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Extracellular and Intracellular Uptake of Zinc in a Photosynthetic Biofilm Matrix

J. M. Morris, J. S. Meyer

Department of Zoology and Physiology, University of Wyoming, Laramie, WY 82071, USA

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During daylight, concentrations of free zinc ion (Zn^{2+}) in the interstitial water in photosynthetic biofilm decrease, but the fate of the Zn^{2+} (e.g., changes in Zn speciation, absorption into algal and bacterial cells, adsorption onto cells, or precipitation as Zn-containing minerals) is unknown. We investigated the mechanism of Zn uptake into biofilm in a previous study by measuring vertical profiles of pH and free Zn^{2+} in biofilm using miniaturized ion-selective electrodes (Morris 2005). In that study, Zn^{2+} concentrations decreased from 1.0×10^{-4} to 4.4×10^{-7} M in the biofilm matrix during photosynthesis while pH increased from 6.7 to 9.7. We concluded that the extremely high pH in the biofilm matrix during photosynthesis creates a steep Zn diffusion gradient from the water column into the biofilm through (1) a shift in Zn speciation in the interstitial water in the biofilm matrix and (or) (2) sorption of Zn to algal and bacterial cells and (or) precipitation of Zn-containing minerals in the biofilm matrix. However, the experimental design of that study did not allow us to conclude whether Zn was accumulating on the inside (i.e., absorbed) or the outside (i.e., adsorbed) of the microbial cells. Therefore, we conducted this experiment under similar conditions with the same biofilm culture and used an EDTA rinse to determine the percentages of internal and external Zn.

MATERIALS AND METHODS

To begin the experiment we placed 12 acid-washed 7.5-cm × 13.5-cm glass plates, which were sandblasted on the top side to provide a rough surface for biofilm attachment, in a mixed-community biofilm culture (described in Morris et al. 2005) growing in a standard algal nutrient medium (Stein 1973). The biofilm colonized the plates for 1 week, producing a thin layer (≤1 mm) of biofilm that could be cut and removed in sections for sampling purposes.

These 12 plates were divided equally among 2 glass exposure chambers (wetted dimensions of each chamber were 110 cm L × 31 cm W × 5.5 cm D), and one chamber was covered in black plastic to occlude light. Each chamber received 2.1 L of test water/min from a common recirculation bath that contained 110 L of the nutrient medium without EDTA and trace metals but with a Zn concentration of 2.3×10^{-5} M (added as $ZnSO_4\cdot7H_2O$). We did not add EDTA and trace metals

Correspondence to: J. M. Morris, Western Research Institute, 365 North 9th Street, Laramie, WY 82072, USA

to the nutrient medium so the results from this experiment could be compared to our previous experiment in which we measured vertical profiles of pH and Zn^{2+} in overlying water and the biofilm matrix (Morris 2005). The pH, temperature, hardness, and alkalinity of the exposure water were 7.46, 26°C, 35 mg/L as $CaCO_3$, and 19 mg/L as $CaCO_3$, respectively.

For the first 12 h of this 24-h experiment, the light and dark chambers were completely dark (Dark pre-treatment). Then the plates in the light chamber were exposed to 130 μmol/m^2/s of photosynthetically active radiation (Dark-Light treatment) and the plates in the dark chamber remained in darkness (Dark-Dark treatment) during hours 12 to 24. At the end of the first 12 h of darkness, 4 plates from the Dark pre-treatment (2 each from the Dark-Light and Dark-Dark chambers) were removed, and the biofilm from each plate was divided into three approximately equal sections and scraped off the plate using the edge of a clean glass slide. Under complete darkness, one of the 3 sections of biofilm from each plate was dipped in deionized water for 5 seconds and placed in an acid-washed 7-ml, plastic scintillation vial; one section of biofilm was soaked in 100 ml of deionized water for 10 minutes and placed in an acid-washed scintillation vial; and the third section of biofilm was soaked in 100 ml of a 4 mM EDTA solution (pH 5.3) for 10 min according to Behra et al. (2002) and placed in an acid-washed scintillation vial. The purpose of the 5-sec rinse in deionized water was to remove dissolved Zn from interstitial water, and the purpose of the 10-min soak in deionized water was to remove loosely sorbed Zn and possibly dissolve some Zn-containing precipitates. We assumed the EDTA soak removed all adsorbed Zn from the cell surfaces. At the end of 24 h, biofilm was collected and washed in the same way under lighted conditions for the 4 remaining plates in the Dark-Light treatment and in complete darkness for the 4 remaining plates in the Dark-Dark treatment. We also sampled 4 plates from our culture stock in the same way under lighted conditions at the end of the 24-h experiment.

All biofilm samples were dried to constant weight at 55°C, digested in concentrated trace-metal grade HNO_3, and analyzed for Zn by flame atomic adsorption spectrophotometry (FAAS; Perkin-Elmer model 372). We collected water samples from each chamber for Zn analysis by FAAS, and we measured water temperature and pH at 0, 12, and 24 h. The instrument detection limit for Zn was 10 μg/L.

We conducted statistical comparisons with ANOVA ($\alpha = 0.05$) followed by Tukey HSD post hoc pairwise comparisons using Minitab™ Version 13.31 (Minitab Statistical Software, Minitab Inc.). To satisfy the homogeneity of variance and normality assumptions of ANOVA, we performed square-root transformations on the data before comparing the residual-Zn content of biofilm among the three treatments; however, we did not need to transform data for comparisons of EDTA-labile and non-labile Zn (see definitions below).

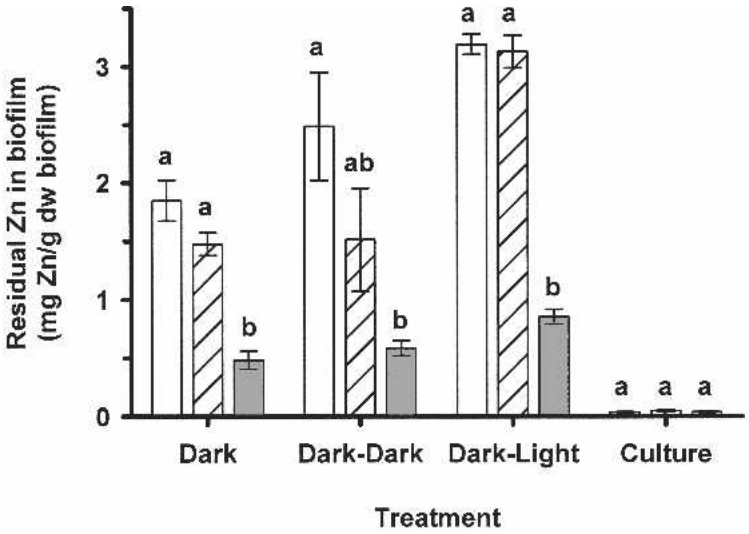

Figure 1. Residual Zn content of biofilm after dipping in deionized water for 5 seconds (open bars), soaking in deionized water for 10 min (hatched bars), or soaking in 4 mM EDTA solution for 10 min (shaded bars). Dark was exposed to 2.3×10^{-5} M Zn in darkness for 12 h, Dark-Dark was exposed to 2.3×10^{-5} M Zn in darkness for 24 h, Dark-Light was exposed to 2.3×10^{-5} M Zn in darkness for 12 h followed by light for 12 h, and Culture was taken directly from the biofilm stock culture (only background Zn exposure). Error bars are ±1 standard error of the mean (n = 4); dw = dry weight. Different letters above bars indicate a significant difference ($P < 0.05$) within each treatment.

RESULTS AND DISCUSSION

In all three treatments, the residual-Zn (Zn that was not removed by dipping or soaking in deionized water or by soaking in EDTA) content of biofilm did not differ significantly ($P < 0.001$) between the 5-second dip in deionized water and the 10-minute soak in deionized water (Fig. 1). Therefore, to calculate the EDTA-labile Zn content of the biofilm, we subtracted the residual-Zn content of biofilm after the 10-minute soak in EDTA from the average residual-Zn content of biofilm rinsed with both the 5-second dip and 10-minute soak in deionized water. The non-labile Zn was the residual-Zn content of biofilm after the 10-minute soak in EDTA.

The biofilm in the Dark-Light treatment accumulated ≥1.6× more EDTA-labile and ≥1.5× more non-labile Zn than biofilm in the Dark pre-treatment or the Dark-Dark treatment ($P < 0.001$; Fig. 2). However, the EDTA-labile and non-labile Zn concentrations in biofilm did not differ significantly between the Dark pre-

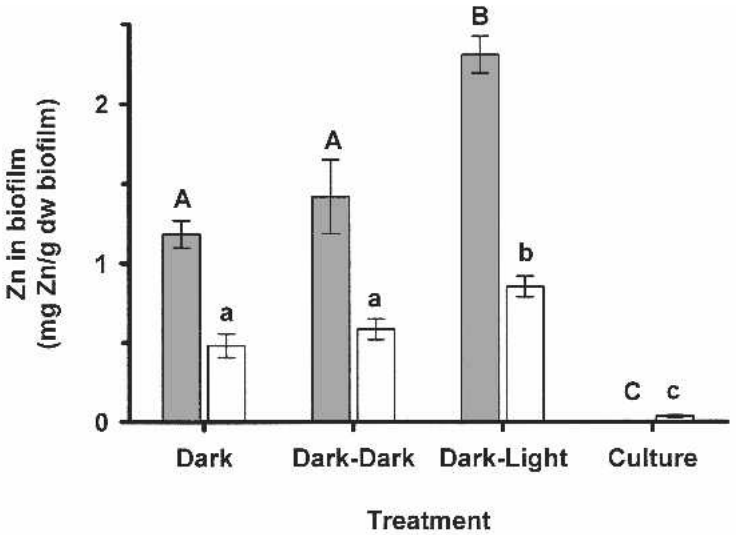

Figure 2. EDTA-labile (shaded bars) and non-labile (open bars) Zn in biofilm. Dark was exposed to 2.3×10^{-5} M Zn in darkness for 12 h, Dark-Dark was exposed to 2.3×10^{-5} M Zn in darkness for 24 h, Dark-Light was exposed to 2.3×10^{-5} M Zn in darkness for 12 h followed by light for 12 h, and Culture was taken directly from the biofilm stock culture (only background Zn exposure). Error bars are ±1 standard error of the mean (n = 4); dw = dry weight. Different uppercase or lowercase letters above bars indicate significant differences ($P < 0.05$) in the EDTA-labile or non-labile Zn content of biofilm among the treatments.

treatment and the Dark-Dark treatment. The average percentages of EDTA-labile and non-labile Zn out of the total Zn measured in biofilm from the 3 treatments were 71 and 29%, respectively (Table 1). Behra et al. (2002) and Meylan et al. (2003) assumed that EDTA-labile Zn is located outside the algal cells in periphyton and non-labile Zn is located within the algal cells; however, we cannot exclude the possibility that EDTA did not remove all the Zn adsorbed to cell surfaces and (or) dissolve all the Zn-containing precipitates. Therefore, the results of this study support our previous conclusion (Morris 2005) that Zn^{2+} uptake in biofilm is primarily due to (1) a shift in Zn speciation in the interstitial water in the biofilm matrix and (or) (2) adsorption of Zn to cells in the biofilm matrix and (or) precipitation of Zn-containing minerals in the matrix, all of which could be caused by the extremely high pH (10-11) in the biofilm matrix during photosynthesis. Based on the results presented herein, we do not believe that a shift in Zn speciation is the major cause of the Zn^{2+} uptake we measured in the biofilm in our previous study (Morris 2005). If a shift in Zn speciation were the cause of this Zn^{2+} uptake we would not expect to measure significant differences between the EDTA-labile and non-labile Zn concentrations of biofilm because dipping or soaking the biofilm in deionized water would rinse away and dilute the

Table 1. Total Zn in (on) biofilm (standard deviation in parentheses, n = 8) from the 12-h dark (Dark), 24-h dark (Dark-Dark), 12-h dark and 12-h light (Dark-Light) treatments, and the culture biofilm.

	Zn in (on) biofilm		
Treatment	mg Zn/g dw biofilm	% EDTA-labile	% non-labile[a]
Dark	1.7 (0.3)	71 (7)	29
Dark-Dark	2.0 (1.0)	70 (7)	30
Dark-Light	3.2 (0.2)	73 (5)	27
Culture	0.04 (0.01)	20 (8)	80

[a] The % EDTA-labile and % non-labile Zn (standard deviation in parentheses, n = 4) correspond to adsorbed and absorbed Zn in the biofilm matrix, respectively. % non-labile Zn = 100 - % EDTA-labile Zn.

aqueous Zn complexes in this interstitial water and little adsorbed Zn would remain to be removed during the EDTA soak.

Interestingly, the ratio of EDTA-labile and non-labile Zn (or adsorbed and absorbed Zn) remained 2.4 in all three treatments in this experiment, even when total Zn concentrations in biofilm increased during the light period in the Dark-Light treatment. This suggests that the internal Zn concentrations of these cells are directly proportional to the number of cell-surface binding sites that adsorb Zn, thus establishing a steady-state between adsorption, absorption, and possibly depuration. At the higher pH in the biofilm layer during lighted conditions, more Zn probably sorbs to cell surfaces (because of less competition with H^+ for binding to surface sites), thus leading to proportionally more internalized Zn but retaining the same EDTA-labile:non-labile Zn ratio. This explains why the EDTA-labile:non-labile Zn ratio remains the same even as total Zn concentrations in the biofilm increase, and it suggests that nearly all of the EDTA-labile Zn adsorbed to cells and little (if any) precipitated out of solution in this experiment. If a substantial amount of the EDTA-labile Zn was in precipitated Zn-containing minerals, we would expect the EDTA-labile:non-labile ratio to increase as the total Zn content of the biofilm increased but the internal Zn remained constant. Therefore, Zn precipitation would not directly influence internal Zn concentrations in cells, and we would expect the EDTA-labile:non-labile ratio to increase as concentrations of EDTA-labile Zn far exceeded concentrations of non-labile Zn. Finally, the low EDTA-labile:non-labile Zn ratio of 0.25 in the culture biofilm reflects the very low aqueous Zn concentration (6.1×10^{-8} M) in our culture

media and suggests that the cells were not depurating Zn and were probably up-regulating their internal Zn concentrations.

We conclude that the majority of the dissolved Zn removed in biofilm during lighted conditions in our laboratory experiments is not inside microbial cells but is adsorbed to the surface of these cells. However, this does not preclude concurrent precipitation of Zn-containing minerals in the thicker layers of biofilm (5-20 mm) we studied in High Ore Creek, Montana (Morris et al. 2005).

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