NOM complex (PbL–R<sub>s</sub>). In addition, the adsorption on SRHA increased significantly with an increase of bulk [Pb] for a constant SRHA of 10 mg C L<sup>-1</sup>, also consistent with the formation of the ternary surface complex, {LPb–R<sub>s</sub>} (data not shown). By assuming that PbL was adsorbed to the same binding sites as Pb<sup>2+</sup>, an average stability constant for the ternary complex of 10<sup>4.7</sup> was estimated.

The values of  $K_{SPbL}$  obtained as above were employed to predict the effect of NOM on Pb uptake under the assumption that the ternary complex could also contribute to uptake. In the presence of 10 mg C L<sup>-1</sup> of NOM,  $K_S/K_{SPbL}K_{PbL}[L]$  was

much smaller than 1 for SRHA (0.045), SRFA (0.18), AA (0.2) and PGA (0.16) corresponding to biouptake increases of 25 (SRHA), 6 (SRFA) and 5 (AA) with respect to "classical" FIAM predictions. On the other hand, for xanthan and succinoglycan, the ratios  $K_S/K_{SPbL}K_{PbL}[L]$  were greater than unity: 34 (XAN) and 6 (SUC) and thus no significant contribution of the ternary metal complex to the bioavailability could be expected. The above model predictions were in good agreement with experimental values for the internalized metal (Figure 3, squares) for a NOM concentration of 10 mg C L<sup>-1</sup> as well as in the presence of increasing NOM concentrations (Figure 4, solid or dashed lines). A slight increase of [Pb]<sub>int</sub> with respect to the "extended" FIAM prediction was observed for [SRHA] > 10 mg C L<sup>-1</sup> likely due to a simultaneous influence increase in membrane permeability and negative surface potential. In most (environmentally relevant) cases, the observed discrepancy between measured Pb uptake and model prediction could be overcome by including the formation of a ternary complex between Pb–NOM and the sensitive sites on the algal surface, thus increasing bound (bioavailable) Pb. Such an approach can also account for the observed higher Pb adsorption (total and transport site bound Pb) in the presence of 20 mg L<sup>-1</sup> SRFA (9). Similarly, the failure of the simple FIAM to explain Pb uptake by *C. kesslerii* at pH > 6.5 (43) was postulated to be due to the contribution of ternary hydroxo and/or carbonate Pb complexes.

In previous studies, the formation of ternary complexes have been hypothesized to explain Al toxicity to *Chlorella pyrenoidosa* in the presence of a soil fulvic acid (27). In addition, ternary complexes with ligands such as F<sup>-</sup> and OH<sup>-</sup> have been demonstrated or hypothesized to explain toxicity and bioaccumulation for both algal and isolated fish gill cells (47, 48). Furthermore, the formation of mixed FAI–R<sub>S</sub> complexes have been demonstrated spectroscopically for fish gill cells using <sup>19</sup>F NMR (48). Finally, ternary Cu surface complexes have been postulated in several cases. For example, mixed OH–Cu–R<sub>S</sub> complexes, with a 2-fold lower binding affinity than that observed for the free ion, were necessary to explain the biological response of the freshwater bivalve, *Hyridella depressa* (46, 49). An improved predictive capacity of a biotic ligand model has also been demonstrated when CuOH<sup>+</sup> and CuCO<sub>3</sub> complexes were taken into account to explain EC<sub>50</sub> values to *Daphnia magna* (50, 51).

**Environmental Implications.** Quantitative data have been presented to demonstrate that the polysaccharide fraction of NOM, especially alginates, may exhibit complexing properties that are comparable to those of fulvic acids at comparable concentrations. In this context, the development of site-specific water quality criteria should take into account not only the fulvic and humic acids, but also the polysaccharides. In this respect, the introduction of correction factors based on measurement of NOM aromaticity (specific absorbance coefficient or SUVA values) (52–55) to account for NOM quality may only be appropriate when the NOM is dominated by humic substances. Future work is needed to determine to what extent and under what conditions the polysaccharides may play an important role in attenuating metal uptake when humic substances are also present.

The important discrepancy between Pb bioaccumulation by algae in the presence of humic substances and alginates and that predicted for similar [Pb<sup>2+</sup>] in the presence of synthetic ligands, raises important new questions about the predictive capacity and environmental relevance of the simplified models (FIAM, BLM), especially when applied to metal bioavailability determinations in natural (i.e., complex) waters. NOM had several simultaneous effects on Pb uptake including complexation in the bulk solution, modifications to membrane permeability, and potential and adsorption via a ternary complex. The contribution of the ternary complex to uptake was dependent on the relative stability

constants. Improved prediction of Pb uptake could possibly be obtained by taking into account the formation of ternary surface complexes. The data confirmed the importance of taking into account site-specific water characteristics, including the composition of NOM, to avoid an overestimation of the protective role of NOM to aquatic organisms.

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