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A Novel In Situ Tool for the Exposure and Analysis of Microorganisms in Natural Aquatic Systems

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To evaluate the effects of contaminants or nutrient limitation in natural waters, it is often desirable to perform controlled exposures of organisms. While in situ exposures are routine for caged organisms or macrophytes, they are extremely difficult to perform for microorganisms, mainly due to difficulties in designing an exposure device that isolates the cells while allowing rapid equilibration with the external media. In this paper, a stirred underwater biouptake system (SUBS) based on the diffusion of chemicals across a semipermeable membrane housing a controlled population of microorganisms is reported. Cd diffusion through the semipermeable membrane was evaluated by voltammetry using a microelectrode. Comparison of stirred and unstirred solutions demonstrated a significantly increased diffusive flux in the presence of stirring. Lab tests using *Chlamydomonas reinhardtii* showed that diffusion across the semipermeable membrane was not limiting with respect to the biouptake of Cd. The SUBS device was field tested and the results of viability studies and trace metal biouptake by *C. reinhardtii* are reported. No diffusion limitation due to the SUBS was observed for Cd under the tested field conditions. The SUBS device was also shown to be useful for field exposures and subsequent measurements of trace metal uptake and viability. The results support the future use of the SUBS for the in situ measurement of phytochelatin/metallothionein production, photosynthetic efficiency, or reporter gene induction of controlled organisms.

Introduction

Novel contaminants are being produced and discharged into the environment on a daily basis. Due to the varying role of the natural environment on contaminant effects, site-specific approaches are becoming more popular for evaluating contaminant risk. Furthermore, microorganisms are routinely used to evaluate the toxicological status of natural waters (*1*−*4*). Indeed, indigenous organisms (*5*) or those taken from culture collections (*2*) have been used to relate the "natural" state of the water to specific toxicological or biological end points. Nonetheless, it is often extremely difficult to obtain representative field samples. Natural waters are always dynamic, and therefore, sampling invariably modifies lighting, temperature, gas and particle concentrations, etc. Such modifications in the physicochemical properties of the water can result in changes to the speciation and bioavailability of both pollutants and nutrients (*6*−*8*). Ideally, sample transport/preservation should be eliminated by conducting *controlled* exposure experiments at the site of interest.

To perform such representative, in situ exposures of microorganisms, it is necessary not to perturb the balances between the contaminant uptake fluxes by the organism (biouptake flux, J_{int}) and the contaminant resupply determined mainly by the diffusive flux, J_{diff}. This implies that it is necessary to isolate the organisms without allowing contaminant (or nutrient) consumption in the exposure solution which can lead to bulk depletion. Indeed, if diffusion into the experimental chamber becomes limiting during exposure then contaminant concentrations will be reduced, leading to an underestimation of potential biological effects (*9*). In this study, the overall diffusive flux, J_{diff}, consisted of at least 3 components: contaminant diffusion in the bulk solution, transport across the semipermeable barrier housing the microorganisms, and diffusion within the exposure chamber (i.e., in the diffusion layer surrounding the microorganisms). Indeed, for some (rapidly accumulating) elements such as phosphate, nitrate, and silver, diffusion in the microlayer has been shown to be rate-limiting with respect to biological uptake (reviewed in *8* and *10*). On the other hand, for most trace metals, internalization fluxes are orders of magnitude lower than diffusive fluxes (*8*) implying that a limited reduction in the diffusive flux is possible without causing a shift in the rate-limiting flux. In this paper, we focused on a target element, Cd(II) that had been previously shown to not be limited by diffusion to the target microorganism, *Chlamydomonas reinhardtii* (*10*−*13*), in bulk solution determinations (*13*). Experiments were designed to test whether the organisms could be held in exposure chambers without causing the overall diffusive flux to become limiting, which would result in metal depletion and an underestimation of bioavailability. This verification is important since a diffusive limitation will not only lead to decreased metal concentrations but also to an increased contribution of the labile trace-metal complexes as their dissociation contributes to the resupply of metals at the surface of the microorganism (*14*). The goal was to design a sampling chamber where mass transport did not limit biological uptake by the suspended microorganisms (i.e., concentration outside the chamber should remain equal to that inside the chamber). Since the system developed in this work has been designed for the simultaneous evaluation of bioaccumulation and stress biomarkers, larger exposure volumes (and cell numbers) were required for exposure post analysis in comparison to previous designs of in situ devices (*11*). As larger volumes and cell numbers increase the risk of a bulk depletion, the system (*S*tirred *U*nderwater *B*iouptake *S*ystem: SUBS) was stirred to (i) provide a more reproducible internal hydrodynamic environment, (ii) suspend the microorganisms and thus avoid fouling or aggregation, and (iii) increase the diffusive flux of contaminants and nutrients into the chamber, thereby reducing the potential for diffusion

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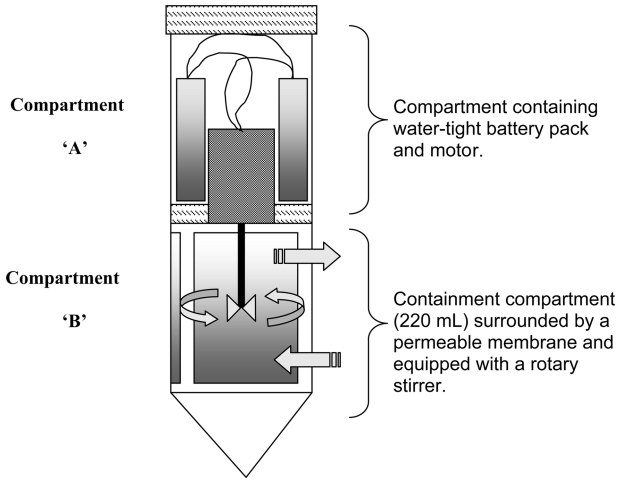

FIGURE 1. Schematic design of the compartmentalized SUBS composed of a control compartment 'A', (motor and battery pack) and a diffusion chamber or compartment 'B' (220 mL volume). A semipermeable Isopore membrane (e.g., 0.4 μm) covers the diffusion windows allowing both contaminants and nutrients to cross into compartment 'B'. In practice, three submersible units (SUBSs) are attached to a deployment rack which is submerged in the natural water to a constant depth. SUBS groupings are used to ensure replication.

limitation to the microorganism during exposure. Diffusion across the semipermeable membrane, bioaccumulation, and cell viability were verified to develop a prototype exposure device that was as minimally perturbing as possible.

Materials and Methods

The SUBS device (Figure 1) is composed of two cylindrical compartments (modified centrifugation tubes) that screw into each other so that the diffusion chamber and its contents are isolated from the external media by an Isopore semipermeable membrane (0.4 μm). In the field experiments, the bottom compartment (B in Figure 1) contained the microorganisms. In a number of laboratory experiments, an agarose-covered, Ir-microelectrode was introduced into the B compartment to allow the real-time measurement of Cd^{2+}. Photos and schema of both the laboratory version of the device and the field deployment are presented in the Supporting Information.

Laboratory Experiments. *Experimental Solutions.* All solutions were prepared using distilled, deionized (Milli-Q, $R > 18$ MΩ cm, TOC < 2 μg L^{-1}) water. Stock solutions used in the experimental reservoir were prepared from their salts: $Cd(NO_3)_2 \cdot 4H_2O$, Aldrich); KNO_3 (Aldrich); $NaNO_3$ (J.T. Baker); 2-[*N*-morpholino]ethanesulfonic acid (MES, Sigma); $NH_4(NO_3)$ (Sigma), $MgSO_4$ (Sigma), and $Ca(NO_3)_2$ (Sigma).

Voltammetric Experiments. An Ecochemie μAutolab type II potentiostat was used with the commercial software (general purpose electrochemical system, v 4.9, Ecochemie). The three electrode configuration consisted of a reference electrode (Metrohm Ag/AgCl/3 M KCl/0.1 M $NaNO_3$), a counter electrode (Metrohm platinum rod), and a working electrode (agarose-membrane-covered, mercury-plated Iridium microelectrode) that was prepared as described in ref *15*. The mercury layer was plated through the agarose onto the Ir substrate at −400 mV (vs Ag/AgCl/3 M KCl/0.1 M $NaNO_3$) in a deoxygenated 5 mM $Hg(CH_3COO)_2$ and 10^{-2} M $HClO_4$ solution. The agarose served to stabilize the Hg electrode as well as protect the Hg from algal deposition and biofouling. Reoxidation of the mercury was accomplished by a linear scan of the potential from −300 to +300 mV at 5 mV/s in a degassed 1 M KSCN solution. Voltammetric measurements were performed in stripping mode using square wave anodic stripping voltammetry (SWASV): deposition potential = −1000 mV; deposition time = 8 min; final potential = −200 mV; pulse amplitude = 25 mV; step amplitude = 4 mV; frequency = 100 Hz; precleaning potential = −100 mV; precleaning time = 12 min. A precleaning step was performed before each voltammetric measurement to ensure uniform spreading of the mercury layer on the iridium surface and complete reoxidation of the Cd (*15*). The precleaning step also corresponded to the delay time between individual measurements. A Leitz Diavert optical inverted microscope was used to monitor the quality of the microelectrode.

In the laboratory experiments, a modified SUBS was placed at the surface of a 70-L reservoir filled with deionized water that was buffered to pH 6.0 with 10^{-2} M MES (Figures S1, S2). The electrodes were mounted in the center of the chamber surrounding the axel and propeller. The first voltammetric measurement was made in the absence of Cd^{2+} while subsequent measurements recorded the Cd signal due to diffusion into the chamber.

Organism Maintenance (Laboratory). C. reinhardtii (wild type C137) was cultured to its late log phase (~2×10^6 cells/mL) in a (4×) diluted Tris-acetate-phosphate (TAP) medium (*16*), under a 12:12 h light:dark regime of 50 μmol photons m^{-2} s^{-1} of fluorescent white light and rotary shaking (100 rpm) at 20 °C. Cultures were isolated from the growth media by centrifugation at 3300*g* (7 min) prior to resuspension of the cells in a diluted (4×) TAP media that did not contain trace metals. This operation was repeated twice to rinse the cells. Following the third centrifugation and resuspension of the pellet, the algal stock solution was diluted into the diffusion chamber (B), which was placed in the 70 L reservoir containing the diluted TAP growth media without trace metals. A final cell surface area of 1 cm^2/mL, corresponding to ca. 6.4×10^5 cells/mL was attained. Cell densities and sizes were evaluated using a Beckman Coulter Multisizer II (50 μm aperture) and cellular volumes were transformed into surface areas by considering the organisms as spheres.

Field Experiments. Experiments were performed in the Riou-Mort River which is located in the upper part of the Lot river watershed, France. The watershed includes a now abandoned Zn ore treatment plant at Viviez (the main source of Cd, Ni, and Zn pollution) and the "La Découverte" mine at Decazeville, France. The testing site, adjacent to the Joanis bridge (44°33′57″ N; 2°12′42″ E) had a depth of approximately 0.5 m and was adjacent to an embankment which allowed easy access and manipulation of the SUBS equipment (Figure S5). The physicochemistry of the river was described by DOC = 4.39 mg C L^{-1}, alkalinity = 124.4 mg $CaCO_3$ L^{-1} (titration), HCO_3 = 2.49 mM, and I = 6.2 mM. Cell separations, filtrations, and other manipulations were performed adjacent to the testing site in a modified van.

Organism Maintenance (Field). C. reinhardtii was cultured to midlog phase (~2×10^6 cells mL^{-1}) in a (4×) diluted TAP medium under a 24 h light regime (50 μmol photons m^{-2} s^{-1} of fluorescent white light) in a portable incubator (110 rpm) at laboratory temperatures (20 °C). Cultures were harvested by centrifugation using a mobile, battery-powered, centrifuge on site (1100*g*, ca. 20 min) then resuspended for 2 min in filtered (0.45 μm) lake water (2×). The resuspended culture was diluted into the SUBS. A calibrated spectrophotometer operating at 665 nm was employed to ensure that cell densities corresponded as closely as possible to surface areas of 1.0 ± 0.1 cm^2/mL.

Cellular Viability. Cellular viability was measured with the Promega CellTiter 96 AQ_{ueous} non-radioactive cell proliferation assay (Cat. G5421). This assay is based on the measurement of dehydrogenase activity in metabolically active cells (*17*). It uses a soluble tetrazolium compound (MTS) and the electron coupling reagent phenazine methosulfate (PMS). MTS is reduced into formazan, an aqueous

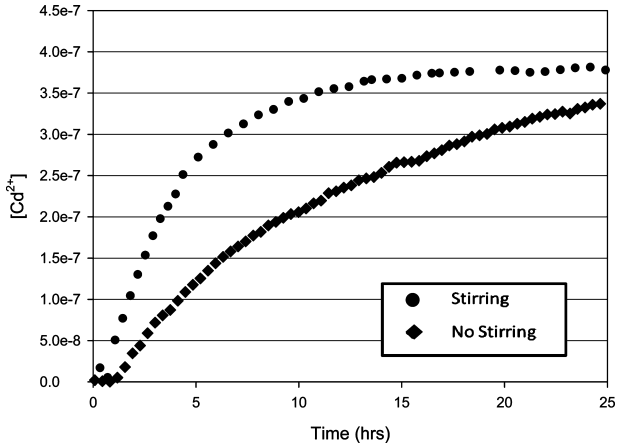

FIGURE 2. Cd^{2+} measurements by an agarose-covered Ir microelectrode in the SUBS in the absence of *C. reinhardtii*. Measurements attest to the diffusion of Cd^{2+} into the chamber with (circles) or without (diamond) stirring.

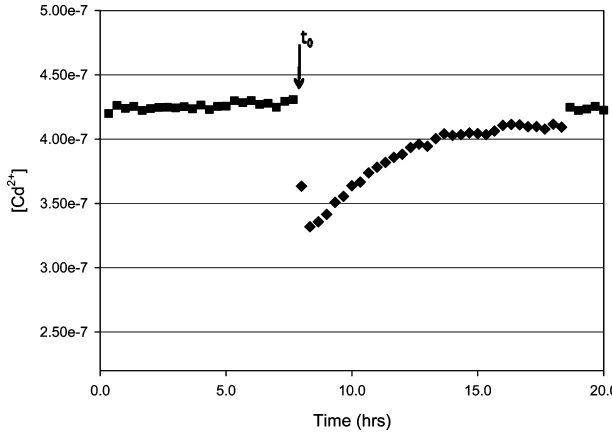

FIGURE 3. Cd^{2+} measurements by an agarose-covered Ir microelectrode in the SUBS containing *C. reinhardtii* with stirring. Squares represent Cd concentration in the interior of SUBS prior to addition of cells (diamond) and following removal of SUBS. Based upon these results, Cd^{2+} diffusion into the chamber is not limiting with respect to biouptake.

soluble compound whose absorbance can be measured at 490 nm. Formazan production is proportional to the number of living cells (*17*). For viability analysis, duplicate algal suspensions (500 μL) were mixed with 100 μL of a MTS/PMS stock solution. After 4 h, the sample was centrifuged at 12280*g* (5 min) and absorbance was measured at 490 nm on a field spectrophotometer. Controls of filtered river water and MTS/PMS were measured to correct for background.

Bioaccumulation. Bioaccumulation experiments consisted of duplicate in situ exposures of *C. reinhardtii* in the Riou-Mort River (France) for 2, 4, or 6 h. Following exposures, the content of the SUBS was recovered and filtered through acid-washed, 0.45 μm Isopore (polycarbonate) filters (Millipore). The cells remaining on the filter were washed twice with 10 mL of 10^{-3} M EDTA to remove metals adsorbed to the biological cell wall (*18*) and then transferred into an acid-washed polycarbonate container (10 mL). In the laboratory, cells were digested in 400 μL of ultrapure HNO_3 at 80 °C overnight. The samples were then diluted to 10 mL with Milli-Q water and analyzed for trace metals by ICP-OES (Vista AX, axial; Varian with CCD simultaneous detector), which had a method detection limit of 0.11 μg Cd L^{-1}. Total and 0.45 μm filtered metal concentrations were also determined in both natural waters. Internalized metal determinations were corrected for the digested, acid-washed filters. Field logistics are described in greater detail in the Supporting Information.

Results and Discussion

Cd Diffusion into the SUBS. To ensure that diffusion into the device was not rate-limiting, Cd^{2+} diffusion into the SUBS was measured over 25 h, with and without microorganisms. In the absence of microorganisms, Cd^{2+} concentrations in the diffusion chamber increased over time, with and without stirring (Figure 2). A much more rapid increase of Cd^{2+} was observed for the stirred system with a nearly complete equilibration observed between 10 and 15 h, whereas in the absence of stirring, 89% equilibration occurred at 25 h.

A second key experiment followed Cd^{2+} concentrations in the diffusion compartment in the presence of *C. reinhardtii*. Prior to the addition of the microorganisms, Cd^{2+} concentrations were constant at 4.2×10^{-7} M (Figure 3). Mass balance calculations indicated that the immediate decrease of Cd^{2+} concentrations upon addition of *C. reinhardtii* was due mainly to dilution (the *C. reinhardtii* stock contained no Cd) and only partly (max. 2−4%) to a fast Cd(II) sorption to the algal cell walls. Most importantly, the increase of Cd^{2+} concentrations from 8.3 to 17.7 h clearly showed that Cd^{2+} replenishment due to diffusion across the semipermeable membrane exceeded consumption due to algal internalization fluxes (J_{int}). After 17.7 h, the diffusion chamber was dismantled and Cd^{2+} concentrations were determined in the bulk solution to confirm that the electrode had maintained its original sensitivity.

The observation of increasing Cd^{2+} concentrations in the SUBS is supported by calculations of Cd consumption by the algae and Cd resupply by diffusion across the semipermeable membrane. Indeed, for the experimental conditions used here ($10^{-6.4}$ M Cd^{2+}, 220 cm^2 algae, J_{int} of 10^{-12} mol cm^{-2} min^{-1} (*13*)), Cd consumption by the algae (ca. 2×10^{-10} mol min^{-1}) could be estimated to be lower than the maximum diffusive flux, (ca. 1.0×10^{-9} mol min^{-1}) calculated using Fick's law principles ($D_{Cd} = 6 \times 10^{-10}$ m^2 s^{-1} (*19*); $\delta = 110$ μm (*11*); SUBS surface/volume ratio of 0.35 cm^{-1}). Since Cd internalization fluxes scale with the concentration of free Cd^{2+} in solution ($J_{int} = f\,[Cd^{2+}]$ (*13*)) while maximum diffusive fluxes scale with the concentration difference of mobile Cd species in relation to their diffusion coefficients (i.e., $\bar{D}_{Cd} \times$ [total Cd]), it is highly likely that Cd replenishment would also occur under field conditions, i.e., for lower Cd^{2+} concentrations. Although diffusive fluxes will decrease as the SUBS equilibrates with the external medium (and the concentration difference disappears), they are predicted to slow until they match the organism's internalization flux. Although there are several other parameters which may plausibly alter the internalization flux, J_{int}, under natural water conditions, e.g., [Ca], T; for the most part, they are expected to result in a disproportionate reduction in J_{int} as opposed to J_{diff}. Note that in the experiments shown here, cells were exposed directly to filtered natural waters which eliminated the requirement for an extended equilibration period. Nonetheless, due to the large number of factors that can influence both J_{int} and J_{diff}, on-site validation is likely required prior to any new deployment of the device.

Metal Bioaccumulation (Field). Bioaccumulation and viability were evaluated for duplicate SUBSs that were deployed in the Riou-Mort River (pH 7.8, 15 °C) over 2, 4, and 6 h using cells resuspended that were in the filtered river water to eliminate a pre-equilibration period (see Supporting Information, Page S7). Internalized Cd ranged from 4.0×10^{-12} to 7.7×10^{-12} mol cm^{-2} and increased with time (estimated internalization flux of 1.0×10^{-14} mol cm^{-2} min^{-1}) (Figure 4a). Dissolved Cd was measured in the filtrates of the algal suspensions for each time point. In this case, results were consistent with a rate-limiting internalization flux, i.e.

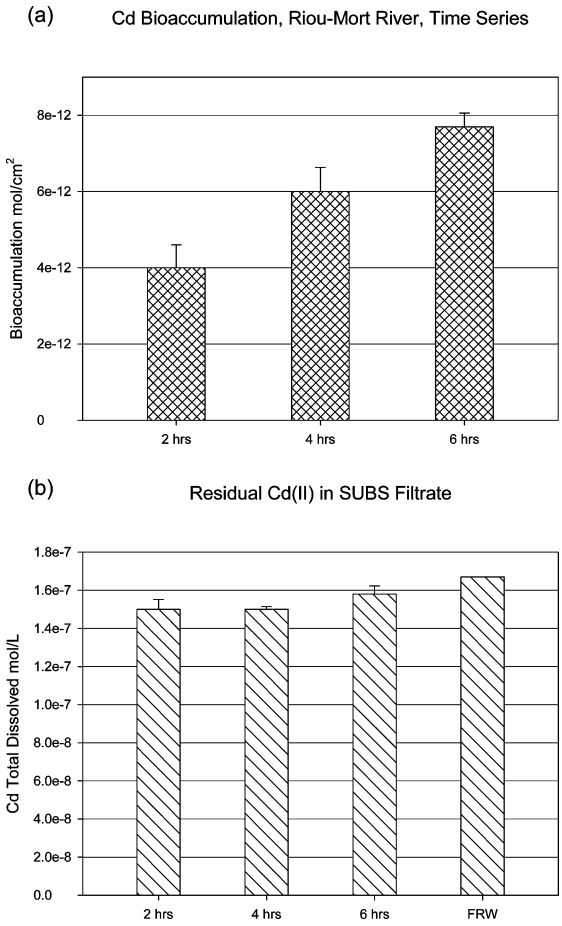

FIGURE 4. (a) Cd(II) Bioaccumulation for *C. reinhardtii* exposed in the Riou-Mort River (France). Duplicate measurements were made for 2, 4, and 6 h. (b) Filtrates obtained from the SUBS exposures for 2, 4, and 6 h. Filtered river water (FRW) represents the total dissolved Cd concentrations obtained prior to resuspending the algae in the in situ exposure.

dissolved Cd concentrations inside the SUBSs were nearly constant; Cd concentrations were between 90 and 95% of those found in the filtered river water (Figure 4b), indicating that J_{diff} well exceeded consumption by the algae, and the risk of bulk depletion of Cd was negligible. The small differences between the internal Cd concentrations and the initial filtered river water concentrations are attributed to the initial dilution of the algae, a slight adsorption of trace metal onto the acid-washed surfaces, and experimental error. The results from this field experiment indicate that biouptake of Cd by the algal cells inside the SUBS is likely representative of cells exposed directly to natural waters.

Cellular Viability. Another concern in this study was whether or not it was possible to maintain viable cells during the exposures. Some points of concern included the fact that they were taken from a growth media at room temperature (20 °C) and placed into a "contaminated" river water at a temperature between 12 and 17 °C. Preliminary trials of the viability test in the laboratory showed that formazan production was completely abolished, as expected, when the cultures were exposed to lethal concentrations of 2% formaldehyde. At the experimental site, samples exposed for 2, 4, and 6 h were compared to controls that consisted of the same exposure solution (e.g., filter river water and cells) maintained in containers kept alongside the river (Figure 5). The viability test demonstrated that despite a decrease in the absolute value of the formazan absorbance with time, algae exposed in the river were alive during the entire exposure. Indeed, at 6 h, a significant viability was observed

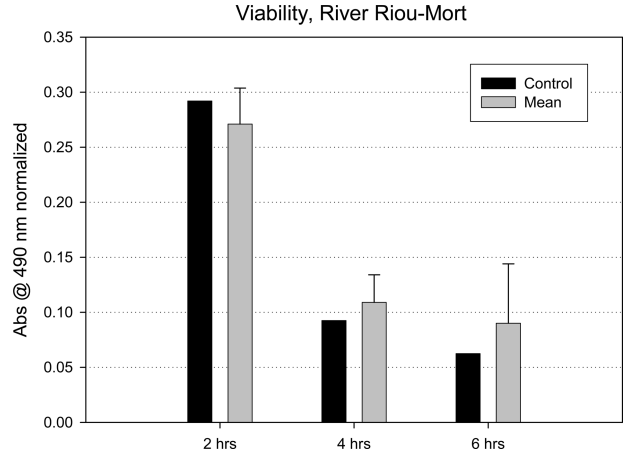

FIGURE 5. Cellular viability assay for *C. reinhardtii* in the SUBS exposed to filtered water from the Riou-Mort River.

and all viability measurements were similar to control values made outside the river. It is nonetheless important to emphasize that while every effort was made to be quantitatively precise, under such field conditions, the formazan test is likely only able to provide a qualitative confirmation of vitality.

Environmental Applications. Ultimately, the SUBS has been developed for use with unicellular bioreporter organisms to evaluate the bioavailability of chemical contaminants in the natural environment (*1*). Indeed, the above results indicate that it should be possible to use standard laboratory cultures to measure trace metal bioaccumulation or biological effects in situ. Nonetheless, two important caveats should be mentioned. As discussed above, the bioaccumulation of a number of elements (e.g., Ag, some nutrients, some organic contaminants; (*8*)) have been shown to be limited by transport through the diffusion layer surrounding the microorganism. In those cases, modification of the overall diffusive fluxes due to suspension of the microorganisms in the SUBS will necessarily result in a further depletion of exposure concentrations and thus an underestimation of bioavailability. While this was shown *not* to be the case for Cd uptake by *C. reinhardtii*, biological contaminant permeabilities depend on the organism, the contaminant, and the precise physicochemical conditions at the study site (e.g., *T*, pH, hardness ions, etc.). A second caveat is that the time required for equilibration of the internal chamber of the SUBS will limit the temporal resolution of data, i.e., short-term changes (ca. < 6 h) in contaminant concentrations will not be "seen" by the SUBS, rather concentrations in the SUBS will be time averaged. This constraint is a similar problem with many of the analytical techniques that are currently available to environmental chemists for measuring contaminant concentrations.

Previous reports on such in situ exposures, under controlled conditions, are scarce (e.g., (*11*)). Traditionally, transplant experiments have been performed with plants (e.g., (*20*), for moss), invertebrates (e.g., (*21*), for mussels), and vertebrates (e.g., (*22*), for fish). In those cases, the experiments have often been successful in relating the effects of contamination to either survival or sublethal responses such as growth, cellular metal content, or enzymatic activity. The proposed SUBS exposure device will extend the feasibility of transplant experiments to microorganisms such as algae and zooplankton—ideal bioindicator organisms for determining the health or toxicological status of aquatic ecosystems (*1, 2*). Furthermore, the use of SUBS should not be restricted to laboratory organisms; likely, they could also be used to transplant natural plankton communities potentially enabling significant advances in ecotoxicology.

Acknowledgments

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Supporting Information Available

Details on the laboratory characterization, construction, and design of the SUBS, as well as further explanation of logistical considerations and photos. This material is available free of charge via the Internet at http://pubs.acs.org.

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