

# DEPOSIT OF ZINC AND MANGANESE IN AN AQUEOUS ENVIRONMENT MEDIATED BY MICROBIAL MATS

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**Abstract.** Microbial mats have been developed to sequester heavy metals from contaminated water. Mixed populations of photosynthetic and heterotrophic bacteria, dominated by *Scillatoria* spp., were developed for metal tolerance and integrated into a durable, self-sustaining community of microbes stimulated by and attached to ensiled grass. The mat was immobilized on glass wool and layered in flow-through baffled tanks. After allowing 8 weeks for the maturation of the mat, mixed solutions of Zn and Mn ( $15\text{--}16\text{ mg L}^{-1}$ ) were passed through a three-tank experimental series. Effluent from each tank was first sampled and then applied to the next tank. This procedure was repeated in triplicate and with six applications of new metal solution per three-tank series. By the third tank, the target metal concentration  $<1\text{ mg L}^{-1}$  was always achieved. Mean percentages of the initial influent concentration removed by tanks 1, 2 and 3, respectively, were 72, 93 and 98 for Zn and 78, 97 and 99 for Mn. Mean metal concentrations in the effluents (average of 6 applications) were, for tank 1: Zn ( $\text{mg L}^{-1}$ ) 5.0, Mn ( $\text{mg L}^{-1}$ ) 4.2; for tank 2: Zn 1.6, Mn 0.75; for tank 3: Zn 0.53, Mn 0.19. Mean effluent concentrations from each of the three sequential treatments (average of 6 applications per tank) were for Zn ( $\text{mg L}^{-1}$ ) 5.0, 1.6 and 0.53; for Mn ( $\text{mg L}^{-1}$ ) were 4.2, 0.75 and 0.19. Thus target concentrations were reached in experimental tank 2 for Mn and tank 3 for Zn. Metal removal in the control tank series, containing glass wool only, was 37% for Zn and 5% for Mn (average of 6 applications). Oxygen and redox potential analyses of the mat/glass wool matrix revealed a heterogenous structure of anoxic and oxic zones. Zeta potential analysis of the mat samples identified a mat surface charge ranging from  $-12.3$  to  $-69.2$  mV. Various metal removal mechanisms possibly involved with metal sequestering include surface binding to the mat or to mat exudates trapped within the glass wool, precipitation of the metals with anions present in the oxic/anoxic zones, mat mediation of the water conditions in favor of metal-oxide precipitation and active transport of the metals into the cell.

## 1. Introduction

Microbial mats are natural communities containing a variety of heterotrophic bacteria and dominated by blue-green algae (cyanobacteria). These ecosystems are stable and resilient communities which self-organize into stratified biofilms arranged for maximum efficiency of interspecies exchanges (Shilo, 1989). During the photo-period, mats maintain aerobic conditions which are closely associated with zones exhibiting highly reducing conditions (Canfield and Des Marais, 1991; Bender *et al.*, 1991). These sharply different environments provide unique opportunities for biotechnology applications.

Microbial mats can be designed for specific remediation tasks by integrating the desired strains into the mats during the early stages of mat formation (Bender *et al.*, 1989a). Metal-sequestering mats were structured for this research by developing elevated metal tolerances in cyanobacteria and mixed populations of soil bacteria, then integrating them into a mat structure by co-culture on ensiled grass clippings.

The objectives of this study were to (1) develop metal-tolerant mats, (2) assess these mats for Zn and Mn sequestering in baffled, flow-through tanks and (3) measure the environmental parameters that develop in and around the mats to identify possible chemical and biological conditions that affect the deposit of Zn and Mn.

## 2. Materials and Methods

### 2.1. DEVELOPMENT OF STRAINS, MAT CONSTRUCTION AND IMMOBILIZATION

Samples of mixed populations of soil bacteria were selected from a variety of sandy and loam soil types from roadside areas, which were expected to have metal contamination from automobiles. Cyanobacteria (*Oscillatoria* spp.) was isolated from pea gravel on a Florida roof surface. Mixed populations of heterotrophic bacteria and *Oscillatoria* spp. were developed separately for elevated metal tolerances by step-wise exposure to increasing metal concentrations (5 mg L<sup>-1</sup> increments). Toxicity was indicated by blanching of the *Oscillatoria* spp. or sedimentation of the bacteria at the bottom of the media flask. Metal tolerances developed to levels expected in the mat after bioconcentration of the metals in the mat. To prevent transfer of metals to the treatment system, stock quantities of the microbes to be used for inocula were first cultured in nutrient medium without the metals. Inocula (3–5 ml L<sup>-1</sup>) from metal-tolerant strains of bacteria and *Oscillatoria* spp. were integrated into a multispecies mat by co-culturing them on a substrate of ensiled grass clippings (1.3 g L<sup>-1</sup> dry wt). Stock mats, after 7–10 days of culture were assessed for retention of metal tolerances and stored in Allen and Arnon mineral media (1955). Mats were immobilized on glass wool by broadcasting sections of the metal-tolerant mats on a 4.5 cm deep layer of glass wool in a baffled tank (61 × 18 cm) saturated with Allen and Arnon media (Figure 1). Illumination was provided by 6 lamps (60 Watt), placed 36 cm from the mat surface (88 lux) and maintained under light/dark cycles of 12/12 h. All sequestering experiments were performed under light conditions.

Within each tank five baffles, separated by 17 cm each, caused the metal solution to flow in an irregular, S-shaped pattern. This maximized exposure of metal solution to the mat. A three-tank series was used such that the effluent from one tank became the influent of the next tank in the series. Average flow rates (mL min<sup>-1</sup>) through tanks 1, 2 and 3 were 3.3, 2.4, and 2.4, respectively.

### 2.2. ENSILING OF GRASS CLIPPINGS

To release the nutrient content of the vegetative material and convert the starch material to organic acids, fresh-cut grass clippings were ensiled according to the procedures of the Iowa State University Cooperative Extension Service (1974). Finished ensiled grass contained lactic and acetic acids, which elicit chemotactic responses in cyanobacteria and several species of heterotrophic bacteria within the mat (Bender *et al.*, 1989b). This material facilitated rapid mat formation.

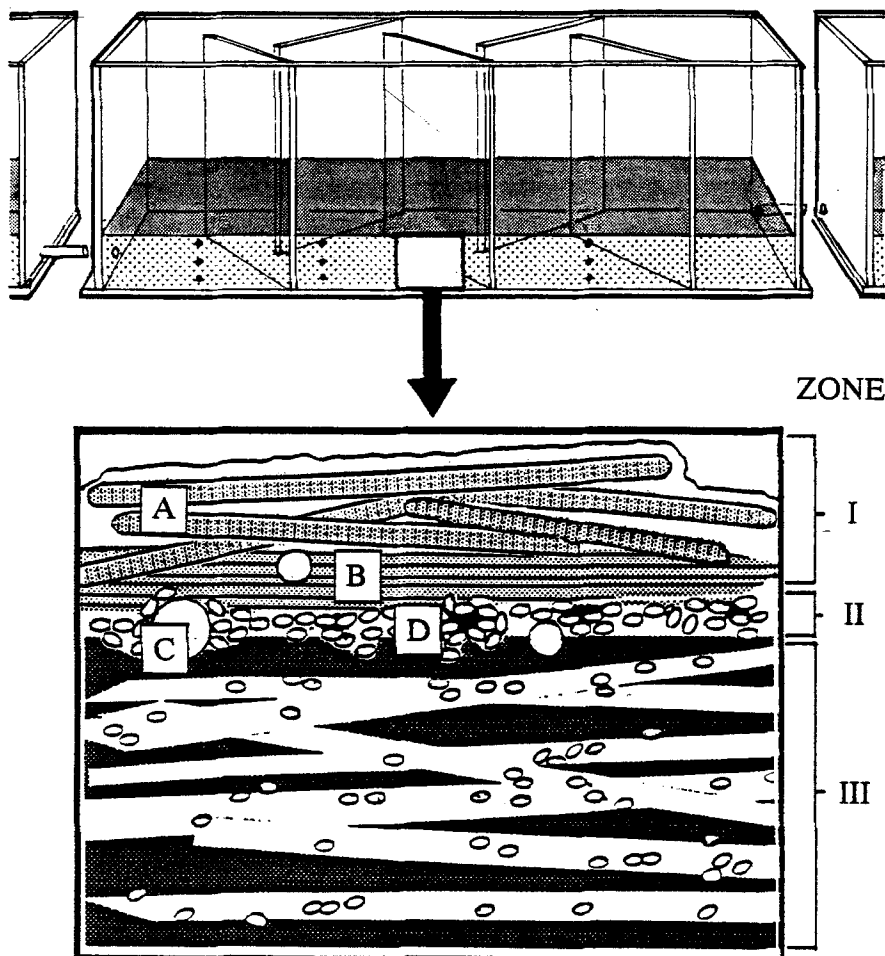


Fig. 1. Diagram of a three unit series of baffled tanks. The tank measured 61 cm (length) by 18 cm (width). The glass wool was layered to a 4.5 cm depth. Black dots represent sampling locations. A schematic diagram of mixed microbial mat immobilized on glass wool is included. Zone I contains *Oscillatoria* spp. (A) and associated bacteria enmeshed with ensiled grass (B). Gases (C) remain entrapped in the slimy matrix for extended periods. Mixed bacteria in zone II include anoxygenic phototrophs, aerobes, anaerobes and facultative bacteria. Zone III contains heterotrophic bacteria and cellular exudates entrapped in the glass wool.

The ensiling *Lactobacillus* spp. probably remained active thereby providing a continuous nutrient feed to the microbes as the grass continued to degrade. Finished ensiled grass was stored in tightly sealed jars at room temperature.

### 2.3. METAL APPLICATION, SAMPLING AND ANALYSIS

Six applications of 0.5 L of mixed solutions, containing  $15.98 \text{ mg Zn L}^{-1}$ ) and  $14.87 \text{ mg Mn L}^{-1}$ ) were flowed through the series of three baffled tanks. Effluents were collected and hydrolyzed by microwave digestion (CEM MDS-2000).

TABLE I

Bacteria and cyanobacteria metal tolerances. The initial tolerance refers to a  $\text{mg L}^{-1}$  metal concentration that the microorganisms tolerated prior to adaptation. Final metal tolerances represent the levels at the time of integration into the mat, not maximum tolerances achieved. ND = not determined

Microbial type	Metal	Metal tolerances, $\text{mg L}^{-1}$	
		Initial	Final
<i>Oscillatoria</i> spp.	Zn	5	100
	Mn	50	150
Bacteria, mixed soil populations	Zn	5	120
	Mn	ND	100

Hydrolysates were analyzed for Zn and Mn concentrations by atomic absorption spectrophotometry (Varian Spectra AA-20 BQ double beam).

#### 2.4. MAT CHARACTERISTICS ANALYSIS

Oxygen concentrations, redox potential and pH were measured in the mat at three locations and three depths (Figure 1) with microprobes (Microprobes), fitted to a pH/mV meter (Orion SA 250). Zeta potentials were measured on three mat samples with a Laser Zee Meter (Pen Kem 501)

### 3. Results and Discussion

Microbial and metal-tolerances, resulting from adaptation procedures, are shown in Table I. Co-culturing the heterotrophic bacteria and *Oscillatoria* spp. with ensiled grass clippings resulted in the spontaneous formation of a metal-tolerant mat. The mat, dominated by *Oscillatoria* spp., was tightly bound to the grass by slimy secretions, forming a sheet 1–2 mm thick. Cyanobacteria occupied the photozone at the surface and several species of heterotrophic and anoxic autotrophic bacteria colonized below the cyanobacteria. Broadcasting only small sections of mat resulted in growth that completely covered the glass wool in approximately two weeks.

The metal content was consistently reduced by flowing contaminated solutions through baffled tanks containing immobilized mat (Figure 2). The control tanks of Figure 2 are displayed separately to illustrate the binding trend of Zn to the glass wool. After the attachment sites became saturated, Zn uptake decreased. Mn showed little wool binding throughout the applications. Cumulative uptake of both metals after flowing through the three immobilized mat tanks was 98–99% of the total metal input (Figures 3 and 4).

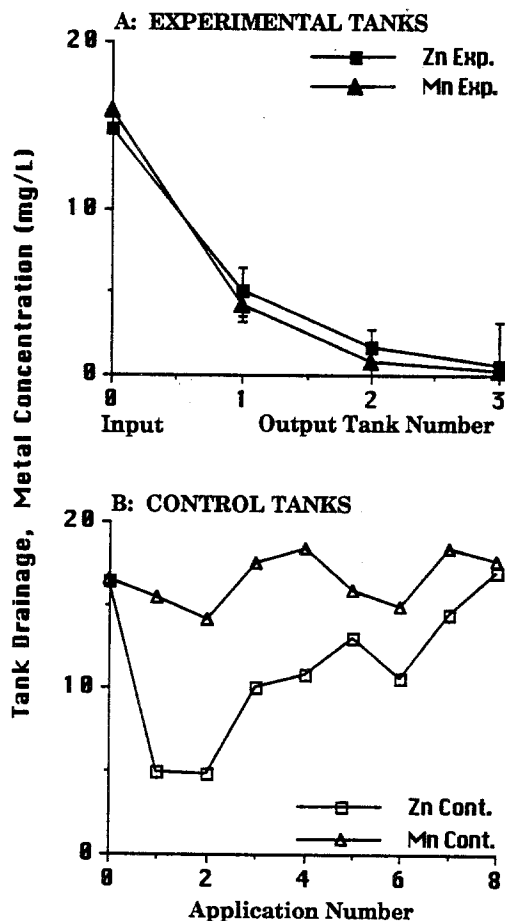


Fig. 2. Experimental tanks (A): Input point represents the initial influent concentration for tank 1. Metal output concentrations for sequential tanks 1, 2 and 3 are indicated as a mean of six applications. Control tanks (B): Eight trials (applications) of metal solution were made with tanks containing only glass wool. Point 0 represents input concentrations. Results of separate applications are present to display the trend of Zn binding to glass wool. Zn showed elevated attachment to the glass wool in the first three applications (60%–70% removal), but showed continuous reduction in binding as applications continued. Mn showed little binding to the glass wool.

Since no attempt was made to augment flow rates by either varying tank slope or pressurizing the flow-through solution, maximum rate of metal uptake has not been determined. Considering the present slow flow rates, potential simultaneous Mn and Zn sequester ( $\text{mg metal m}^{-2} \text{ h}^{-1}$ ) is 28.5 for Zn and 34.0 for Mn (values represent an average of 6 applications in tank 1 with a flow rate of  $3.3 \text{ mL min}^{-1}$ ).

A parallel field study using pilot-scale ponds, was conducted based on the results of this laboratory research. The field ponds received an average flow rate of  $3.3 \text{ L min}^{-1}$  acid mine drainage. Microbial mat, attached to green filamentous algae and limestone substrate, removed  $2.6 \text{ g m}^{-2} \text{ day}^{-1}$  of manganese at temperatures as

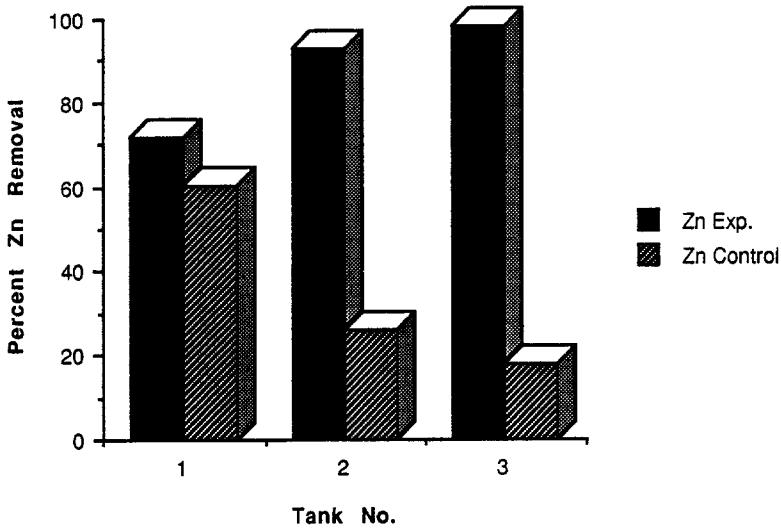


Fig. 3. Cumulative percent removal of Zn in three treatment tanks connected in series. Effluent from tank 1 was applied in series to tanks 2 and 3. Percents represent the total cumulative removal of the influent metal concentrations.

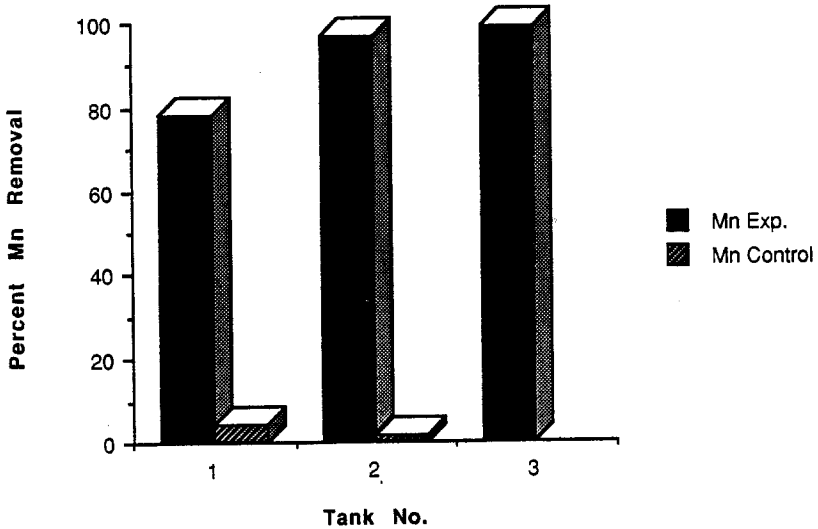


Fig. 4. Cumulative percent removal of Mn in three treatment tanks connected in series. Effluent from tank 1 was applied in series to tanks 2 and 3. Percents represent the total cumulative removal of the influent metal concentrations.

low as 4 °C. Control ponds removed approximately 0.6 g m<sup>-2</sup> day<sup>-1</sup> (Phillips *et al.*, 1994).

The metal sequestering mechanisms in a mixed microbial system are expected to be complex. Widely varying zones of oxygen and redox conditions are present in the

TABLE II

Environmental conditions of the immobilized mat. Oxygen, Redox and pH levels are expressed as ranges of values from three points along the mat. Each sample point was analyzed at vertical depths of 1, 2 and 3 cm from surface. Samples selected for zeta potential measurements were surface mat sections taken from three locations after completion of the metal applications

	Light	Dark
Mat parameter		
Oxygen, mg L <sup>-1</sup>	0 to 18	0
Redox, mV	+164 to -303	-248 to -407
pH	7.2 to 8.6	9.0
Sample	Medium pH	Zeta potential $\pm$ 5
1	6.57	-69.2
2	6.84	-53.0
3	6.75	-12.3

mat (Table II). Thus, the metal solution is exposed to these heterogenous conditions as it passes through a labyrinthine path of bacteria and cyanobacteria. The specific mechanisms of metal deposition within the mat are not known. However, the environmental conditions mediated by the unique physiology of the component microbes suggest several processes that may be operative in the sequester of the metals. The laminated structure of the mat creates discrete regions of unique chemistry characterized by oxic and anoxic regions in close proximity (Bender *et al.*, 1989). Reducing zones contain sulfur-reducing bacteria (Archibold *et al.*, 1989) and probably concentrations of sulfide ions which can precipitate the zinc. Oxygen generated during the photosynthetic period becomes entrapped in the surface slime covering the cyanobacteria. These high concentrations of oxygen would be expected to react with Mn, likely producing MnO<sub>2</sub>. Zn could also be deposited in the oxygenic zones as ZnO. In the field study, Mn is removed as effectively at night as during daylight. Redox and oxygen measurements after 10 hours of darkness confirmed that anoxic conditions did not develop during the night. This suggests that oxygen retained by the microbial mat remains at a high enough level during dark periods to continue MnO<sub>2</sub> deposit (Phillips and Bender, 1993). Additionally, the expected pH depression during dark periods was absent in both the laboratory experiments (Table II) and the field study. This may be due to the extensive buffering effect of organic releases by the mat into the water column (manuscript in preparation).

It is important to note that the proposed mechanisms suggest that metal deposit occurs outside the cells, thereby accounting for the observed high tolerance and resiliency of the mat after metal loading. Although the individual microbial components were not tolerant outside the mat structure and required adaptation to the metals, the multi-species mat, with its zonal structure, may achieve tolerance through community-level protective mechanisms that limit cell exposure. X-ray microanalysis of the mat surface showed a lack of congruency between the cell filaments and the metal deposit (Bender *et al.*, in press). Metals were layered on the mat surface as amorphous deposits or crystals.

The formation of manganese oxides, specifically  $\text{MnO}_2$ , would be expected under the environmental conditions generated by the mat. Stumm and Morgan (1981) have described the autocatalytic oxidation of  $\text{MnO}_2$  after an initial deposit of the oxide became available for a substrate. The reaction rates were strongly dependent on pH, requiring values above pH 8.5. High pH levels and concentrations of photosynthetic oxygen, entrapped in the surface slime, are available along the path of the flowing metal solution in both light and dark conditions. Thus initial deposits of  $\text{MnO}_2$  will form in these zones and subsequently serve as surfaces for autocatalysis in continued deposition of the precipitate.

Zinc will also oxidize in the oxic zones present in the mat. Additionally, unlike manganese,  $\text{ZnS}$  is insoluble, and might deposit in the zones of low redox potentials containing pools of sulfide ions released by sulfur-reducing bacteria (Bender *et al.*, 1989a). Table II illustrates the heterogenous nature of the mat which might contribute to the simultaneous precipitation of oxides and sulfides within the same microbial community and during the same photoperiod.

Taylor and Marshall (1967) suggested that manganese-oxidizing microorganisms include a variety of genera that are distributed worldwide. Schutt and Ottow (1977) and Douka (1980) identified *Pseudomonas*, a genus commonly found in our mats, as a taxon capable of manganese oxidation as an energy source. The oxide accumulation was a byproduct of bacterial metabolism.

Certain species of cyanobacteria are known to produce bioflocculents (Bar-Or and Shilo, 1988; Fattom and Shilo, 1984). Their production by our mats was stimulated by the presence of  $\text{Mn}^{+2}$  (Rodriguez-Eaton *et al.*, 1994). Data (not shown) indicated that the metal-tolerant mats, used in these experiments produced negatively charged bioflocculents, which may have become entrapped in the glass wool. These materials may have bound the metals in the matrix below the mat, thereby protecting the cells. Although the production of the flocculent correlates with the rate of deposit of the  $\text{Mn}^{+2}$  and might be seen as an advantage for rapid deposition of the metal, these organic materials may also retard the autocatalysis reactions by complexing the oxidizable species (Wilson, 1980). Metal/bioflocculent complexes may not have the same long-term stability as  $\text{MnO}_2$ .

The negative charges on the mat surfaces suggest that some of the metals may have been bound to the outer layers of the mat, probably to the slimy matrix which covers the cyanobacteria cells.



A number of mechanisms for metal deposit are presented by the mats, including community-level alterations in the micro-environment, cell sorption, biological oxidation and biofloculent production. The fact that mature mats typically demonstrate much higher metal tolerances than do the component microbes suggests that the community-level mechanisms may be dominant over cell sorption interactions in metal removal.

#### 4. Summary

Constructed mats, immobilized on glass wool, were effective in sequestering Zn and Mn simultaneously. Metal solutions were decontaminated by flowing through a series of three treatment tanks, producing a final effluent with metal concentrations  $<0.6 \text{ mg L}^{-1}$ . Mixed microbial mats, although complex and difficult to elucidate, may represent important material for bioremediation because they offer a variety of mechanisms for metal sequester. The fact that these mechanisms seem to be protective of the total microbial community represents a second advantage in terms of long-term cell survival and high loading potential of the system.

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