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Discriminating between intra- and extracellular metals using chemical extractions

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Abstract

Several washing agents (EDTA, CDTA, NTA, 8 HQS, Ti-EDTA-citrate, Ca^{2+}, and H^+) were tested for their ability to extract adsorbed metal from biological surfaces to distinguish between internalized and total cellular metal (Pb, Ni, Cd, Zn, and Cu) for a green alga, *Chlorella kesslerii*. Although a single extraction protocol cannot be provided that would be acceptable for all biological species and metals, several guidelines are proposed that should facilitate the interpretation of metal bioaccumulation data. It was shown that for the studied metals, a thermodynamic explanation was generally sufficient to explain the metal extraction results. At pH 6.0, a solution of 5×10^{-3} M ethylenediaminetetraacetic acid (EDTA) was an acceptable washing agent in most cases, whereas Ca or proton exchange solutions did not appear to quantitatively remove adsorbed metals. Conditions of ligand excess could be attained with relatively small concentrations of added ligand, effectively minimizing potential damage to the organism, including modifications in membrane permeability. Potential deleterious effects of the wash agents were verified. For example, a Ti-EDTA-citrate or an EDTA wash at high concentrations resulted in perturbations of algal membrane permeability. In spite of potential difficulties interpreting extraction data, the usefulness of the extraction protocols was clearly demonstrated for several organisms.

The bioaccumulation of trace metals is most often described by the sum of several metal body burdens including metal bound to the cell wall, the cell membrane, specific carrier proteins, and intracellular ligands. On the contrary, acute effects due to trace metals are generally best related to either the concentration of free metal ion in solution (basis of the free ion activity model [FIAM]; Morel 1983) or to the metal bound to sensitive (biologically active) sites at the biological surface (basis of the biotic ligand model [BLM]; Di Toro et al. 2001; Hassler et al. 2004). Both the FIAM and BLM are based upon the underlying hypotheses that metal internalization fluxes are a good indicator of metal toxicity (Campbell 1995) and that the metal must cross or react with sensitive sites on a biological membrane to invoke a toxic effect (Lindon and Henriques 1993; Rand et al. 1995). The differentiation between metal that has been internalized by the cell (i.e., biologically active) and that adsorbed indiscriminately to cell components is thus extremely relevant and necessary when relating biological effects to measurements of chemical speciation.

Although a majority of the bioaccumulation literature continues to examine total body burdens or total cell contents, a number of techniques including cation exchange (e.g., Crist et al. 1981, 1990), acid washes (e.g., Xue and Sigg 1990; Chu et al. 1997), isotopic exchange (e.g., Hudson and Morel 1990), and the use of complexing agents such as ethylenediaminetetraacetic acid (EDTA) (e.g., Bates et al. 1982; Chu et al. 1997; Britta et al. 2002), 8-hydroxyquinoline-5-sulfonic acid (e.g., Price and Morel 1991) or Ti-EDTA-citrate (e.g., Hudson and Morel 1989) have been employed to distinguish surface bound metal from internalized metal. Of the above techniques, the use of the EDTA extraction appears to be the most widespread even though systematic verifications of ligand efficiency, desorption kinetics, or secondary effects are rarely performed. The present work aims to examine, in detail, some of the important factors that influence the common metal extraction procedures including the nature and concentration of the washing agent, the contact time with the microorganism, and the effect of the agent on organism metabolism and membrane

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permeability. Several recommendations allowing for the critical evaluation and extension of the experimental protocol to other organisms are given at the end of the paper.

Materials and procedures

Organism maintenance—Chlorella kesslerii (UTCC 266, University of Toronto, ON, Canada) is a unicellular, spherical, freshwater green alga with an average diameter of 3.6 µm under the examined conditions. *C. kesslerii* was grown in OECD media (Organization for Economic Cooperation and Development Test Guideline 201 1984) in an incubation chamber (Multitron, Infors, Switzerland) at 20°C and 100 rpm rotary shaking under a 12:12 dark:light (50 µmol photons m^{-2} s^{-1}) regime. After 7 d of incubation, corresponding to the attainment of the mid-exponential growth phase, the algae were isolated by gentle filtration on 3 µm nitrocellulose filters.

Experimental solutions—Experimental solutions generally contained 10^{-2} M 2-[*N*-morpholino]ethanesulfonic acid (MES) (Sigma) at pH 6.0 and 10^{-6} M of the metal of interest. Some comparative experiments were performed at pH 7.0 in 10^{-2} M *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES). Five metals of variable reactivity and toxicity were examined: Pb, Ni, Cd, Zn, and Cu. Under the studied conditions, each of the five trace metals was present mainly as free hydrated ion in the experimental medium (>97% at pH 6.0) as determined by MINEQL+ (Version 3.01a) using updated stability constants (NIST 2003). Solution pH was measured using a H^+ selective electrode (Metrohm Herisau, pH electrode model 6.0204.100) calibrated with buffers at pH 4.00 ± 0.02 (25°C) and pH 7.00 ± 0.02 (25°C).

Metal washing agents—Stock solutions (10^{-1} M) of each of the washing agents were prepared in 10^{-2} M MES at pH 6.0. Further dilution was performed in 10^{-2} M MES at pH 6.0. The extraction efficiencies of EDTA, trans-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetracetic acid (CDTA) (Fluka), nitrilotriacetic acid (NTA) (Sigma), and 8-hydroxyquinoline-5-sulfonic acid (HQS) were examined at various concentrations. In addition, solutions of dilute HNO_3 (pH 3.0), 5×10^{-3} M $Ca(NO_3)_2$ at pH 6.0 and a mixture of Ti(III)-EDTA-citrate, prepared as recommended by Hudson and Morel (1989) at pH 8.0, were also tested. In one experiment, cells that had been exposed to 10^{-6} M Zn containing a ^{65}Zn spike (Perkin-Elmer Life Sciences; specific activity of 185 GBq/g) were washed with an excess (10^{-6}, 10^{-5}, 10^{-4} M) of non-radioactive Zn.

Extraction of surface bound metal—After isolation of the algal cells from their growth media and washing with 3 volumes (5 mL) of a metal-free experimental solution, algae (10^7 cells mL^{-1}) were resuspended in an experimental solution containing 10^{-6} M of the trace metal of interest. After a 30-min uptake phase, algal cells were filtered and rewashed (3 times) with 5 mL of the metal-free solution. Algae were subsequently resuspended in each of the wash media (4 to 5×10^6 cells mL^{-1}) for different extraction times (0.5, 1, 2, 5, 10, 20, 40, and 60 min.). Following the extraction, 20 mL aliquots of the algal suspension were filtered and once again rinsed (3 times) with 5 mL of the metal-free experimental solution. The filter and algae were transferred to a 10-mL plastic (low-density polyethylene) tube where they were digested with 250 µL of suprapure HNO_3 (70%, J.T. Baker) for 1 h at 90°C. Control samples of algae that were not exposed to metal were treated in the same manner. Cell densities, sizes, and surface areas were determined using a particle counter (50 µm orifice, Beckman Coulter Multisizer II).

For Pb, Ni, Cd, and Cu, internalized, extractable, and dissolved metal concentrations were determined by ICP-MS (Hewlett Packard 4500). Platinum sampling and skimmer cones were employed during Ni ICP measurements to reduce Ni contamination. Experiments with Zn were performed using a high activity ^{65}Zn spike so as to attain 1.2 MBq mL^{-1}. ^{65}Zn was measured with a γ-counter (Perkin-Elmer) and transformed in zinc concentrations using the measured isotopic ratio. Internalized (nonextractable) concentrations of trace metals were calculated on a surface area basis and normalized for the initial concentration of dissolved metal. Mass balances were performed by comparing initial added metal quantities to the sum of extractable and nonextractable metal. Data were rejected if the mass balance exceeded a 10% variation from the initial dissolved concentration. Each experimental condition was repeated in triplicate.

CO_2 uptake and membrane permeability measurements—Modifications to the algal CO_2 uptake or membrane permeability were determined by incubating the algae with a spike of $NaH^{14}CO_3$ (2 GBq $mmol^{-1}$, Anawa) or ^{14}C-D-sorbitol (10 GBq $mmol^{-1}$, Anawa). Sorbitol is a lipophilic molecule that is accumulated by passive diffusion, allowing for an evaluation of lipidic membrane integrity for the algae (Parent et al. 1996; Slaveykova et al. 2003). As in the extraction experiments, algae were collected in their mid-exponential growth phase, filtered, rinsed, and resuspended in a medium containing the washing agents and either [^{14}C]-D-sorbitol or $NaH^{14}CO_3$. Twenty milliliter samples were filtered after a contact time of 10 min. Algae and filters were rinsed using 10^{-2} M of nonlabeled D-sorbitol or $NaHCO_3$ (pH 6.0, 10^{-3} M MES). ^{14}C activities were quantified following digestion in a scintillation cocktail (Packard Biosciences Filter count) prior to analysis on a Beckmann LS 6500 scintillation counter. Algal ^{14}C concentrations were normalized to initial concentrations on a surface area basis.

Experimental precautions—Algae were manipulated under laminar flow conditions. Containers and solutions were autoclaved prior to use. To avoid contamination, all manipulations were performed in polymer (low-density polyethylene or polycarbonate) containers that were soaked in 0.1 M HNO_3, and then rinsed (3 times) in deionized water and a further 4 times in Milli-Q water (Millipore, R > 18 mΩ cm; total organic carbon < 2 µg C L^{-1}). The pH of the experimental solutions was verified immediately before and after experiments

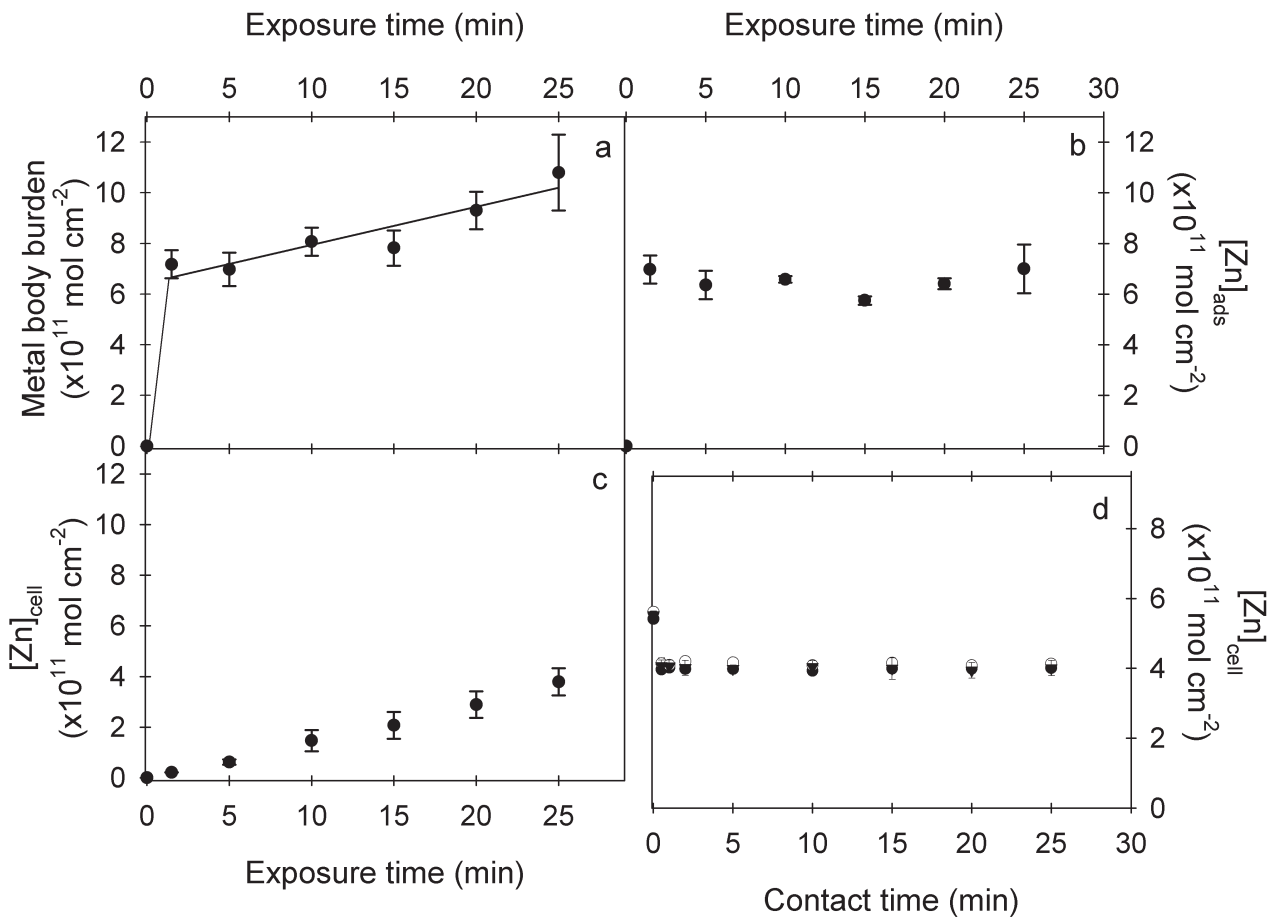

Fig. 1. (a) Total Zn body burdens (b) adsorbed (5×10^{-3} M EDTA extractable) Zn, (c) internalized (non-EDTA extractable) Zn as a function of exposure times. (d) Cellular ^{65}Zn following a wash (variable times) with non-radiolabelled Zn, (θ) 10^{-4} M, (▲) 10^{-5} M and (○) 10^{-6} M, after a 30-min exposure to ^{65}Zn. In (a) to (d), algae were incubated in 10^{-6} M Zn at pH 7.0. Error bars represent standard deviations (n = 3).

using a calibrated H^+ selective electrode. Nitrocellulose filters were also acid-washed and rinsed prior to their use. Metal losses due to adsorption on the membrane filters were assessed by superimposing two membranes during filtration of the algae. The metal content of the lower filter always represented less than 1% of the measured content of cellular metal. Verifications of the trace metal contamination of the suprapure HNO_3 and the various washing agents were performed for each experiment.

Significant differences among the data were identified using a one-way analysis of variance (ANOVA) with $\alpha = 0.05$. Control and treatment values were distinguished using a Student-Newmann-Keuls comparison ($P < 0.05$). Comparisons were performed using SigmaStat software (Version 1.03).

Assessment

The principal assumptions of the extraction techniques are that equilibrium is rapidly attained between the metal in solution and that adsorbed to biological surface sites and that internalization is a slow process with a constant positive internalization flux over short times (Bates et al. 1982; Xue et al. 1988; Klimmek et al. 2001; Slaveykova and Wilkinson 2002; Hassler and Wilkinson 2003). In such a case, it should be possible to distinguish metal adsorption to the cell surface (rapid) from metal internalization (slow) based upon the kinetics of the reactions. Indeed, total metal uptake curves (Fig. 1a) can be separated either theoretically (Galceran et al. 2004) or experimentally (Bates et al. 1982) into two separate components that include a fast (Fig. 1b) and slow accumulating fraction (Fig. 1c). In the presence of a strong ligand such as EDTA in the bulk solution, desorption from the surface of the organism should be rapid, with most of the extraction occurring in the first few minutes and a significant quantity of metal, i.e., the internalized fraction, being not accessible to the washing agent. The rapid exchange of surface bound radioactive Zn by nonradioactive Zn in the bulk medium was shown in Fig. 1d, with desorption occurring as fast as the first data point could be measured (≤30 s). In that case, no additional ^{65}Zn was removed following longer equilibration times or by increasing Zn concentrations in the wash solution.

Evaluation of the wash efficiencies can be made on the basis of thermodynamic or kinetic arguments. In the first case, if the wash agents are strong ligands, equilibrium will be shifted from predominantly surface-bound metal toward the metal-ligand complex in solution. For the extraction to be quantitative, the wash agent must be in excess and the product of the stability constant and the free ligand concentration for the complex in solution ($K_{ML}[L]$) must largely exceed the product of stability constant (K_{MS}) and the cellular ligand concentration ($[A_{cell}]$) for the surface complex. In such a case, no significant differences in extraction efficiency should be observed for different choices or concentrations of ligand. On the contrary, for $K_{ML}[L] < K_{MS}[A_{cell}]$, extraction efficiency (at equilibrium) will depend on both the ligand and ligand concentration employed. In that case, significant differences would be observed when comparing extractions using equimolar concentrations of the different ligands. For example, for Pb at pH 6.0, the conditional stability constants decrease in the order: CDTA > EDTA > NTA > 8HQS which, under these conditions, should reflect the effective extraction efficiencies.

On the other hand, in the case where desorption kinetics limit the extraction efficiency of the adsorbed metal, it is necessary to estimate the time required for the extraction to be quantitative. Based upon the assumption that water loss from the inner coordination sphere of the metal is the rate-limiting step for the complexation of the metal with the biological surface (Eigen mechanism; Morel and Hering 1993), it is possible to estimate a maximum forward rate constant, k_f, for the complexation reaction. In the case of Pb^{2+}, k_f can be estimated to be 3×10^{10} L mol^{-1} s^{-1} for a singly charged anionic ligand and 3×10^{11} L mol^{-1} s^{-1} for a doubly charged one (Wilkinson and Buffle 2004). Because for Pb at pH 6.0, the stability constant (reciprocal of the Michaelis-Menten constant) is $10^{5.5}$ M^{-1} (Slaveykova and Wilkinson 2002) and because the forward and reverse rate constants, k_r, are related through their equilibrium constant ($K_{MS} = k_f/k_r$), the maximum predicted value for the reverse rate constant can be calculated to be 10^6 s^{-1}. For a more slowly reacting metal such as Ni^{2+}, k_r values of approximately 10^{-1} s^{-1} can be predicted (based upon a $K_{MS} \approx 10^7$ M^{-1}, unpubl. data). According to these arguments, the rate of complex dissociation should decrease in the order: Pb > Cu ≈ Cd > Zn >> Ni. Lower limits for the dissociation rate constants are much more difficult to obtain. In the case of Pb, k_r values of 10^{-2} to 10^{-3} s^{-1} have been estimated (Slaveykova and Wilkinson 2002) based upon the reasonable assumption (Hudson and Morel 1993) that the dissociation rate constant is larger than the internalization rate constant k_{int} (4.3 to 6.6 $\times 10^{-4}$ s^{-1}). Based upon the above arguments, it is possible to estimate that the time required for a 50% dissociation of the Pb surface complex ($\ln 2/k_r$) will be no greater than 700 s, and much less (fraction of second) if the dissociation reaction is governed by the Eigen mechanism. These calculations also imply that the metal with potentially the slowest desorption kinetics, i.e., Ni, would have half dissociation times between 10 s and 10 min.

Based upon the above thermodynamic and kinetic considerations, the extraction efficiency of the wash agents should be evaluated by examining the effects of both thermodynamic (ligand choice and concentration) and kinetic (extraction time) factors. In this study, the metal extraction efficiency of 5×10^{-3} M solutions of EDTA, CDTA, NTA, 8HQS, and calcium nitrate; a Ti(III)-EDTA-citrate mixture and dilute HNO_3 (pH 3.0) were evaluated for a fixed contact time of 1 min. For EDTA, the role of ligand concentration (10^{-4} to 10^{-1} M) was also evaluated for a constant contact time (1 min) with the algae. Contact times of 0.5 to 60 min were evaluated for a 5×10^{-3} M EDTA solution. Finally, an assessment was made of the effects of the washing agents on both $^{14}CO_2$ uptake and membrane permeability for the unicellular green alga, *C. kesslerii*.

Effect of the nature of the washing agent—Extraction efficiencies of the ligands were compared to initial values of total internalized metal and to a 1-min control rinse in 10^{-2} M MES at pH 6.0 (Fig. 2). In the absence of added ligand, MES was not an efficient washing agent. No significant differences in extraction efficiencies (ANOVA, $P < 0.05$) were observed for any of the washing agents that formed strong complexes (EDTA, NTA, CDTA, 8HQS, Ti-EDTA-citrate) with the studied metals: Cd, Cu, Ni, Pb, and Zn. On the other hand, $Ca(NO_3)_2$ and the dilute acid, which have also been employed by past studies to indicate competitive binding of the metals (Crist et al. 1994; Chu et al. 1997; Xu et al. 1988), extracted less Pb and Cu than the complexing agents (Fig. 2a to 2e), whereas the presence of $Ca(NO_3)_2$ actually increased internalized Ni by a factor of more than 4.5. On the other hand, no significant extraction of Ni by EDTA was observed (ANOVA, $P > 0.05$). For the most part, the results appear to be consistent with the presence of a large excess of strong washing agent, as discussed above. Indeed, based upon the cell, metal, and ligand concentrations that were employed, 1 L of solution containing 5×10^{-3} moles of the wash agents would be in large excess over the total quantity of extractable metal (e.g., 4.3×10^{-8} moles of Zn; 8.6 $\times 10^{-8}$ moles of Pb).

Effect of the EDTA concentration—Given that the extraction efficiency was nearly independent of the nature of the strong ligands, the effect of the concentration of EDTA on the metal extraction was studied. Although the data suggested a slight increase in extraction efficiency, no significant effect was observed when the EDTA concentration was increased from 10^{-4} to 10^{-1} M for Pb, Cd, or Ni (Fig. 3a). In contrast, a significant increase in the Cu and Zn extraction efficiency was observed for EDTA concentrations exceeding 10^{-2} M EDTA (Fig. 3b). Note that the observed increases in extracted metal were small and not always consistent with purely thermodynamic arguments that would support a single, fairly steep decrease in the extraction curves with the steepest slope at the inflexion point corresponding to $K_{ML}[L] = K_{MS}[A_{cell}]$.

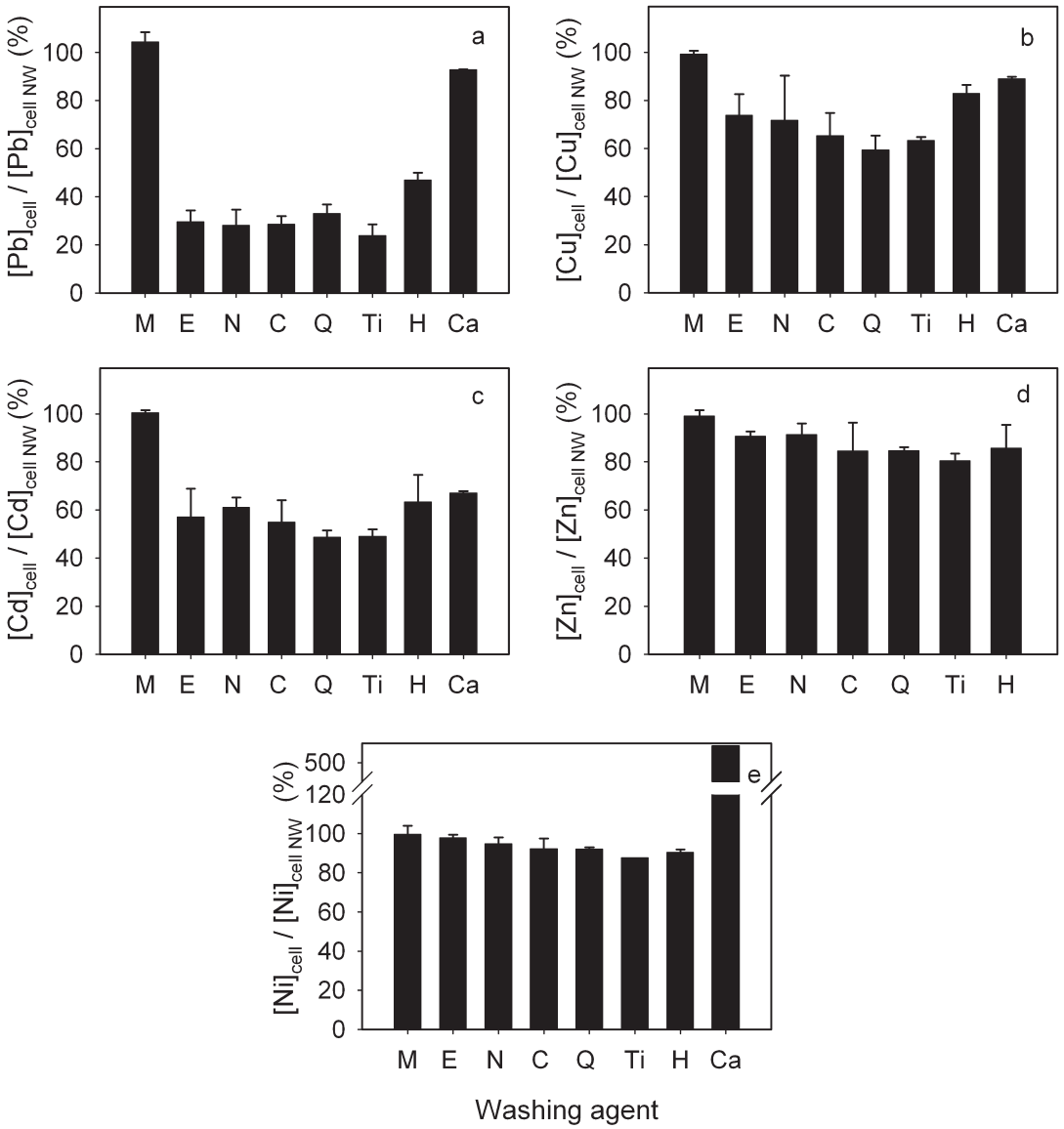

Fig. 2. Effect of several washing agents on the cellular content of (a) Pb, (b) Cu, (c) Cd, (d) Zn, and (e) Ni for a 1-min contact time. All the compounds (MES [M], EDTA [E], NTA [N], CDTA [C], 8HQS [Q], Ti-EDTA-citrate [Ti], and calcium [Ca]) were employed at a concentration of 5×10^{-3} M in 5×10^{-3} M MES at pH 6.0, except for the dilute HNO_3 solution at pH 3.0 (H). Data were normalized by dividing by the cellular metal concentration measured prior to initiation of the wash procedure. Error bars represent standard deviations (n = 3).

Effect of contact time—Extraction by 5×10^{-3} M EDTA revealed a fast initial decrease in the organism-associated metal, followed by the stabilization of metal content that is interpreted to correspond to internalized metal (i.e., non-EDTA accessible metal fraction; Fig. 4a and 4b). A similar observation of a fast initial decrease followed by the stabilization of the internalized metal was observed for each of the metals and has also been seen for other species of algae (e.g., Zn binding by *Chlamydomonas variabilis* [Bates et al. 1982]; Ni, Pb, Zn, Cd binding by *Chlorella vulgaris* or *Lyngbya taylorii* [Klimmek et al. 2001]). Plateau levels depended on the ratio between surface-bound and internalized metal and were different for each of the five metals. Due to the dissimilarity in the kinetics of metal internalization (slow, linear) and adsorption (rapid attainment of equilibrium), the ratio of adsorbed to internalized metal could be maximized by decreasing the length of the metal uptake phase. For Pb and Cd, a larger proportion of metal (80% to 90%) was extracted from the cell surface as compared to Zn and Cu (40%; Fig. 4a). Although these proportions are in line with the relatively higher internalization fluxes that would be expected for the essential metals Zn and Cu, a similar explanation does not hold for the low pro-

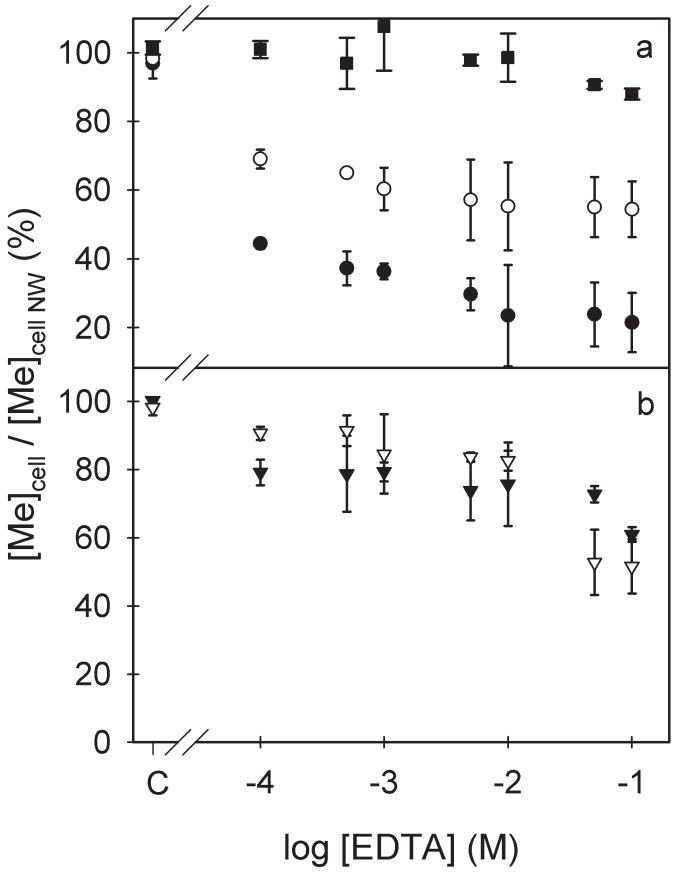

Fig. 3. Effect of the EDTA concentration on the normalized cellular contents following an exposure to (a) Pb, ●; Cd, ○; and Ni, ■; and (b) Cu, ▼; and Zn, (∇). All EDTA concentrations were prepared in 5×10^{-3} M MES at pH 6.0. Data were normalized by cellular metal concentrations obtained prior to the washing procedures. Control values (C) were obtained by washing the cells with 5×10^{-3} M MES. Error bars represent standard deviations (n = 3).

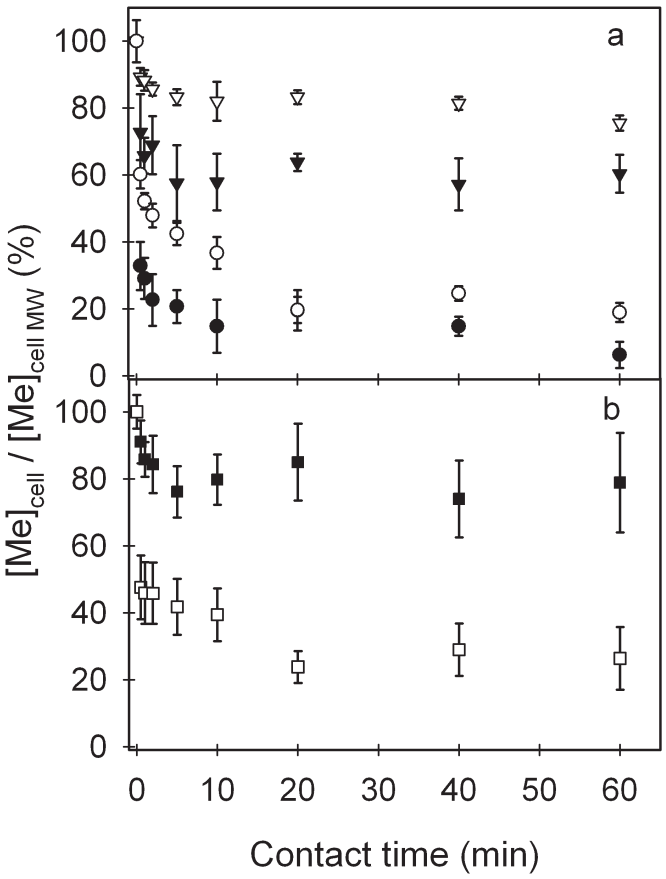

Fig. 4. Effect of 5×10^{-3} M EDTA (in 5×10^{-3} M MES, pH 6.0) on the normalized cellular contents of (a) Pb, ●; Cd, ○; Cu, ▼; Zn, ∇; and (b) Ni, ■ as a function of the contact time. A Ni extraction (□) was also performed following a 10-min accumulation of 5×10^{-5} M Ni^{2+}. Data were normalized by cellular metal concentrations obtained prior to the washing procedure. Error bars represent standard deviations (n = 3 to 4).

portion of extracted Ni (20%; Fig. 4b) because no known biological function has been documented for Ni in *C. kesslerii*. To ensure that the lower concentrations of extracted Ni were not due to slow extraction kinetics (as discussed above), algae were exposed to 5×10^{-5} M dissolved Ni for a shorter 10-min contact time (cf. previous 30-min exposure to 10^{-6} M Ni) to increase the adsorbed to internalized metal ratio. This modification in experimental design resulted in an increase to 60% EDTA extractable Ni (Fig. 4b, open squares). In this case, the attainment of a plateau value of EDTA extractable Ni strongly suggested that the kinetics of Ni desorption was not limiting. For the examined conditions, no further Cu, Pb, or Zn was extracted after 0.5 min (ANOVA, $P < 0.05$; Fig. 4a). On the other hand, 20 min were required for Cd and Ni to reach constant internalized concentrations (Fig. 4a, 4b). Because the observed variations in metal distribution and concentration depended on both the affinity of the metal for the surface and the precise experimental conditions, these results demonstrate that contact times must be adapted for the precise conditions of the experiment.

Distinguishing cellular efflux—Microorganisms are well known to be able to exclude metals including Cd, Co, Cu, and Zn (Nies 1999; Cavet et al. 2003). In such cases, it is often difficult to distinguish between metal desorption from the cell surface and cellular efflux based simply on the shape of the temporal extraction curve. As discussed above, desorption generally occurs for short contact times while metal efflux is thought to be initiated following the (slower) internalization of metal. Based upon such a two-step adsorption-internalization, in most cases, it is often possible to distinguish between desorption and efflux based upon the measured rates of decreasing internalized metal content. In addition, given the typical shape of the internalization and adsorption curves (Fig. 1a, 1b) and because cellular efflux is often inducible and described by a first-order dependence on internalized metal contents (Hassler and Wilkinson 2003), the ratio of desorbed to excluded

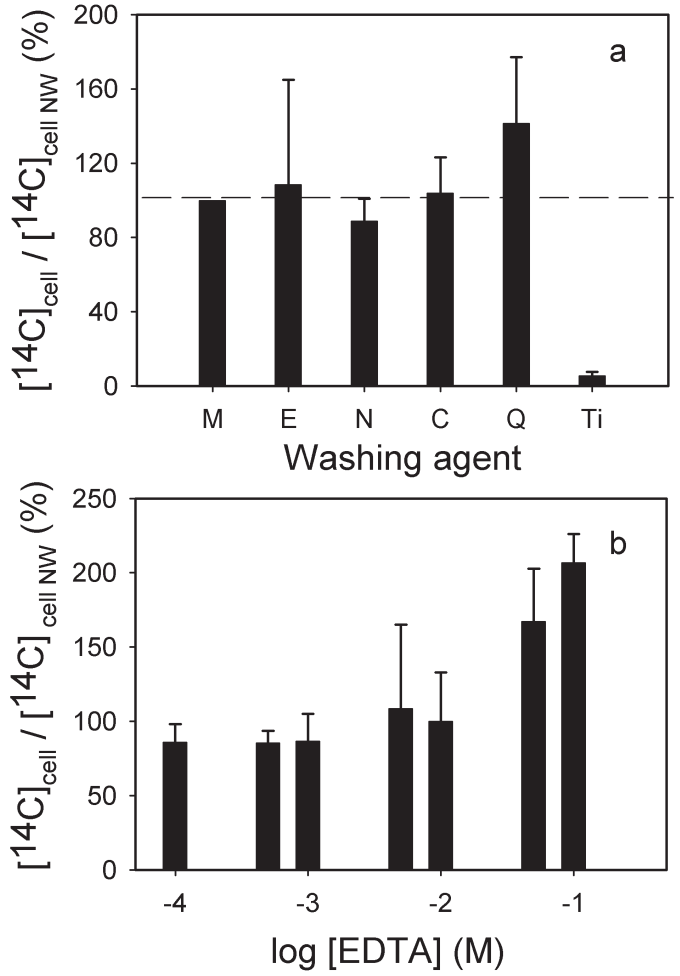

Fig. 5. $NaH^{14}CO_3$ cellular content after a 10-min contact time with (a) several washing agents (MES [M], NTA [N], CDTA [C], 8-hydroxyquinoline-5-sulfonic acid [Q], and Ti-EDTA-citrate [Ti]) at concentrations of 5×10^{-3} M or (b) several EDTA concentrations. All washes were performed in 5×10^{-3} M MES at pH 6.0. Dotted line indicates control values in absence of the washing agents. Error bars represent standard deviations (n = 4).

metal should be maximized for short metal exposure times. Typically, for microorganisms, efflux is induced following relatively long exposures (>1 h) to relatively high concentrations of metal (Wolterbeek et al. 1995; Mirimanoff and Wilkinson 2000; Smiejan et al. 2003). For example, for green alga, an efflux has been reported for Cu (*Chlorella vulgaris* [Foster 1977]) and Zn (*Selenastrum capricornutum* [Wolterbeek et al. 1995]; *Chlorella kesslerii* [Hassler and Wilkinson 2003]) with a first-order efflux rate constant of 5.0×10^{-6} to 4.3×10^{-5} s^{-1}. For a 30-min accumulation of 10^{-6} M Zn^{2+} by *C. kesslerii* (i.e., efflux rate constant of 4.3×10^{-5} s^{-1}, [Hassler and Wilkinson 2003]), this corresponds to an efflux of 1.5×10^{-15} mol cm^{-2} s^{-1}, equivalent to an 8% or 15% decrease of internalized Zn following a 30-or 60-min contact time with EDTA, respectively. These calculations suggest that EDTA contact times of less than 5 min will result in negligible decreases of internalized Zn due to efflux.

Effect of the washing agents on CO_2 uptake—Measurements of $^{14}CO_2$ allow for an assessment of the global metabolic effects that are susceptible to affect short-term uptake or efflux due to an induced biological stress on the organism provoked by the use of the washing agents. For short-term (i.e., 10 min) incubations with the washing agent and $NaH^{14}CO_3$, large standard deviations of ^{14}C uptake were observed. Nonetheless, no significant decreases were observed in ^{14}C incorporation for 5×10^{-3} M of any of the washing agents (Fig. 5a) except for the Ti-EDTA-citrate treatment. This decrease was most likely due to a pH effect: the Ti-EDTA-citrate extraction is performed at pH 8 rather than at pH 6. At pH 8, a shift in the equilibrium partitioning of the carbonates results in significantly less dissolved CO_2 available for uptake. On the other hand, increasing the EDTA concentration beyond 10^{-2} M decreased ^{14}C uptake with respect to the control (Fig. 5b). Although there is no evidence demonstrating that EDTA is metabolized or internalized by algae, Britta et al. (2002) have observed increased algal growth in the presence of 5×10^{-5} M EDTA (5 to 63 d exposure) while Chu et al. (1997) reported a 15% to 30% loss of algal biomass following either an EDTA (3×10^{-3} M) or HCl (pH 2) wash. A 2-min treatment of Gram-negative bacteria with 1 to 2×10^{-4} M EDTA was shown to increase by 1.5- to 200-fold the toxicity of substances affecting membrane permeability (*Salmonella typhi* [Muschel and Gustafson 1968]; *Escherichia coli* [Reid and Speyer 1970]). In those cases, EDTA was postulated to chelate cations of the lipopolysaccharide layer of the outer membrane (Vaara 1992) leading to an increase in the permeability of the outer membrane.

Effect of the washing agents on membrane permeability—In spite of the large error bars due to the short incubation period (10 min) of ^{14}C-sorbitol, EDTA, and Ti-EDTA-citrate washes resulted in significant decreases in membrane permeability while NTA resulted in an increased incorporation of sorbitol (Fig. 6a). At all examined concentrations, lipophilic membrane permeability decreased in the presence of EDTA with respect to the control, especially for concentrations exceeding 5×10^{-3} M (Fig. 6b). The smallest effects were observed for the 8-hydroxyquinoline-5-sulfonic acid and CDTA at both 5×10^{-3} M (Fig. 6) and 10^{-2} M (data not shown).

No significant differences in extraction efficiencies were observed following the addition of identical concentrations of strong ligands (Fig. 2) even though, for a given metal, the conditional stability constants for the complexes varied by 11 orders of magnitude. This result implies that metal adsorption at the cell surface is either of relatively low affinity or that small numbers of sites (at this cellular concentration of ca. 5×10^6 mL^{-1}) are involved in the process (or that both explanations operate simultaneously). In the presence of metal chelates, metal desorption rates may also depend greatly on the associated cation and may, in some cases, be extremely slow. Nonetheless, the extraction of surface bound metal was not significantly increased (i.e., decreased cellular metal) after

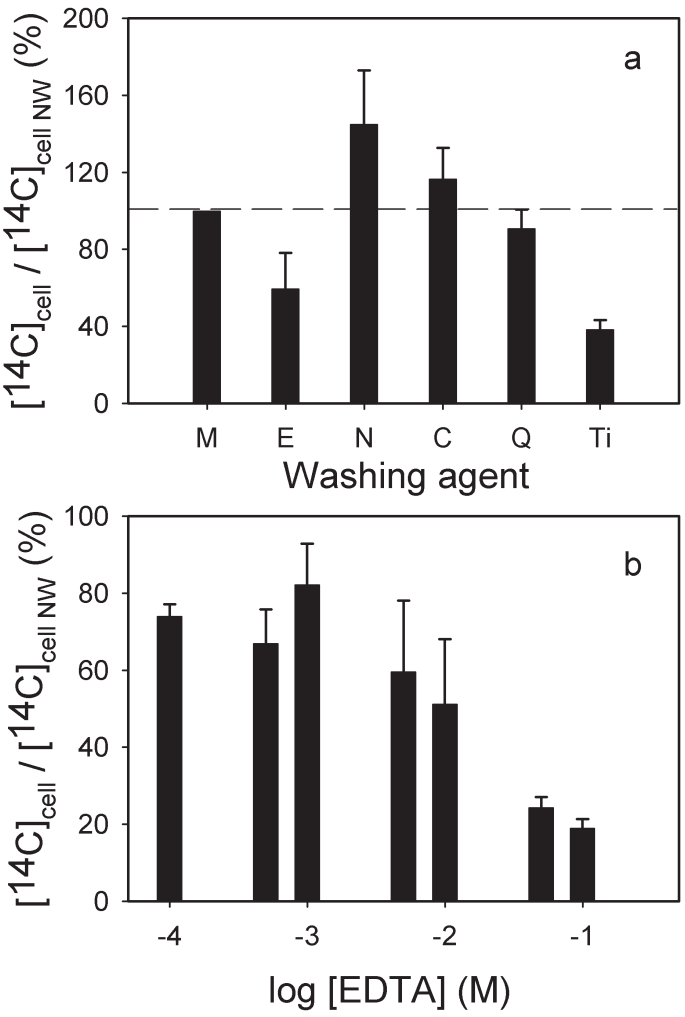

Fig. 6. Cellular ^{14}C-D-sorbitol content after a 10-min contact time with (a) several washing agents (MES [M], NTA [N], CDTA [C], 8-hydroxyquinoline-5-sulfonic acid [Q], and Ti-EDTA-citrate[Ti]) at a concentrations of 5×10^{-3} M or (b) several EDTA concentrations. All washes were performed in 5×10^{-3} M MES. Dotted line indicates control values in absence of the washing agents. Error bars represent standard deviations (n = 4).

20 min for any of the five metals studied (Fig. 4). In most cases, a constant value of nonextractable metal was obtained following even shorter extraction times (i.e., < 0.5 min), in line with rapid desorption kinetics. In similar work, Crist et al. (1994) showed that the desorption of Mg, Cd, Cu, and Pb from heat killed *Vaucheria* cells was very rapid with constant values of extracted metal being obtained following 10 to 15 s of contact with 1×10^{-3} M Li-EDTA (pH 9). Furthermore, Chu et al. (1997) have demonstrated a constant adsorption of Cd to algal cells (*Sargassum baccularia*) following 5 successive adsorption/desorption cycles using 3×10^{-3} M EDTA, suggesting both that desorption was complete and that the algal surface was not significantly modified by the EDTA treatment.

Discussion

Measurements of transporter bound metal, generally a small fraction of total adsorbed metal, cannot be determined directly using this method. This is unfortunate because discrimination of the metal bound to sensitive sites such as transporters from the more generalized binding of metal to cell wall components such as polysaccharides and phospholipids would provide a direct verification of one of the key hypotheses underlying the biotic ligand model (Di Toro et al. 2001). On the other hand, an indirect determination of metal bound to transport sites has been made recently using the EDTA extraction (Slaveykova and Wilkinson 2002; Hassler and Wilkinson 2003) by plotting the consistently observed (but small) intercept in the temporal plots of internalized metal (e.g., Fig. 1c) as a function of bulk solution $[M^{z+}]$. In those cases and for all data obtained here, the intercepts were significantly larger than control values of non-EDTA extractable metal for algae that were not exposed to metal (i.e., control solutions containing algal cells, ultrapure acid, and filters). Furthermore, in the case of Pb, stability constants obtained from a Langmuir treatment of the intercepts as a function of $[Pb^{2+}]$ were identical to those obtained independently from the reciprocal of the Michaelis-Menten constant determined from Pb uptake fluxes (Slaveykova and Wilkinson 2002). In the case of Zn, preincubation of the algae under starvation conditions had no effect on total adsorbed Zn but did increase both biological uptake fluxes and the y-intercept on the temporal plots of internalized Zn by 100 fold (Hassler and Wilkinson 2003). These papers and others (Alsop et al. 1999; Alsop and Wood 2000; Slaveykova et al. 2003) have interpreted a seemingly irreversible metal adsorption to transport sites, possibly due to conformational changes of the transport ligand (protein) upon binding of the metal. If so, the EDTA extraction may also provide uniquely obtained information that is extremely difficult to obtain in other manners, i.e., the concentration of the "biotic ligand." Such an important use of the technique should be verified in future research using other organisms and ligands.

The applicability of the extraction technique would be extremely limited if it were only valid for *C. kesslerii*. In fact, kinetic desorption curves are available for several organisms, albeit for slightly different conditions (Fig. 7). Although it is apparent that the results support the use of the EDTA wash for several organisms in addition to the algae (e.g., Gram-negative bacteria: *Rhodospirulum rubrum* [Smiejan et al. 2003] and Gram-positive bacteria: *Rhodococcus opacus* [Mirimanoff and Wilkinson 2000]; fish: *Salmo salar*; Fig. 7a), other results demonstrated clearly that the technique would be difficult, if not impossible to apply without important modifications (e.g., *Chlamydomonas reinhardtii* and *Rhodospirulum rubrum* at 10^{-5} M; Fig. 7b). For example, for *C. reinhardtii*, no plateau in extracted Cd was observed during the EDTA wash. In that case, a continuous synthesis of cell wall components (Monk 1988)

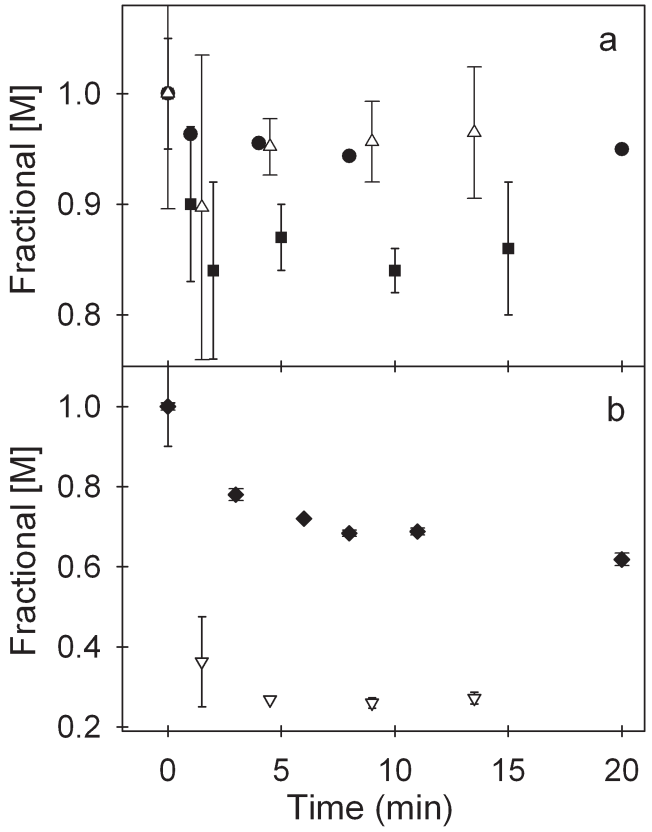

Fig. 7. Desorption kinetics from several organisms for various metals: (a) ■, Al extraction by 10^{-2} M EDTA from fish (*Salmo salar*); ●, Zn extraction by 5×10^{-3} M EDTA from Gram-positive bacteria (*Rhodococcus opacus* [Mirimanoff and Wilkinson 2000]); △, Cd extraction by 2×10^{-2} M EDTA from Gram-negative bacteria (*Rhodospirillum rubrum* [Smiejan et al. 2003]) in absence of cellular efflux. (b) ▽, Cd extraction by 2×10^{-2} M EDTA from Gram-negative bacteria (*Rhodospirillum rubrum* [Smiejan et al. 2003]) in presence of cellular efflux, ◆, Cd extraction by 10^{-2} M EDTA from green alga (*Chlamydomonas reinhardtii*).

resulted in a cell wall renewal and production of new Cd adsorption sites. For *R. rubrum*, as for many prokaryotes, efflux is initiated much more rapidly than is generally observed for eukaryotes. Although cellular efflux was not observed following exposure to 10^{-7} M Cd (Fig. 7a), efflux rather than desorption was responsible for the important decrease in internalized cadmium for cells exposed to 10^{-5} M Cd (Smiejan et al. 2003). This result demonstrates that, in some cases, the apparent plateau value in the EDTA desorption curve is not sufficient evidence of the efficiency of the EDTA extraction.

Comments and recommendations

The use of washing agents has substantial potential to allow for a kinetic and possibly functional distinction among cellular pools. Despite its widespread use, there is no common protocol nor systematic verification of the extraction protocols prior to their use when distinguishing organism-bound metal fractions. Furthermore, potential deleterious effects of the ligands are rarely verified. Due to the interspecies variability of the biological interface, it is impossible to provide a single extraction protocol that would be acceptable for all biological species. Nonetheless, it is possible to recommend a set of guidelines that should be followed to fully exploit extraction data of a given bioaccumulation experiment:

(1) The selected ligands should form metal complexes with high stability constants and must be in excess compared to the molar concentration of metal on the biological surface. If necessary, cell concentrations can be reduced while maintaining a large effective stability constant for the extracting ligand ($K_{ML}[L]$) over the surface complex ($K_{MS}[A_{cell}]$). Successive extractions can also be performed to increase the ligand to metal ratio with each rinse. Note that the effective concentration of [L] (and thus extraction efficiency) will be reduced in solutions containing Ca or other potentially interfering cations.

(2) Concentrations of the washing ligands need to be optimized (minimized) to limit biological effects, especially effects that are likely to modify bioaccumulation on the time scale of the uptake experiment. For a given experiment, a lower limit should be identified for which the wash ligands remain under conditions of ligand excess as defined by cellular and metal concentrations. While the photosynthetic activities and membrane permeabilities were followed in this paper, other relevant measurements such as enzymatic induction or respiration rates could be useful sublethal parameters of interest.

(3) The presence of a metal efflux should be verified. While metal efflux is often difficult to discriminate from metal desorption, it can generally be verified by increasing exposure times to increase the ratio of adsorbed: internalized metal. Isotopes can be used to distinguish efflux from metal desorption by varying exposure conditions in the presence of different metal loadings. Desorption of radioactive or stable isotopes using an excess of nonlabeled metal for short exposure times is also a useful means to distinguish between equilibration at the cell surface and efflux.

These experiments have demonstrated that the extraction techniques require significant optimization for the organism and for the specific experimental conditions that are being examined. Nonetheless, it is clear that, with proper care, it is possible to gain a much greater insight into organism bioaccumulation than can be gained from measurements of total metal body burdens.

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