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Microbial manganese(II) oxidation in the marine environment: a quantitative study†

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Abstract. A great number of important chemical reactions that occur in the environment are microbially mediated. In order to understand the kinetics of these reactions it is necessary to develop methods to directly measure in situ reaction rates and to develop models to help elucidate the mechanisms of microbial catalysis. The oxidation of Mn(II) in a zone above the O_2/H_2S interface in Saanich Inlet, B.C., Canada is one such reaction. We present here a method by which in situ rates of microbial Mn(II) oxidation are measured and a model based on our experimental results to describe the general mechanism of Mn(II) oxidation. We propose a two step process in which Mn(II) is first bound by a site on the bacterial surface and then oxidized. The model is analogous to the Langmuir isotherm model for surface catalyzed gas reactions or the Michaelis-Menten model for enzyme kinetics. In situ Mn(II) oxidation rates were measured during five cruises to Saanich Inlet during the summers of 1983 and 1984. We use the model to calculate the apparent equilibrium binding constant ($K_s \approx 0.18\,\mu M$), the apparent half saturation constant for biological Mn(II) oxidation ($K_m = 0.22$ to $0.89\,\mu M$), the maximum rate of Mn(II) oxidation ($V_{max} = 3.5$ to $12.1\,nM \cdot h^{-1}$) and the total microbial surface binding site concentration ($\Sigma E \approx 51\,nM$). V_{max} for Mn(II) oxidation agrees with the rates calculated from the value of the flux of Mn(II) to the oxidizing zone using the Mn(II) gradient and estimates of the eddy diffusion coefficient. This consistancy verifies our methodology and indicates that the rate of Mn(II) oxidation is nearly equal to the V_{max} for the reaction. We conclude that in this environment the Mn(II) oxidation rate is more a function of the total number of surface binding sites than the Mn(II) concentration.

Introduction

Saanich Inlet is a seasonally anoxic fjord on the southeast corner of Vancouver Island in British Columbia, Canada. Based on concentration profiles and flux arguments it was suggested that the rate of Mn(II) removal from solution immediately above the O_2/H_2S interface was too rapid to be oxidation by abiological processes and microbial catalysis was suggested (Emerson et al., 1979). That the removal of dissolved Mn(II) was due to oxidation was confirmed by measuring the oxidation state of particulate

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material (Tebo et al., 1984; Kalhorn and Emerson, 1984). The microbial oxidation hypothesis was tested by incubating water samples from the depth of the manganese particulate maximum with ^{54}Mn(II) and measuring the binding of the tracer in the presence and absence of poisons that inhibit biological activity without measurably interfering in the solution chemistry of manganese (Emerson et al., 1982; Tebo 1983; Rosson et al., 1984; Tebo et al., 1984). These results clearly indicated that a portion of the Mn(II) removed from solution was blocked by metabolic inhibitors and thus biologically catalyzed. Bacterial structures that are coated with manganese oxide were identified (Emerson et al., 1982; Tebo, 1983) indicating that bacteria are important in mediating Mn(II) oxidation in this environment.

In order to obtain quantitative in situ rates of Mn(II) oxidation we recently refined our earlier radiotracer method, eliminating the addition of poisons which in some unmeasurable way could interfere with precise rate determinations, and conducted the incubations under in situ conditions of temperature, pH and oxygen tension (Tebo and Emerson, 1985). In this method incubations are conducted in the presence and absence of oxygen; the no-oxygen incubations serve as controls and a measure of exchange of ^{54}Mn(II) between the dissolved and solid Mn(II) pools. We interpret the difference between the oxygen-containing and no-oxygen experiments to be a measure of Mn(II) oxidation. Justification for this interpretation is presented in the methods section of this paper.

In a separate paper (Tebo and Emerson, 1985) we report results that indicate high (18 μM) concentrations of manganese and temperatures above 20 °C inhibit the manganese oxidation rate in Saanich Inlet and that the rate was almost twice as fast under near air saturation oxygen tensions versus the in situ oxygen tension. Furthermore, in no-oxygen incubations ^{54}Mn(II) binding was 85% inhibited by sonication suggesting that most of the Mn(II) binding occurs on biological binding sites since sonication should not affect the binding capacity of inorganic surfaces. Here we present results of field work conducted in Saanich Inlet during the summers of 1983 and 1984 and a model based on the experimental observations to describe the mechanism of microbial catalysis of Mn(II) oxidation.

Methods

Experimental procedure for measuring the binding of ^{54}Mn(II)

Details of the method used are presented in Tebo and Emerson (1985). Briefly, water samples were collected from various depths with Niskin or Go-Flo bottles. Duplicate samples were taken without contaminating them with oxygen, placed in acid-washed Pyrex Erlenmeyer flasks that had been previously flushed with N_2/CO_2 and then, using acid washed gas dispersion tubes (Kimex) inserted through two-holed stoppers, vigorously sparged with

N_2/CO_2 to remove all oxygen. One flask was connected to the "no-oxygen" gas mixture and the other to the low "in situ" oxygen mixture (~ 15 μM O_2). The CO_2 tension in both incubations was adjusted with gas proportioners to maintain the in situ pH (7.4). The samples were sparged continuously for about 1 h until the oxygen tension and pH stabilized. Carrier-free ^{54}Mn(II) (as $MnCl_2$, 6 μCi, 35.37 mCi $\cdot$ mg^{-1}; New England Nuclear Corp.) was added to each flask and in a time course fashion, triplicate samples (1 ml) were removed directly to vials for measurement of total radioactivity and triplicate samples (5 ml) filtered through 0.2 μm membrane filters (Gelman Metricel GA-8). The filters were washed (by filtration) with 5 ml of seawater to remove any non-specific ^{54}Mn(II) adsorption and the samples (totals and filtrate) were counted on a gamma counter. The percent of radioactivity bound times the measured Mn(II) concentration gave the amount of Mn(II) bound at a given time. All incubations were carried out at the in situ temperature (9° ± 1 °C) and initiated within 2 hours after sampling.

An example of a typical experiment is presented in Figure 1. In Figure 1a the total amount of Mn(II) bound (trapped by the filters) was measured under conditions of 5% air saturation (~ 15 μM O_2) and no oxygen. The amount of Mn(II) bound includes that portion which (1) exchanges with Mn on the surface of the particulate material, (2) adsorbs to inorganic or biological surfaces, (3) is transported into the bacteria and (4) oxidizes during the course of the experiment. We suggest that the uptake of the ^{54}Mn(II) tracer in the absence of oxygen represents exchange between the dissolved and solid pools of Mn(II); notice that the uptake reaches a plateau after 8 hours (Figure 1a). The difference between the no-O_2 and low O_2 experiments is interpreted as oxidation of manganese. This approach corrects for the relatively long time it takes for the tracer to achieve exchange equilibrium with the different Mn(II) pools. Further justification of this interpretation is presented below.

The initial rate of Mn(II) oxidation (V_o) is nearly linear for the first 4 hours; we derive the rate from the slope of the regression line drawn through the initial timepoints (Figure 1b). The decrease in Mn(II) removal after 8 hours in the presence of O_2 may in part be due to nutrient limitation in the experimental system. It is not due to Mn(II) limitation since less than 10–20% of the ^{54}Mn(II) is bound in a given experiment.

Justification of the method used

The interpretation of the results of our experiments depends on the validity of our assumption that the rate derived from the difference in the amount of ^{54}Mn(II) bound between oxygen-containing and no-oxygen incubations is actually the in situ Mn(II) oxidation rate rather than a result due to one or a combination of the following factors: (1) a difference in the rate of exchange of the ^{54}Mn(II) tracer and the background manganese in solution, (2) adsorption onto particle surfaces, (3) transport into cells, (4) the

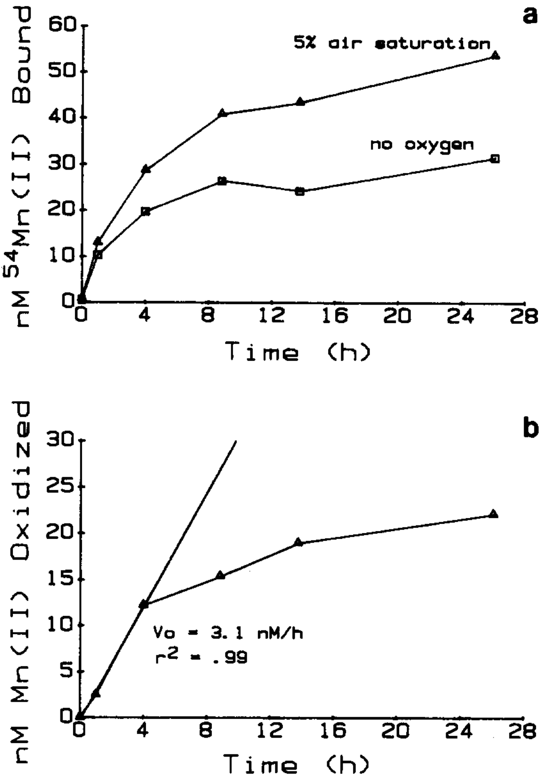

Figure 1. (a) An example of the time course of ^{54}Mn(II) binding under conditions of no oxygen and 5% air saturation (the in situ oxygen tension. (b) The same data displayed in (a) except that the no-O_2 values have been subtracted from the 5% air saturation value. We interpret this as the amount of Mn(II) oxidized. Data were obtained as described in Methods.

oxidation of Mn(II) by electron acceptors other than oxygen or (5) manganese reduction in the no-oxygen incubations. We address each of these points individually:

(1) We tested the degree to which the ^{54}Mn tracer was behaving as the non-radioactive Mn(II) by comparing the rate derived from a radioactive experiment with the rate of change determined directly by measuring the Mn(II) concentration. Within the analytical precision of the Mn(II) measurements (± 10%) there was no measurable difference (Tebo and Emerson, 1985).

(2) Since the O_2/H_2S interface in Saanich Inlet is near steady state with respect to Mn oxidation (see Emerson et al., 1979), particulate material should be near equlibrium with regard to simple inorganic adsorption. Consequently, most of the ^{54}Mn(II) removal observed in the no-oxygen incubations should be due to isotopic exchange and there is no reason to expect more enhanced adsorption in experiments

containing oxygen. We showed in a previous paper (Tebo and Emerson, 1985) that sonication inhibited 85% of the binding in no-oxygen experiments indicating that the binding is primarily biological; sonication should, if anything, increase adsorption to inorganic surfaces by increasing the surface area. Thus, adsorption can only represent a small portion of the total binding observed.

(3) Since Mn(II) is a required trace element it could be argued that the observed differences are due to oxygen dependent Mn(II) transport into bacterial cells. We believe this to be unlikely because our observations indicate that the manganese oxides always form on the outside surface of bacteria (Nealson and Tebo, 1980; Emerson et al., 1982; Tebo, 1983). The oxidized Mn is often associated with bacterial extracellular matrices (e.g., polysaccharides, sheaths, etc.; see Tebo (1983) and Ghiorse (1984) and references cited therein) although in some instances proteins or other metabolites excreted into the medium can oxidize Mn(II) (Adams and Ghiorse, 1985; Marshall, 1979). Particulate Mn has never been observed within cells. Although some Mn(II) uptake undoubtedly occurs because Mn(II) is required as a cofactor for many enzymatic reactions, the rates we observe in Saanich Inlet can not be explained by the intracellular transport of Mn(II). The most extreme case of an internal Mn(II) concentration known is for the lactic acid bacterium *Lactobacillus plantarum*, an aerotolerant obligate fermentor. *L. plantarum* can have an intracellular Mn(II) concentration as high as 25 mM; the Mn(II) is used to protect the cells from the toxic effects of superoxide radicals (Kono and Fridovich, 1983). Assuming these bacteria are 1 μm spheres, even if all 10^9 cells per liter in Saanich Inlet (Emerson et al., 1982) were this one species, the contribution to the particulate Mn would only be 12.5 nM and thus a doubling time of 2 h would be needed to account for the uptake rate. This a rather fast growth rate even for nearshore environments and all 10^9 bacteria in Saanich Inlet are not the same species (Tebo, 1983). Thus, the rate of Mn(II) oxidation that we measure is much too fast to be explained by cellular transport, whether such a process is affected by oxygen tension or not.

(4) There is the possibility that Mn(II) is being oxidized by an alternate electron acceptor in the no-oxygen experiments. Since the concentrations (dissolved or particulate) of other metals (e.g., Fe) in the particulate layer are extremely low (Emerson et al., 1979; Jacobs, 1984), the only other plausible electron acceptor would be nitrate. This type of artifact would lead to an underestimation of the oxidation rate as we measure it. However, additon of azide to our no-oxygen incubations had little or no effect on the rate or extent of Mn(II) binding (data not shown). Similarly, previous experiments with closed bottle incubations (the water contained ~ 25 μM nitrate)

indicated that low level Mn(II) binding in the absence of oxygen was not inhibited by azide (Tebo et al., 1984). Thus, if Mn(II) is being oxidized by nitrate it is only a negligible contribution to the total oxidative process.

(5) Finally, there is the possibility that Mn reduction was occurring in the no-oxygen incubations but not in the oxic incubations thereby causing us to overestimate the oxidation rate. If Mn reduction were occurring we would expect to see a decrease in ^{54}Mn(II) bound (after an initial increase due to isotopic exchange) with time. This was not observed. Secondly, in the experiment to ensure that ^{54}Mn was a tracer for total Mn(II) in the samples (see (1) above), we did not see an increase in dissolved Mn(II) in the no-oxygen experiment indicating that Mn oxide reduction was not occurring. Since this experiment was conducted with water from just above the particulate Mn maximum which is the region we are investigating in this paper, we feel confident that our rate determinations for the oxidizing zone (above and including the particulate Mn maximum) are accurate.

While this method provides a rapid and sensitive way of measuring the in situ Mn(II) oxidation rate, it does not, by itself, demonstrate that the process is microbially catalyzed. Microbial catalysis has been demonstrated previously by showing inhibition of Mn(II) oxidation by high Mn(II) concentrations, high temperature (Tebo and Emerson, 1985) and poisons which inhibit microbial activity without interfering in the solution chemistry of manganese (Emerson et al., 1982; Tebo, 1983; Rosson et al., 1984; Tebo et al., 1984).

Chemical analyses

To be sure the N_2/CO_2 gas mixture used in the no-O_2 experiments was devoid of traces of oxygen we purged a Mn(II) reagent used for Winkler oxygen analysis with 50 to 100 liters of the gas mixture and titrated the reagent to measure the amount of O_2 which has been reduced by the reagent. The concentration of O_2 in the control gas mixture was always less than 4.5 nM and below the detection limit (0.5 μM) as measured (Broenkow and Cline, 1969) directly in the no-oxygen incubations. The level of O_2 in the other experiments was regulated to in situ levels of $\sim 15\,\mu$M (measured) with gas mixtures and a gas mixing pump. Mn(II) was analyzed for each water sample either colorimetrically (Brewer and Spencer, 1971) or by direct injection into graphite furnace with Zeeman background correction on a Perkin Elmer 5000 atomic adsorption spectrophotometer. Analysis of oxygen (Broenkow and Cline, 1969) and hydrogen sulfide (Cline, 1969) were by spectrophotometric methods.

Results and discussion

The depth distribution of manganese (dissolved and particulate) for three cruises to Saanich Inlet in the summer of 1983 are presented in Figure 2.

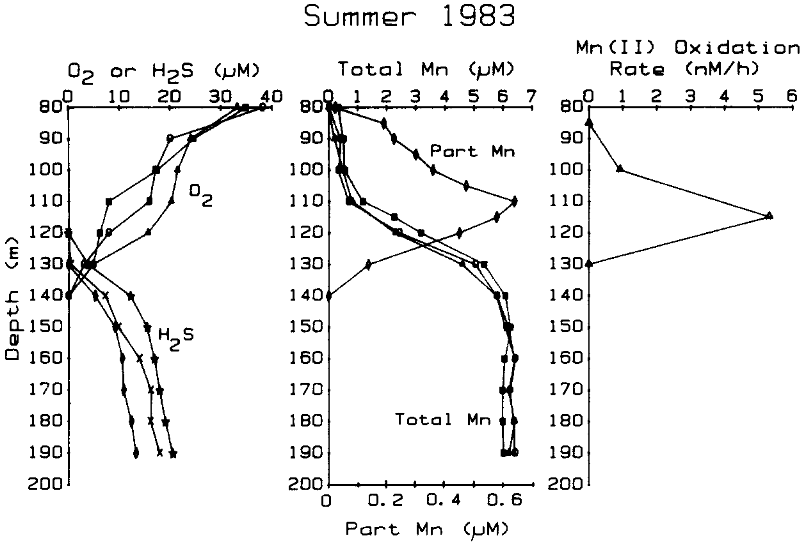

Figure 2. Summary of Mn(II), particulate Mn, O_2, and H_2S for 3 cruises in the summer of 1983 (△, ◇ 7/7/83; ○, x 7/19/83; □, ☆ 8/12/83). The particulate Mn and oxidation rate data as a function of depth is presented for the August cruise.

The maximum oxidation rate in August 1983 was at or just below the Mn particulate peak and well above the O_2/H_2S interface. This value was about $6\,\mathrm{nM \cdot h^{-1}}$ which is very similar to the rates measured in 1981 using an in situ sampler incubation device (Taylor et al., 1983; Tebo et al., 1985).

As a first step in quantifying the experimental observations from Saanich Inlet we use a model suggested by the data and analogous to the Langmuir isotherm for surface catalyzed gas reactions or the Michaelis-Menten Model for enzyme kinetics (Hiemenz 1977; Lehninger, 1975). There is nothing inherently unique about this model as similar mathematics have been applied to describe both homogenous and heterogeneous reaction catalysis for many years. Since microbial Mn(II) oxidation appears to be a surface mediated process (Mn oxides are usually found coating the bacteria) such a model seems particularly suited to this study. We assume a two step process in which dissolved manganese is adsorbed by an exchange site, E, and then oxidized:

$$\mathrm{Mn(II)} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} \mathrm{Mn(II)\text{-}E} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} \mathrm{Mn_{oxidized}} + \mathrm{E} \qquad (1)$$

This reaction sequence is supported by laboratory studies of Mn(II) oxidation by spores of a marine bacillus (Hastings and Emerson, 1984).

Since Mn oxide reduction can occur both chemically and biologically in the presence of low levels of oxygen (Stone and Morgan, 1984; Marshall, 1979), we choose to model only the oxidizing zone, the region above and including the depth where the Mn(II) oxidation rate is fastest (this approximately coincides with the region at and above the particulate Mn maximum). At these depths k_{-2} should be much less than k_2, and the equation for the change in [Mn(II) – E] with respect to time is:

$$\frac{d[\mathrm{Mn(II)}-\mathrm{E}]}{dt} = k_1\,[\mathrm{Mn(II)}]\,[\mathrm{E}] - k_{-1}\,[\mathrm{Mn(II)}-\mathrm{E}] \qquad (2)$$
$$- k_2\,[\mathrm{Mn(II)}-\mathrm{E}]$$

Mass balances for the exchange sites and manganese are:

$$\begin{aligned} \Sigma\mathrm{E} &= [\mathrm{E}] + [\mathrm{Mn(II)}-\mathrm{E}] \\ \Sigma\mathrm{Mn} &= [\mathrm{Mn(II)}] + [\mathrm{Mn(II)}-\mathrm{E}] \end{aligned} \qquad (3)$$

Since the amount of ^{54}Mn(II) bound is always less than 20% of the total, ΣMn is approximately equal to [Mn(II)]. Now, at steady state, the ratio of the rate constants is defined as:

$$\frac{[\mathrm{Mn(II)}]\,(\Sigma\mathrm{E} - (\mathrm{Mn(II)}-\mathrm{E}]\,)}{[\mathrm{Mn(II)}-\mathrm{E}]} = \frac{k_{-1} + k_2}{k_1} = K_m \qquad (4)$$

where K_m is the half saturation constant for Mn(II) oxidation (the Michaelis-Menten constant; Lehninger 1975). The initial (V_o) and maximum (V_{max}) rates of manganese oxidation,

$$\begin{aligned} V_o &= k_2\,[\mathrm{Mn(II)}-\mathrm{E}] \qquad (5) \\ V_{max} &= k_2\,[\Sigma\mathrm{E}] \end{aligned}$$

can be combined with equation (4) to give:

$$V_o = \frac{V_{max}\,[\mathrm{Mn(II)}]}{K_{m+}[\mathrm{Mn(II)}]} \qquad (7)$$

which is the Michaelis-Menten equation (Lehninger, 1975).

We use equation (7) to interpret the data from the Saanich Inlet radiotracer experiments. V_o is the difference in the rate of ^{54}Mn(II) removal between oxygen containing and no-O_2 experiments. Since Mn(II) varies with depth, profiles provide a measure of V_o as a function of dissolved manganese; however, we must assumed that ΣE is relatively constant. We make this assumption rather than follow the alternative approach in which exogenous additions of substrate are made to incubations (Parsons and Strickland, 1962; Wright and Hobbie, 1965, 1966). Williams (1973) has pointed out that for mixed populations of bacteria additions of high substrate concentrations can lead to large errors in estimating turnover times and K values and we have shown that addition of 10–18 μM Mn(II) inhibits Mn(II) oxidation (Tebo et al., 1984; Tebo and Emerson, 1985). Considering the nature of the environment that we study, which is probably as biologically stratified as it is chemically, we are undoubtedly introducing some error in the interpretation by assuming that ΣE is constant over the oxidizing zone. However, we model the data over a narrow depth range in which the particulate Mn concentration varies by only a factor of two or three.

Table 1. Summary of experimentally derived model parameters

Expt # (date)	# of data points	depth range (m)	$^*k_2$ range (h^{-1})	K_m (μM)	V_{max} ($nM \cdot h^{-1}$)	ΣE (nM)	$^\dagger r^2$
[1]II, III, V–VII (1983)	7	95–115	0.05–0.70	0.59	7.41	11–148	0.82
[2]VIII–X (July 1984)	5	100–115	0.05–0.76	0.43	4.52	6–90	0.86
[3]XI (Aug 1984)	5	85–105	0.04–0.21	0.22	3.50	17–84	0.92
[4]XII (Aug 1984)	9	90–100	0.09–0.28	0.89	12.05	44–127	0.82
[5]XI–XIII (Aug 1984)	9	85–105	0.04–0.28	0.62	9.43	34–224	0.96
[6]XI–XIII (Aug 1984)	17	85–105	0.04–0.28	0.51	7.69	28–183	0.90

*calculated from equation (5), where [Mn(II)–E] is the amount of Mn(II) bound in the no oxygen experiments.

†least squares correlation coefficeint for the double reciprocal plot from which K_m and V_{max} are derived.

[1] results from experiments with no added Mn(II) conducted on three different cruises in 1983.

[2] results from three experiments with no added Mn(II).

[3] results from an individual experiment with no added Mn(II).

[4] results from an individual experiment with added Mn(II).

[5] results from three experiments with no added Mn(II).

[6] results of all experiments in Aug 1984 (including the Mn(II) addition experiment).

Values of K_m and V_{max} were derived from double reciprocal plots of the results obtained during the summers of 1983 and 1984 (Table 1). Also included in this table are the data derived from experiments in which Mn(II) concentrations were only slightly increased (by 0.1 and 0.2 μM) at three different depths (experiment #XII). (Data for all the experiments conducted in 1983 and 1984 are presented in a separate paper, Tebo and Emerson, 1985). K_m and V_{max} vary by a factor of three to four over the three different cruises in two summers: $K_m = 0.22–0.89\,\mu M$ and $V_{max} = 4.5–12.1\,nM \cdot h^{-1}$. We believe that this shows remarkable consistancy and indicates that the experimental approach provides reproducible results.

The flux of Mn(II) to the oxidizing zone can be calculated using the manganese gradients from 1983 (Figure 2) and a value for the eddy diffusion coefficient of $1\,cm^2 \cdot s^{-1}$ (Emerson et al., 1982). The flux is between 4.6 and $12.7\,nM \cdot h^{-1}$. This calculation demonstrates the internal consistency between the experiments and flux estimates and suggests that the bacteria are working near their V_{max}. If this is true then the manganese oxidation rate in this environment is a function of the value for total binding sites (ΣE) as well as the manganese concentration. At the depth where Mn(II) oxidation is most rapid, the bacteria are working at Mn(II) concentrations higher than their apparent half saturation, K_m, and thus out of the range of substrate concentration where the reaction is first order.

The value of the oxidation rate constant, k_2, can be calculated by rearranging equation (5), i.e., $k_2 = V_o/[Mn(II)–E]$ where $[Mn(II)–E]$ is the amount of $^{54}Mn(II)$ bound in the no-oxygen experiments and V_o is the initial oxidation rate. Since these values vary with depth they are given as a range in Table 1. Then, using the results for V_{max} and k_2, a value for ΣE can be calculated using equation (6). The range in ΣE is also presented in Table 1.

In the no oxygen experiments $k_2 = 0$ so equation (4) becomes:

$$\frac{[Mn(II)](\Sigma E - [Mn(II)–E])}{[Mn(II)–E]} = \frac{k_{-1}}{k_1} = K_s \qquad (8)$$

or

$$\frac{[Mn(II)]}{[Mn(II)–E]} = [Mn(II)](1/\Sigma E) + K_s/\Sigma E \qquad (9)$$

where K_s is the apparent equilibrium binding constant and $[Mn(II)]/[Mn(II)–E]$ is the reciprocal of the fraction of $^{54}Mn(II)$ bound in the absence of oxygen in the radiotracer experiments. By plotting 1/(fraction bound) versus the Mn(II) concentration we can determine K_s and ΣE (Figure 3). The value for K_s ($= 0.18\,\mu M$) is close to the lower limit of the range of values for K_m and, considering the error of K_s, probably overlaps. If $K_s \approx K_m$ then the oxidation rate term, k_2, is small with respect to k_{-1} and the oxidation

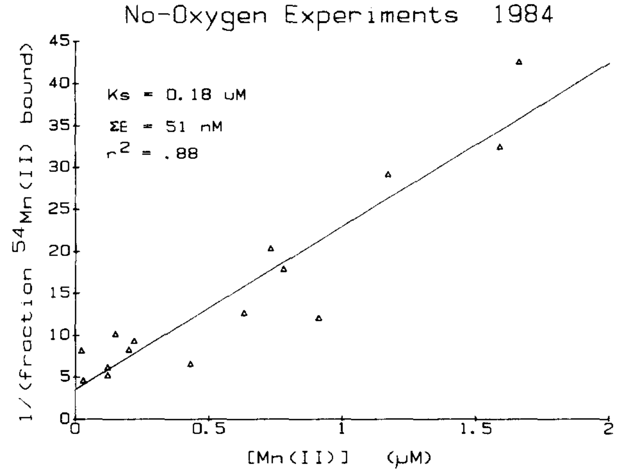

Figure 3. Dissolved Mn(II) plotted vs 1/(fraction bound) for all the experiments with no oxygen. The linear correlation ($r^2 = 0.88$) has a slope = 0.194 and y-intercept = 0.035. From these values the total binding sites (ΣE) and apparent equilibrium binding constant (K_s) were determined.

rate, rather than adsorption or exchange is the rate limiting mechanism for the bacterial catalysis of manganese oxidation. ΣE calculated from these no oxygen experiments is ~ 51 nM, in agreement with the values estimated from the oxic experiments (Table 1).

The data interpretation by itself, does not demonstrate biological oxidation; however, this has been well established in Saanich Inlet by other means. The success of a simple kinetic model supports the two step mechanism of Mn(II) oxidation and suggests that oxidation rather than adsorption or exchange is the rate limiting step. This approach leads to the conclusion that the Mn(II) oxidation rate in Saanich Inlet is limited by the number of microbial Mn(II) binding sites (or total number of Mn(II) binding and oxidizing bacteria). For this to be true, oxygen or some nutrient other than Mn(II) must be limiting the growth of Mn(II)-oxidizing bacteria or alternatively, Mn(II) oxidation in this environment is not providing the energy necessary for growth of the bacteria which catalyze the reaction.

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