Article

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Abstract

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SLAVEYKOVA, Vera, *et al*. Role of fulvic acid on lead bioaccumulation by Chlorella kesslerii. *Environmental science & technology*, 2003, vol. 37, no. 6, p. 1114-1121

DOI : 10.1021/es025993a
WOS : 000181629700010

Environ. Sci. Technol. **2003**, *37,* 1114−1121

Role of Fulvic Acid on Lead Bioaccumulation by *Chlorella kesslerii*

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To better understand the relationship between lead speciation and bioavailability in natural freshwaters, the interaction of lead with the freshwater alga *Chlorella kesslerii* was studied in the presence of the Suwannee River fulvic acid (SRFA). Special attention was paid to direct interactions of the fulvic acid on the algae, as well as potential physiological (membrane permeability and algal metabolism) influences. Lead-free ion concentration measurements were carried out using a novel ion-selective electrode. Pb uptake decreased in the presence of SRFA with respect to noncomplexed Pb, but uptake fluxes, cellular Pb, Pb bound to the transport sites, and total adsorbed Pb were all higher than predicted from Pb^{2+} activities, in accordance with the free ion activity model (FIAM). The discrepancies between the observed values and those predicted by the FIAM in the presence and absence of synthetic ligands increased with increasing concentration of SRFA. Several hypotheses were examined to explain the observed differences. No contributions of labile and/or hydrophobic Pb−SRFA complexes were found. Furthermore, direct biological effects, including variations in membrane permeability or algal metabolism, could not account for the observations. On the other hand, changes in the algal surface charge due to SRFA adsorption seemed to account, at least partially, for the observed increase in lead uptake in the presence of SRFA as compared to that corresponding to the same Pb^{2+} concentration in the presence of synthetic ligands.

Introduction

According to the free ion activity model [FIAM (*1*)], the role of ligands in solution is assumed to be limited to their participation in complexation reactions in the bulk solution. In this case, the complexation of a metal is predicted to reduce bioaccumulation (and the resulting biological effects) in direct proportion to the concentration (activity) of free ions, M^{z+}, for concentrations below the saturation of biological transport sites. Indeed, in the presence of small synthetic ligands, Pb uptake (Pb bound to transport sites and Pb uptake fluxes) by the unicellular green alga *Chlorella kesslerii* was shown to be directly proportional to the free lead ion concentration, $[Pb^{2+}]$, in accordance with the FIAM (*2*).

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On the other hand, there is currently no consensus in the literature as to whether the role of humic substances (HS) is limited to trace metal complexation in the bulk solution (*3*). Deviations from the FIAM and the closely related biotic ligand model [BLM (*4*)] might be expected because of the polyfunctionality and polydispersity of the HS. Furthermore, HS could indirectly affect trace metal bioavailability (i) by adsorbing to the surfaces of the plankton (*5*−*10*); (ii) by providing nutrient C (*11*, *12*), N (*13*−*15*), or P (*8*, *16*); (iii) by complexing exoenzymes (*17*); (iv) by modifying the membrane permeability (*5*, *8*, *18*); or (v) by modifying the algal surface activity (*19*).

Nonetheless, because of the absence of trace metal speciation measurements in the vast majority of published reports, only qualitative data are generally available to examine the effect of HS on trace metal bioavailablity. Among the exceptions are recent studies on Al toxicity to *Chlorella pyrenoidosa* in the presence of a soil fulvic acid (*8*); Hg uptake by *Chaoborus* (*20*); and several studies of Cu^{2+} toxicity (e.g., ref *21*), in which a Cu^{2+}-selective electrode was used to monitor free copper in the presence of HS (for a review, see ref *22*). Of the papers that have measured both trace metal bioavailability and speciation in the presence of HS, no consensus has emerged as to whether the data conform to the FIAM (*22*). Although Hg uptake was directly correlated to the concentration of Hg^{2+} (*20*), examples of enhanced toxicity (*20*, *22*) and enhanced protection (*22*) have also been documented. In contrast, the complexation of trace metals by aquatic humic substances has been widely studied from the point of view of metal speciation. For example, fairly complete compilations of conditional stability constants and effective ligand concentrations can be found in some recent reviews (*23*, *24*) for trace metals in natural waters. The competitive effects of Ca(II) (*25*) and Al(III) (*26*) on Pb(II) binding by humic substances have also been studied.

The aim of the present work was to investigate the nature of the interactions in a system containing algae, fulvic acid, and lead. Initial efforts were focused on a determination of the relationship between Pb(II) speciation and biological uptake fluxes in the presence of fulvic acid to verify the applicability of the FIAM and BLM. Pb^{2+} concentrations were quantified using a recently developed polymeric membrane electrode with low detection limits (*27*, *28*). Special attention was also paid to the interactions between the algae and the fulvic acid, as well as the direct metabolic and physiological influences of the HS on the algae.

Experimental Section

Choice of Humic Substance and Microorganism. A standard fulvic acid isolated from the Suwannee River, Georgia [Suwannee River fulvic acid (SRFA)], having well-characterized properties, composition of major and minor constituents, molecular weight, size, density, etc., was obtained from the International Humic Substances Society (Colorado School of Mines, Golden, CO) (*29*). Stock solutions of 1 g L^{-1} were prepared from the freeze-dried SRFA in Milli-Q water adjusted to pH 9.0 with dilute NaOH. Solutions were stored at 4 °C in the dark for at least 24 h to ensure equilibration before further dilution. Fluorescence correlation spectroscopy (FCS) was used to estimate the hydrodynamic sizes of the fulvic acid molecules from their diffusion coefficients (*30*). Calibration of the confocal volume of the FCS instrument was performed using Rhodamine 6G (R6G), which has a known diffusion coefficient, D, of 2.8×10^{-6} cm^2 s^{-1} (*31*).

Chlorella kesslerii (previously *Chlorella vulgaris*) was obtained from the University of Toronto culture collection

10.1021/es025993a CCC: $25.00
Published on Web 02/06/2003

(UTCC 266) and cultured in OECD (*32*) medium at 20 °C, with rotary shaking (100 rpm) and a 12/12-h light/dark regime (50 mmol of photons m^{-2} s^{-1} provided by fluorescent tubes). Each time the cells were sampled for an experimental measurement, cell densities, sizes, and surface distributions were determined by a Coulter Multisizer II particle counter (50-μm orifice, Coulter Electronics). To reduce or eliminate contamination (bacterial or chemical), all solutions and experimental containers were autoclaved, and experiments were performed under laminar flow conditions. *Chlorella* is particularly suitable for use in biouptake experiments because the cells are spherical, with little variation in cell size or morphology among the different experiments.

Pb(II) Speciation Measurements. A Pb^{2+}-selective electrode with an optimized membrane based on the ionophore 4-*tert*-butylcalix[4]arenetetrakis thioacetic acid dimethylamide was employed to measure Pb^{2+} activities. The detailed membrane composition and the membrane and electrode preparation procedures are described in detail elsewhere (*33*). An inner electrolyte composition of 10^{-6} M $Pb(NO_3)_2$ and 20 mg L^{-1} SRFA in 10^{-2} M MES [2-(*N*-morpholino) ethanesulfonic acid], nearly matching that of the measured sample, was employed to minimize zero-current ion fluxes. Potentials were measured with a 16-channel electrode monitor (Lawson Labs Inc., Malvern, PA) at room temperature in stirred solutions by simultaneously recording data from three electrodes. An internal filling solution of 10^{-3} M NaCl was used in contact with the reference half-cell Ag|AgCl. The electrodes were preconditioned for 12 h in a mixture of 10^{-6} M $Pb(NO_3)_2$ and 20 mg L^{-1} SRFA and used to measure standards and samples. Experimental selectivity coefficients for Pb^{2+} over a number of potentially interfering ions are given in ref *33*. The polymeric membrane electrodes were calibrated with a series of Pb solutions in the concentration range of 10^{-7} to 5×10^{-5} M. The slope of the response curve was 29.6 mV, as predicted by the Nernst equation. The drift during measurements was less than 1 mV h^{-1}. All activity measurements obtained with the ion-selective electrode (ISE) were performed at a constant ionic strength of 5×10^{-3} M. The results that follow are presented as concentrations.

In addition to the experimental measurements with the ISE, the Pb^{2+} concentration was predicted by two chemical speciation models. WinHumic V (*34*) incorporates a humic ion binding model (*35*, *36*) that uses a bimodal distribution of discrete binding sites. The ECOSAT program (*37*) uses a NICA–Donnan approach with a bimodal continuous distribution of sites. Both models take nonspecific electrostatic interactions into account. In both cases, model calculations were made using the default database sets (*38*, *39*): (i) For the WinHumicV model, the density of carboxylic sites, n_A, is 4.73 mol kg^{-1}; the density of phenolic sites, n_B, is $n_A/2$; the intrinsic proton binding constants are $pK_A = 3.26$, with a range (ΔpK_A) of 3.34, and $pK_B = 9.64$, with $\Delta pK_B = 5.52$; the intrinsic exchange constant, pK_{Pb-HA}, is 0.9; and the parameter P that determines the electrostatic interaction factor is −103. (ii) For the ECOSAT model, the density of carboxylic sites, Q_{1max}, is 5.88 mol kg^{-1}, with a distribution width (heterogeneity), m_{H1}, of 0.59; the density of phenolic sites, Q_{2max}, is 1.86 mol kg^{-1}, with $m_{H2} = 0.70$; the median intrinsic proton/Pb binding constants are $\log K_{H1} = 2.34$, $\log K_{H2} = 8.6$, $\log K_{Pb1} = -1.22$, and $\log K_{Pb2} = 6.87$ for the carboxylic and phenolic groups, respectively; and a parameter $b = -0.57$ was used for determination of the Donnan volume of the fulvic acid.

The hydrophobicity of the Pb–fulvic acid complex was estimated by flux measurements across a hydrophobic permeation liquid membrane (PLM) system containing lauric acid in a 1:1 mixture of toluene and phenylhexane. The PLM system was prepared as described in detail in ref *40*, with the exception that no ion carrier was added to the liquid membrane.

Bioaccumulation Studies. Cells in their mid-exponential-growth phase were harvested by gentle filtration, washed, and then resuspended in a defined experimental medium to a final algal cell density of ca. 10^7 cell mL^{-1}. The experimental medium consisted of 10^{-2} M MES [2-(*N*-morpholino] ethanesulfonic acid, Sigma] buffered to pH 6.0 and containing known quantities of Pb and/or SRFA. Fifty-milliliter aliquots of the algal solution were filtered (3.0-μm nitrocellulose filters, Millipore) 1, 10, 20, 30, 40, 50, and 60 min after resuspension in the experimental medium. Cells were rinsed twice with the experimental medium. Surface-bound Pb was distinguished from cellular (internalized) Pb using a 1-min extraction in 10^{-2} M EDTA (*2*, *41*, *42*). This wash procedure has been optimized with respect to the nature of the complexing ligands NTA (nitrilotriacetic acid), CDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetracetic acid), and EDTA (ethylenediaminetetraacetic acid) and the contact time with the cells (*2*). In summary, the concentration of cellular (post-washing) Pb was independent of the ligand used for the wash and stable following brief contact times (<1 min.). Cellular Pb was determined following digestion of the filtered, EDTA-washed algae with 1 mL of concentrated, ultrapure HNO_3 (Baker Instra-Analyzed Reagents). The dissolved, adsorbed, and cellular Pb fractions were measured by inductively coupled plasma mass spectrometry (Hewlett-Packard 4500 series).

Fulvic Acid Adsorption onto the Cell Surface. In parallel with the bioaccumulation experiments, fulvic acid adsorbed onto the algal surface was determined from the difference between bulk SRFA concentrations before and after algal addition (*5*). SRFA concentrations in solution were determined by UV–vis absorbance measurements at 280 and 324 nm (Lambda 4 UV–vis spectrophotometer, Perkin-Elmer). The concentration of SRFA was varied from 2 to 30 mg L^{-1}, and experiments were carried out in the presence and absence of 10^{-6} M Pb. Two types of controls were run in each experiment. The first control contained SRFA and/or Pb in the absence of algae and was used to estimate potential adsorptive losses to the filters. The second set of controls contained algae and Pb and was used to determine the possible influence of extracellular products on the absorbance signal.

To estimate modifications of the surface charge of *Chlorella kesslerii* in the presence of fulvic acid and/or Pb, electrophoretic mobilities (EPMs) were measured by laser Doppler velocimetry (Zetasizer 2000, Malvern). EPMs of the cells were measured following a 30-min exposure to different concentrations of SRFA. ζ-Potential latex particle standards (Malvern) were used for calibration.

Algal Metabolic Activity and Membrane Permeability. The influence of Pb and fulvic acid on C assimilation was determined by [^{14}C]$NaHCO_3$ short-term uptake experiments. The bicarbonate salt (specific activity 7.5 mCi $mmol^{-1}$, Perkin-Elmer life science products) was diluted in Milli-Q water, and the pH was adjusted to 9.0. Algae were collected in their mid-exponential-growth phase, filtered, rinsed, and resuspended in 10^{-2} M MES at pH 6.0 containing [^{14}C]$NaHCO_3$, 10^{-6} M Pb(II) as $Pb(NO_3)_2$, 30 mg L^{-1} SRFA, or their mixtures. Twenty-milliliter aliquots were filtered following contact times of 5, 10, 20, 30, and 40 min. A solution of 10^{-2} M nonlabeled $NaHCO_3$ was used to rinse the algae and filters.

To study the effects of SRFA and Pb on membrane permeability, cells were incubated in the presence of [^{14}C]-D-sorbitol, which has previously been shown to be taken up by *Chlorella pyrenoidosa* by passive diffusion without being metabolized (*41*). Radiolabeled [^{14}C]D-sorbitol with a specific activity of 370 mCi $mmol^{-1}$ was obtained from ANAWA Biomedical Services & Products and was diluted with 10^{-2}

M nonlabeled D-sorbitol. As in the bioaccumulation experiments, algae were collected in their mid-exponential-growth phase, filtered, rinsed and resuspended in the experimental media containing [^{14}C]D-sorbitol. Twenty-milliliter portions were filtered after contact times of 5, 10, 20, 30, 40, 50, and 60 min. Subsequently, 10^{-2} M of nonlabeled D-sorbitol was used to rinse the algae on the filters. ^{14}C activity in the filtrates was employed to quantify the dissolved sorbitol, whereas rinsed, digested filters and algae were used to determine cellular [^{14}C]D-sorbitol. For both [^{14}C]D-sorbitol and [^{14}C]-$NaHCO_3$ uptake experiments, the measured signal of ^{14}C was normalized to its initial concentration in solution. Data were rejected if the ^{14}C mass balance in the initial, dissolved, adsorbed, and cellular fractions was not recovered to within ±10%.

General Treatment of the Results. Short-term uptake of Pb was quantified using a linear regression of cellular Pb versus accumulation time. The slopes were used to obtain internalization fluxes, J_{int}. J_{int} values were fitted by a nonlinear regression of the Michaelis−Menten flux equation (*1*)

$$J_{int} = J_{max}\frac{[Pb^{2+}]}{K_M + [Pb^{2+}]} \quad (1)$$

where J_{max} is the maximal uptake flux and K_M is the Michaelis−Menten half-saturation constant, corresponding to the Pb^{2+} concentration for $J_{int} = {}^1/_2 J_{max}$. Under steady-state conditions, the reciprocal value of K_M provides the stability constant for complexation of the metal by the transport sites. The intercepts in the cellular Pb versus accumulation time plots were used as a surrogate of the concentration of the metal bound to transport sites {Pb−L_{tr}}. It has been shown previously (*2*) that, at low lead concentrations, the intercepts are directly related to the concentration of Pb^{2+} in solution with a plateau occurring above 10^{-5} M Pb, in agreement with the obtained saturation of the maximum uptake flux. Indeed, the apparent stability constant obtained from a Langmuir-type treatment of the intercepts (versus $[Pb^{2+}]$) gave a value ($10^{5.54}$ M^{-1}) that was almost identical to the reciprocal value of Michaelis−Menten constant, $10^{5.52}$ M^{-1}. This intercept-based technique for determining the metal-bound carrier concentrations has also been tested for Zn uptake by *C. kesslerii*, where it was shown to reflect the physiological state of the algae (i.e., carrier number), a distinction that is not seen with nonspecific Zn adsorption (*42*).

All experiments were repeated at least three times. Mean values and standard deviations are given throughout the text. All results were normalized by measured values of algal surface areas.

Results and Discussion

Determination of Pb Speciation. Experiments with the polymeric membrane Pb^{2+}-selective electrode showed that the proportion of complexed lead increased with increasing SRFA and decreasing total Pb concentrations. Indeed, under the experimental conditions employed a total Pb concentration of 10^{-6} M and a SRFA concentration of between 2 and 30 mg L^{-1}, $[Pb]_{tot}/[Pb^{2+}]$ increased from 3 to 55. Furthermore, for a constant SRFA concentration of 20 mg L^{-1} and a total Pb concentration ranging from 1×10^{-7} to 1×10^{-5} M, $[Pb]_{tot}/[Pb^{2+}]$ ranged from 90 to 5, corresponding to Pb^{2+} concentrations in the range of 1.1×10^{-9} to 2×10^{-6} M. In this case, the experimentally obtained conditional stability constants varied between log $K = 6.0$ and log $K = 5.2$ for a degree of site occupation, θ = [PbFA]/[SRFA], ranging from 10^{-3} to $10^{-1.6}$, assuming 6.1 mmol g^{-1} of carboxyl groups (*29*, *44*) and 82% deprotonation at pH 6.0 (*44*). Although it is very difficult to compare stability constants obtained under variable experimental conditions (pH, ionic strength, metal/fulvic acid ratio, source of HS, experimental technique), the values appear consistent with the literature. For example, a conditional logarithmic stability constant of 6.0 (pH 6.7) was determined using a Pb^{2+}-selective electrode for the complexation of Pb with a fulvic acid isolated from a bog (*45*). Reverse pulse polarography for $[Pb]_{tot} = 2.8 \times 10^{-5}$ M, $I = 0.1$ M, and pH 4.8 gave a log K value between 4.79 and 5.05 for 32 mg L^{-1} SRFA and between 5.39 and 5.6 for 92 mg L^{-1} SRFA(*46*).

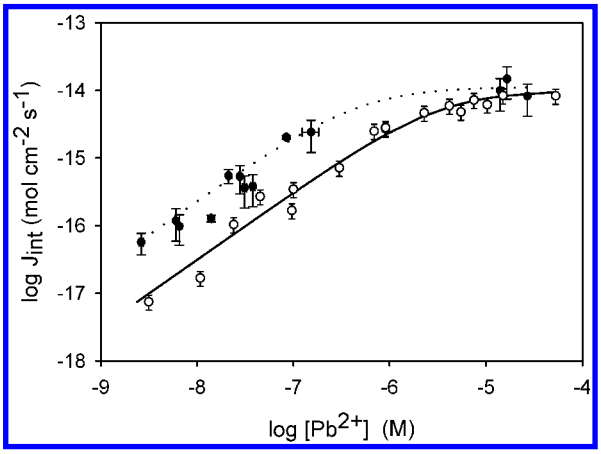

FIGURE 1. Dependence of the Pb internalization fluxes on the free lead ion concentration, $[Pb^{2+}]$, in the presence of 20 mg L^{-1} SRFA at pH 6.0. The dotted line represents a Michaelis−Menten plot for $K_M = 4.8 \times 10^{-7}$ M and $J_{max} = 1.1 \times 10^{-14}$ mol cm^{-2} s^{-1}. Standard deviations are given when larger than the symbol size. For comparison, uptake fluxes corresponding to the $[Pb^{2+}]$ in a reference system in the presence of synthetic ligands (*2*) are given by the open points and solid line (Michaelis−Menten plot).

To ensure that no aggregation of the fulvic acid was occurring in the presence of Pb, FCS measurements were performed under experimental conditions identical to those applied above. The mean values of the diffusion coefficient for the Pb−SRFA complex was estimated to be $(2.4 \pm 0.1) \times 10^{-6}$ cm^2 s^{-1}, independent of the metal-to-ligand ratio and in agreement with previous measurements of the fulvic acid alone (*30*). No aggregation of SRFA was observed following a 1-day equilibration of the Pb−fulvic acid mixtures.

Pb Bioaccumulation in the Presence of Fulvic Acid. Three different kinds of biouptake experiments were performed in parallel with measurements of Pb^{2+} activities. First, to avoid any influence of the fulvic acid on the algae, the concentration of Pb^{2+} was varied by modification of the total lead concentration for a constant SRFA concentration of 20 mg L^{-1}. In the second set of experiments, the concentration of Pb^{2+} was varied by varying the total SRFA concentration between 2 and 30 mg L^{-1} for a constant total Pb concentration of 10^{-6} M. In a final set of experiments, the Pb^{2+} concentration was kept constant at 5×10^{-8} M by increasing the concentrations of total Pb and SRFA in parallel. The results presented below for Pb biouptake by *Chlorella kesslerii* in the presence of fulvic acid are compared to the biouptake of Pb in the absence of ligand and in the presence of synthetic ligands, work that has previously been studied in detail in our laboratory (*2*) and is noted below as the reference system.

(i) Lead Bioaccumulation in the Presence of 20 mg L^{-1} Fulvic Acid. Pb internalization fluxes were *reduced* significantly by the addition of 20 mg L^{-1} fulvic acid with respect to a system without fulvic acid. Nonetheless at low Pb^{2+} concentrations ($[Pb^{2+}] < K_M$), Pb biouptake fluxes were *higher* in the presence the fulvic acid than those observed for equivalent concentrations of free Pb^{2+} in the reference system (Figure 1). Although both cellular lead, Pb_{cell}, and uptake fluxes, J_{int}, were proportional to the concentration of Pb^{2+} in solution (slope of 1 on the log J_{int} vs log $[Pb^{2+}]$ plot in Figure

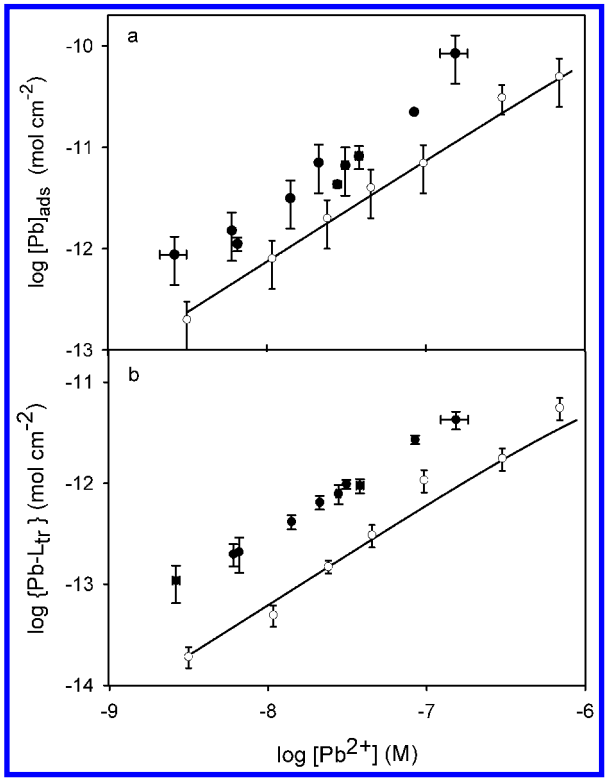

FIGURE 2. Dependence of the (a) adsorbed ("EDTA-extractible") Pb and (b) Pb bound to transport sites {Pb−L_{tr}} (obtained from the intercept of a cellular Pb vs time plot) on the Pb^{2+} concentration in the presence of 20 mg L^{-1} SRFA at pH 6.0 (solid points). For comparison, adsorbed Pb and Pb bound to transport sites corresponding to the Pb^{2+} concentration in a reference system in the presence of synthetic ligands (*2*) are given by the open points and solid line.

1), values in the presence of SRFA were about 7 times higher than those observed for the same Pb^{2+} concentration in the presence or in the absence of synthetic ligands.

In the presence of 20 mg L^{-1} SRFA, the best fit of the J_{int} versus $[Pb^{2+}]$ plot using the Michaelis−Menten equation gave a value of K_M of $(4.8 \pm 0.5) \times 10^{-7}$ M as compared to $(3.0 \pm 0.5) \times 10^{-6}$ M for the reference system. On the other hand, maximum biouptake fluxes, J_{max}, were equal in the absence [$J_{max} = (1.0 \pm 0.2) \times 10^{-14}$ mol cm^{-2} s^{-1}] and presence [$J_{max} = (1.1 \pm 0.2) \times 10^{-14}$ mol cm^{-2} s^{-1}] of SRFA. Under the assumption that K_M is equal to the reciprocal of the thermodynamic binding constant to the transport sites, this result suggested that the fulvic acid increased the apparent stability constant for the binding of Pb to transport sites, K_{Pb-tr}. In other words, for low Pb^{2+} concentrations ($[Pb^{2+}] < K_M$), the membrane permeability of *Chlorella kesslerii* to Pb^{2+}, $P_{Pb} = J_{int}/[Pb^{2+}]$, increased by a factor of ca. 7 from $(3.3 \pm 0.5) \times 10^{-6}$ cm s^{-1} to $(2.3 \pm 0.5) \times 10^{-5}$ cm s^{-1} in the presence of 20 mg L^{-1} SRFA.

A similar tendency of enhanced response with respect to the values obtained in the presence of synthetic ligands was also observed for adsorbed (i.e., EDTA-extractible) Pb (Figure 2a) and Pb bound to transport sites, Pb−L_{tr} (Figure 2b). Below transporter saturation, concentrations of adsorbed Pb and Pb bound to transport sites were proportional to the Pb^{2+} concentration but higher than values observed in the reference system, in agreement with the results obtained for the biouptake fluxes.

(ii) Lead Bioaccumulation in the Presence of Increasing Amounts of Fulvic Acid. The addition of 2−30 mg L^{-1} SRFA to 10^{-6} M Pb decreased Pb uptake by *Chlorella*. As can be seen in Figure 3, in the presence of increasing amounts of

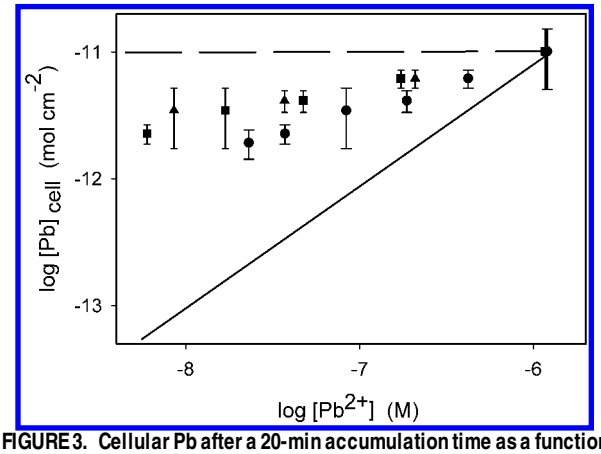

FIGURE 3. Cellular Pb after a 20-min accumulation time as a function of the Pb^{2+} concentration. The Pb^{2+} concentrations were measured by ISE (●) and predicted by WinHumicV (▲) and ECOSAT (■) model calculations. The total Pb concentration was maintained at 10^{-6} M while the concentration of SRFA was varied among the values 0, 2, 5, 10, 20, and 30 mg L^{-1} (pH 6.0). Standard deviations are given when larger than the symbol size. For comparative purposes, cellular Pb corresponding to the same Pb^{2+} concentration in the presence of synthetic ligands (*2*) and to the total Pb concentration are given by full and dashed lines, respectively.

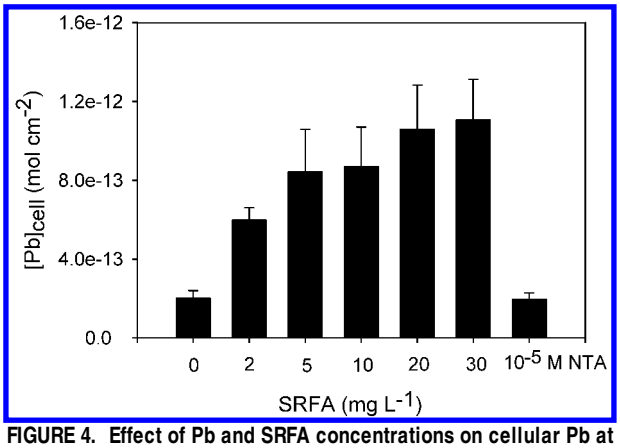

FIGURE 4. Effect of Pb and SRFA concentrations on cellular Pb at pH 6.0 for a 50-min accumulation time. Total concentrations of Pb and SRFA were increased in parallel to maintain a constant Pb^{2+} concentration of 5×10^{-8} M. For comparison, cellular Pb concentrations corresponding to the same Pb^{2+} concentration in the absence and in the presence of NTA are also given. All values are given as mean ± standard deviation, $n = 3$.

fulvic acid (from right to left in Figure 3), the cellular Pb concentration was lower than expected on the basis of the total Pb concentration (dashed line) but higher than that corresponding to the Pb^{2+} concentration in solution (solid line). Moreover, the difference between the cellular Pb concentration predicted on the basis of the solution Pb^{2+} concentration and the observed cellular Pb concentration increased with increasing concentration of fulvic acid.

(iii) Lead Bioaccumulation at Constant Pb^{2+} Concentration. In the third set of experiments, the total Pb and fulvic acid concentrations were varied in a manner that kept the concentration of Pb^{2+} constant. Under these conditions, the FIAM predicts a constant cellular Pb concentration. Contrary to this expectation, an increasing concentration of SRFA resulted in a significant increase in cellular Pb with respect to solutions of 5×10^{-8} M Pb^{2+} containing either no ligand or NTA as the complexing ligand (Figure 4).

Possible Explanations for the Enhanced Bioaccumulation of Pb in the Presence of Fulvic Acid. To explain the higher than expected bioaccumulation of Pb in the presence of fulvic acid with respect to Pb in the presence of synthetic

TABLE 1. Parameters Used To Calculate the Lability, L, of the Pb−SRFA Complexes for a Total Pb Concentration of 10^{-6} M and a SRFA Concentration of 2−30 mg L^{-1}

parameter, meaning (units)	values
k_a, formation rate constant (M^{-1} s^{-1})	1×10^{10}
K, conditional stability constant of Pb−SRFA complex (determined using polymeric membrane ISE measurements) (M^{-1})	$10^{5.67}$
k_d, dissociation rate constant, $k_d = k_a/K$ (s^{-1})	2.14×10^4
r, radius of the microorganism (measured by particle counter) (cm)	1.8×10^{-4}
D_{Pb}, diffusion coefficient of Pb^{2+} (cm^2 s^{-1})	9.5×10^{-6} [a]
$D_{Pb-SRFA}$, diffusion coefficient of Pb−SRFA complex (measured by FCS) (cm^2 s^{-1})	2.4×10^{-6}
c^*_{FA}, bulk concentration of SRFA (M)	varied

[a] Ref *52*.

ligands, several hypotheses were examined: (i) underestimation of the Pb^{2+} concentration by the ISE, (ii) contribution of labile hydrophilic Pb−SRFA complexes to the uptake, (iii) uptake of lipophilic Pb−SRFA complexes, (iv) adsorption of SRFA on the algae with resulting modifications to the (v) algal physiology (membrane permeability, algal metabolism) and/or (vi) surface charge (so as to modify the effective affinity of the transport sites with respect to Pb).

(i) Underestimation of the ISE-Determined Pb^{2+} Concentration. The larger than expected internalization fluxes observed in Figure 3 could be explained by a systematic underestimation of the Pb^{2+} concentration by the ISE. The most obvious interference, i.e., that of the fulvic acid, was unlikely to be a problem because a symmetrical electrode setup was employed in which the concentration of the fulvic acid was the same on both sides of the ion-selective membrane. Furthermore, the accuracy of the Pb^{2+} measurements was evaluated by comparing the ISE-determined Pb^{2+} concentration with values predicted by the WinHumic V and ECOSAT models. For both models, the calculated Pb^{2+} concentration was smaller than that measured by the ISE, further accentuating the difference between the predicted line (based on the Pb^{2+} concentration) and the experimental points (Figure 3). Similar results were also obtained using a permeation liquid membrane (*47*) as the Pb speciation technique. For all four determinations of the Pb^{2+} concentration (ISE, PLM, WinHumicV, and ECOSAT), cellular Pb was significantly higher than would be predicted on the basis of the FIAM alone.

(ii) Lability of the Pb−Fulvic Acid Complex. Over the past few years, greater attention has been paid to the role of the dynamics of mass transport and the kinetics of association/dissociation reactions in attempts to understand metal bioavailability in complexing media (*21*, *48*, *49*). With respect to metal bioavailability, a complex is considered to be labile when it can dissociate and re-form many times during its transport through the steady-state diffusion layer of the organism. In this case, the resulting metal supply to the biological surface can be determined by a coupled flux of the free metal and labile species (*48*, *49*). For radial diffusion and first-order association−dissociation kinetics, as can be assumed to be the case here, complexes can be considered labile if the value of the lability criterion, L, is much greater than 1 (*50*, *51*)

$$L = \frac{k_d^{1/2} r D_{Pb}^{1/2}}{K^{1/2} D_{PbFA} c^{*1/2}_{FA}} \quad (2)$$

The meaning and values of the different parameters applied to calculate L are compiled in Table 1. The validity of this equation, the basic assumptions for its derivation, and its applicability to microorganisms have been discussed elsewhere (*49*).

The value of L for Pb−SRFA complexes was calculated to be 16 for a SRFA concentration of 2 mg L^{-1} and 4 for 30 mg L^{-1} SRFA. Nonetheless, such a calculation of the lability criterion is strictly valid only for chemically homogeneous systems. For heterogeneous systems, such as for fulvic and humic acids, the lability of the metal complexes can also be influenced by the degree of heterogeneity and the degree of occupation of the complexing sites. In this case, complex lability is predicted to decrease for an increasing degree of heterogeneity and for increasing concentrations of HS (for a given total metal concentration) (*53*). The heterogeneity of the Pb−SRFA complexes can also be described by a corresponding distribution of the dissociation rate constants. For example, for a fixed SRFA concentration of 20 mg L^{-1}, L varies from 4 to 13 for an increasing site occupation of Pb corresponding to total Pb concentrations from 1.0×10^{-8} to 2.5×10^{-6} M, respectively. In summary, in all cases, the estimated lability criterion was higher than 1, indicating that, for the studied conditions, the Pb−SRFA complexes can all be considered labile.

Although the lability criterion calculated above suggested that the complexes were able to dissociate during their transport through the diffusion layer, their contribution to the biouptake flux also depends on the capacity of the organism to internalize the metal (*48*, *49*). In this case, observed internalization fluxes were much smaller than the theoretical limiting diffusive fluxes corresponding to Pb^{2+} alone (i.e., $J_{diff} = 5.2 \times 10^{-14}$ mol cm^{-2} s^{-1} for $[Pb^{2+}] = 10^{-9}$ M), strongly suggesting that the supply of Pb^{2+} alone was sufficient to satisfy the algal demand (in a chemical sense) and that no contribution of the complexes was necessary because of the already significant concentration gradient of Pb^{2+} in the diffusion layer. Under the conditions examined here, complex lability cannot explain the increase in J_{int} in the presence of SRFA. A similar theoretical analysis has shown that diffusion could not be limiting for Cd uptake by carp (*Cyprinus caprio*) (*54*), whereas uptake fluxes for Zn(II) by mussels (*Mytilus edulis*) and freshwater algae (*Chlorella kesslerii)* appeared to be diffusion-limited at low concentrations (*42*, *54*).

(iii) Contribution of a Hydrophobic Fraction of Pb−Fulvic Acid Complexes. HS are a chemically heterogeneous mixture of molecules with a continuum of chemical properties. Because the transport of hydrophobic Pb complexes by passive diffusion is likely to be faster by several orders of magnitude than carrier-mediated transport (*55*), even a small proportion of hydrophobic Pb−SRFA complexes could result in a significant increase in the observed uptake flux. The presence of hydrophobic complexes was thus verified by the PLM technique in the absence of a carrier. In the case of a significant hydrophobicity of the Pb−fulvic acid complex, a flux of Pb should have occurred across the hydrophobic PLM membrane. In contrast to such an expectation, no PLM fluxes were detected in the concentration range of 2−30 mg L^{-1} fulvic acid for a total Pb concentration of 10^{-6} M. This

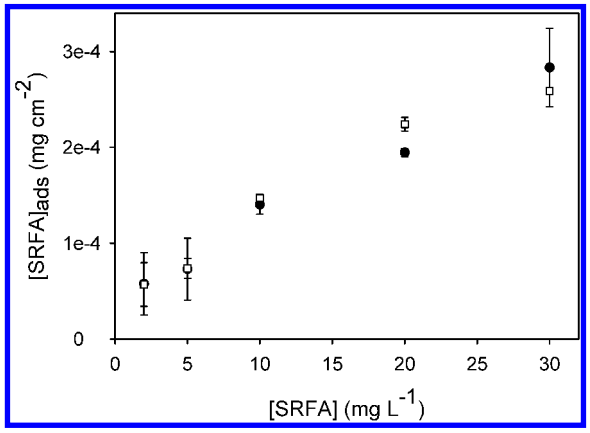

FIGURE 5. SRFA adsorbed to the surface of *Chlorella kesslerii* as a function of fulvic acid solution concentration (pH 6.0, contact time of 30 min) in the absence of Pb (●) and in the presence of 10^{-6} M total Pb (□). Standard deviations are given when larger than the symbol size.

result strongly suggests that no hydrophobic Pb species contribute to Pb bioaccumulation in the presence of SRFA.

(iv) Adsorption of Fulvic Acid on the Algal Surface. Adsorption of SRFA to the surface of *Chlorella* increased with its concentration in solution up to 30 mg L^{-1} (Figure 5). This tendency is in reasonable agreement with observations for other algal species. Although it is difficult to compare HS adsorption data because of strong dependences on the pH and ionic strength of the experimental media and the algal species, the obtained values of adsorbed SRFA were higher than those observed for *Chlamydomonas reinhardtii* (*10*) but lower than those obtained for the more hydrophobic soil (*8*) and Armadale (*5*) fulvic acids. The addition of 10^{-6} M Pb did not significantly influence the adsorption of SRFA, possibly because the adsorbed fulvic acid was always in molar excess to both the quantity of Pb bound to transport sites and the total quantity of adsorbed Pb (ca. 10-fold molar excess at 10 mg L^{-1} SRFA and 10^{-6} M Pb). Using the greatly simplified assumption that each fulvic molecule covers 1 nm^2 of a smooth algal surface (*54*), the amounts of adsorbed SRFA correspond to a monolayer surface coverage for bulk solution concentrations of 10 mg L^{-1} SRFA.

(v) Influence of Adsorbed Fulvic Acid on Membrane Permeability and Carbon Assimilation. Experiments with ^{14}C-labeled D-sorbitol were performed to estimate the influence of Pb and fulvic acid on membrane permeability. *Chlorella pyrenoidosa* accumulates D-sorbitol by passive diffusion without metabolizing the substrate (*43*). If this is also the case for *C. kesslerii*, then changes in sorbitol uptake should reflect overall membrane permeability. Indeed, the presence of 10^{-6} M Pb increased membrane permeability from the control value of $(2.4 \pm 0.3) \times 10^{-7}$ to $(2.8 \pm 0.1) \times 10^{-7}$ cm^{-1} s^{-1}, whereas the presence of fulvic acid alone had no observable effect. When fulvic acid was added to solutions containing 10^{-6} M Pb, permeability was restored to the values observed in the control (absence of both Pb and fulvic acid). A similar restoration of membrane permeability due to the addition of a soil fulvic acid has previously been observed by Parent et al. (*8*); however, in that case, Al^{3+} decreased the membrane permeability, whereas the addition of fulvic acid restored (increased) it to control values. In another related study, the addition of fulvic acid increased the passive diffusion of the lipophilic fluorescein diacetate at pH 5.0, although no effect was observed at pH 7.0 (*9*).

The influence of Pb and fulvic acid on nutritive carbon assimilation was studied using ^{14}C-labeled $NaHCO_3$. At pH 6.0, the addition of 30 mg L^{-1} SRFA did not significantly (Student *t*-test, $P < 0.05$, data not shown) influence carbon

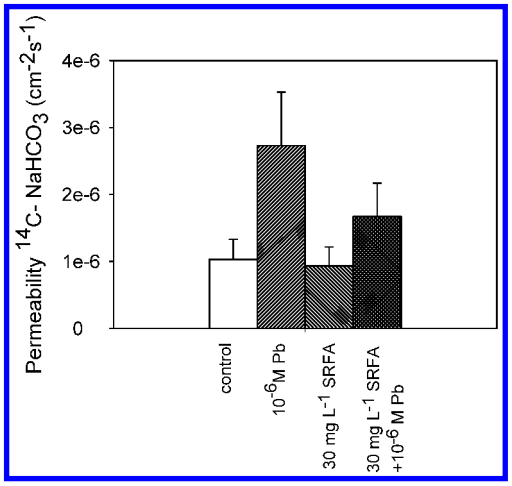

FIGURE 6. Influence of Pb and SRFA on uptake of [^{14}C]$NaHCO_3$ expressed in terms of membrane permeability, *P*, with *P* defined by $P = J/c$. In this case, *J* is the flux of ^{14}C determined for the first 20 min, and *c* is the solution concentration of $NaH^{14}CO_3$.

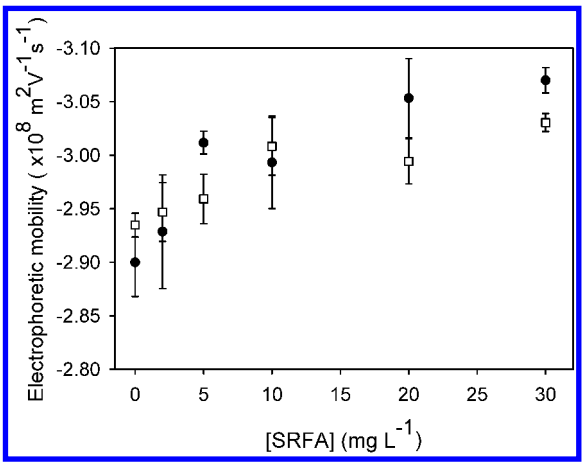

FIGURE 7. Variation of the electrophoretic mobility (EPM) of *Chlorella kesslerii* as a function of the SRFA concentration (pH 6.0, contact time of 30 min) in the absence of Pb (●) and in the presence of 10^{-6} M total Pb concentration (□). Standard deviations are given when larger than the symbol size.

assimilation. This observation is in agreement with data for *S. subspicatus*, where, at pH 6.0 and 7.0, no enhancement of carbon assimilation was observed in the presence of 10 mg L^{-1} SRFA (*10*). In contrast, enhanced carbon assimilation was observed in response to high concentrations (10^{-6} M) of Pb. This effect could be related to the increase in membrane permeability observed in the D-sorbitol uptake experiments. Moreover, the addition of 30 mg L^{-1} SRFA to solutions containing 10^{-6} M Pb significantly decreased the C assimilation rate with respect to that in Pb solutions containing no SRFA (Figure 6). The decrease in C assimilation could be related to a decrease of the Pb^{2+} activity as a result of either complexation with the fulvic acid or direct interaction of SRFA with the algae.

(vi) Modifications of Algal Electrophoretic Mobilities in the Presence of SRFA. Modifications of the algal surface charge due to the presence of SRFA were estimated from the electrophoretic mobilities (EPMs). An increase in the SRFA concentration, in the presence or absence of Pb, resulted in more negative values of EPM (Figure 7) similar to previous observations (*5*, *10*). Given that the experiments were performed at constant ionic strength and pH, it could be assumed that the EPM was directly related to the cell surface potential and thus the surface charge. As a first approximation, it was assumed that the algae behaved as rigid spheres with

homogeneously distributed charges on the cell wall and a cellular diameter that was much larger than the Debye length (*57*). In such a case, Smoluchowski's equations can be used to estimate the ζ-potential (*58*). By assuming that the distance between the algal surface and the shear plane is known, it is possible to estimate the surface potential of the algae. For example, for a shear plane that is 0.2 nm (1−2 water layers) from the algal surface, it is possible to estimate an algal surface potential of −39.0 mV in the absence of SRFA and −39.5 mV in the presence of 2 mg L^{-1} SRFA. On the other hand, if the shear plane is assumed to be 2.0 nm from the algal surface, the estimated values of surface potential would be significantly more negative. This significant negative surface potential would result in surface concentrations of Pb^{2+} that are significantly higher than those in the bulk solution. Note that this mechanism would also be expected to apply to other cations.

Although the adsorption of SRFA increased the negative surface potential with a resulting increase of surface-bound Pb, the surface potentials estimated above are not sufficient to explain the 5−10-fold increase of surface-bound Pb or Pb internalization fluxes observed in the presence of the fulvic acid (cf. Figures 1 and 2). To explain a 7-fold increase of the internalization flux by a surface charge effect, it would be necessary for the fulvic acid to increase the surface potential by a factor of 1.7 or to displace the shear plane from 0.2 to 2 nm from the algal surface. The second possibility is not as unrealistic if it is taken into account that the hydrodynamic diameter of SRFA is about 2 nm and that, for 30 mg L^{-1} SRFA in the bulk solution, mass balance calculations indicate that two molecules of fulvic acid are available for adsorption for each square nanometer of algal surface. On the other hand, given the current lack of theory relating electrophoretic mobilities to surface potentials (especially for microorganisms), any further calculations would be speculative only.

The results from the bioaccumulation experiments revealed that the fulvic acid decreased Pb bioaccumulation but less than would be predicted on the basis of Pb^{2+} measurements in relation to previous biouptake experiments in the absence and presence of synthetic ligands. The difference between predicted values of the Pb^{2+} internalization flux and the observed values increased with increasing concentrations of SRFA. Furthermore, increases in transporter-bound Pb and total adsorbed lead (Figure 3) paralleled the observed increase in internalization flux, reinforcing the importance of an explanation based on the surface chemistry of the algae as opposed to Pb solution chemistry. Such results would suggest that a BLM-type model relating adsorption rather than bulk solution chemistry to biological effects might be better adapted to bioavailability determinations in complex natural environments. Indeed, the BLM approach assumes that the biological response (i.e., metal uptake) depends on the concentration of metal bound to transporters at the biological "interphase". Unlike the observations for Pb^{2+} (Figure 1), uptake fluxes in both the presence and absence of SRFA were directly related to transport-site-bound Pb (Figure 8), in accordance with a such an approach.

Environmental Implications. Although it was impossible to quantitatively explain the role of SRFA with respect to Pb uptake, a chemical change to the biological membrane due to SRFA adsorption (cf. Figures 5 and 7) was the most reasonable hypothesis considered. Other explanations, especially those examining physiological changes of the microorganism (overall membrane permeability, nutrient accessibility) did not appear to be responsible for the observed results. In any case, the effect of the adsorbed SRFA is important and different from that of the typical synthetic ligands that are most often employed in laboratory bioaccumulation experiments. Such fundamental differences will need to be taken into account as new models are developed for understanding the uptake process and predicting the effects of trace metals in the natural environment. The important discrepancy between Pb bioaccumulation in the presence of SRFA and that predicted for similar concentrations of Pb^{2+} in the presence of synthetic ligands raises important questions about the predictive capacity and environmental relevance of the simplified models, especially when applied to determine metal bioavailability in natural waters. Indeed, such results suggest that, whereas the introduction of speciation-based regulations governing trace metal concentrations will be an improvement over the use of total metal concentrations, further investigations are still required prior to their implementation. For instance, in the specific example examined here, the concentration of Pb bound to transport sites better reflected Pb uptake than did free ion concentrations, suggesting that the BLM would be a more suitable scientific framework for regulations than the FIAM. On the other hand, several recent examples have been documented for which neither of the steady-state models (FIAM, BLM) could predict uptake or biological effects (e.g., refs *22*, *42*, *59*). It is clear that the complex relationships between trace metal speciation and bioavailability and the complex nature of the heterogeneous metal complexing ligands will need to be addressed further if we are to understand the effects of metals on biota in natural waters.

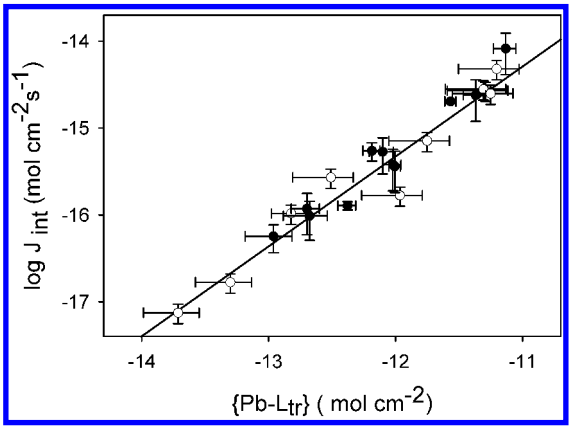

FIGURE 8. Dependence of the Pb internalization fluxes on Pb bound to transport sites ($Pb-L_{tr}$) in the presence of 20 mg L^{-1} SRFA at pH 6.0. For comparison, uptake fluxes corresponding to Pb bound to transport sites in a reference system in the presence of synthetic ligands (*2*) are given by the open points.

Acknowledgments

This work was supported, in part, by the Swiss National Funds 2000-050529.971 and 20-64829.01, the National Institutes of Health (Grant R01-GM59716), and the European framework project BIOSPEC (EVK1-CT-2001-00086). We appreciate the helpful critical readings of earlier versions of the paper by C. Hassler and H. van Leeuwen and "lability" discussions with J. P. Pinheiro. Extremely helpful technical assistance was provided by S. Closter, M. Martin, and M. Tuveri. Finally, we also thank N. Parthasarathy, who kindly performed the PLM measurements.

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Received for review July 24, 2002. Revised manuscript received December 10, 2002. Accepted December 19, 2002.

ES025993A