# Cadmium and zinc interactions with a Gram-positive soil bacterium

from variable charging behavior of the cell wall
to
bioavailability of heavy metals in soils

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The bacterium is obtained from Dr. H.H.M. Rijnaarts (at that time, Department of Microbiology, Agricultural University Wageningen). The experiments with the intact, living cells are performed at the Department of Ecotoxicology of the Institute of Forestry and Nature Research in Arnhem, The Netherlands. Experimental work with the isolated cell wall material was done at several departments of the Agricultural University in Wageningen, The Netherlands. Destruction of the cells was performed at the Department of Microbiology, with help of ir. A. van der Wal of the Department of Physical and Colloid Chemistry, who offered the procedures and materials for isolating the cell walls and for analyzing the quality of the isolated material. Proton and metal ion titrations with the isolated cell wall material were performed at the Department of Soil Science and Plant Nutrition. Toxicity experiments are performed by Dr. P. van Beelen and A. Fleuren-Kemilä of the Department of Ecotoxicology of the National Institute of Public Health and Environmental Protection, Bilthoven, the Netherlands.



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# Stellingen

1. De biologische beschikbaarheid van zware metalen in de bodem is niet volledig gekarakteriseerd door de activiteit van het vrij metaal-ion in de bodemoplossing.
   *Dit proefschrift.*

2. Om factoren als pH te verwerken in ecotoxicologisch onderbouwde normen voor zware metalen in de bodem, moet ook de invloed van deze factoren op de sorptie en opname door de toetsorganismen worden bestudeerd.
   *N.M. van Straalen and W.F. Bergema, Ecological risks of increased bioavailability of metals under soil acidification. Pedobiologia, 39: 1 (1995).*

3. Het gedrag van een metaal-ion in de bodem lijkt soms onvoorspelbaar, maar is na studie van de metaalbinding door de afzonderlijke bodemcomponenten niet onverklaarbaar.

4. Modelberekeningen ver buiten de range aan condities waarbij metingen zijn verricht, kunnen snel tot onjuiste voorspellingen leiden.
   *Dit proefschrift.*

5. Numerieke procedures voor parameterschatting zijn slechts beperkt bruikbaar.
   *Dit proefschrift.*

6. Experimenten t.a.v. initiële bacteriële hechting, uitgevoerd in een 1:1 elektrolyt milieu, kunnen een verkeerde inschatting geven van hechting in een natuurlijk bodemmilieu.
   *Dit proefschrift, n.a.v.: H. Rijnaarts, Interactions between bacteria and solid surfaces in relation to bacterial transport in porous media, Thesis W.A.U. (1994); F.Huysman and W. Verstraete, Biol. Fertil Soils, 16: 21 (1993).*

7. Het $K_D$-concept doet geen recht aan de niet-lineariteit van de meeste sorptieprocessen.

8. "Le sol est un être vivant".

9. Arbeidsparticipatie gaat moeilijk samen met mobiliteitsreductie.

10. "Roeien met de riemen die je hebt", is een mooi streven, maar kan fors beperkend zijn voor de voortgang.

11. Stilte kan meer zeggen dan woorden.

12. In wezen is schrijven niets anders dan de lezer verleiden tot het uitlezen van een bepaalde tekst.
    *Sarah Verroen, Volkskrant 31 dec 1993.*

Stellingen behorend bij het proefschrift "Cadmium and zinc interactions with a Gram-positive soil bacterium, from variable charging behavior of the cell wall to bioavailability of heavy metals in soils". A.C.C. Plette, Wageningen, 10 mei 1996.

# Voorwoord

Het is inmiddels al weer ruim 5 jaar geleden dat ik mijn eerste werkdag begon op het IBN in Arnhem. Het was snijdend koud buiten, als ik opkeek van het leeswerk, keek ik uit over een besneeuwd dak, en kale bossen die goudachtig leken in het late winterzonnetje. In die vijf jaar heb ik heel wat uitzichten gehad vanaf diverse werkplekken. En zo heb ik in de loop van de tijd lief en leed gedeeld met een groot aantal mensen. Eerst anderhalf jaar in Arnhem. Deels op het "Lab van Lieuwe", waar later ook René Rietra wat ruimte claimde voor dode wormen en vieze grond (ongelooflijk, dat dat samen kon gaan met steriel werken!). Deels op een kamertje aan de andere kant van het gebouw, dat ik heel genoeglijk deelde met Nico van den Brink. Daarna wisselde ik elke paar maanden van werkplek. Dan weer wat rekenen en schrijven in Wageningen op de vakgroep, of titraties uitvoeren op het lab van René Janssen of in het klimaathok van Peter Venema, dan weer extra data vergaren in Arnhem. In Wageningen eerst een gezellige drukte met Maarten Nederlof, Ron Beuger en Wilma Visser op één kamer. Gedurende de tijdelijke verhuizing naar het Staringgebouw heb ik een zonnige kamer gedeeld met Hans van der Lee, en uiteindelijk, in de aangenaam veranderde kelder van het scheikunde gebouw, heb ik alle perikelen rondom de laatste loodjes kunnen delen met Wendela Schlebaum. Hoewel ik om de paar maanden van werkplek veranderde, was het altijd meteen weer alsof ik nooit weg was geweest. Iedereen, van het IBN en de vakgroep, enorm bedankt voor alle gezelligheid en het telkens wederkerende warme welkom.
Terwijl ik dit schrijf, kijk ik uit over de rode pannendaken van Harderwijk.
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De afgelopen vijf jaar gaven me niet alleen steeds wisselende uitzichten, ze hebben ook tot nieuwe inzichten geleid, waarvan dit boekje deels een overzicht geeft.
Ik koester goede herinneringen aan de oude uitzichten, en verheug me op nieuwe vergezichten.

Sandra.

# Abstract

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A detailed study is presented on the cadmium and zinc sorption to both isolated cell walls and intact, living cells of the Gram-positive soil bacterium *Rhodococcus erythropolis* A177. Acid/base titrations were performed on isolated cell wall material to characterize the type and amount of reactive sites on the cell wall. The proton binding was described with a three modal Langmuir-Freundlich equation, combined with a Donnan model to correct for the electrostatic interactions. Cadmium and zinc sorption to the isolated cell walls was reduced with increasing proton or calcium concentration. During the metal ion sorption, desorption of protons and calcium ions was observed. Calculations showed, that at high coverage with bivalent ions, charge reversal takes place. On the basis of the charging behavior, a competitive binding model, the NICA equation, was selected to describe the sorption data. The model is used to predict the sorption of cadmium and zinc to intact, living cells of the bacterium. The trends of sorption studies with intact cells at two exposure times suggest competitive interactions, not only for the adsorption of the metal ions to the cell wall, but also for the uptake into the cell. The impact of this competitive binding is reflected in the different levels of toxicity experienced for different pH conditions. A good correlation was found between cell wall adsorption and toxicity, indicating that for this organism, total body burden is a good indicator for potential effects, resulting from exposure to heavy metals. In combination with model descriptions of cadmium binding to a sandy and a clay soil, the influence of pH and calcium concentration on sorption to the bacterium when present in soil is predicted. Results show, that the impact of pH and calcium concentration is of the same order as the impact of soil type. It is quite obvious, that bioavailability is not only determined by the free metal ion concentration in solution, but that other parameters like the pH and the concentration of competing ions in solution play an important role as well.

Additional index words: Donnan volume, Donnan potential, peptidoglycan, chemical heterogeneity, intrinsic proton affinity distribution, specific ion binding, speciation, copper, soil biota.

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# Chapter 1

# General introduction

# General introduction

Total heavy metal contents of soil are often used to express the degree of contamination. However, they are of little value to judge environmental effects. Many environmental factors influence the availability of the metal in the soil for the biota present in that soil. Apart from the soil type, the pH and the composition of the soil solution (e.g., the concentration calcium) are important factors that influence the mobility, and thus the availability, of metal ions in soil. In agricultural soils, these factors are kept within certain limits via liming and the addition of fertilizers. In natural ecosystems, these factors may vary widely. Relatively extreme changes can take place in agricultural land that is temporarily set aside, or that is transformed into "new nature". Especially in these area's, it is of major importance that the ecological processes continue, and that they are not disturbed due to the increased availability of the heavy metals present in the soil. In order to be able to predict the impact of changing environmental conditions, a quantitative and mechanistic insight in the influence of soil parameters (like the pH and the concentration competing ions) on the metal sorption behavior of both the abiotic and the biotic components must be obtained. Concerning the metal ion binding to the major abiotic soil components, numerous studies have been performed. However, for biota, little research has been done on the binding and uptake of metal ions as a function of environmental conditions.
In this chapter, a short outline is given of the nature and origin of heavy metals, of the function of soil biota, and of the interactions that may take place between heavy metal ions in solution and soil bacteria. An introduction will be given on the subjects that will be treated in detail in the other chapters of this thesis.

## Heavy metals; cadmium and zinc

Heavy metals are elements like cadmium, copper and lead. They belong to the so-called trace elements, i.e., elements that occur in natural and perturbed systems in small amounts. "Heavy metals" usually refers to elements having densities greater than 5.0 (1). At low concentrations, the metals will not have an impact on living organisms. A number of heavy metals are even essential micronutrients (e.g., Cu, Zn, Co). For other heavy metals (e.g., Cd, Pb) no function in living organisms is found yet. But, when present in sufficient concentrations, all heavy metals are toxic to living organisms.
Apart from the metals present in soil parent material, all sources of heavy metals are anthropogenic in nature. On agricultural lands, the heavy metal content of the

soil is increased due to the application of commercial fertilizers, liming materials, sewage sludge and animal wastes. More diffuse sources of heavy metals are, e.g., the metallurgic industries, waste burning and auto exhaust emissions. These emissions may have an impact on natural ecosystems, as well as on urban and rural areas. Zinc smelters have often lead to a sizable input of metals like Zn, Cd, Cu and Pb into the environment. The metals emitted in the air are readily deposed via raindrops and dust. The impact of atmospheric discharges from smelters can be detected within several kilometers from the point of release. Roadside soils have been shown to be contaminated with various trace metals primarily from automobile exhaust. It includes Pb, Zn, Cd, Cu, and Ni, the more important being Pb from fuel and Zn from tires. Depending on the location and traffic intensity, contaminated zones can extend up to several hundred meters from the road (1, 2). A third source of heavy metals are contaminated river waters, from which metal loaded particles are settled in river flood plains.

From 1970, a reduction of the emission of heavy metals into the environment has been realized. But, since metal ions in soil are strongly held on inorganic and organic exchange sites on the soil components, and since the metals are not biodegradable, they are very persistent in nature, and thus still present in contaminated areas. A change in environmental conditions, e.g., a decrease of the soil pH, may result in mobilization of the retained heavy metals, leading to an increased exposure for soil biota.

In this thesis, the experimental work is confined to cadmium and zinc. Cadmium and zinc share a number of physical and chemical characteristics. In addition, they are usually mined and refined together, since cadmium is essentially an impurity (in the ratio 1:200, Cd:Zn) in zinc ores (3). In the Netherlands, wet deposition of cadmium in 1991 was on average 12 $mmol.ha^{-1}.year^{-1}$. In the province of Zuid-Limburg the highest values were obtained: in this region the average Cd-deposition was 18-21 $mmol.ha^{-1}.year^{-1}$. Wet deposition of zinc in 1991 was on average 2600 $mmol.ha^{-1}.year^{-1}$, with a maximum in the south-west of the Netherlands of almost double this amount (2).

Usually, cadmium in soil is accompanied by a much larger quantity of zinc. Field data concerning uncontaminated soils has shown that both for total metal present in soil (4), and for the concentration in soil solution (5) the difference in cadmium and zinc concentrations is about two to three orders of magnitude, depending on the soil type. In polluted soils, the ratio cadmium to zinc is shifted generally more towards the cadmium.

As for the biological function, the two elements are quite different. Zinc is an essential micronutrient for microorganisms, plants and animals. However, in high

concentrations zinc is also toxic for living systems. Cadmium has no known biological functions. Bååth (6) compiled data on effects of heavy metals in soil on microbial processes and populations. Cadmium commonly was found to be more toxic than zinc (on a $\mu g.g^{-1}$ soil basis). Cadmium and zinc appear to compete for certain biological ligands, accounting in part for the toxic effects of cadmium and the ameliorative effects of zinc on cadmium toxicity (1). Due to their different function, it will be interesting to compare binding and uptake of these two metals.

## Soil biota; ecological function

In agricultural systems, soil fertility can be increased by application of inorganic or organic fertilizers. However, the fertility of natural ecosystems depends almost entirely on natural biological processes, e.g., $N_2$-fixation, N-mineralization and organic matter transformations, mediated by the microbial biomass. In order to give an impression of the type and amount of organisms, a diagram is presented below, indicating the weight percentage of all (biotic and a-biotic) components in a soil:

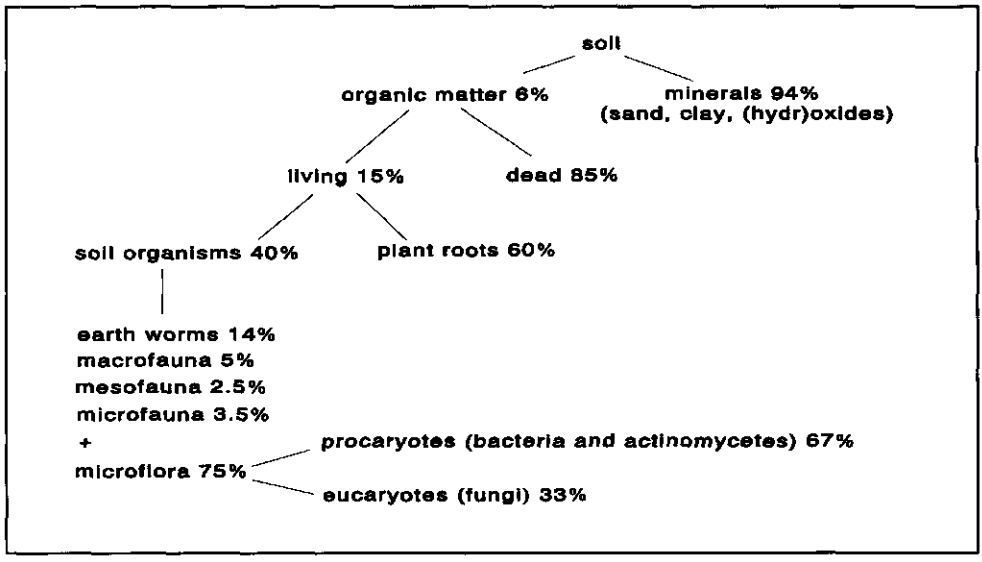


**Fig. 1** Weight percentage of all (biotic and a-biotic) components in a soil (adapted from v.d. Drift (7)).

The main function of the soil fauna is the mechanical fractionation of dead plant and animal material, and the mixing of the organic material through the soil profile. The final mineralization of the organic matter is almost exclusively a microbial process (7). In turn, predators of microorganisms may attribute to the turnover of microbial organic material. E.g., soil mites are capable of digesting fungal material (8). Heavy metal contamination may inhibit the microbial activity, and thus reduce this natural fertility (9). In addition, heavy metals may accumulate in the microorganism, which may result in an increased exposure for their predators as well. In general, prokaryotes (bacteria and actinomycetes) are more sensitive to heavy metal pollution of soil than eukaryotes (fungi), and bacteria are more sensitive than actinomycetes (10).

A method to determine the impact of heavy metal pollution on soil microbial communities is to asses the number of metal-resistant bacteria isolated from an environment affected by heavy metals, as bacteria are capable of rapidly responding to changes in their environment. If a significant proportion of the bacterial population is resistant to a high concentration of the metal, the judgement is made that the soil is negatively affected by the presence of this metal (11, 12). A loss of biochemical capacities has been observed in metal contaminated soils. It has been shown repeatedly that sensitive bacteria grow significantly better than resistant bacteria on a range of selected aromatic compounds (12). Thus, resistance for heavy metals may lead to a reduced natural fertility, and to a reduction of the bioremediation capacity towards organic contaminants (13, 14).

In search of uniform criteria for the judgement of the seriousness of soil contamination, target and intervention values for a wide number of pollutants have been introduced for the Netherlands environmental policy. Target values are based on measurements of pollutant concentrations in soils of natural environments. Intervention values are based on estimates of the actual risk for both man and ecosystem due to exposure to soil pollution. At the intervention value, the maximum permissible risk level (MPR) is exceeded. Above the intervention value, the soil is judged seriously contaminated (15).

The ecological risk of a chemical in the environment can be estimated as the probability that a randomly selected species is being exposed to a concentration greater than its no-effect-level for that chemical. It is assumed that the ecosystem is protected when 95% of the species in the ecosystem is not negatively affected by the pollutant. Thus, the MPR for ecosystems is defined as the concentration at which for 5% of the species the NOEC (no observed effect concentration) is exceeded (16). The intervention value is established at the concentration at which 50% of the species experience harmful effects, which is far beyond the MPR. In the

case that the pollutant concentration in soil or in the ground water exceeds the concentration as defined, soil will have lost important functional characteristics which are essential for man, plant or animal. Target and intervention values are dependent on the soil type; the weight percentage of clay and organic matter is included in the calculation of these soil dependent values. For a standard soil (25% clay, 10% organic matter), target values for cadmium and zinc are respectively 0.8 and 140 $mg.kg^{-1}$ dry soil, and 0.4 respectively 65 $\mu g.l^{-1}$ for groundwater. Intervention values are respectively 12 $mg.kg^{-1}$ and 720 $mg.kg^{-1}$. Intervention values for groundwater are respectively 6 $\mu g.l^{-1}$ and 800 $\mu g.l^{-1}$ (15).
Van Straalen and Bergema (17) used relationships between soil pH and metal concentrations of earthworms to adjust NOEC-values from toxicity-experiments. The analysis showed that present soil quality standards for cadmium and lead are insufficient to protect the community of soil invertebrates at low pH.
Toxicity of cadmium to *Aspergillus niger* was increased when pH of the soil was increased, however, some other fungi were more tolerant in an acidic soil than in a more alkaline soil. The toxicity of cadmium for some bacteria and fungi was reduced when calcium was added to the solution (18).
More examples could be given here that show that environmental parameters like the soil pH and the calcium concentration in the soil solution have an impact on the toxicity experienced by the organisms present in a heavy-metal contaminated soil. The impact of these factors can be explained using the key words "speciation" and "bioavailability".

## Heavy metals in soil; Speciation and bioavailability

The relationship between the total metal content of the soil and its positive or negative effects on soil biota is not straightforward. It is well recognized that the speciation of metal ions, i.e., the distribution of the total metal content over all possible chemical forms (species) either in the solid, the liquid or the biotic phase of the soil, plays an important role with respect to bioavailability. This distribution is influenced by a great number of processes like adsorption, complexation and precipitation, which on their turn depend on properties of the metal, soil properties, and environmental conditions like pH, salt level, redox potential, concentration complexing ligands in solution, and the concentration of other metal ions that may compete for the same binding sites. In addition, specific characteristics of the biota may play an important role as well.

According to Morel (19), trace metal interactions with organisms can be understood by considering an organism as an assemblage of reactive ligands. The degree of complexation of these ligands with particular metals is reflected in measurable physiological effects. The amount of metal bound to an organism depends not only on the free metal ion concentration in solution, but also on the pH and on the concentration of other ions in solution that may compete for the binding sites on the organism. All interactions with the metal ion are considered to take place via the liquid phase. Interaction between a soil organism and metal ions present in soil can be easily understood by considering the organism to be an additional reactive soil component, with its own (pH and other environmental-factor dependent) binding characteristics. Sorption of the metal ion by the organism is thus the result of competition for that metal ion by all reactive components (including the organism) present in the system.

Major abiotic soil components involved in metal ion binding are clay minerals, metal (hydr)oxides of iron, aluminum and manganese, and soil organic matter (humic and fulvic acids). Already, considerable knowledge is available on metal ion binding to these soil components.

The functional groups present on soil organic matter supposedly resemble those present on roots of plants or on the surface of soil microorganisms. The organic matter in soil has a strongly pH dependent charge. With rising pH, more sites on the organic structure become available for metal ion binding. The metal ion binding to this type of materials has been studied in detail during the last decade (e.g., (20-23)). On the basis of the present knowledge, models could be developed that can describe and predict metal ion sorption to humic materials for a wide range of conditions (e.g., (24)). These models regard the material as a chemically strongly heterogeneous sorbent, of which each reactive site has it's own proton and metal ion affinity. The various ions present in solution may compete for binding to these sites. Since for soil biota, little information is available on the influence of factors like pH and concentration calcium on the sorption and uptake of the metal ions, models derived for metal ion binding to soil organic matter may be of value for the interpretation of data on metal ion binding by soil microorganisms.

## Interactions of heavy metals with a soil bacterium

Due to their function in recycling elements, their abundance, their shape and small size, and, consequently, their high surface area to volume ratio, microorganisms are the first group of soil organisms to be affected by diffuse soil pollution (12).

These characteristics make bacteria convenient organisms to study metal ion binding.

Two main types of bacteria can be distinguished on the basis of the structure of their outer layers, which can be identified by their reaction to Gram staining. For Gram-positive bacteria, this layer is a rather amorphous matrix of about 25 nm thick. The main component, forming the frame of the cell wall, is Peptidoglycan. Peptidoglycan is a very common structure, since it is a cell wall component in almost all procaryotic organisms (25).

Cell walls of Gram-negative bacteria are complex multilayered structures. They consist of an external outer membrane that overlies a thin layer of peptidoglycan (26).

In this thesis, most experimental work is performed with a Gram-positive, rod-shaped coryneform soil bacterium, isolated from river Rhine sediment (27). At that time, this bacterium was still considered to be an Arthrobacter. At present, the bacterium is classified as *Rhodococcus erythropolis* A177. Members of the genus Arthrobacter are among the dominant bacteria in soil, at least as assessed by the usual plating methods (28).

In Figure 2, all interactions between a Gram-positive soil bacterium and a metal ion in solution are visualized. First of all, the metal ion "meets" the cell wall.

The Gram-positive bacterial cell wall consists of a number of polymers containing various acidic groups, which may deprotonate with rising pH. Due to the deprotonation, an electronegative potential is developed in the cell wall. The extent to which this potential may develop, is dependent on the salt level and the valency of the ions in the bulk solution. This leads to a pH and salt dependent variable charge. On the basis of the charging behavior the type and number of reactive sites can be characterized. In chapter 2, a detailed study of the pH dependent charging behavior of isolated cell walls of R.A177 is presented.

The reactive sites on the cell wall may bind metal ions from solution. Two mechanisms of ion binding can be distinguished. The ion can be attracted by the negative potential in the cell wall. This electrostatic binding is non-specific, and only influenced by the valency of the ion. The second mechanism is specific binding of the ion to the reactive sites on the cell wall. This type of binding may even take place in the presence of a repulsive potential. In natural systems, a number of different cations is present in solution. Competition between these ions for binding to the cell wall will play a role of interest for the binding of the heavy metal ions. Chapter 3 deals with the competitive binding of protons, calcium, cadmium and zinc to isolated cell walls.

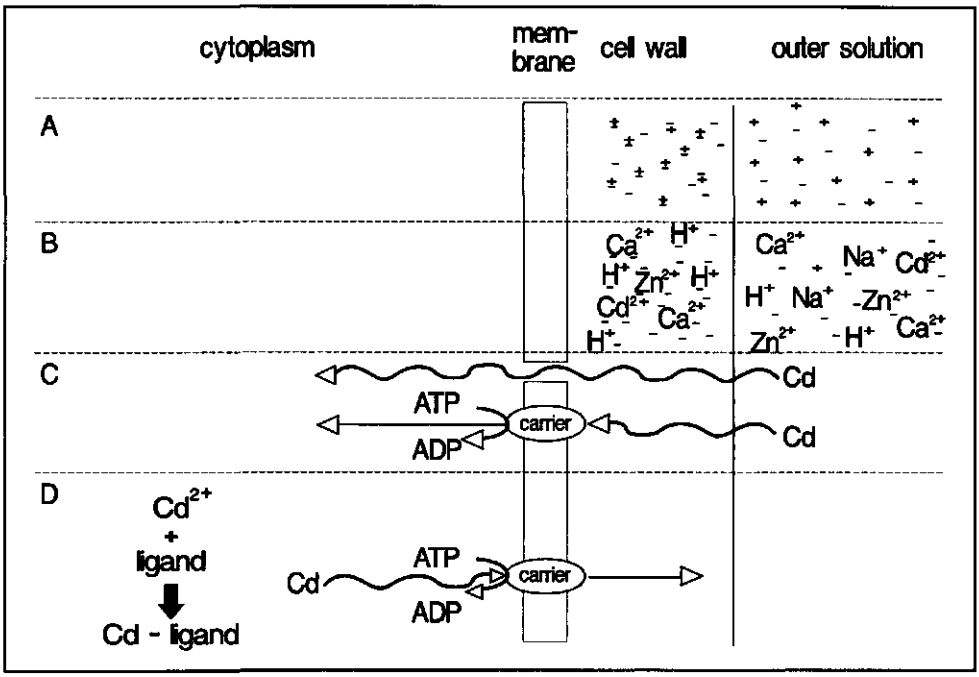


**Fig. 2** Schematic representation of the interactions of cadmium ions with a Gram-positive soil bacterium. A) pH dependent charge of the cell wall. B) Competition of cations for the binding sites on the cell wall. C) Passive and active uptake of cadmium into the cell. D) Detoxification mechanisms; facilitated precipitation with organic and/or inorganic ligands and active exclusion.

As long as an ion is bound to the reactive groups of the cell wall polymers, it will probably not have an important influence on the bacterium. But, once a metal ion arrives at the cytoplasmic membrane, it may react with functional groups on this membrane and it may be transported into the cell. The uptake may be passive, via diffusion through membrane pores, or active, via energy-dependent carrier-mechanisms. Due to the uptake, the internal concentration of the metal ion may reach toxic levels, leading to disfunctioning of the cell metabolism. However, concentrations of the free metal ion in the cytoplasm may be reduced due to complexation by inorganic or organic ligands (e.g., facilitated precipitation with phosphates and sulfates, complexation with proteins), or by active exclusion. In chapter 4 and 5, the work performed on intact, living cells of R.A177 is presented. Metal ion sorption to intact cells is the sum of two processes: the adsorption to the reactive groups on the bacterial cell wall, and uptake into the cell. Since the latter

process will depend on time, the kinetics of sorption to the intact cells are studied as well.
Up to here, all interactions studied took place via the soil solution. What will happen if a soil micro-organism is exposed to metal ions in its own environment, the soil? The metal ion sorption by organisms present in soil, and the influence of changing environmental conditions (e.g., pH), can be predicted once the binding of the metal ion to all components separately is known. In chapter 6, model predictions are presented, concerning metal ion binding from soil for a number of soil biota, among which the *Rhodococcus erythropolis* A177.

## References


1. Adriano, D.C., Trace elements in the terrestrial environment. Springer Verlag, New York, 533p (1986).
2. RIVM, Metingen van radioactiviteit en xenobiotische stoffen in het milieu in Nederland 1991. CCRX-report, Bilthoven, (1993).
3. Higgins, I.J., and Burnes, R.G., The chemistry and microbiology of pollution. Academic Press, London, 248p (1975).
4. Heinrichs, H., Schultz-Dobrick, B., and Wedepohl, K.H., Terrestrial geochemistry of Cd, Bi, Tl, Pb, Zn, and Rb. *Geochimica Cosmochimica Acta* **44**, 1519 (1980).
5. Goody, D.C., Shand, P., Kinniburgh, D.G., and van Riemsdijk, W.H., Field-based partition coefficients for trace elements in soil solutions. *European J. Soil Sci.* **46**, 265 (1995).
6. Bååth, E., Effects of heavy metals in soil on microbial processes and populations (a review). *Water, Air and Soil pollution*, **47**, 335 (1989).
7. van der Drift, J. Bodemfauna. In: van den Berg, C., de Haan, F.A.M., and Zeilmaker, D.A., (Eds.), Handboek voor milieubeheer, chapter IV-2.2.3.1 (1984).
8. Siepel, H., Are some mites more ecologically exposed to pollution than others? *Exp. Appl. Acarol.* **19**(7), 000 (1995).
9. Brookes, P.C., and Verstraete, W., The functioning of soil as an ecosystem. In: de Haan, F.A.M., *et al.*. (Eds.) Soil Quality Assessment, State of the art report on soil quality. EC Report XII/523/89. (1989).
10. Doelman, P., Resistance of soil microbial communities to heavy metals. In: Jensen, V., Kjöller, A., and Sörensen, L.H. (eds.) Microbial communities in soil, Elsevier, London 447p (1985).
11. Angle, J.S., Chaney, R.L., and Rhee, D., Bacterial resistance to heavy metals related to extractable and total metal concentrations in soil and media. Soil *Biol.Biochem.* **25**(10), 1443 (1993).
12. Doelman, P., Jansen, E., Michels, M., van Til, M., Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. *Biol. Fertil. Soils* **17**, 177 (1994).
13. Starzecka, A., and Bednarz, T., Comparison of development and metabolic activity of algae and bacteria in soils under the influence of short- and long-term contamination with metallurgic industrial dusts. *Algological studies* **70**, 71 (1993).
14. Burckhardt, C., Insam, H., Hutchinson, T.C., and Reber, H.H., Impact of heavy metals on the degradative capacities of soil bacterial communities. *Biol. Fertil. Soils* **16**, 154 (1993)
15. Leidraad bodembescherming, aflevering 10, juni 1995. Sdu Uitgeverij Den Haag, (1995).
16. van Straalen, N.M., and Denneman, C.A.J., Ecotoxicological evaluation of soil quality criteria. *Ecotox. Environ. Saf.* **18** 241 (1989).
17. van Straalen, N.M., and Bergema, W.F., Ecological risks of increased bioavailability of metals under soil acidification. *Pedobiologia* **39**, 1 (1995).
18. Babich, H., and Stotzky, G., Environmental factors that influence the toxicity of heavy metal and gaseous pollutants to microorganisms. *CRC Critical Reviews in Microbiology*, oktober (1980).

19. Morel, F.F.M., Trace Metals and Microorganisms. In: Morel, F.F.M. (ed.), Principles of Aquatic Chemistry. Wiley Interscience, New York, 446p (1983).
20. Tipping, E., Modeling the competition between alkaline earth cations and trace metal species for binding by humic substances. *Environ. Sci. Technol.* **27**, 520 (1993).
21. Marinsky, J.A., A two-phase model for the interpretation of proton and metal ion interaction with charged polyelectrolyte gels and their linear analogues. In: Stumm, W. (ed.), Aquatic surface chemistry, chemical processes at the particle-water interface. Wiley-Interscience, New York 520p (1987).
22. Westall, J.C., Jones, J.D., Turner, G.D., and Zachara, J.M., Models for association of metal ions with heterogeneous environmental sorbents. 1. Complexation of Co(II) by Leonardite humic acid as a function of pH and $NaClO_4$ concentration. *Environ. Sci. Technol.* **29**, 951 (1995).
23. de Wit, J.C.M., Nederlof, M.M., van Riemsdijk, W.H., and Koopal, L.K., Determination of $H^+$ and metal ion affinity distributions for humic substances. *Water, Air, and Soil Pollution* **57-58**, 339 (1991).
24. Benedetti, M.F., C.J. Milne, D.G. Kinniburgh, W.H. van Riemsdijk, and L.K. Koopal, Metal Ion Binding to Humic Substances: Application of the Non-Ideal Competitive Adsorption Model. *Environmental Science and Technology* **29**, 446 (1995).
25. Schleifer, K.H., and Kandler, O., Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**(4), 407 (1972).
26. Beveridge, T.J., Metal ions and bacteria. In: Beveridge, T.J., and Doyle, R. J. (eds.), Metal ions and bacteria. Wiley and Sons, New York, 461p (1989).
27. Schraa, G., Bethe, B.M., van Neerven, A.R.W., van den Tweel, W.J.J., van der Wende, E., and Zehnder, A.J.B., Degradation 1,2-dimethylbenzene by *Corynebacterium* strain C125. *Anthonie van Leeuwenhoek* **53**, 159 (1987).
28. Buchanan, R.E., and Gibbons, N.E., Bergey's manual of determinative bacteriology, 8th ed. Williams & Wilkins, Baltimore, 1268p (1974).

# Chapter 2

# Bacterial cell wall charge

pH dependent charging behavior of isolated cell walls of a Gram-positive soil bacterium

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# Bacterial cell wall charge

The cell walls of Gram-positive bacteria are highly porous structures. Peptidoglycan, the main component of these cell walls, contains many acidic groups, leading to a pH- and salt-dependent charge. This charge is involved in many processes, such as the attachment to surfaces and the binding of metal ions. Acid-base titrations are performed on cell wall material from *Rhodococcus erythropolis* A177, a Gram-positive soil bacterium. To describe proton binding to this material both salt effects and pH effects must be taken into account. A stepwise analysis, made by first eliminating the influence of the salt level and then analyzing the intrinsic protonation behavior, leads to a very good description of the original data. The specific volume of the cell walls is derived from charging curves measured at different salt levels using a master-curve approach in combination with a Donnan model to account for electrostatic effects. Results indicate that swelling becomes significant for salt levels below 0.1 M, and that above pH 5 at a given salt level the volume is independent of the particle charge. Donnan potentials obtained with this approach are close to potentials derived from electrophoretic mobility measurements.
The intrinsic proton affinity distribution of the protonation constants is obtained. Results indicate the presence of chemical heterogeneity. At least three different groups are distinguished. On the basis of this affinity distribution analysis a binding model, the Langmuir-Freundlich equation, is selected. The combination of the proton binding model and the Donnan model gives a very close reproduction of the observed charging behavior. All model constants obtained are physically realistic.

## Introduction

The physical and chemical characteristics of bacterial cell walls have received some attention in the literature. These characteristics are of interest for the interpretation of the attachment of bacteria to surfaces and of the binding of ions. The structure and the chemical components of cell wall material have been determined. It is possible to separate the cell wall from the cytoplasmic membrane for Gram-positive bacteria. The main component, forming the frame of the cell walls of these organisms, is peptidoglycan (PG). Peptidoglycan is a heteropolymer built out glycan strands crosslinked through short peptides. It is a very common structure, since it is a cell wall component in almost all procaryotic organisms (1).
This PG-matrix is a highly porous three-dimensional structure (20 to 80 nm in thickness), containing up to more than 80% water, into which ions can readily diffuse without actually reacting with the polymeric compounds of the cell wall (2).

In Gram-positive organisms the cytoplasmic membrane is the site of much enzymatic activity; there is little or no activity in the wall (3).
The cell wall components contain several acidic groups. With increasing pH these groups may deprotonate and thus form negatively charged sites. The main reactive groups on PG are carboxylic and amino groups (e.g., 1, 4-6). For cell walls containing teichoic acids, phosphate groups are important for the charge development as well. The negatively charged groups of the polymer matrix may be neutralized by (hydrated) counterions in the double layer and/or by positively charged amino groups which are part of the cell wall structure. In the latter case crosslinking may occur, which may lead to contraction of the wall material (2).

Up to now, most studies have been made by measuring the electrophoretic mobility of the cell wall material. These measurements give qualitative information on the influence of pH and electrolyte composition of the solution, growth stage, etc. on the charging behavior. Data are, however, difficult to translate into absolute amounts of charge present on the material. The interpretation of data obtained by electrophoretic mobility or zeta-potential measurements is therefore the subject of debate (7, 8). Nevertheless, this information is very relevant for microbial adhesion studies, as the zeta potential provides information on the net effective charge at the cell wall surface (3, 4).
Yet for studies of metal ion binding to cell walls, the knowledge of the total cell wall charge is needed. The reactive sites present inside the cell wall can interact with all ions present in the solution phase, since the pores in the cell wall are large enough for the transport of hydrated ions (2, 4, 6, 9). Acid-base titration in the presence of a background electrolyte is a means to measure effectively the dissociation behavior of the reactive groups in the cell wall. The absolute total structural charge of the cell wall can be determined once the point of zero charge is known.
Some data have been published on proton titrations of microbial cell walls. Crist *et al.* (10) studied the binding of metal ions to algal cell walls in combination with proton titrations. Between pH 3 and 10 protons were expected to be released by amino and carboxylic groups. Carstensen and Marquis (4) performed acid-base titrations on isolated *Micrococcus lysodeikticus* cell walls. The total amount of protons that were released by raising the pH from 2 to 11 was about 2 meq.g$^{-1}$ dry weight cell wall material. The ratio of carboxylic to amino groups was 144:90. Van der Wal (11) found a good agreement between the number of reactive groups derived from acid-base titration data and analysis of the chemical composition of bacterial cell wall material. Up to now, no quantitative mechanistic description of the pH and salt dependent charging behavior of bacterial cell walls has been given.

The purpose of this study is to measure the charging behavior of bacterial cell wall material in a 1:1 background electrolyte at different salt levels and to develop a physical-chemical model that can describe the data.

## Materials and methods

### The cell wall material

*Rhodococcus erythropolis* A177 (12) was grown in batch to the late-exponential growth stage in a synthetic medium containing 1.46 $g.l^{-1}$ $KH_2PO_4$, 2.86 $g.l^{-1}$ $Na_2HPO_4.2H_2O$, 1.0 $g.l^{-1}$ $NH_4NO_3$, 0.1 $g.l^{-1}$ $MgSO_4.7H_2O$, 0.05 $g.l^{-1}$ $Ca(NO_3)_2.4H_2O$, and 1 $ml.l^{-1}$ of a micronutrient solution and a vitamin solution. Ethanol was the sole carbon and energy source.

Cells were harvested, washed three times in a 0.05 M $NaNO_3$ solution, and kept in a freezer until a sufficient quantity of material was collected. For the destruction of the cells, the isolation of the cell wall material, and the tests for the purity and composition of the cell walls, a method developed by van der Wal (13) was used. Before destruction, the cells were resuspended in a solution containing 382 g guanidine hydrochloride per kg cell suspension, in order to prevent autolysis of the cell wall material. The cells were destroyed with a French Press, and washed several times during the destruction to remove the released cell contents. After destruction, cell wall material was washed several times in a slightly acidic 0.05 M $NaNO_3$ solution and kept in a freezer until experiments started.

The purity of the recovered cell wall material is assessed by measuring the remaining amount of phospholipids and comparing it with the amount of phospholipids in intact cells. In addition, the total phosphate of both cell walls and intact cells was determined. D-lactate is a specific component of PG. The content of D-lactate of both cell wall material and intact cells was measured and used to determine the cell wall content of intact cells and the PG content of the cell walls. For more detailed information on these procedures we refer the reader to van der Wal (13).

### The titration

The acid-base titrations were performed using a fully automated titration system (14) under nitrogen and at a constant temperature of 25±1°C.

Proton activity was measured with a half-cell glass electrode in combination with a double-junction Ag/AgCl sleeve electrode (Orion), the outer junction of which was filled with an equitransferent solution containing 0.125 M $NaNO_3$ and 0.875 M $KNO_3$ (15). Solutions were made in preboiled, double-distilled water.

Before titrations started, 96 mg dry weight cell wall material was washed once in an acidified (0.001 M $HNO_3$) 0.01 M $NaNO_3$ solution, and three times in a 0.01 M $NaNO_3$ solution, in order to remove all other nonstructural cations in the cell wall material. Then the material was suspended in a starting volume of 17.123 ml and left over under nitrogen for a few hours at pH 5, to ensure $CO_2$ removal from the solution. With this batch of cell wall material, titrations were performed at three salt levels: 0.01 M $NaNO_3$, 0.1 M $NaNO_3$, and 1 M $NaNO_3$. For each salt level a base titration was performed with NaOH (depending on the salt level a concentration 0.0102 ± 0.0001 or 0.0522 ± 0.0006 M was used). Backwards titrations were performed with $HNO_3$ (0.0510 ± 0.0002 M). During the titrations the salt level was kept constant by adding $NaNO_3$. Drift criterium for the pH electrode was set to 0.15 $mV.min^{-1}$. Addition of the $HNO_3$ and NaOH was adjusted to obtain a constant change of 5 mV for each titration step, in order to obtain a good distribution of the data points over the pH-range ($3 \leq pH \leq 10.5$).
After the titrations, the NaOH solutions were calibrated by titrating with 0.1 M HCl (standardized, titrisol) and phenolphtaline as indicator. The $HNO_3$ solution that was used for the titration was calibrated with the 0.0522 M NaOH solution.

## Results and discussion

We will first briefly describe the quality of the material recovered after the destruction procedure; then the results of the titrations will be shown and analyzed by means of the calculated proton affinity distributions. A good description of the data is achieved only if the effects both of salt level and pH are taken into account; this will be discussed in detail below.
The isolation of the cell wall material removed 83% of the phospholipids. The purity based on total phosphate measurements was equal to the purity derived from phospholipid measurements. Total phosphate of the recovered cell wall material was 0.19 ± 0.02 $mmol.g^{-1}$. The cell wall content of intact cells is 31% (g dry weight per g dry weight), and the cell wall material itself consists of 93% PG.
In Fig. 1a the results of the acid-base titrations, i.e., the relative charge as a function of the pH at three ionic strengths, are shown. Some hysteresis can be observed between the acid- and base titrations at the same ionic strength.
For reasons of clarity, all subsequent figures show only the results obtained from acid titrations. Calculations, however, are made using the entire dataset.

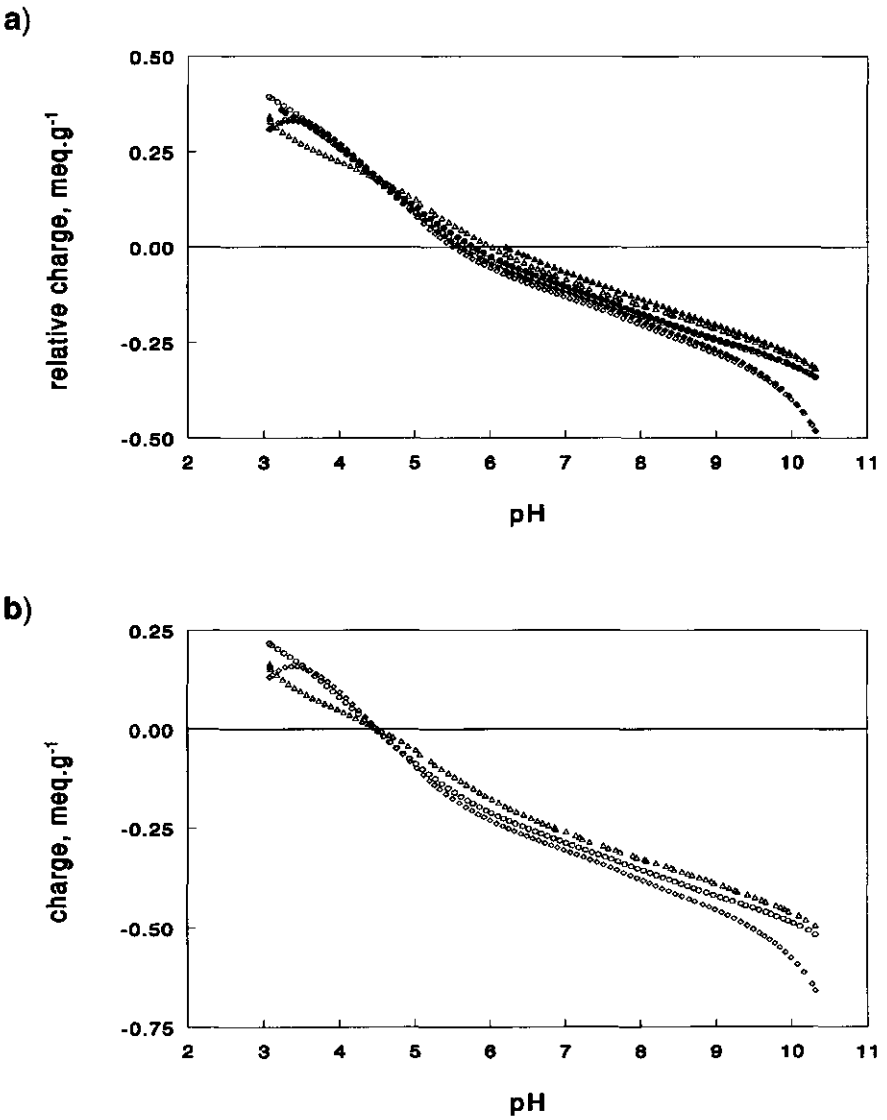


**Fig. 1** a) Relative charge of isolated cell walls as a function of pH, data obtained at three different salt levels: 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○) and 1 M $NaNO_3$ (◊). Closed symbols: data obtained from base titrations. Open symbols: data obtained from acid titrations. **b)** Absolute cell wall charge as a function of pH at three different salt levels: 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○) and 1 M $NaNO_3$ (◊). Only the results of acid titrations are shown.

The data show an intersection point at pH 4.5 ± 0.1. At this pH there is no effect of the salt concentration on the charge and it may therefore be set equal to the point of zero charge (pzc) of the cell wall material. The change in charge measured from the titration can therefore be converted into the absolute particle charge, as has been done in Fig. 1b.

The charge changes gradually with pH. This gradual change can only occur in combination with a single intrinsic proton affinity constant for all carboxylic groups if it is combined with a very strong electrostatic effect. The relatively small effect of the salt levels on the charging behavior, however, suggests a relatively small electrostatic effect (16). The gradual change observed is therefore most likely caused mainly by a distribution of proton affinity constants for the carboxylic groups. A change in the volume of the cell wall can also affect the charging behavior.

In the following we will analyze all effects seperately, before we select a mechanistic model to describe the data. First, a global idea of protonation is derived from the data. Then, the influence of the salt level will be eliminated in order to obtain the intrinsic protonation behavior, i.e., the protonation when no electrostatic interactions are present. From the recalculated data intrisic protonation constants can be derived.

**Initial estimation of concentration of reactive groups and the proton affinity constant of the carboxylic groups**

Assuming all the material consists of the PG primary structure (neglecting phosphate-containing impurities and other minor components), the amount of potentially deprotonating groups, as well as the type of groups, is known. In total 1 gram of PG (molar weight equals 3744) contains 0.8 mmol carboxylic groups and 0.267 mmol amino groups. In general, the log $K_H$ for amino groups in amino acids is about 9.7 (17). This means that at the observed pzc all R-$NH_2$ are protonated to R-$NH_3^+$ ($\theta_{H,n}$=1). Then, for reasons of electroneutrality the following relationship is valid at the pzc

$$Q_{max,c}(1-\theta_{H,c})=Q_{max,n} \qquad [1]$$

where $Q_{max,c}$ is the total amount of carboxylic groups (mmol.$g^{-1}$), $Q_{max,n}$ is the total amount of amino groups, and $\theta_{H,c}$ is the fraction of protonated carboxylic groups.

The degree of protonation of the carboxylic groups follows from a Langmuir-type equation if, as a first estimate, it is assumed that all carboxylic sites are characterized by the same proton affinity constant.

According to the Langmuir equation,

$$\theta_{H,c}=\frac{K_{H,c}(H^+)}{1+K_{H,c}(H^+)} \qquad [2]$$

in which $K_{H,c}$ is the protonation constant of the carboxylic group and $(H^+)$ is the proton activity in solution. The combination of Eqs. [1] and [2] together with the given values for $Q_{max,c}$ and $Q_{max,n}$ gives at the pH of the pzc ($(H^+) = 10^{-4.5}$) an estimation of the value of the proton affinity constant of the carboxylic groups: log $K_{H,c}$ = 4.8. This value is in the range of the proton affinity constants for carboxylic groups found in literature (18). Since the protonation constants for both carboxylic and amino groups are known, a description of the original data with a two-pK discrete model can be given. Agreement between data and the model, however, was very poor. Therefore, the proton affinity distribution requires a more detailed study.

**Heterogeneity analysis**

The protonation behavior can be influenced by more than one reactive group. This binding heterogeneity can be described by a set of discrete affinity constants or by a distribution of affinity constants, since the different reactive groups have different affinities for the proton. To describe overall protonation behavior, each group is assumed to have its own local isotherm with its own affinity constant.

Various techniques exist to derive estimates of the various affinity constants from proton binding data (19). The simplest technique is the condensation approximation (CA) method. This method replaces the local isotherm by a step function. The method leads to a reasonable estimate of the affinity distribution provided that the data are characterized by a wide and relatively smooth distribution. In other cases it would lead to a distribution that is too smooth and too wide compared to the real underlying distribution. A more detailed discussion about heterogeneity analysis can be found in Nederlof *et al.* (20).

Applying heterogeneity analysis to ion binding data leads to a so-called apparent affinity distribution. The apparent affinity distribution is affected by the underlying intrinsic affinity distribution and by electrostatic effects due to variable charge characteristics of the adsorbent, in this case, the cell wall material.

In the present study we used the CA method. The distribution function is approximated with the first derivative of the adsorption isotherm. All inflections that lie within the range of experimental error are removed with a smoothing spline technique (21) (in combination with cross validation to determine the smoothing parameter, and the constraint that the first derivative of the charging curve always had to be positive).

Consequently, spurious peaks that are not related to chemical heterogeneity are removed. For these calculations, software has been used that was developed by Nederlof (20).
Figure 2 shows the apparent affinity distribution derived from the proton binding behavior at three salt levels.

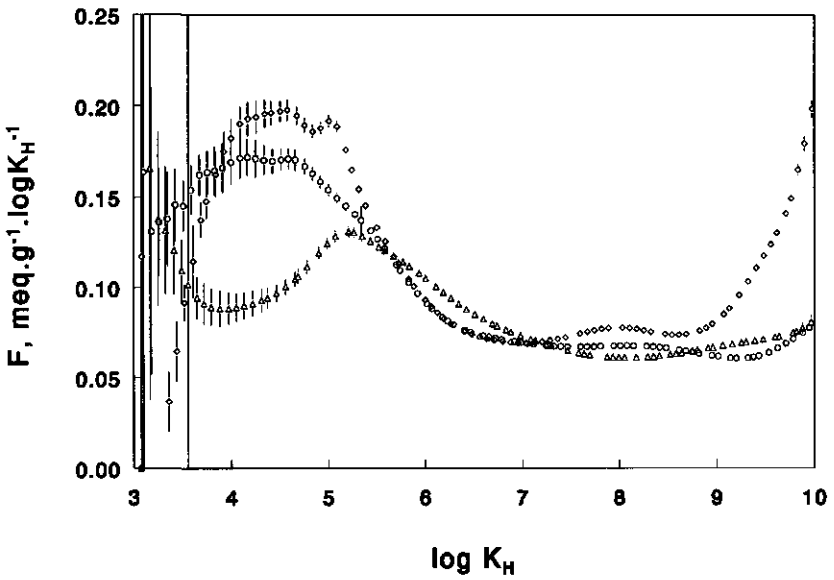


**Fig. 2** Apparent proton affinity distribution derived from the spline of the acid titrations at three different salt levels: 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○), and 1 M $NaNO_3$ (◇).

Due to electrostatic effects a different distribution is found for each salt level. At 1 M salt a broad peak is observed with an average peak position around pH 4.5. This finding corresponds quite well with the first estimate of the proton affinity constant given previously. The highest salt level shows also a very small peak around log $K_H$ = 8. The sharp rise in reactive site density above log $K_H$ = 9 is most likely due to the start of significant deprotonation of the amino groups. The position of this increase corresponds quite well with the affinity constants of these groups (17). Due to the very high affinity constant it is, in practice, unfortunately very difficult to get reliable information about these groups from acid-base titration data. As can be seen from the error bars, results below pH 4.5 are less reliable.
The apparent affinity distribution obtained at high salt level agrees relatively well with the first-order estimate of the expected behavior. The electrostatic effects on the

distribution are smallest at the higher salt levels. The change in shape with salt level may be ascribed mainly to electrostatic effects. For instance, the peak around pH 4.5 at 1 M salt is shifted towards log $K_H \approx 5.5$ for an ionic strength of 0.01 M.
To remove the salt dependency on the apparent affinity distribution, an electrostatic model is needed to describe the effect of the salt level on the proton binding behavior. Once the appropriate double layer model is chosen, it is possible to plot the charge as a function of the local pH. In such a graph all salt level data should merge into one and unique curve; the so-called "master-curve" (22). This approach will allow the determination of intrinsic protonation constants, i.e., constants that are unaffected by electrostatic interactions.

**Double layer model: The Donnan model**

The electrostatics involved in the charging behavior of fulvic acids are succesfully described with a double layer model that attributes all charge to the surface of this material (16). The geometry of the cell walls makes the use of a flat-plate double layer model most likely. However, this model leads to a strong overestimation of the salt effect. A more realistic model for cell wall material is to consider a Donnan-type model. In this approach the reactive sites are assumed to be equally distributed all over the cell wall volume, leading to a spatial charge density. This spatial charge density leads to an electrostatic potential in the aqueous part of the Donnan phase that is approximated by a constant potential. This Donnan potential is a function of the spatial charge density and the salt level. The spatial charge density is influenced by the specific volume of the cell wall material and by the degree of dissociation of the reactive groups. It is further assumed that the particle charge is fully neutralized inside the cell wall and that osmotic effects can be neglected (23). The latter was already found by Marquis (24), since his results indicated that even for intact cells, changes in the specific volume when changing the salt level could mainly be ascribed to electrostatic interactions.
Assuming the structural charge $Q_0$ to be fully compensated for by the positive adsorption of cations in the Donnan phase and by the repulsion of anions, the countercharge $Q_D$ is given by

$$Q_D = -Q_0 = v_D(\Sigma C_D^+ - \Sigma C_D^-) \qquad [3]$$

where $v_D$ is the specific volumetric water content of the cell wall material in ml water per g dry weight material. $C_D^+$ and $C_D^-$ are the concentrations of cations and anions in the Donnan phase or gel phase expressed in $mol.l^{-1}$, and Q is expressed in meq of charge per g dry weight material. The ion concentration inside the cell wall ($C_D$) is

related to the concentration in the bulk solution ($C_B$) via the Boltzmann accumulation factor X

$$C_D = XC_B \quad [4]$$

in which

$$X = \exp\left(\frac{-zF\psi_D}{RT}\right) \quad [5]$$

where $\Psi_D$ is the Donnan potential (V), z is the ionic charge, F is the Faraday's constant ($C.mol^{-1}$), R is the gas constant ($J.mol^{-1}.K^{-1}$), and T is the temperature (K). The accumulation factor for anions is related to the cation accumulation factor for a single 1:1 monovalent salt by:

$$X^- = \frac{1}{X^+} \quad [6]$$

In the case of a 1:1 monovalent salt $C^+ = C^- = I$, where I is the ionic strength. Thus the combination of Eqs. [3], [4], and [6] results in

$$Q_0 = \nu_D I\left(\frac{1}{X^+} - X^+\right) \quad [7]$$

In principle, the concentration of protons and hydroxyl ions should also be included in Eq. [7]. However, for our experimental conditions these terms can be neglected. The Boltzmann accumulation factor X can be calculated from Eq. [7] for a given salt level and particle charge $Q_0$, provided that the specific volume is known. It is then possible to calculate for each datapoint the pH in the Donnan phase ($pH_D$):

$$pH_D = pH - \log X^+ \quad [8]$$

The combination of Eqs. [5] and [7] allows us to calculate the Donnan potential as well. In order to obtain the mastercurve, the Boltzmann accumulation factor for the proton ($X^+$) has to be known. The validity of the approach mentioned above to derive $X^+$ is strongly related to a realistic estimate of the water content of the cell wall material.

**Donnan volumes**

Due to electrostatic repulsion, the Donnan volume, and thus the total cell wall volume ( $v = v_D + v_{dry\ weight\ cell\ walls}$) may depend on the charge present on the structural components of the cell wall and on the ionic strength. With the help of the master-curve approach the Donnan volumes can be estimated. It follows from Eq. [8] that for a given particle charge and salt level the Boltzmann factor X can be derived from the difference between pH and $pH_D$, where the value of pH - $pH_D$ equals the shift of a charging curve at a given salt level and particle charge compared with the master-curve. This concept is illustrated in Fig. 3.

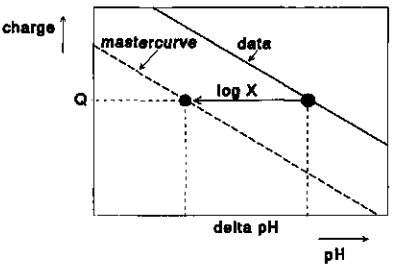


**Fig. 3** Schematic presentation of the calculation of the Boltzmann accumulation factor for a set of data for which the location of the master-curve is known. For a given charge Q the log(X) equals delta pH, i.e., the difference between the pH of the original data set and the pH of the master-curve belonging to that value of Q. With this method, the Boltzmann factor, and thus the Donnan potential, can be calculated for all data points.

Rewriting Eq. [7] leads to:

$$v_D = \frac{Q_0}{I(\frac{1}{X^+} - X^+)} \qquad [9]$$

Then the combination of Eqs. [8] and [9] enables the calculation of $v_D$ for each salt level and particle charge, provided that the location of the master-curve is known. However, the position of the master-curve is not known a priori. Calculation of the potential with a spherical diffuse double layer model for a fulvic acid shows that the electrostatic effect is the smallest at the highest salt levels (16). In other words, the higher the salt level, the closer is the charging curve to the master-curve. It is often assumed that the electrostatic effects are negligible at 1 M salt (23). So as a first approximation it is assumed that at 1 M salt all electrostatic effects are suppressed; at 1 M salt $\Psi_D = 0$ so pH = $pH_D$ or $X^+ = 1$.

In this way the volumes corresponding to lower salt levels (0.1 M and 0.01 M) can be calculated. Results of these calculations are given in Fig. 4.

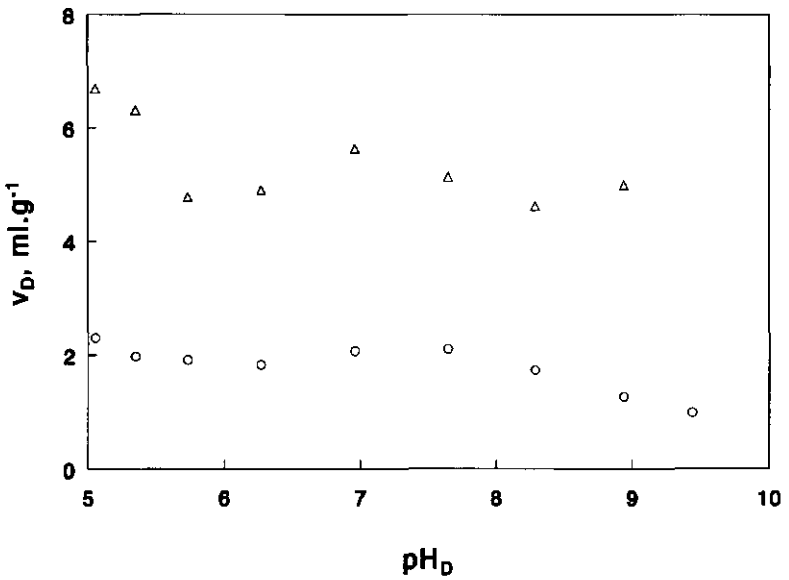


**Fig. 4** Water content of the cell wall material as a function of the calculated local pH ($pH_D$). To calculate the volumes, the 1 M curve is assumed to be equal to the master curve. Data shown are averages of values obtained from acid and base titrations. 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○).

Equation [9] is not determined at the pzc and results of the calculations close to the pzc give different results depending on whether the acid or the base titration data are used. For pH > 5, calculations from both acid and base titrations are in agreement. It follows then that the calculated volumes for I = 0.1 and 0.01 M salts are hardly dependent on the particle charge. Results show that the cell walls may swell considerably at low ionic strength for a monovalent salt, since $v_D$ values are equal to 2.3 to 4.8 ml.g$^{-1}$ for I = 0.1 and 0.01 M respectively. These results imply that the volume of the material at 1 M must be smaller than 2.3 ml.g$^{-1}$, since it is expected that the cell walls shrink with increasing salt level due to decreased electrostatic repulsion. Using the results obtained with the assumption that at 1 M salt the electrostatic effects are negligible, we can now construct a master-curve.
In order to construct the master-curve we have assumed that the Donnan volume is constant for a given salt level, independent of the charge (see Fig. 4). The maximum

value that the Donnan volume can have for the 1 M salt is 2.3 $ml.g^{-1}$. The position of the master-curve can be derived from the 1 M charging curve once a specific volume has been chosen for the material at 1 M salt using Eqs. [8] and [9]. The Donnan volumes at the lower salt levels can then be calculated using the master-curve as described above. This has been done for a range of specific volumes for the 1 M salt level ($0.5 \leq v_D \leq 2.3$ $ml.g^{-1}$), which resulted in a number of combinations for the values of the Donnan volumes for the three salt levels. The results are given in Table 1. An upper limit for the volume at 1 M salt can be established, since it is physically quite unrealistic that cell wall volumes at 1 M salt will be larger than at lower salt levels. It therefore follows from Table 1 that the upper limit of the specific volume at 1 M salt is around 1.8 $ml.g^{-1}$. A lower limit for the volume cannot be obtained from this approach. Van der Wal (11) calculated a total cell wall volume for *Rhodococcus erythropolis* A177 of 3.1 $ml.g^{-1}$. Marquis (24) measured the Dextran-impermeable volume of cell walls of *Bacillus megaterium* at different salt levels. He obtained specific volumes ranging from 3 $ml.g^{-1}$ at 0.1 M NaCl to 6 $ml.g^{-1}$ at 0.01 M NaCl. The volumes we obtained are well in agreement with values given by Marquis (24) and van der Wal (11).

**Table 1**

**Donnan volumes calculated using the master-curve approach for given values at 1 M salt**

| $v_D$ (1 M salt) $ml.g^{-1}$ | $v_D$ (0.1 M salt) $ml.g^{-1}$ | $v_D$ (0.01 M salt) $ml.g^{-1}$ |
|---|---|---|
| 2.3 | 1.9 | 5.5 |
| 1.8 | 1.8 | 5.4 |
| 1.0 | 1.6 | 5.1 |
| 0.5 | 1.3 | 4.4 |

**Calculation of the Donnan potentials**

Once we have chosen a value for the specific volume at 1 M salt, the Boltzmann factor, and thus the Donnan potentials, can be calculated for all data points with Eq. [7]. The Donnan potentials are calculated as a function of pH and salt level for three values of $v_D$ at 1 M salt (Fig. 5).

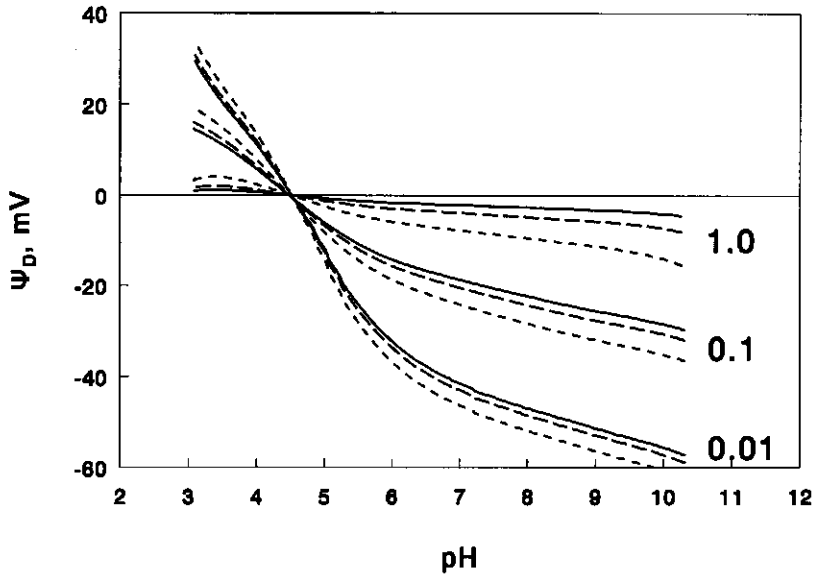


**Fig. 5** Donnan potential as a function of pH, salt level, and volume chosen for the 1 M data set. The salt levels are indicated in the figure (1 M, 0.1 M and 0.01 M). Results from the calculations are compared for three values of $v_D$ (1 M): 1.8 ml.g$^{-1}$ (——), 1.0 ml.g$^{-1}$ (– – –), and 0.5 ml.g$^{-1}$ (- - - -). Only data of acid titrations are shown.

The results clearly show that the calculated potentials for 1 M salt are relatively small whereas at lower salt levels considerable potentials develop. When the highest 1 M specific volume (1.8 ml.g$^{-1}$) is chosen, the former assumption that the 1 M curve is close to the master-curve is justified. When a smaller value is chosen for this volume, higher potentials are calculated and the 1 M curve will thus deviate more from the master-curve.

It is of interest to compare these calculated potentials with measured values. Since the ζ-potential is defined as the potential at the plane of shear, it will be somewhat smaller than the Donnan potentials inside the cell wall structure. Nevertheless, they should be of the same order of magnitude. ζ-potential measurements on cell walls of A177 are not available at present, but ζ-potentials of similar cell material have been obtained by Rijnaarts *et al.* (25). They calculated ζ-potentials of -50 mV at 0.01 M salt and -40 mV at 0.1 M salt at pH 7 (intact cells, DSM 44016). Within experimental uncertainties a reasonable agreement between these data and our calculated potentials can be observed.

In the following we have chosen a Donnan volume of 1.8 $ml.g^{-1}$ at 1M salt. The resulting charging curves as a function of the pH in the Donnan phase ($pH_D$) are shown in Fig. 6. The results show that the three curves merge quite well into a single master-curve. Only at the extremes do some deviations still exist that are not accounted for by the present approach. It can be concluded that the Donnan model can explain the salt dependency of the charging curves satisfactorily, provided that some swelling with decreasing salt level is taken into account.

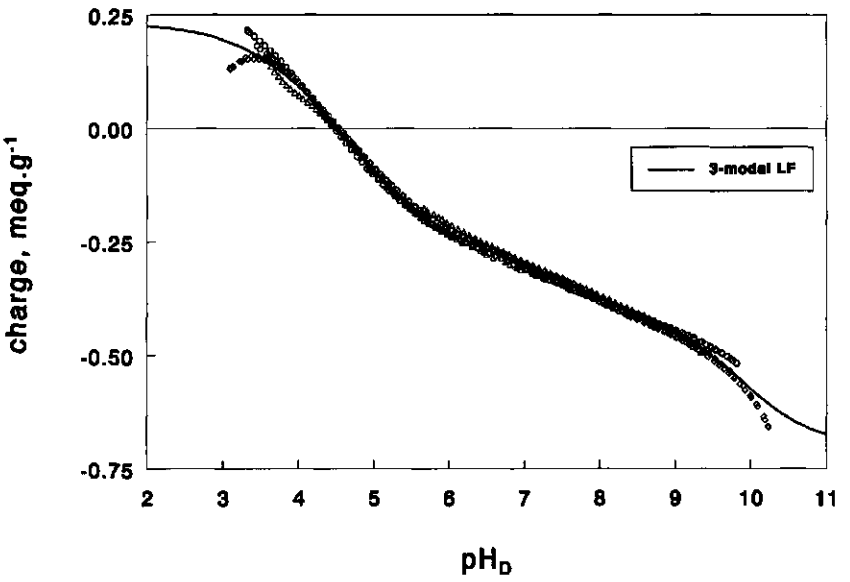


**Fig. 6** Master-curve, obtained by eliminating the influence of the salt level with the Donnan model. Only results of acid data are shown. 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○), and 1 M $NaNO_3$ (◊). The lines show the model-description given by the trimodal Langmuir-Freundlich equation.

**Intrinsic proton affinity distribution**

It is now possible to derive the intrinsic proton affinity distribution from the data in Fig. 6. In Fig. 7 these recalculated distributions are shown for each salt level.

For pH-values larger than 5 the distribution curves now merge quite well; below pH 5 the differences between the three salt level curves illustrate the large uncertainty of the data at these pH values, which was already observed with the apparent proton affinity distribution (Fig. 2).

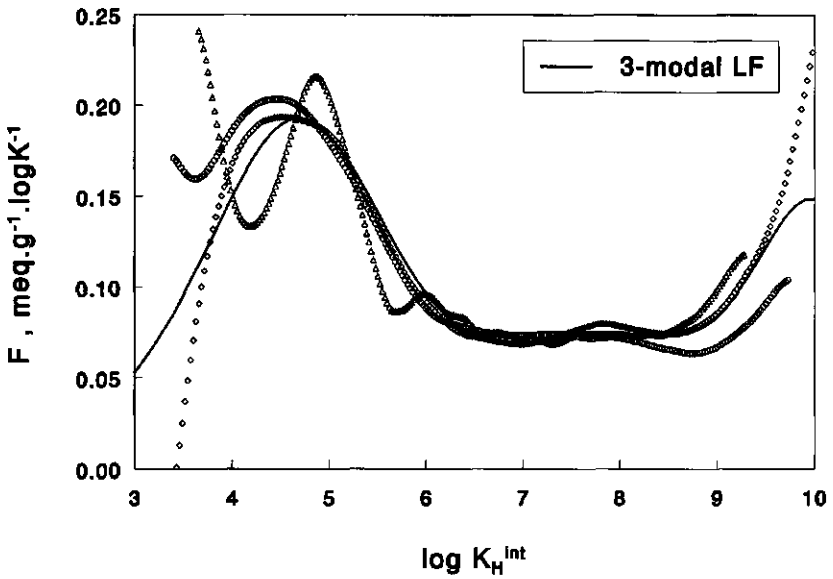


**Fig. 7** Nonnormalized intrinsic proton affinity distribution, derived from the mastercurve, compared to the trimodal LF model-distribution. 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○), and 1 M $NaNO_3$ (◊).

The distributions clearly show a relatively wide peak that corresponds to the carboxylic acid groups, a smaller peak around log $K_H$ = 8, and the steep rise at high log $K_H$ values, which is probably due to the deprotonation of amino-groups. The peak around log $K_H$ = 8 must be related to a different type of acidic group. Pure cell wall material was not recovered; 17% of the membrane material was not removed, so some phospholipidic material must be present. In addition, cell walls contain 7% of other components. The total phosphate content of the isolated cell walls was measured; the cell wall material contained 0.193 mmol P per gram material (dry weight). This amount cannot be neglected, as it is on the same order of magnitude as the estimated contents of carboxylic and amino groups. Thus the peak centered around log $K_H$ = 8 could be associated to phosphate-type groups.

Based upon the heterogeneity analysis, a proton binding model can be chosen. The simplest option would be to use three discrete ligands with intrinsic proton affinity constants ($K_H^{int}$) that correspond to the peak positions. The individual charging curves can then be described by combining the proton site binding model with the Donnan model. Using this approach, it is not possible to obtain a good description of the

original data (not shown here). This result is expected since the affinity distribution shows relatively broad peaks.

More groups could be taken into account. PG contains three different carboxylic groups, phospholipids may also contain some carboxylic and aminogroups, and each group may have its own log $K_H$. The more groups are taken into account, the better the quality of the data description will be. However, when insufficient information concerning composition of the material and proton affinity constants of the different reactive groups is available, we prefer to use the Langmuir-Freundlich (LF) equation, in which heterogeneity is taken into account (18), because with this approach the number of unknown parameters is limited.

As three peaks were observed, a trimodal LF equation is used, in which the protonation of each type of reactive group is described by a LF binding equation.

The LF equation can be represented as

$$\theta_{H,i}=\frac{(\tilde{K}_{H,i}H_D)^{m_i}}{1+(\tilde{K}_{H,i}H_D)^{m_i}} \qquad [10]$$

where $\tilde{K}_{H,i}$ is the average intrinsic proton affinity constant for the reactive groups of type i (which corresponds to the peak position in the affinity distribution) and $m_i$ is a parameter that characterizes the width of the distribution ($0 < m_i \leq 1$). In the case of a homogeneous group of sites, $m_i = 1$. The charge of the cell walls can be calculated using

$$Q=Q_{max,c}(1-\theta_{H,c})+Q_{max,p}(1-\theta_{H,p})+Q_{max,n}\theta_{H,n} \qquad [11]$$

in which c, p and n are subscripts for, respectively, the carboxylic, phosphate and amino groups. Numerical optimization of the parameters was performed using nonlinear least-squares with the computerprogramme FIT developed by Kinniburgh (26). During the first optimization procedure all parameters ($\tilde{K}_{H,i}$, $Q_{max,i}$ and $m_i$) were free to vary. Though a very good description of the master-curve was obtained ($r^2$=0.997), the optimization procedure lead to a highly overestimated value for the maximum charge of phosphate-like groups (-0.73 meq.g$^{-1}$) and to an unrealistic value for the exponent m for the amino groups ($m_n > 1$). The resulting affinity distribution was more or less a broad peak around log $\tilde{K}_H = 8$, which is in contradiction with the affinity distribution we obtained with the CA method. Experimentally, only part of the amino peak could be determined, which makes interpretation quite uncertain. Therefore, for the final numerical optimization of the parameters, we decided to set the value of $m_n$ equal to 1. In addition, the maximum charge of the phosphate-like groups

was set equal to the total amount of phosphate (0.193 mmol.g$^{-1}$). The final results are shown in Table 2.

**Table 2**

**Parameters derived with the trimodal Langmuir-Freundlich equation**

| | log $\tilde{K}_H$ | $Q_{max}$ (meq.g$^{-1}$) | m |
|---|---|---|---|
| 1) carboxylic | 4.62 ± 0.02 | -0.503 ± 0.015 | 0.66 ± 0.03 |
| 2) phosphatic | 7.83 ± 0.13 | -0.193 | 0.58 ± 0.02 |
| 3) amino | 9.96 ± 0.08 | 0.235 ± 0.007 | 1 |

The affinity constants of the first two groups are characterized by relatively broad distributions. Fitted intrinsic protonation constants can be compared to literature data. Affinity constants (log $\tilde{K}_H$) for the carboxylic and amino groups are quite similar to values given in literature for other organic structures (17, 18). Less data are available for the log $\tilde{K}_H$ of the phosphatic group of phospholipids. Simply regarding the protonation of this group as the second protonation step of ortho-phosphate would lead to a log $\tilde{K}_H$ of about 7.2 (27). Van Dijck *et al.* (28) determined an apparent log $K_H$ ranging from 8.1 to 8.5 for the second deprotonation step of phosphatidic acid, a rather common phospholipid.

Though our log $\tilde{K}_H$ is of the same order, the fact that the given range is for the second deprotonation step implies that a first step must already have taken place. Van Dijck *et al.* (28) give a log $K_H$ for this first step of about 4. The CA method is not very well suited to distinguish between groups whose log $\tilde{K}_H$ values differ less than two unities, so if indeed this type of phospholipidic material is titrated, the first deprotonation step of these phospholipidic groups will fall in the range of the broad "carboxylic" peak.

The total amounts of carboxylic and amino groups are on the same order of magnitude as was estimated on the basis of the composition of peptidoglycan. The material used contained some impurities. Ignoring the possible presence of carboxylic groups on these unidentified components, would lead to an amount of 0.62-0.74 mmol carboxylic groups per gram material, which is close to the value obtained by fitting.

Nevertheless, some interesting differences can be observed: The ratio carboxylic groups:amino groups (2:1) is different from what was expected at the basis of the

structure of PG (3:1). However, Carstensen and Marquis (4) found for *Micrococcus leisodeikticus* a ratio carboxylic to amino-type groups of 1.6:1.
The affinity distribution that can be derived from the trimodal LF model is drawn in Fig. 7 (solid line). It follows that the model gives a good description of the affinity distribution, indicating that the CA method gives in this case a reliable estimate of the underlying distribution. The model also gives a good representation of the master-curve, as can be seen in Fig. 6. If the Donnan model is used to convert $pH_D$ to pH values a good description of the original charging curves is obtained (Fig. 8). Some deviations can be observed at extreme pH-values. This may be the result of the use of a constant volume for each salt level; as can be seen in Fig. 4, deviations from the average water content increase at the extreme pH-values.

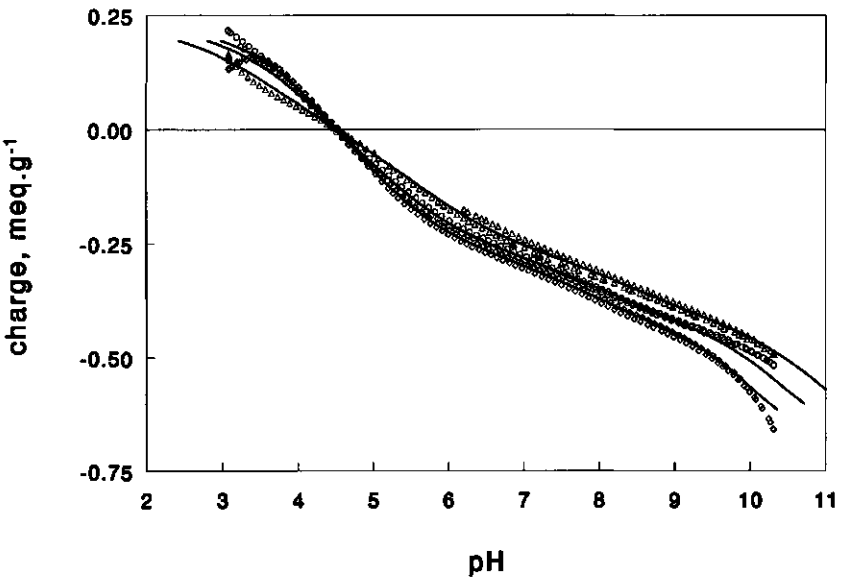


**Fig. 8** Comparison between original data and model description, based on a trimodal LF proton binding model in combination with a Donnan-type electrostatic model. Data: 0.01 M $NaNO_3$ (△), 0.1 M $NaNO_3$ (○), and 1 M $NaNO_3$ (◇). The lines show the model-description given by the trimodal Langmuir-Freundlich equation.

With the help of a so-called Henderson-Hasselbalch-plot (HH plot) van der Wal (11) analyzed the protonation of carboxylic groups for *Rhodococcus erythropolis* A177 at 1 M salt. It can be shown that the slope of a HH-plot is equal to the exponent $m_i$ used in the Langmuir-Freundlich equation. In case of ideal behavior, i.e., in the absence of

electrostatic interactions and for a chemically homogeneous composition of the material, the slope of a HH plot should equal 1. From the HH plot given by van der Wal (11) a slope of 0.63 can be derived for *Rhodococcus erythropolis* between pH 4 and 5, which is in close agreement with the value of 0.66 for the heterogeneity parameter $m_c$ obtained by the LF-fit presented in this paper.

## Conclusions

Charging behavior of this bacterial cell wall material is influenced both by pH and salt level. The techniques for analyzing such data for electrostatic effects and heterogeneity effects as developed for the description of the charging behavior of humic acids (18, 19, 21, 22) proved to be very valuable tools for the analysis of the data obtained with the bacterial cell walls. The analysis indicates that it is likely that the volume of the cell walls is dependent on the salt level. The volumes obtained are physically realistic. It is shown that heterogeneity analysis is a very valuable tool in selecting an appropriate proton binding model. Simple unconstrained numerical parameter optimization may lead to physically irrealistic parameter values. The combination of a trimodal Langmuir-Freundlich model in combination with a Donnan model gives an excellent description of the charge of the cell wall as a function of pH and salt level. Comparison with data from literature shows that all model constants, as well as the values obtained for the Donnan volumes and the Donnan potentials, are physically quite realistic.

## References


1. Schleifer, K.H., and Kandler, O., Peptidolican types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**(4), 407 (1972).
2. Marquis, R.E., Mayzel, K., and Carstensen, E.L., Cation exchange in cell walls of Gram-positive bacteria. *Can. J. Microbiol.* **22,** 975 (1976).
3. James, A.M., The electrical properties and topochemistry of bacterial cells. *Adv. Colloid Interface Sci.* **15**, 171 (1982).
4. Carstensen, E.L., and Marquis, R.E., Passive electrical conductivity properties of microorganisms: III. Conductivity of bacterial cell walls. *Biophys. J.* **8**(5), 536 (1968).
5. Beveridge, T.J., Bioconversion of inorganic materials - Mechanisms of the binding of metallic ions to bacterial walls and the possible impact on microbial ecology. In "Current Perspectives in Microbial Ecology" (M.J. Klug and C.A. Reddy, Eds.), p.601. Am. Soc. Microbiol., Washington D.C., (1984).
6. Beveridge, T.J., The bacterial surface: general considerations towards design and function. *Can. J. Microbiol.* **34**, 363 (1988).
7. Einolf, C.W., Jr., and Carstensen, E.L., Bacterial conductivity in the determination of surface charge by microelectrophoresis. *Biochim. Biophys. Acta* **148**, 506 (1967).
8. Neihoff, R., and Echols, W.H., Physicochemical studies of microbial cell walls. I. Comparitive electrophoretic behavior of intact cells and isolated cell walls. *Biochim. Biophys. Acta* **318**, 23 (1973).
9. Doyle, R.J., Matthews, T.H., and Streips, U.N., Chemical basis for selectivity of metal ions by the *Bacillus subtilus* cell wall. *J. Bacteriol.* **143**(1), 471 (1980).
10. Crist, R.H., Oberholser, K., Shank, N., and Nguyen, M., Nature of bonding between metallic ions and algal cell walls. *Environ. Sci. Technol.* **15**(10), 1212 (1981).
11. van der Wal, A., Determination of the total charge in the cell walls of Gram-positive bacteria, In: Electrochemical characterization of the bacterial cell surface. Ph.D. thesis WAU chapter 3 (1996).
12. Rijnaarts, H.H.M., Norde, W, Bouwer, E.J., Lyklema, J., and Zehnder, A.J.B., Bacterial Adhesion under static and dynamic conditions. *Appl. Environ. Microb.* **59**, 3255 (1993).
13. van der Wal, A., Chemical analysis of isolated cell walls of Gram-positive bacteria and the determination of the cell wall to cell mass ratio. In: Electrochemical characterization of the bacterial cell surface, Ph.D. thesis WAU chapter 2 (1996).
14. Kinniburgh, D.G., and Milne, C.J.,"Guide to the Wallingford Titrator". Technical Report WD/92/14, British Geological Survey, Keyworth, Nottinghamshire, (1992).
15. de Keizer, A., Electrosorption of tetra-alkyl ammonium ion on silveriodide. Ph.D. thesis, chapter 4.4 Wageningen Agricultural University, (1981).
16. de Wit, J.C.M., van Riemsdijk, W.H., and Koopal, L.K., Proton binding to humic substances. *Environ. Sci. Technol.* **27**, 10, 2005 (1993).
17. Martell, A.E., and Smith, R.M., "Critical Stability Constants, Volume 1: Aminoacids". Plenum, New York, 469p (1974).
18. van Riemsdijk, W.H., and Koopal, L.K., Ion binding by natural heterogeneous particles. In "Environmental Particles". (J. Buffle and H.P. van Leeuwen, Eds.), Vol. 1. 455p. Lewis, London, (1992).

19. Nederlof, M.M., van Riemsdijk, W.H., and Koopal, L.K., Determination of adsorption affinity distributions: A general framework for methods related to local isotherm approximations. *J. Colloid Interface Sc.* **135**, 410 (1990).
20. Nederlof, M.M., van Riemsdijk, W.H., and Koopal, L.K., Comparison of semianalytical methods to analyse complexation with heterogeneous ligands. *Environ. Sci. Technol.* **26**, 763 (1992).
21. Nederlof, M.M., van Riemsdijk, W.H., and Koopal, L.K., Heterogeneity Analysis for binding data using an adapted Smooting Spline Technique. *Environ. Sci. Technol.* **28**(6), 1037 (1994).
22. de Wit, J.C.M., van Riemsdijk, W.H., Nederlof, M.M., Kinniburgh, D.G., and Koopal, L.K., Analyses of ion binding on humic substances and the determination of intrinsic affinity constants. *Anal. Chim. Acta* **232**, 189 (1990).
23. Marinsky, S.A., Wolf, A., and Bunll K., The binding of trace amounts of lead(II), copper(II), zinc(II) and calcium(II) to soil organic matter. *Talanta* **27**, 461 (1980).
24. Marquis, R.E., Salt induced contraction of bacterial cell walls. *J. Bacteriol.* **95**(3), 775 (1968).
25. Rijnaarts, H.H.M., Norde, W., Lyklema, J., and Zehnder, A.J.B., DLVO and Steric contributions to bacterial depositions in different ionic strength environments. *Environ. Sci.Technol.* submitted (1994).
26. Kinniburgh, D.G., and Milne, C.J., "Fit User Guide". Technical Report WD/93/23, British Geological Survey, Keyworth, Nottinghamshire, (1992).
27. Smith, R.M., and Martell, A.E., "Critical stability constants, Volume 4: Inorganic complexes". 257p. Plenum, New York, (1976).
28. van Dijck, P.W.M., de Kruijff, B., Verkleij, A.J., van Deenen, L.L.M. van, and de Gier, J., Comparative studies on the effects of pH an $Ca^{2+}$ on bilayersof various negatively charged phospholipids and their mixtures with phosphatidylcholine. *Biochim. Biophys. Acta* **512**, 84 (1978).

# Chapter 3

## Competitive metal ion binding to cell walls

Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a Gram-positive soil bacterium

Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a Gram-positive soil bacterium
*Environmental Science & Technology*, in press

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# Competitive metal ion binding to cell walls

Metal ion binding to the bacterial cell wall is the first step in the interactions of a metal with a bacterium. Cadmium and zinc ion binding to isolated cell walls of *Rhodococcus erythropolis* A177 have been studied for a wide range of proton and calcium concentrations. The release of calcium ions and protons during metal ion binding is an indication of the competitive nature of the binding. Calculations, based on the metal binding data and the pH-dependent charging behavior, reveal that charge reversal may take place at high coverage of the cell wall with bivalent ions. A cooperative effect of the presence of calcium on metal ion binding was observed. Apparently, the cell wall structure is altered in the presence of bivalent ions. Since calcium is the dominant bivalent cation in natural systems one may regard the calcium as a "structure determining ion". On the basis of a qualitative interpretation of the data, the NICA model was selected for a quantitative description of the data.
In the cell wall material used, in addition to the carboxylic and amino-type groups of peptidoglycan, phosphate-type groups originating from phospholipidic materials were also present. The model developed is able to discriminate between the binding of metal ions to both the carboxylic and phosphatic sites, and therefore allows us to extrapolate our experimental results to metal ion binding to the cell walls of intact cells.

## Introduction

Trace metal interactions with organisms can be understood by considering an organism to be an assemblage of reactive ligands. Since binding is a first step towards uptake and toxicity, the degree of complexation of these ligands with particular metals is reflected in measurable physiological effects (1).
Heavy metal toxicity for an organism in a soil environment is influenced by soil parameters like pH and composition of the soil solution (2). This influence can largely be understood by assuming competition for a metal ion between biota and other, abiotic, components of the system. Nederlof *et al.* (3) have shown that, if factors such as pH are included in the model, studying metal ion binding to soil and to biological surfaces separately may enable a good estimate of the amount of metal bound to the biological surfaces in a soil system to be made. In order to be able to use this concept, a quantitative understanding of the binding mechanisms of metal ions to the biological surfaces is required. In natural systems, metal ion binding will be influenced by a number of processes and environmental parameters. Soil solutions contain a variety of ions that will compete to a greater or lesser extent for the available binding sites. Apart from the protons, inevitably present in aqueous solutions, calcium is known

to be an important competitor for metal ion sorption, since in general it is the major bivalent cation in soil solutions. Usually, cadmium in soil is accompanied by a much larger quantity of zinc. Field data has shown that the difference in cadmium and zinc concentrations in these situations is about two orders of magnitude. The influence of this excess of zinc on cadmium binding may also be important.
Cell walls of Gram-positive bacteria are highly porous structures. Peptidoglycan, the main component of these cell walls, contains many acidic groups (mainly carboxylic), leading to a pH- and salt dependent charge. Information on the metal ion binding characteristics can be derived from the charging behavior of the material as determined from proton titrations. Therefore, acid/base titrations have already been performed on cell wall material from *Rhodococcus erythropolis* A177 (4).
In this paper, data are presented for the binding of calcium, cadmium and zinc to cell walls of the Gram-positive soil bacterium *Rhodococcus erythropolis* A177. The metal ion binding has been studied over a range of proton and calcium concentrations. In addition, cadmium binding is studied in the presence of both calcium and zinc.
An attempt is made to model the observed metal ion binding, as well as the release of other cations due to this metal ion binding. The aim of the modelling is to describe metal ion binding at different concentrations of protons and other competing ions using a multicomponent competitive binding model that can account for both ion-specific non-ideality and heterogeneity.

## Materials and methods

### Materials

Cell wall material from *Rhodococcus erythropolis* A177 was obtained following the method developed by van der Wal (5). The major component of the cell wall (93%) is peptidoglycan. The purity of the material was derived from the phospholipidic phosphate content; at the end of the preparation of the cell wall material 83% of the original phospholipidic material had been removed. All cations from the nutrient solution in which the bacteria were grown (including the calcium), are expected to be extracted during the purification procedure. The purified material was kept in batches (containing approximately 100 mg dry weight cell wall material) in a freezer until required.
Metal ion titrations were performed with the same material as used for the proton titrations described in Plette *et al.* (4). The charging behavior of this material could be described well with a three site Langmuir-Freundlich proton binding equation in combination with a Donnan model to account for the electrostatic effects.

All reagents used were p.a. quality (Merck). Solutions were prepared from preboiled, double-distilled water.

**Preparation of the cell wall suspension, conditions for the titrations**

The cell wall material was washed one time in an acidified $NaNO_3$ solution (0.001 M $HNO_3$ in 0.01 M $NaNO_3$), then three times in the electrolyte solution that was to be used during the experiment, i.e. 0.01 M $NaNO_3$ with 0.001 M $Ca(NO_3)_2$ or 0.1 M $NaNO_3$. The material was resuspended in a thermostatted reaction vessel (Metrohm). The starting volume was made up to about 20 ml.

Titrations were performed under $CO_2$-free air and at a constant temperature of 25 ± 1°C. Proton activity was measured with a glass electrode (Orion) in combination with a double-junction Ag/AgCl sleeve reference electrode (Orion) in which the outer junction was filled with an equitransferent solution containing 0.125 M $NaNO_3$ and 0.875 M $KNO_3$ (6). The pH was kept at a constant value with help of a Metrohm titroprocessor 686 with an automated buret (Metrohm Dosimat 665). After each 2 or 3 additions of metal ion solution, a sample was taken from the vessel and centrifuged at 6400 rpm (Beckman centrifuge, model TJ-6) and 3 ml of 0.1 M $HNO_3$ in 0.01 M $NaNO_3$ was added to the supernatant. This acidified supernatant was used to analyze metal ion (Cd, Zn) and calcium concentration by flame-AAS. During the calcium and cadmium titrations, the change in concentration of the titrated ion was monitored by an ion-specific electrode (Orion). The cell wall material turned out to influence the signal of the ion selective electrodes (ISE). Therefore, the ISE could not be calibrated accurately for the experimental conditions. The ISE was calibrated by relating the readings (Cd or Ca) to the ion concentration measured by flame-AAS in samples periodically taken from the vessel. For zinc, no ion-specific electrode is available. Therefore, for those experiments, the zinc concentration in the acidified supernatant was measured by flame-AAS.

Ion binding is always expressed in $\mu mol.g^{-1}$ dry weight cell wall material.

**Calcium titrations**

Calcium is an important cation in natural aqueous media, and it can be a relatively strong competitor with respect to the binding of metal ions such as $Cd^{2+}$ and $Zn^{2+}$. Therefore, most metal ion titrations were performed in the presence of approximately 1 mM $Ca(NO_3)_2$. In addition, some titrations were performed without calcium ions in solution.

In order to take into account the effect of calcium on the binding of other metal ions, it is necessary to know how the calcium binds to the cell wall material. A calcium ion titration was performed at pH 5 as described above. The calcium concentration ranged

from 10 μM to 1 mM. After the titration, the entire content of the reaction vessel was centrifuged, the supernatant removed and analyzed for its calcium content. The calcium concentration in this supernatant was used to calculate the total amount of calcium remaining in the suspension. This suspension was used to perform a calcium titration at pH 6.5. Before the start of the titration, after equilibration at pH 6.5, the concentration of calcium in solution was measured. Thus, the amount of calcium sorbed at the start of the titration at pH 6.5 could be calculated. This gave the first data point of the calcium titration curve at pH 6.5. Due to the washing procedure and the increase in pH, the calcium concentration at the start of the new titration was lower than at the end of the titration at pH 5. After the titration at pH 6.5, the procedure was repeated once more to perform a calcium titration at pH 8.

**Metal ion titrations (Cd and Zn) in presence of a variable Ca concentration**

A series of cadmium titrations were performed at pH 5, 6.5 and 8 in the presence of approximately 1 mM $Ca(NO_3)_2$ in a 0.01 M $NaNO_3$ background electrolyte. In addition, a cadmium ion titration was performed at pH 8 in a 0.1 M $NaNO_3$ background electrolyte. All titrations were duplicated except the Cd-titration at pH 8 in the presence of calcium.

During the titrations, the amount of NaOH needed to keep the pH at a constant value was measured. In addition, the calcium concentration was measured in the supernatant at each sampling point by flame-AAS. Thus, information was obtained about the release of calcium and protons from the cell wall material during the cadmium-titration.

Before titrating in the presence of calcium, the cell wall material was washed three times with an electrolyte solution containing calcium ions. Due to the adjustment to higher pH-values of the washed suspensions, part of the calcium present in solution was sorbed to the material, leading to a lower calcium concentration at the start of the titration than had been used during the washing. For example, at the start of the (duplicate) pH 5 titrations, the calcium concentration was 0.99 ± 0.03 mM, whereas the titrations at pH 6.5 started with a calcium concentration of 0.75 ± 0.05 mM, and at pH 8 the concentration calcium was reduced to 0.65 mM at the start of the titration.

During the titration with cadmium and/or zinc, calcium ions were released into solution. The variation of the concentration of calcium during the cadmium titrations was greatest for the pH 8 titration; the standard deviation for this data range was 0.14 mM. In the text we will refer to these experiments as being carried out in approximately 1 mM $Ca(NO_3)_2$. The exact concentrations were known and all calculations were carried out using the measured calcium concentrations. Zinc may form hydroxides at lower pH than Cd (7). Therefore, in order to avoid precipitation of zinc, titrations were

performed at pH 5, 6 and 7, in a 0.01 M $NaNO_3$ background electrolyte in the presence of 0.70 to 1.09 mM $Ca(NO_3)_2$. In addition, a titration was performed at pH 7 in the presence of a lower concentration of calcium (0.22 ± 0.06 mM). All Zn-titrations were duplicated.

**Zinc-cadmium competition experiment**

The influence of Zn on Cd binding to the cell wall material was assessed at pH 6.5 in a 0.01 M $NaNO_3$ background electrolyte in the presence of approximately 1 mM $Ca(NO_3)_2$. A duplicate titration was made with a solution containing both $Cd^{2+}$ and $Zn^{2+}$. The concentrations of $Cd^{2+}$ and $Zn^{2+}$ in the titrating solution were 0.05 mM and 5 mM, respectively. As in the other titrations, samples were taken periodically to measure the metal ion concentrations after centrifugation in the supernatant. Cadmium was measured by GF-AAS, zinc and calcium by flame-AAS. In addition, the cadmium concentration was monitored by ISE.

## Results

**Speciation in solution**

The influence of pH on the solution speciation of the metals was checked with the help of the computer program ECOSAT (8). For the combinations of pH and metal ion concentration used in the experiments, the reduction of the free metal ion concentration due to hydrolysis was less than 2% in most cases. Only at a high pH and metal ion concentration this reduction was larger; for cadmium at pH 8, the reduction increased to 8% at the end of the titration. For the zinc titration at pH 7, the reduction in the concentration free $Zn^{2+}$ was slightly greater than 10%. This reduction was due to the formation of dissolved metal-hydroxide complexes; precipitation was predicted not to occur. Since the hydrolysed species may also bind to the cell wall, and since the concentration of this species was relatively low, we decided to ignore hydrolysis for the description in the metal ion binding to the cell wall material.

**Calcium sorption to cell walls**

The results of the calcium titrations at pH 5, 6.5 and 8 are given in Fig. 1a and b. The binding of calcium, $Q_{Ca}$ ($\mu mol.g^{-1}$), is given as a function of the calcium concentration ($mol.l^{-1}$) in solution in Fig. 1a. Sorption of calcium increases with increasing pH. The initial slope of the sorption at pH 5 is high and is almost equal to 1. At higher coverage this slope is lower, indicating effects of non-ideality, chemical heterogeneity, or saturation of readily available binding sites.

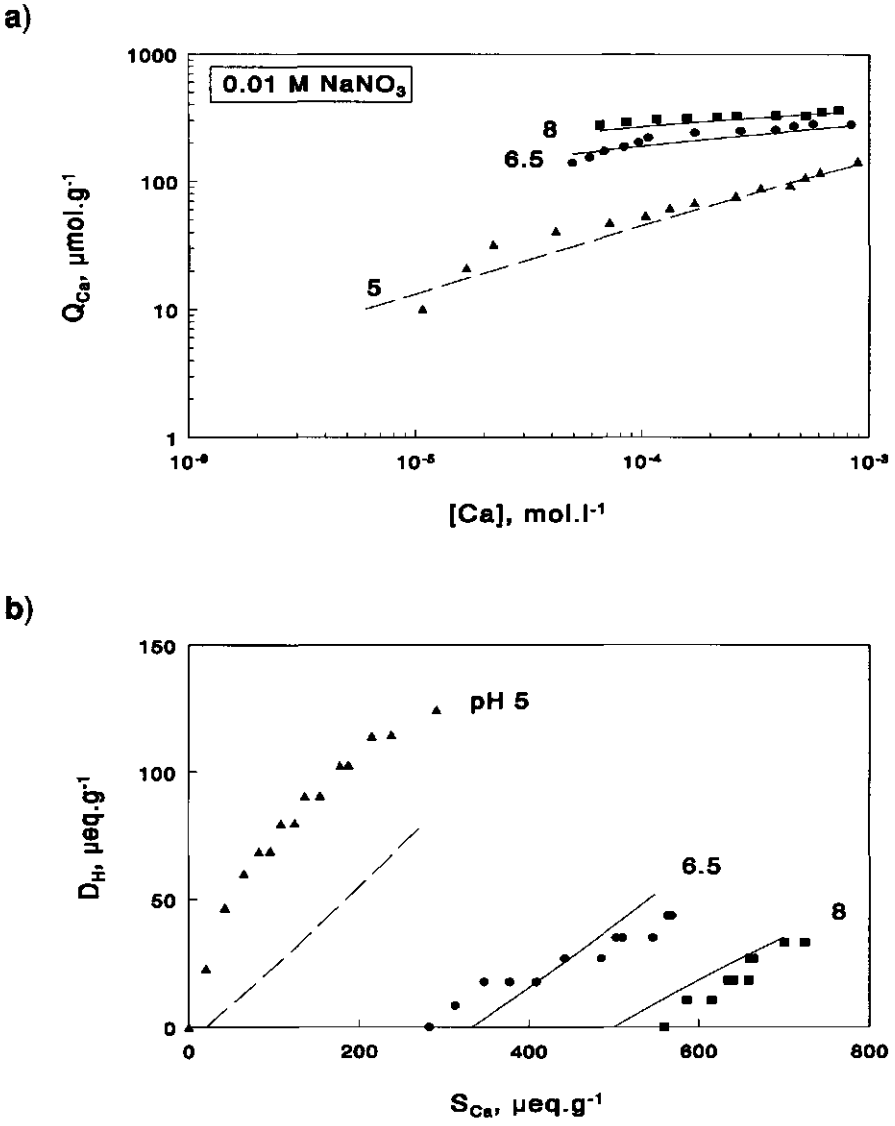



**Fig. 1** Calcium sorption to cell walls at pH 5, 6.5 and 8. Titrations performed in 0.01 M $NaNO_3$. Solid lines: model prediction based on parameter set 1. Broken line: model prediction based on parameter set 2. **a)** Calcium binding isotherms. ▲ pH 5, ● pH 6.5, ■ pH 8. **b)** Proton release ($D_H$) due to calcium sorption ($S_{Ca}$); amount of protons released (µeq.g$^{-1}$) as a function of the calcium sorbed (µeq.g$^{-1}$). ▲ pH 5, ● pH 6.5, ■ pH 8. For the model predictions, the predicted proton desorption is plotted as a function of the predicted calcium sorption.

In Fig. 1b, the proton release during the titration, $D_H$ ($\mu eq.g^{-1}$), is given as a function of the number of equivalents of calcium bound ($S_{Ca}$, $\mu eq.g^{-1}$). Since for the curves at pH 6.5 and 8 some calcium is already sorbed at start of the titration, an intercept on the x-axis for these curves has arisen. For all conditions used, protons are released upon calcium binding. As can be observed in Fig. 1b, proton desorption is most important at pH 5; at low calcium ion concentrations, the ratio of H desorbed to Ca sorbed ($eq.eq^{-1}$) then equals 1.

**Metal ion ($Cd^{2+}$ and $Zn^{2+}$) sorption to cell walls in the presence of calcium**

Fig. 2 shows the results of cadmium sorption experiments performed in a 0.01 M $NaNO_3$ background electrolyte in the presence of approximately 1 mM $Ca(NO_3)_2$ at pH 5, 6.5 and 8. As for the calcium sorption, cadmium ion sorption increases with increasing pH. The initial slopes of these cadmium sorption isotherms are smaller than 1.

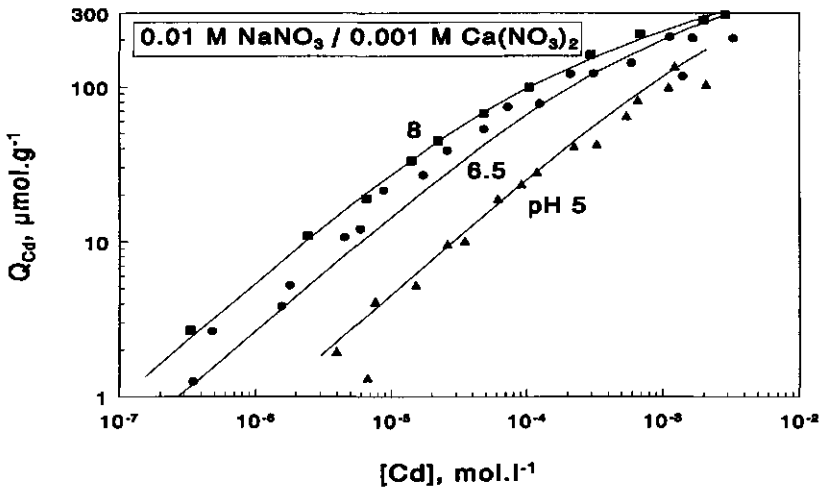
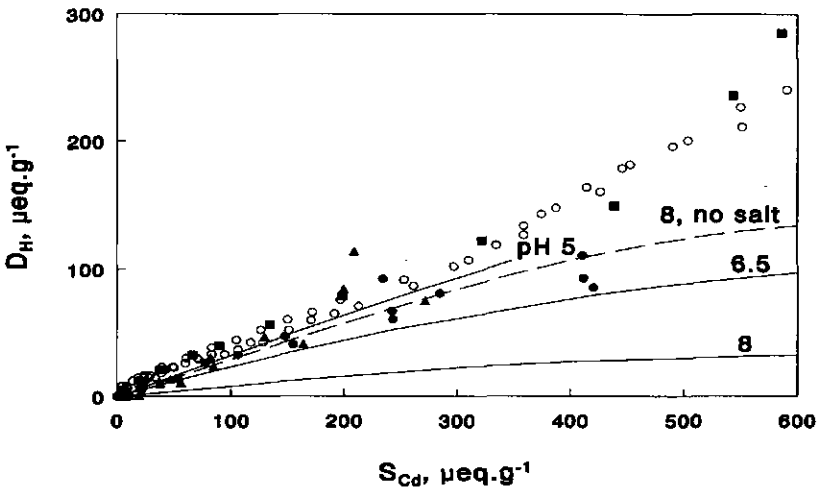



**Fig. 2** Cadmium sorption at pH 5, 6.5 and 8. Titrations performed in 0.01 M $NaNO_3$ in the presence of calcium. ▲ pH 5, ● pH 6.5, ■ pH 8. Solid lines: model prediction based on parameter set 1.

a)




b)

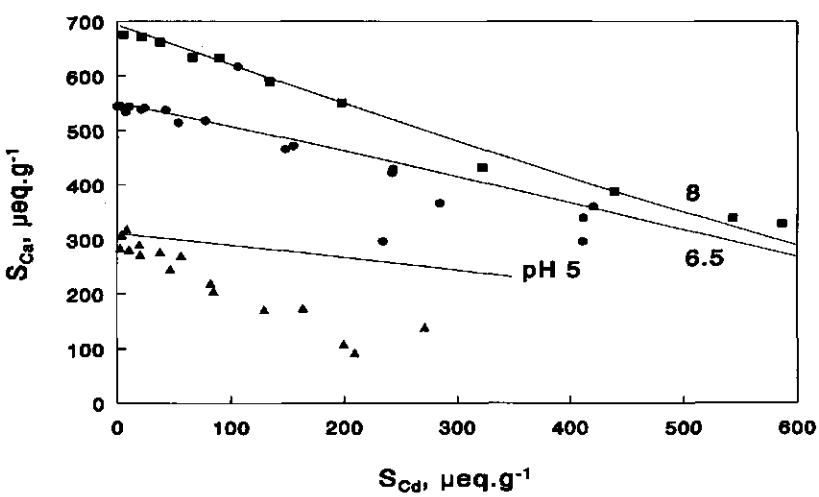



**Fig. 3** Proton and calcium release due to the binding of cadmium. **a)** Amount of protons released (µeq.g$^{-1}$) as a function of the cadmium sorbed (µeq.g$^{-1}$). **b)** Calcium release due to cadmium sorption; amount of calcium sorbed ($S_{Ca}$, µeq.g$^{-1}$) as a function of the cadmium sorbed ($S_{Cd}$, µeq.g$^{-1}$). ▲ pH 5, ● pH 6.5, ■ pH 8, ○ pH 8 in the absence of calcium (0.1 M $NaNO_3$). Solid lines: model predictions based on parameter set 1. Broken line: model predictions of the proton desorption based on parameter set 2, this line is corresponding to the data obtained in the absence of calcium (○). For the model lines, the predicted proton desorption or calcium adsorption is plotted as a function of the predicted cadmium sorption.

c)

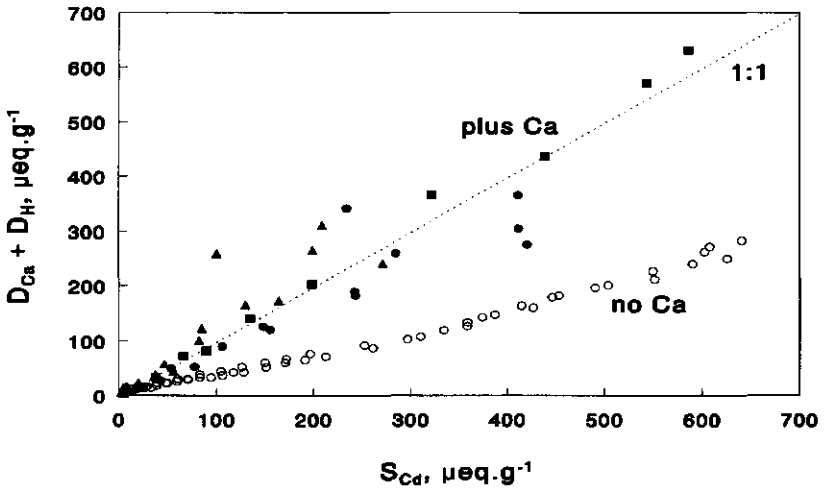



**Fig. 3c** Proton and calcium release due to the binding of cadmium. Sum of protons released and calcium released as a function of the cadmium sorbed. ▲ pH 5, ● pH 6.5, ■ pH 8, ○ pH 8 in the absence of calcium. The dotted line is indicative for an equivalent exchange.

Due to the cadmium sorption, protons and calcium ions are released (Fig. 3a and 3b respectively). The ratio of protons released/cadmium sorbed (eq.eq$^{-1}$) is between 0.5 and 0.25. A similar proton/metal exchange ratio was obtained for the titrations at pH 8 in the 0.1 M $NaNO_3$ background electrolyte (Fig. 3a).

The total release of calcium ions and protons was almost equal to the amount of cadmium sorbed in the Na/Ca environment (Fig. 3c). Apparently, cadmium sorption in this system is largely an exchange reaction; protons and calcium ions compete with cadmium for binding to the cell wall material.

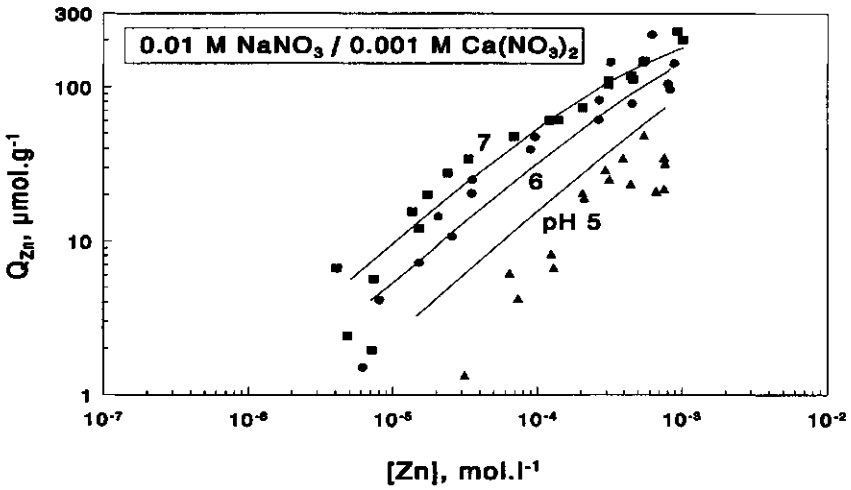



**Fig. 4** Zinc sorption to the cell wall material in the presence of calcium (the electrolyte solution contains 0.01 M $NaNO_3$ with approximately 1 mM $Ca(NO_3)_2$). ▲ pH 5, ● pH 6, ■ pH 7. Solid lines: model prediction based on parameter set 1.

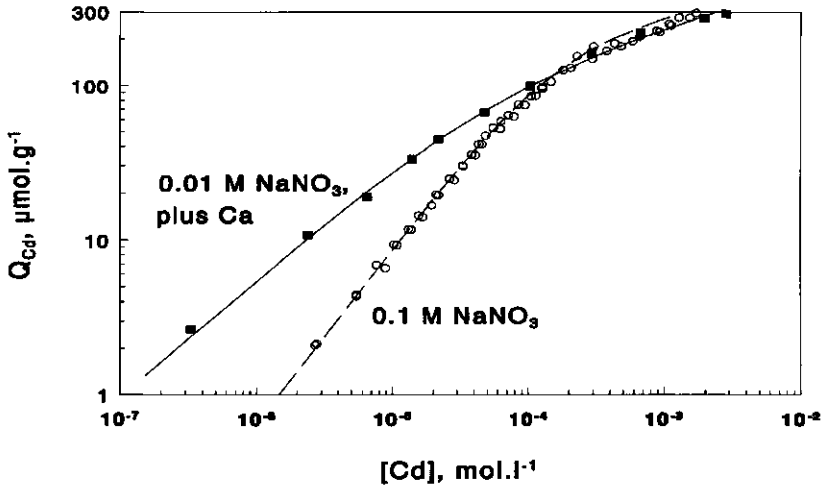
Fig. 4 shows the Zn sorption to the cell walls at pH 5, 6 and 7. Zinc behaves quite similar to cadmium, though sorption is somewhat smaller for zinc than for cadmium.

**Influence of calcium on the metal ion sorption to the cell walls**

The cadmium sorption at pH 8 in the presence of calcium (in a background electrolyte of 0.01 M $NaNO_3$) is compared to the cadmium sorption at pH 8 in a 0.1 M $NaNO_3$ background electrolyte without calcium (Fig. 5a). Surprisingly, Cd sorption is favoured in conditions where calcium is present. In other words, the calcium sorbed to the cell walls promotes Cd sorption. At higher free cadmium concentrations (>$10^{-4}$ M), the effect of calcium was negligible.

This counter-intuitive behavior is also shown for Zn, at a different pH (pH 7) and salt level (0.01 M $NaNO_3$ instead of 0.1 M $NaNO_3$) (Fig. 5b). The sorption of Zn is promoted by calcium as the concentration of free calcium in solution is raised from 0.2 to approximately 1 mM. Surprisingly, the enhancement in binding is still observed at higher concentrations of zinc since the zinc binding curves do not coincide.

a)




b)

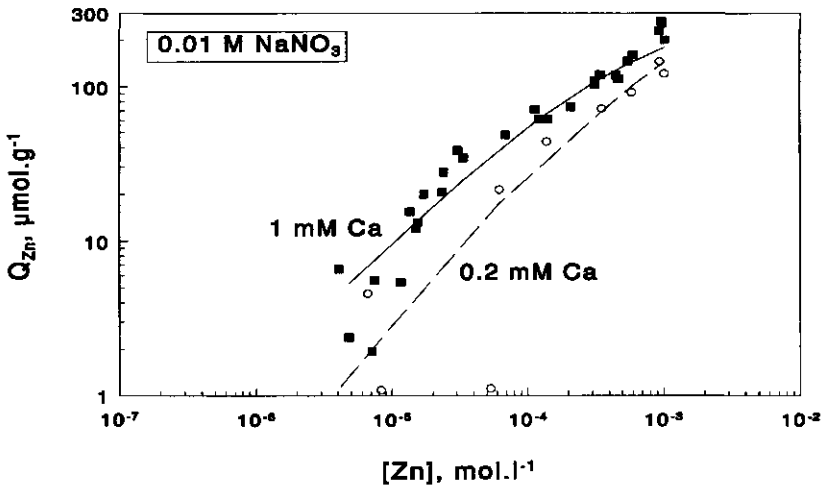



**Fig. 5** Effect of calcium on cadmium and zinc binding to cell walls. **a)** Cadmium sorption at pH 8 in different electrolyte solutions: ■ 0.01 M $NaNO_3$ with approximately 1 mM $Ca(NO_3)_2$, ○ 0.1 M $NaNO_3$. Solid line: model prediction based on parameter set 1. Broken line: model prediction based on parameter set 2. **b)** Zinc sorption at pH 7 in different electrolyte solutions: ■ 0.01 M $NaNO_3$ plus 1 mM $Ca(NO_3)_2$, ○ 0.01 M $NaNO_3$ plus 0.2 mM $Ca(NO_3)_2$. Solid line: model prediction based on parameter set 1. Broken line: model prediction based on parameter set 2.

**Cd/Zn competition experiment**

Fig. 6 shows the results of the Cd/Zn competition experiment at pH 6.5 in the presence of calcium. The Cd binding in the absence of Zn at the same pH is also shown. The results show that the zinc ions compete with cadmium ions for binding sites on the cell walls since the Cd sorption is reduced by a factor 3 to 4 on a linear scale. Conversely, the sorption of zinc to the cell walls is not affected by the Cd sorption to the cell walls due to the much lower Cd concentration used in the titration solution (the Cd-concentration is a 100-fold smaller than the Zn concentration).

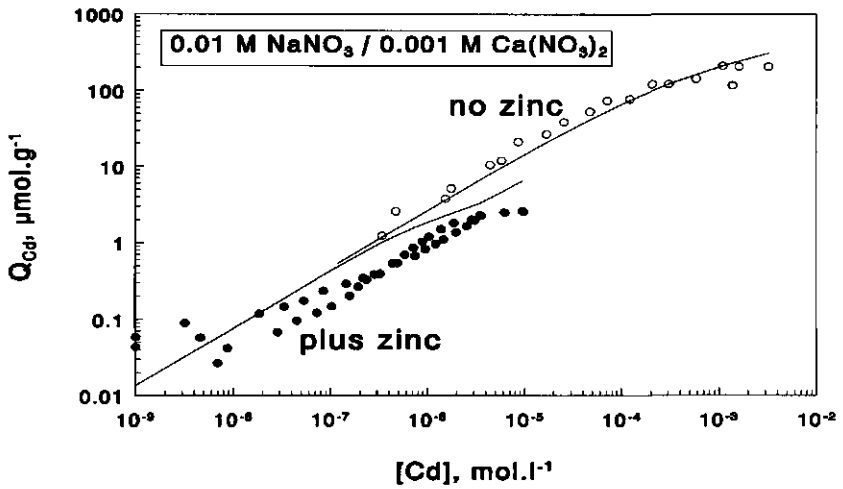



**Fig. 6** Influence of zinc on cadmium sorption at pH 6.5 in an electrolyte solution containing 0.01 M $NaNO_3$ and ca. 0.001 M $Ca(NO_3)_2$. ○ no zinc present, ● [Zn] 100-fold higher than [Cd]. Solid lines: model prediction based on parameter set 1.

**Charge balance calculations**

The initial charge of the cell walls, prior to the metal sorption experiments, is derived from the proton titrations as presented by Plette *et al.* (4). Metal ions bound in the diffuse double layer (non-specific binding) could affect the charge balance calculations. However, it is possible to estimate the amount of ions bound in the diffuse double layer using a Donnan model (4, 9, 10). Donnan-type calculations (not given here) for cadmium binding to the cell wall material at 0.1 M salt show that the non-specific binding is never greater than a few percent of the total amount of sorbed metal ions. Most other cadmium and zinc binding data were obtained in an environment with approximately 1 mM calcium, where charge reversal already has taken place. In these

systems, binding is occurring despite the repulsive potential; the concentration of cadmium in the Donnan phase will be lower than in the bulk solution. Therefore, the binding must be related to specific binding to reactive groups of the cell wall material. Thus, the net charge ($q_{net}$) can be calculated at each data point according to:

$$q_{net}=q_{init}+S_{Ca}+S_{Cd}+D_H \qquad [1]$$

where $q_{init}$, expressed in $\mu eq.g^{-1}$, is the initial charge, present on the cell wall material before addition of any bivalent ion. $S_{Ca}$ and $S_{Cd}$ are the amounts of calcium and cadmium sorbed to the cell wall in $\mu eq.g^{-1}$. $D_H$ is the net amount of protons desorbed during the calcium and cadmium sorption experiments in $\mu eq.g^{-1}$.

The results of the charge balance calculations are shown in Fig. 7. The first data points represent the situation at the start of the calcium or cadmium ion titration. For the cadmium titrations, some protons have already been released due to the calcium binding. Therefore $D_H$ does not start at zero for these titrations.

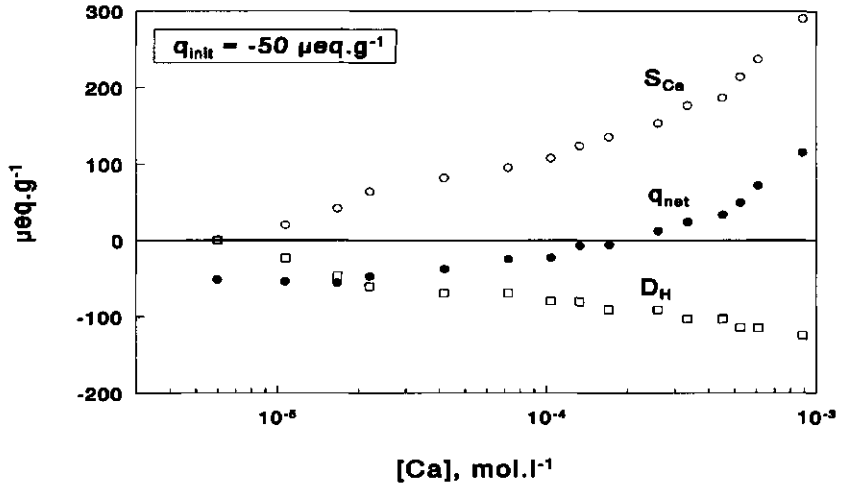
During the calcium titration at pH 5 (Fig. 7a), the amount of protons released equals the amount of calcium sorbed for calcium concentrations up to 20 $\mu mol.l^{-1}$, which leads to a constant net charge. At higher free calcium concentrations, the proton desorption does not fully compensate for the charge added by the sorbed calcium. Consequently, the number of positively charged sites must increase in the cell walls. This leads to a gradual decrease of the negative charge of the cell wall.

At calcium concentrations above 0.2 $mmol.l^{-1}$, the high calcium coverage leads to a charge reversal; the net charge of the cell walls becomes positive.

Fig. 7b shows the results for the calcium ion titration at pH 6.5. This sorption experiment was made with the cell walls which had previously been reacted with Ca at pH 5. Due to the amount of Ca that has already sorbed at pH 5, the net charge of the cell walls is slightly positive at the start of this experiment. The number of protons released during this experiment is small compared to the amount of calcium bound. The net charge of the cell walls therefore becomes even more positive. Similar results were obtained for the calcium sorption experiment at pH 8 (not shown here).

Fig. 7c shows the results for the calculations of $q_{net}$ for the cadmium ion titration performed in the presence of calcium at pH 5. Due to the large amount of calcium ions sorbed to the cell walls, the net charge of the material at the start of the sorption experiment is already positive. Cadmium sorption under these conditions is an equivalent exchange between calcium and protons (see Fig. 3c). Therefore the net charge of the cell walls remains positive and nearly constant over most of the studied cadmium concentration range. Similar results were obtained for the $Cd^{2+}$ sorption experiments made in the presence of calcium at pH 6.5 and 8 (not shown here).

a)




b)

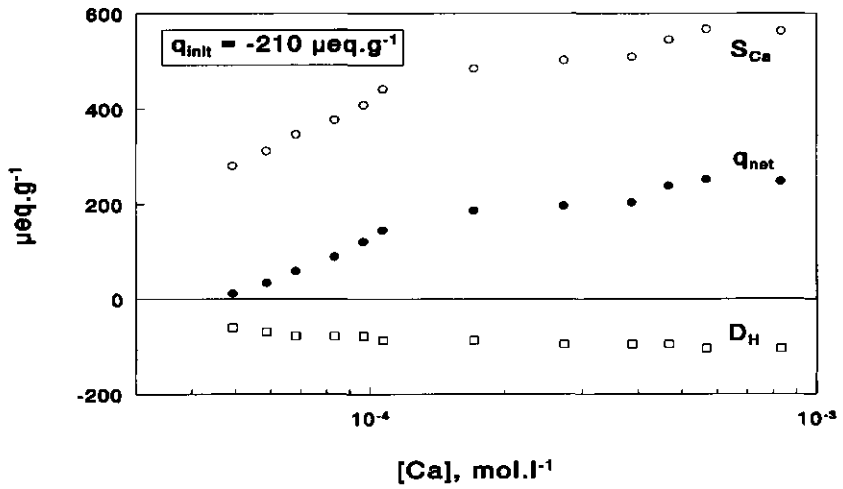
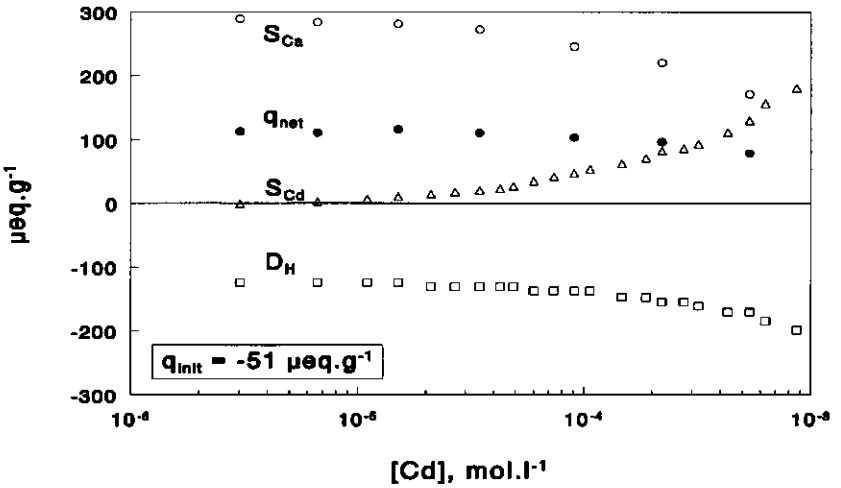



**Fig. 7** Net charge, calculated on the basis of measured sorption and desorption data. ○ Ca sorbed, □ H desorbed, △ Cd sorbed, ● calculated net charge. **a)** calcium titration, pH 5. **b)** calcium titration, pH 6.5.

c)




d)

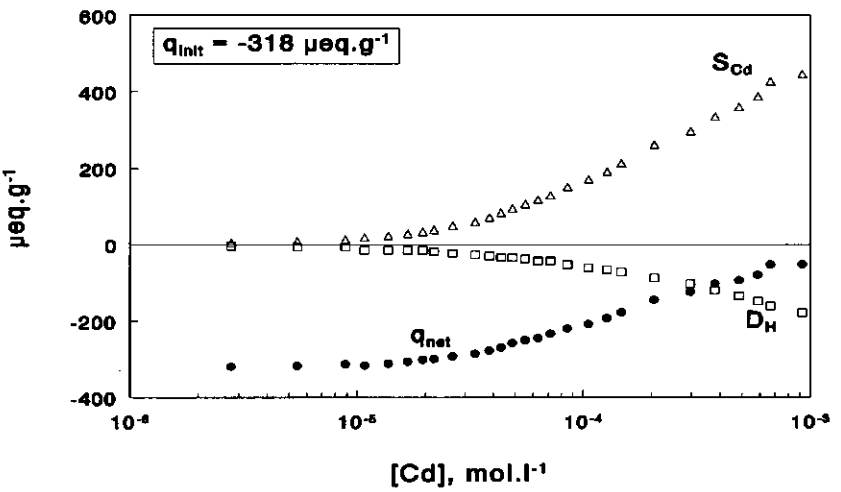



**Fig. 7** Net charge, calculated on the basis of measured sorption and desorption data. ○ Ca sorbed, □ H desorbed, △ Cd sorbed, ● calculated net charge. **c)** cadmium titration, pH 5. **d)** cadmium titration in the absence of calcium, pH 8, 0.1 M $NaNO_3$.

Fig. 7d shows the charge balance calculations for the cadmium sorption experiment at pH 8 in the absence of calcium in a 0.1 M $NaNO_3$ background electrolyte. At the start of the experiment the cell walls were negatively charged. The number of protons released during the titration was always smaller than the amount of Cd sorbed. Hence, during the titration the negative charge of the cell wall decreased. At the end of the titration the net charge of the cell walls was close to zero. If higher concentrations of free Cd were used, a charge reversal might be expected.
Binding of metal ions still occurs when charge reversal has taken place. This clearly shows that calcium, cadmium and zinc ions are specifically bound to reactive groups. This result supports *a posteriori* the Donnan calculations which already indicated that most of the binding to the cell walls was specific. Ke and Rayson (11) also observed that metal ion binding cannot be described with a model that does not take into account specific binding.

## Qualitative analysis of the data

Specifically bound protons, calcium and metal ions compete for the same binding sites on the cell wall material, since the metal ion sorption increases with increasing pH and since calcium ions and protons are released from the cell wall material as a result of metal ion sorption.
Before selecting a model to describe this competitive binding, two questions need to be addressed: i.e., which types of sites are involved in the metal ion binding and how can we explain the enhancing effect of calcium on binding of cadmium and zinc?

### Cell wall composition; reactive sites

Results from the proton titrations for the cell wall material (4) indicate the presence of strong chemical heterogeneity and/or non-ideality. Three different types of sites were distinguished which differ in the median value of their intrinsic proton affinity constants: carboxylic (log $\tilde{K}_{H,int}$ = 4.6), phosphatic (log $\tilde{K}_{H,int}$ = 7.8) and amino-type sites (log $\tilde{K}_{H,int}$ = 10.0).
Both calcium and cadmium binding are strongly pH dependent. If the carboxylic sites were the only sites involved in the metal ion binding, the majority of binding sites would be deprotonated at a pH value of 6.5, and increasing the pH from 6.5 to 8 would not lead to an enhancement of the metal ion binding. Since the data (Figs. 1a, 2 and 4) show that the ion binding is still increasing with increasing pH, it may be expected that at higher pH a second type of sites is involved in metal ion binding. These sites are probably the phosphatic groups. The amino groups are positively

charged over the whole pH-range of the metal ion titrations and are therefore not expected to coordinate strongly with the bivalent ions. In the following, we will assume that two site types are involved in metal ion binding to the cell wall material. The carboxylic sites (site-type 1) and the phosphatic sites (site-type 2).

**Cooperative effect of binding of bivalent ions**

A large difference in the sorption of cadmium and zinc in the presence and absence of calcium was observed (Fig. 5). Surprisingly, binding in the presence of calcium was higher than in the absence of calcium. In the case of cadmium, two conditions were changed simultaneously: the calcium ion concentration and the sodium ion concentration, the latter leading also to a difference in total salt concentration in solution. Calcium usually acts as a competitor (12-14), so one would expect a reduction of the binding in the presence of calcium. The ions of the "indifferent electrolyte" $NaNO_3$ are not expected to bind specifically and will mainly affect the binding via the influence on the electrostatic potential. From the results of the calculations of the net charge, as presented in Fig. 7, it was shown that the net charge of the "back-bone" was always positive during the metal ion binding experiments in the presence of calcium. At high salt concentrations and in the absence of calcium, the charge will be negative for the major part of the titration, leading to an attractive electrostatic effect. In the presence of calcium, the charge reversal due to calcium binding will lead to a repulsive electrostatic effect. This electrostatic effect makes the enhanced cadmium binding even more striking. We therefore assume that processes other than electrostatic interactions and competition for binding sites must be involved. The structure of the cell wall material may be significant.

The main component of Gram-positive bacterial cell walls is peptidoglycan, which has as its main reactive groups carboxylic- and amino-type groups. The $COO^-$ and the $NH_3^+$ of the peptidoglycan may react and form internal linkages (cross-linking) (15-17). In the absence of bi-valent ions, a significant number of the carboxylic groups are likely to be involved in this cross-linking. This leads to a condensed structure, and probably to structural hinderance for the binding of cadmium and calcium.

Once some bi-valent ions are bound, cross-linkages begin to be broken, resulting in an increase in the number of accessible binding sites for the bivalent ion and probably in an increase of the chemical heterogeneity and/or non-ideality. Due to the change in structure and conformation the apparent metal ion affinity constants may change as well. This can be regarded as a kind of cooperativity of metal ion binding: binding of one ion enhances the binding of another ion. This is most clearly reflected in the steep slope (greater than 1) of the isotherm obtained for the cadmium binding at pH 8 in the absence of calcium (Fig. 5a). At high concentrations of cadmium, the structural

differences disappear, and the binding becomes similar to that in the presence of calcium.
For the calcium ion titration at pH 5 (Fig. 1a), the slope of the binding curve at low calcium coverage almost equals 1. However, this slope is reduced at a much lower concentration than in the case of cadmium. This different behavior with respect to the influence of the structure of the cell wall on the ion binding can be explained when the conditions of the two titrations are taken into account. The cadmium titration was performed at pH 8. Therefore, at the start of the cadmium ion titration, the cell walls were highly negatively charged and, though the negative charge was reduced due to the sorption of cadmium, charge reversal did not take place. Therefore, over the full concentration range, the cadmium ions experienced an attractive electrostatic potential. The calcium titration on the other hand, was performed at pH 5, and though the charge was still negative at the start of the titration, charge reversal already took place at rather low coverage with calcium. The calcium ion will then experience a repulsive potential, which may explain the reduction of the slope of the isotherm at low coverage.
We have already mentioned that two types of sites are probably involved in the metal binding. Phospholipids are known to interact with sugars (18). It is therefore not unlikely, that, due to the breaking of internal linkages, the phosphate groups are also involved in the promotive effect.
Summarizing, the concept implies that in the absence of bivalent ions, reactive groups are internally linked, the structure is probably more condensed and binding of bivalent ions is hindered. In the presence of bivalent ions, internal cross-linkages are broken and more sites become accessible for the binding of bivalent ions. Thus, binding of cadmium and zinc ions is enhanced in systems where bivalent ions (in this case calcium) are already present at relatively high concentrations.

## Model description

Results from the proton titrations (4) indicate that it is necessary to take into account the chemical heterogeneity of the cell wall material. Probably, two types of sites (carboxylic and phosphatic) are involved in metal ion binding to the cell walls. In addition, the metal ion binding model should take into account competition between metal ions and protons and be able to describe the enhanced binding of both Cd and Zn in the presence of calcium. Moreover, as the affinity depends on both the characteristics of the sorbing ion and the adsorbate, affinity distributions for various ions on the same surface may differ (19).

**NICA Model**

For the description of metal ion binding to humic substances, good results have been obtained with the recently developed NICA (acronym for Non-Ideal Competitive Adsorption) model (19, 20). The model is based on a quasi-Gaussian affinity distribution for all ions. Apart from a general heterogeneity part that is common for all ions, the NICA model takes into account ion-specific heterogeneity or non-ideality. The metal ions may bind to two different groups of sites on the cell walls (carboxylic (1) and phosphatic (2)); $Q_{i,t}$ is the sum of the amount of ion i sorbed by site 1 and site 2:

$$Q_{i,t}=Q_{max,1}*\theta_{i,1}+Q_{max,2}*\theta_{i,2} \qquad [2]$$

in which $Q_{max,1}$ and $Q_{max,2}$ are the maximum number of carboxylic and phosphatic sites respectively in $\mu mol.g^{-1}$, and $\theta_i$ is the fraction of sites occupied by component i. The full bimodal NICA equation is:

$$Q_{i,t}=Q_{max,1}*\frac{(\tilde{K}_{i,1}c_i)^{n_{i,1}}}{\sum_j(\tilde{K}_{i,1}c_j)^{n_{i,1}}}*\frac{[\sum_j(\tilde{K}_{i,1}c_j)^{n_{i,1}}]^{p_1}}{1+[\sum_j(\tilde{K}_{i,1}c_j)^{n_{i,1}}]^{p_1}}$$

$$+\ Q_{max,2}*\frac{(\tilde{K}_{i,2}c_i)^{n_{i,2}}}{\sum_j(\tilde{K}_{i,2}c_j)^{n_{i,2}}}*\frac{[\sum_j(\tilde{K}_{i,2}c_j)^{n_{i,2}}]^{p_2}}{1+[\sum_j(\tilde{K}_{i,2}c_j)^{n_{i,2}}]^{p_2}} \qquad [3]$$

in which $\tilde{K}_i$ is the median affinity constant for component i, $c_i$ is the total concentration of component i in solution ($mol.l^{-1}$). The exponent $n_i$ (component specific non-ideality) accounts for electrostatic effects and other "non-ideal" behavior of component i. For $n_i = 1$ the NICA equation simplifies to a multi-component Langmuir Freundlich equation. In cases where $0 < n_i < 1$, non-ideality is involved. The value of p determines the width of the distribution due to the intrinsic chemical heterogeneity of the sorbent and is the same for all ions. For a chemically homogeneous surface, p would equal one. In the case of chemical heterogeneity, $0 < p < 1$. For monocomponent sorption, as is used for description of proton binding in the absence of bivalent metal ions, one can only determine the combined effect of $n_i$ and p, i.e., $m_H = n_H * p$. For a detailed discussion on the NICA model we refer to Koopal *et al.* (19), Benedetti *et al.* (20) and Kinniburgh *et al.* (10).

**Derivation of the parameters**

The binding of calcium could change the structure of the cell wall material. Therefore we have to consider two types of cell wall material. In the absence of calcium, part of the reactive groups is still involved in cross-linking, leading to a rather condensed structure. In the presence of calcium, these linkages can be broken and the structure may be more open. We will therefore use two sets of parameters to describe the data. The maximum number of sites ($Q_{max}$,μmol.g$^{-1}$) for both types of sites are taken from the acid-base titrations (4). The median proton affinity constants ($\tilde{K}_H$, L mol$^{-1}$) and the width of the distribution ($m_H$) are derived from the proton titration data at 0.01 M salt. The number of carboxylic sites ($Q_{max,1}$) is estimated as 503 μmol.g$^{-1}$, the number of phosphatic sites ($Q_{max,2}$) is estimated as 193 μmol.g$^{-1}$. Conditional constants at 0.01 M salt for these groups of sites are: $\log\tilde{K}_{H,1}$ = 4.68, $m_{H,1}$ = 0.45, $\log\tilde{K}_{H,2}$ = 8.66 and $m_{H,2}$ = 0.54.

Calcium is assumed not to affect proton binding and so these conditional constants were used for both parameter sets. The only adjustable parameters are those concerning metal ion binding and p. These parameters may differ between the two parameter sets.

We firstly described the data sets obtained on material for which structural change already had taken place, i.e., cadmium and zinc binding in the presence of calcium. Since the structure changed during the calcium titrations, the calcium parameters ($\tilde{K}_{Ca,1}$, $\tilde{K}_{Ca,2}$, $n_{Ca,1}$ and $n_{Ca,2}$) for this parameter set were obtained by optimizing the description of the desorption of calcium due to the sorption of cadmium. During the optimization procedure, priority was given to the description of the binding of cadmium to the cell wall material (Fig. 2). Next, parameters were adjusted in order to obtain a good description of the calcium desorption due to cadmium sorption (Fig. 3b) as well, without reducing the quality of the description of the cadmium binding. Once the calcium-associated parameters were obtained, we checked if the proton desorption due to cadmium binding (Fig. 3a) and the calcium binding at high pH (Fig. 1a) were also predicted reasonably well.

Next, the zinc binding in the presence of approximately 1 mM calcium was described. Since the structure of the material was the same as during the cadmium titrations, the proton and calcium parameters and p are known and only the zinc binding parameters were adjusted ($\tilde{K}_{Zn,1}$, $\tilde{K}_{Zn,2}$, $n_{Zn,1}$ and $n_{Zn,2}$).

This parameter set (Set 1) was used as a first estimate for the description of the data for which structural changes take place during the titrations (i.e.; the binding of cadmium at pH 8 in the absence of calcium, the binding of zinc in the presence of 0.22 mM calcium, and the calcium titrations). Again, priority was given to the description of the metal ion binding. The parameters so derived were adjusted slightly

to improve the description of the calcium binding and the proton desorption, provided that the description of the metal ion binding did not deteriorate. This gave Set 2. The values of the parameters for these two data sets are given in Table 1.

**Table 1**

**Parameters used in the conditional bimodal NICA equation**

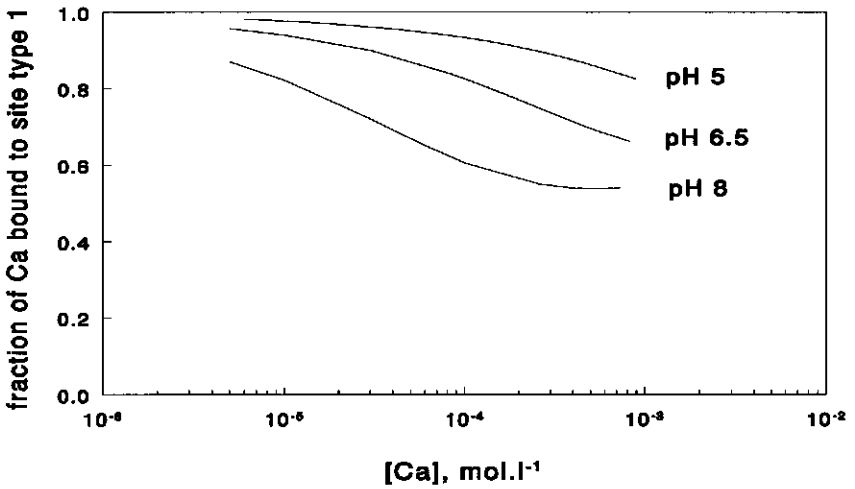
Set 1 is used to describe the data obtained in the presence of calcium; Set 2 is used for the data obtained in the absence of calcium. Site type 1 refers to the carboxylic sites, site type 2 to the phosphatic sites. $Q_{max}$ is expressed in µmol.g$^{-1}$.

| | Set 1 with calcium | | Set 2 no calcium | |
|---|---|---|---|---|
| site type | 1 | 2 | 1 | 2 |
| parameter | | | | |
| $Q_{max}$ | 503 | 193 | 503 | 193 |
| log $\tilde{K}_H$ | 4.68 | 8.66 | 4.68 | 8.66 |
| log $\tilde{K}_{Cd}$ | 2.3 | 5.3 | 2.2 | 4.2 |
| log $\tilde{K}_{Ca}$ | 2.3 | 5.3 | 2.5 | 4.2 |
| log $\tilde{K}_{Zn}$ | 2 | 4.9 | 1.9 | 4.1 |
| $m_H$ | 0.45 | 0.54 | 0.45 | 0.54 |
| p | 0.6 | 1 | 0.75 | 1 |
| $n_H$ | 0.75 | 0.54 | 0.6 | 0.54 |
| $n_{Cd}$ | 0.8 | 0.75 | 1 | 1.2 |
| $n_{Ca}$ | 0.3 | 0.45 | 0.55 | 1 |
| $n_{Zn}$ | 0.8 | 0.85 | 0.9 | 1 |

The model description of the cadmium, calcium and zinc binding, as well as the predicted desorption of protons, are shown as model lines in Figs. 1-5. Solid lines are used for the description based on the parameter set 1. Dashed lines are used for the description based on the parameter set 2. The sorption of cadmium in the presence

of an excess of zinc and calcium was predicted with the derived parameters. Results are shown in Fig. 6 (solid line).
Calcium is a strong competitor for cadmium and zinc binding. This is reflected in the high affinity constants obtained for calcium, which are similar to those for cadmium and greater than those for zinc.
Cooperativity is mainly expressed in the values for the ion specific non-ideality. Set 1 describes the data for which structural changes already have taken place, i.e., the structure and the conformation are the same over the full titration range. Thus, here the exponent n for the bivalent ions has values < 1, which reflects non-ideality and electrostatic effects. However, the metal ion binding described by parameter set 2, experienced the impact of structural changes during the titrations. This is reflected in an element of cooperative ion-binding. For cadmium and zinc, the non-ideality in the presence of calcium, was (more than) fully compensated for by these cooperative effects since the values for n needed to describe their binding were $\geq 1$ ($n_{Cd} = 1.2$; $n_{Zn} = 1$).
The apparent affinity constants for zinc are somewhat smaller than for cadmium and calcium for both site types. Apart from the pH 5 data, a reasonable description of the zinc binding was obtained.
The predicted desorption of protons due to the sorption of cadmium (Fig. 3a), was much smaller than the measured proton desorption. Since the predictions were deviating most at high pH, this discrepancy might be because the amino-type sites were not taken into account in the model. At high pH, these groups may also deprotonate, especially when the charge of the cell wall material has been reversed, leading to a proton repulsive potential and a positive surface potential created. This would lead to the repulsion of protons.
The prediction of calcium sorbed during the cadmium sorption experiments (Fig. 3b) was good at pH 6.5 and 8. At pH 5, the amount of calcium sorbed at start of the cadmium titration was predicted well, but the release of calcium ions during the cadmium titration was underestimated by the model.
The model was used to calculate the distribution of the calcium and cadmium over both site types. The calcium binding, as described by parameter set 2, is dominated by the binding to the carboxylic sites (Fig. 8a). At high pH and high calcium concentrations, the phosphatic sites also contribute to the calcium ion binding (up to 50%).

a)




b)

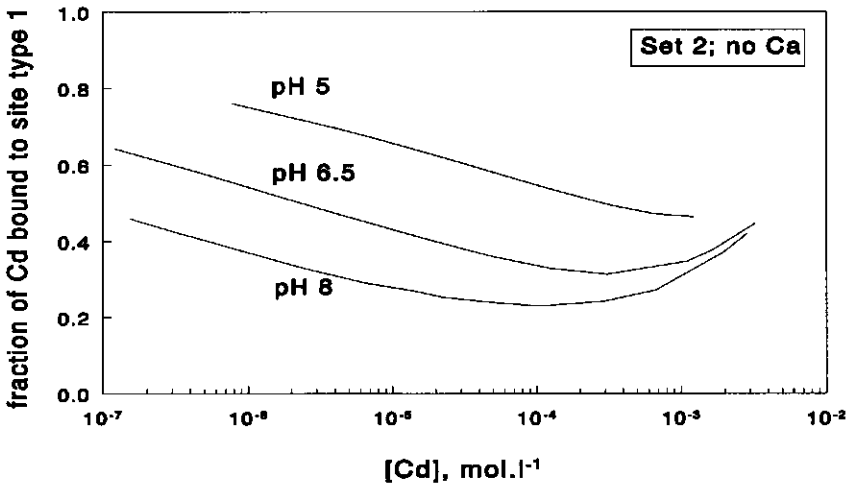



**Fig. 8** Fraction of total calcium or cadmium ion bound to the first group of sites, as calculated by the NICA model. **a)** calcium binding in 0.01 M $NaNO_3$. Parameters of set 2 are used for the calculations. **b)** fraction of cadmium bound to first site type, as calculated by using parameter set 2.

c)

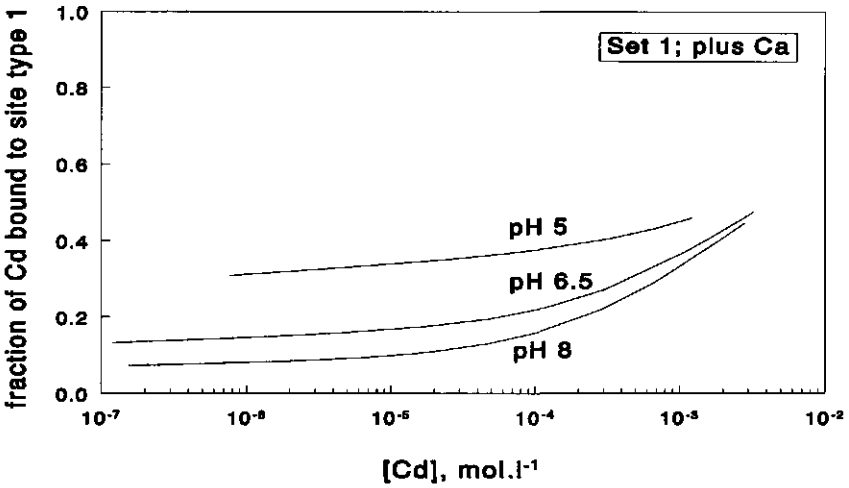



**Fig. 8c** Fraction of total calcium or cadmium ion bound to the first group of sites, as calculated by the NICA model. **c)** fraction of cadmium bound to first site type, as calculated by using parameter set 1.

In the absence of calcium and at low pH, cadmium ion binding was also dominated by the carboxylic sites (Fig. 8b). At higher pH, the binding to the phosphatic sites becomes more important. In the presence of calcium, the distribution of cadmium over the two site types is quite different. Cadmium ion binding is dominated by the phosphatic sites for the major part of the pH and cadmium concentration range studied (Fig. 8c). However, the contribution of the carboxylic site to the cadmium bound is also larger at low pH than at high pH.

## Discussion

### Specific binding leading to charge reversal

The assumption that most binding of bivalent cations to the cell wall material is related to specific binding appears to be confirmed. This enabled us to calculate charge balances for all titration data, leading to the striking result that at high coverage with these ions charge reversal probably takes place. The calculated charge reversal may be partly related to some specific characteristics of the material used, since there were reactive groups (amino-type groups) present that remained positively charged even at high pH. The results of the charge balance calculations are supported by the work

of Doelman (21), who measured the electrophoretic mobility of Gram-negative bacteria in solutions containing varying concentrations of $Pb^{2+}$. At low concentrations, a negative mobility was obtained. With increasing lead concentration, the mobility reduced, becoming zero at approximately 0.3 mM $Pb^{2+}$. At higher lead concentrations, even a positive mobility was measured.
In soil, where calcium, magnesium and other bivalent ions are abundant, this might have an impact on the attachment of bacteria to soil particles. In a sodium environment, bacteria experience a repulsive electrostatic potential when approaching a negatively charged clay particle (22). In a calcium-rich environment, the bacterium will be positively charged, and therefore will tend to be attracted to the clay particle. Hence, in many natural soil systems, the impact of electrostatic interactions may be completely the reverse of what might be expected from attachment studies performed in sodium-rich environments. Huysman and Verstraete (23) have recently observed an enhanced attachment of bacteria to soil particles in the presence of bivalent ions.

**Electrostatic interactions**
For reasons of simplicity, electrostatic interactions have not been taken into account explicitly, even though the impact of the electrostatic potential on the metal ion binding is recognized. The electrostatic interactions are included implicitly in the exponent n, which is the parameter characterizing all component-specific non-idealities.
This might also explain the high value obtained for the exponent $n_{Cd}$ for cadmium binding at pH 8 in 0.1 M $NaNO_3$ (Set 2, site 2): due to the higher salt level, the impact of the electrostatic potential is limited, leading to more ideal behavior than in the presence of a lower salt level.
We assumed that $Na^+$ does not bind specifically and thus does not directly influence the specific binding other than via electrostatic effects. This assumption is supported by the work of Doyle *et al.* (12) and Crist *et al.* (24). Recently, Westall *et al.* (25) described pH- and salt dependent cobalt binding by humic acid by suggesting that sodium ions compete with metal ions for the binding sites. In order to show such an effect, it has to be assumed that the affinity for sodium ions is very high, which is rather unlikely. Moreover, the binding of sodium ions will only affect the location of the predicted binding curve, not it's shape. Therefore, the concept as presented by Westall *et al.* (25) can not explain the different slopes obtained in the 0.01 and 0.1 M $NaNO_3$ environment.

**Composition and structure of the cell wall**

Beveridge and Murray (26) found carboxylic groups to be the dominant functional groups responsible for binding of $Cd^{2+}$ to the cell walls of *Bacillus subtilis*. At pH 6, diamine groups have been observed to be involved in the binding of $Cd^{2+}$ to the biomass of the higher plant *Datura innoxia* (11). In the material we used, in addition to carboxylic and amino-type groups, phosphate-type groups originating from phospholipidic materials (4) were present. The maximum degree of cross-linking between a carboxylic and an amino-group can be estimated from the proton titration data (4). At pH 8, all carboxylic sites (0.5 meq.$g^{-1}$) and half of the amount of phosphatic sites (in total 0.19 meq.$g^{-1}$) are deprotonated whereas there are 0.23 meq.$g^{-1}$ of positively charged amino-type sites. Even if all amino-groups were involved in the cross-linking with carboxylic groups, there would still be "free" carboxylic sites available for ion binding.

From the results of the distribution calculations (Figs. 8 a-c), it can be seen that in the absence of calcium and at low cadmium concentrations the cadmium ion mainly binds to the carboxylic site. The contribution of the phosphatic sites to cadmium binding increases once some cadmium has been bound. In a calcium-rich environment, apparently all phosphatic sites are available for binding, since here, at low cadmium concentrations, cadmium binding is dominated by the phosphatic sites. Apparently, the presence of calcium ions not only affects the availability of carboxylic sites but it also affects the phosphatic sites.

The phospholipids may also interact with sugar molecules (18). The mechanism suggested (18), is that the phosphate group of the phospholipid is linked to the -OH groups of the sugar via hydrogen bonding. Biondi (27) concluded that the sugars interact with the same groups to which the calcium ion binds. Structural changes of the lipid membranes were observed that are coincident with the increase in the electrostatic repulsion due to calcium sorption (27). Indeed, as is predicted by our model, at pH 8 and in a 1 mM calcium solution, 80% of all phosphatic sites are occupied by a calcium ion, and it therefore may be expected that under these conditions most hydrogen bonds are broken, thus facilitating further metal ion binding. The promotive effect of calcium could thus also be due to the breaking of the hydrogen bonds between the peptidoglycan and the phospholipid.

Another mechanism for the facilitation of metal ion binding by calcium is presented by Bush (28). He observed cooperative binding of calcium to cell walls of maize roots. On the basis of cell wall structure studies he assumed that chains of polygalacturonic acid (the main component of this material) are bridged by calcium ions such that binding of the first cations between any pair of chains causes an alignment which facilitates binding of further ions. This concept might explain our observations. The formation of

bidentate complexes also cannot be ruled out. However, the assumption that all $Me^{2+}$ form bidentate complexes, would lead to a reduction of the maximum amount of cadmium and calcium that may be bound ($Q_{max}$) by a factor 2. In that case, for some conditions, the amount of calcium and cadmium ions bound exceeds the number of theoretically available binding sites.
The increase in affinity of the phosphatic sites for cadmium and calcium when the structure is more open is remarkable. This might indicate the formation of bidentate complexes under these conditions. Therefore, whether or not the binding ion induces bridging between different binding sites is at present an open question.
Although the exact mechanism by which calcium enhances the binding of cadmium and zinc remains unclear, the change in structure of the cell wall and/or the change in conformation of the reactive groups, appears to play an important role.

**Extrapolation to intact, living cells**

Two stages can be distinguished in metal sorption by intact, living cells. A fast stage, representing sorption to the cell wall, is followed by a much slower stage, representing uptake into the cell. The phosphatic sites are probably located on the membrane or cytoplasmic organelles, and thus will not contribute to metal sorption to the intact cell wall. It is therefore important to be able to discriminate between the binding of metal ions to both the carboxylic and phosphatic sites on the isolated cell wall material. The model developed explicitly accounts for the metal ion binding by this impurity, and therefore enables our experimental results to be extrapolated to metal ion binding to the cell walls of intact cells.

## References


1. Morel, F.M.M., Principles of Aquatic Chemistry. Wiley: New York, chapter 6 (1983).
2. Collins, Y.E., and G. Stotzky, Factors affecting the toxicity of heavy metals to microbes, In: Metal ions and Bacteria. Beveridge, T.J., Doyle, R.J., (eds.), Wiley Interscience, New York, chapter 2 (1989).
3. Nederlof, M.M., van Riemsdijk, W.H., Effect of natural organic matter and pH on the bioavailability of metal ions in soils. In: Environmental impact of soil component interactions, P.M. Huang et al. (eds.), CRC Press, Boca Raton, 73 (1995).
4. Plette, A.C.C., van Riemsdijk, W.H., Benedetti, M.F., van der Wal, A., pH-dependent charging behavior of isolated cell walls of a Gram-positive soil bacterium. *J. Colloid Interface Sci.* **173**, 354 (1995).
5. van der Wal, Chemical analysis of isolated cell walls of Gram-positive bacteria and the determination of the cell wall to cell mass ratio. In: Electrochemical characterization of the bacterial cell surface, Ph.D. thesis WAU chapter 2 (1996).
6. de Keizer, A., Electrosorption of tetra-alkyl ammonium ion on silveriodide. Ph.D. thesis, chapter 4.4 Wageningen Agricultural University, (1981).
7. Lindsay, W.L., Chemical Equilibria in Soils. Wiley Interscience, New York, 449 (1979).
8. Keizer, M.G., van Riemsdijk, W.H., ECOSAT: a computer programme for the calculation of speciation and transport in soil-water systems. Manual, Department of Soil Science and Plant Nutrition, Agricultural University, Wageningen (1995).
9. Benedetti, M.F., van Riemsdijk, W.H., Koopal, L.K., Humic substances considered as a heterogeneous Donnan gel phase. *Environ. Sci. Technol.*, in press (1996).
10. Kinniburgh, D.G., Milne, C.J., Benedetti, M.F., Pinheiro, J.P., Filius, J., Koopal, L.K., van Riemsdijk, W.H, Metal ion binding by humic acid: application of the NICA-Donnan model. *Environ. Sci. Technol.*, accepted (1996).
11. Ke, H.Y., Rayson, G.D., Characterization of Cd binding sites on *Datura innoxia* using $^{113}Cd$ NMR spectrometry. *Environ. Sci. Technol.* **26**, 1202 (1992).
12. Doyle, R.J., Matthews, T.H., Streips, U.N., Chemical basis for selectivity of metal ions by the *Bacillus subtilis* cell wall. *J. Bacteriol.* **143** (1), 471 (1980).
13. Xue, H.B., Stumm, W., Sigg, L., The binding of heavy metals to algal surfaces. *Wat. Res.* **22** (7), 917 (1988).
14. Flemming, C.A., Ferris, F.G., Beveridge, T.J., Bailey, G.W., Remobilization of toxic heavy metals adsorbed to bacterial wall-clay composites. *Applied Environmental Microbiol.* **56** (10), 3191 (1990).
15. Marquis, R.E., Mayzel, K., Carstensen, E.L., Cation exchange in cell walls of Gram-positive bacteria. *Can. J. Microbiol.* **22**, 975 (1976).
16. Marquis, R.E., Bender, G.R., Compact structure of cortical peptidoglycans from bacterial spores. *Can. J. Microbiol.* **36**, 426 (1990).
17. Koch, A.L., Growth and form of the bacterial cell wall. *American Scientist* **78**, 327 (1990).
18. Lee, C.W.B., Das Gupta, S.K., Mattai, J., Shipley, G.G., Abdel-Mageed, O.H., Makriyannis, A., Griffin, R.G., Characterization of the $L_\lambda$ phase in trehalose-stabilized dry membranes by solid-state NMR and X-ray diffraction. *Biochemistry* **28**, 5000 (1989).

19. Koopal, L.K., van Riemsdijk, W.H., de Wit, J.C.M., Benedetti, M.F., Analytical isotherm equations for multicomponent adsorption to heterogeneous surfaces. *J. Colloid Interface Sci.* **166**, 51 (1994).
20. Benedetti, M.F., Milne, C.J., Kinniburgh, D.G., van Riemsdijk, W.H., Koopal, L.K., Metal binding to humic substances; Application of the Non-Ideal Competitive Adsorption model. *Environ. Sci. Technol.* **29**, 446 (1995).
21. Doelman, P., Effects of lead on properties of lead-sensitive and lead-tolerant cells. In: Krumbein, W.E. (ed.), Environmental biochemistry and geomicrobiology, Vol. 3, Ann Arbor Science Michigan, (1978).
22. Rijnaarts, H.H.M., Norde, W., Lyklema, J., and Zehnder, A.J.B., DLVO and steric contributions to bacterial deposition in different ionic strength environments. In: Rijnaarts, H.H.M., Interactions between bacteria and solid surfaces, in relation to bacterial transport in porous media. Ph. D. thesis, Wageningen Agricultural University (1994).
23. Huysman, F., Verstraete, W., Effect of cell surface characteristics on the adhesion of bacteria to soil particles. *Biol. Fertil. Soils* **16**, 21 (1993).
24. Crist, R.H., Oberholser, K., Shank, N., Nguyen, M., Nature of bonding between metallic ions and algal cell walls. *Environ. Sci. Technol.* **15**, 1212 (1981).
25. Westall, J.C., Jones, J.D., Turner, G.D., Zachara, J.M., Models for association of metal ions with heterogeneous environmental sorbents. 1. Complexation of Co(II) by Leardonite Humic Acid as a function of pH and $NaClO_4$ concentration. *Environ. Sci. Technol.* **29**, 951 (1995).
26. Beveridge, T.J., Murray, R.G.E., Sites of metal deposition in the cell wall of *Bacillus subtilis*. *J. Bacteriol.* **141**, 876 (1980).
27. Biondi, A.C., Arroyo, J., Diaz, S., Disalvo, E.A., Effect of H-bonding compounds on the adsorption of $Ca^{2+}$ to lipid membranes in the gel state. *J. Coll. Interface Sci.* **171**, 28 (1995).
28. Bush, D.S., Cation and water sorption in the leaf apoplast of *Brassica oleracea* var. *acephala*. Ph.D. thesis, University of California at Berkeley (1983).

# Chapter 4

# Sorption of metal ions by intact cells I

Preliminary experiments

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# Sorption of metal ions by intact cells I

The study of metal ion sorption to living bacterial cells may be complicated by metabolic processes. Living cells will respirate and produce $CO_2$ from endogeneous substrates. Under extreme conditions, the lysis of bacterial cells may lead to an increased concentration of small organic molecules in solution. The use of pH-buffers in batch experiments may influence metal ion speciation and bacterial metabolism. Some experiments were performed to study the impact of these processes on the metal sorption by the bacteria.

## Introduction

The metal ion sorption to isolated cell wall material of *Rhodococcus erythropolis* A177 (R.A177) is studied and described in detail (1). It is a great challenge to study these interactions on living bacterial cell material. Some questions that rise are: Is sorption behavior to intact cells similar to the adsorption of the metal ions to the isolated cell walls (pH-dependency, influence of the calcium concentration), can this sorption be predicted with the binding model derived for the metal ion binding to isolated cell walls, and can similar trends be distinguished between sorption and toxic effects, for changing environmental conditions?

The study of metal sorption to living bacterial cells may be complicated by metabolic processes. A possible complication is the $CO_2$-production due to endogeneous respiration by the bacteria. Since the sorption experiments have to be performed in rather thick cell suspensions, this $CO_2$-production may be significant. The dissolved $CO_2$ is partly hydrated, thus forming carbonic acid, $H_2CO_3$. The carbonic acid will release protons at high pH. Below pH 5, practically all carbonate species are fully protonated. Around pH 8, the main carbonate species is $HCO_3^-$, whereas at pH values greater than 8 even $CO_3^{2-}$ starts to become a significant species in solution. The dissociation of carbonic acid will acidify the solution, which is rather inconvenient for experiments performed in simple batch systems, where pH cannot be controlled. Cadmium and zinc ions may form complexes with $HCO_3^-$ and $CO_3^{2-}$, at high carbonate concentrations even precipitation of the metals as a carbonate salt may take place. Therefore, conditions that may lead to high carbonate concentrations in solutions must be avoided. The $CO_2$ production by R.A177 cells was studied at pH 5 and pH 8. In addition, the effect of stress (cadmium addition) on the $CO_2$ production was tested.

During preliminary experiments at pH 8 a slight yellow colouring and increased viscosity of the supernatant was observed. At a lower pH, no change in colour was

observed. Two processes may be involved in this phenomenon: the living bacteria may produce and excrete small organic ligands (e.g., results of the work of Xue and Sigg (2) indicate a relation between the copper concentration and the exudate production by algae), or the bacteria are killed due to the presence of cadmium, which is followed by lysis of the bacterial cells. In case of the first process, the supernatant will probably contain sugar-type molecules. In case of lysis of the cells, the nature of these products will be protein-like. An experiment was performed in order to obtain information on the nature of these components and on the time dependency of the production of these components.
The influence of pH on the toxicity zinc for R.A177 was determined by van Beelen and Fleuren-Kemilä (3). They studied the reduction in the respiration of radioactive labelled acetate due to the presence of cadmium and zinc at pH 6, 7 and 8 in dilute cell suspensions. All experiments were performed in a buffer solution, in order to avoid pH-fluctuations during the experiments. Tris-HCl (Tris = tris(hydroxymethyl)aminomethane) was used as the buffering agent. They did not add any other salts as background electrolyte. In addition, they performed similar experiments with cadmium (unpublished data).
In contrast, data on cadmium and zinc sorption to isolated cell walls were obtained from experiments performed in a $NaNO_3$ environment, using a pH-stat setup. Tris may influence the metal ion sorption to the bacteria, since the concentration of free metal ions in solution may be reduced due to complex formation with Tris, and since the Tris-molecule may be too large to act properly as a counterion in the cell wall. In addition to the influence on the metal ion speciation, Tris may affect the metabolism of micro-organisms (4, 5), thus influencing the interaction between the metal ions and the bacteria. In order to test for the impact of Tris on the sorption of metal ions by R.A177, some experiments are performed both in $NaNO_3$ and in Tris solutions.
The results of these experiments are used for the assessment of the limiting conditions and the experimental setup of the sorption experiments that are described in chapter 5.

## Materials and methods

### *Rhodococcus erythropolis* A177, growth conditions

*Rhodococcus erythropolis* A177 (6) was grown in batch culture till the late-exponential growth stage at 28°C, in a mineral medium as was described by Schraa *et al.* (7), except that yeast extract was not added. The medium contained

1.46 g.l$^{-1}$ $KH_2PO_4$, 2.86 g.l$^{-1}$ $Na_2HPO_4.2H_2O$, 1.0 g.l$^{-1}$ $NH_4NO_3$, 0.1 g.l$^{-1}$ $MgSO_4.7H_2O$, 0.05 g.l$^{-1}$ $Ca(NO_3)_2.4H_2O$ and 1 ml.l$^{-1}$ of a micro-nutrient solution and a vitamin-solution. Ethanol was the sole carbon- and energy source.

**Preparation of the cell suspension**

The cell suspension was washed three times in the electrolyte solution that was to be used for the experiment. For the experiments performed in 0.01 M Tris or 0.005 M Tris with 0.005 M $NaNO_3$, washing was performed twice in 0.01 M $NaNO_3$, and after that only the last washing step was performed in a solution containing Tris. The washed suspension was stirred under $CO_2$-free air in a thermostatted reaction vessel at pH 4.7 at 25 ± 1°C for 1 hour, in order to remove all $CO_2$ from the suspension. Then the pH was raised to the value at which the experiment was to be performed, and the suspension was allowed to equilibrate at this pH for at least another hour. The pH was kept constant either by the Tris buffer or by a pH-stat setup.

**Preparation of solutions and analysis of metal, calcium and proton concentration in solution**

Metal ion solutions were made in the same electrolyte solution as the solution in which the bacterial cells were suspended. All solutions were made in preboiled, double-distilled water. All chemicals used were p.a. quality (Merck).

For the determination of the total metal and calcium concentration in solution, the following procedure was used. 2 ml samples were taken from the vessel or from the batches, put into 4 ml polypropylene tubes, and centrifuged at 6000 rpm (Denley BM402) for 10 minutes. 3 ml of acid (0.1 M $HNO_3$ in 0.01 M $NaNO_3$) was added to 1 ml of the supernatant. The acidified supernatants were kept at 4°C until the metal content of the supernatant was analyzed. The total metal concentration was measured with flame AAS.

The proton activity was measured with a half cell glass electrode in combination with a double-junction Ag/AgCl sleeve electrode (Orion) of which the outer junction was filled with an equitransferent solution containing 0.125 M $NaNO_3$ and 0.875 M $KNO_3$ (8). For the pH-measurements in the batches, a micro pH-electrode was used (Orion).

During the titrations of Tris with cadmium the change in free cadmium ion concentration was measured using an ion selective electrode (ISE, Orion), which was calibrated in 0.01 M $NaNO_3$ with 0.001 M $HNO_3$ at $10^{-6}$, $10^{-5}$, $10^{-4}$ and $10^{-3}$ M $Cd^{2+}$. The calibration solutions were dilutions of a standardized 0.1 M $CdCl_2$ solution (Titrisol).

**Determination of the dry weight content of the bacterial cell suspension**
The dry weight of bacterial cell material used in the experiments was obtained from the pellets after centrifugation as described above. The pellets of moist bacterial cell material were dried at 60°C for 24 hours. To test the quality of the drying, some samples were left over at 60°C for another 24 hours, which did not influence the outcome of the measurements.

**Measurements of $CO_2$ produced in batch systems**
Samples, taken from the suspension in the thermostatted reaction vessel, were put in 35 ml glass tubes closed with a septum (inner surface teflon, outer surface silicone). The tubes were shaken for some minutes on a vortex to equilibrate the initially $CO_2$-free air with the $CO_2$-containing suspension. A 2 ml sample of the air was taken and used for $CO_2$ analysis on the gas chromatograph (Chrompack Gas Chromatograph CP9001, oven temperature 45°C, detector 130°C and injector temperature 55°C). The GC was calibrated with 404 ppm $CO_2$ in $N_2$ (AGA gas). To release all $CO_2$ from the suspension, 2 ml buffer (1 M $KH_2PO_4$, pH 4) was added to the tube, and after shaking another 2 ml sample was taken from the superposing air to measure the concentration $CO_2$. In this way, approximately all $CO_2$ is released from the suspension, and thus the total amount of $CO_2$ produced during the experiment is measured.

**$CO_2$ production during batch experiments**
A bacterial suspension was prepared as described above. First the pH was raised from 4.7 to 5. Four samples of the bacterial cell suspension were taken from the vessel with the help of a syringe and put in glass tubes, closed with a septum. The samples were used to measure the $CO_2$ production in duplicate, both immediately after the sampling from the vessel, and after two hours of incubation on a thermostatted shaking machine at 25°C.
Next, the pH of the suspension in the reaction vessel was raised to 8 and allowed to equilibrate at this pH for 1 hour. 6 samples were taken from the suspension in the reaction vessel. The $CO_2$-production was measured in duplicate immediately after the sampling, after two hours of incubation and after two hours incubation in the presence of $Cd(NO_3)_2$. Total Cd concentration in these tubes was 2.6 (2.64 ± 0.02) mM, the concentration in solution was determined by flame-AAS after centrifugation of a part of the suspension. The Cd-activity in these solutions was measured by ISE.
The whole experiment was repeated with a $NaNO_3$ solution (without bacterial cells), in order to test the experimental setup for other $CO_2$-sources.

**Nature and quantity of exudates formed during exposure to $Cd^{2+}$ at pH 8**
The experiment was performed in the thermostatted reaction vessel. Duplicate samples were taken from the vessel during 24 hours after addition of 2 ml 0.02 M $Cd(NO_3)_2$. Total volume of the suspension at the start of the experiment was 67 ml. Concentration cadmium in solution was 60 µM at the end of the experiment. In the supernatant the protein concentration was determined according to Lowry (9). The amount of glucose was determined by enzymatic oxidation and coloration with o-dianisidine (Sigma method n° 510).

**Cadmium ion titrations of $NaNO_3$/Tris solutions**
Cadmium ion titrations were performed in a thermostatted reaction vessel under $CO_2$ free air. The pH was kept at a constant value with a pH-stat setup. The titrations were performed in a 0.01 M Tris (tris(hydroxymethyl)aminomethane, $C_4H_{11}NO_3$, Merck, p.a. quality) solution at pH 5, 6.5 and 8 and in a 0.01 M $NaNO_3$ solution at pH 5 and 8. Small aliquots of cadmium solution (0.01 M $Cd(NO_3)_2$ in 0.01 M $NaNO_3$) were added to the solution in the reaction vessel. For these additions a Gilson dilutor (401) was used. During the titrations the total cadmium concentration ranged from 10 µM to 1 mM.

**Impact of the presence of Tris on the cadmium sorption to R.A177 intact cells**
Batch experiments were performed to study the $Cd^{2+}$ sorption to intact cells at pH 6.5 in 0.01 M $NaNO_3$, in 0.005 M $NaNO_3$ with 0.005 M Tris, and in 0.01 M Tris. The sorption to the bacterial cells was determined at two free cadmium concentrations (approximately 10µM and 100µM Cd) and at two exposure times (30 and 90 minutes). Duplicate batches were made for all combinations. The pH was measured in the suspension immediately after the exposure. After centrifugation, the supernatant was collected in order to determine the exact total cadmium concentration in solution by flame-AAS.

## Results

### $CO_2$-production during batch experiments

The total amount of carbonate species and $CO_2$ present in the system can be calculated on the basis of the concentration $CO_2$ in the superposing air, as measured after acidification of the suspensions in the batches. Concentrations of $CO_2$ in the blank experiments (performed in 0.01 M $NaNO_3$, without bacterial cells) were negligible. In the batches with bacterial cells, $CO_2$ production obviously took place. Even before acidification of the suspension, $CO_2$ concentration in the superposing air ranged from 800 to 16000 ppm, depending on the conditions. The concentration carbonate species and $CO_2$, present in the suspensions, ranged from 0.63 to 4.2 mM.

Despite the flushing with $CO_2$-free air, a lot of carbonate species had accumulated in the suspension in the reaction vessel, since the suspensions already contained 0.63 mM $CO_2$ at the start of the batch experiment at pH 5. After two hours of incubation in batch the concentration in the pH 5 suspension had increased to 2 mM. At pH 8, the initial $CO_2$ concentration was 1.4 mM and this concentration increased during incubation to 4.2 mM. In the presence of cadmium, at pH 8, $CO_2$-production was smaller as compared to the batches without cadmium; after two hours the total $CO_2$ concentration had increased from 1.4 to 2.2 mM. Total cadmium concentration in these tubes was 2.64 ± 0.02 mM, the concentration in the supernatant was 0.394 ± 0.002 mM Cd. The Cd-activity in these solutions was 0.323 ± 0.01 mM, which is of the same order as the effect concentration (50% reduction of the acetate respiration) that was determined for this bacterium by van Beelen and Fleuren-Kemilä (unpublished data).

### Exudates formed at pH 8

During preliminary studies of cadmium and zinc sorption to intact cells, a change in colour of the supernatant was observed for the batches at pH 8. As compared to the protein concentration, the concentration of glucose is negligible. This may be due to the method chosen for the analysis: the determination of the glucose concentration is performed without a hydrolysis step, and thus is highly specific, only reacting with free glucose in solution.

In Fig. 1 the protein concentration ($mg.l^{-1}$) in the samples is shown. The concentration protein is already quite high before addition of the cadmium. This might indicate that it is rather the high pH that causes the lysis of cells, instead of the cadmium. This was also observed by Ledin (10). She found that, at metal ion concentrations of $10^{-8}$ M, survival of the bacteria was highly pH dependent, but was

not influenced by the presence of the metal, and that above pH 7 the concentration of organic components in the solution increased with increasing pH.
The amount of bacteria that did not survive this treatment can be estimated from the concentration proteins in solution; the percentage lysis was less than 1% of the cells present.

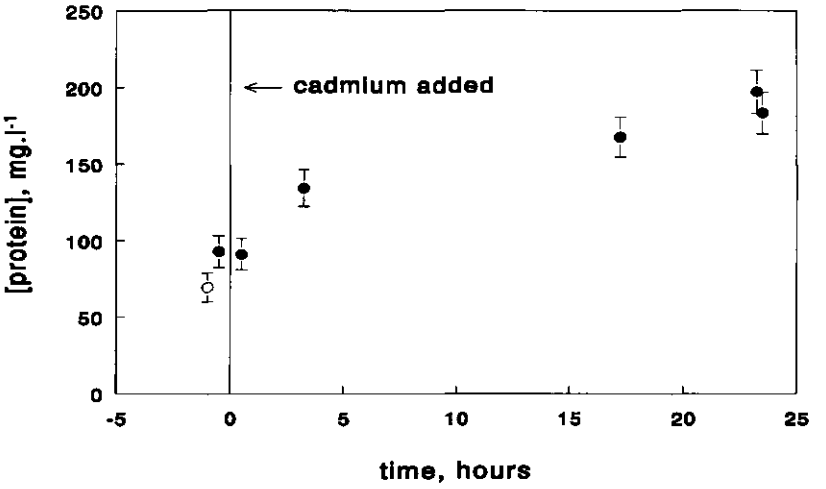


**Fig. 1** Protein production by living R.A177 cells at pH 8 in presence of cadmium; concentration protein ($mg.l^{-1}$) in solution as a function of exposure time (hours). Negative values are used to indicate the time before addition of the cadmium. o pH 5. • pH 8.

**Influence of the presence of Tris on the speciation of Cd in solution, on the sorption of Cd, and on the pH at sampling time**

Tris may form complexes with cadmium ions, and thus influence the speciation of cadmium in solution. The complex formation will depend on the pH. Therefore, cadmium ion titrations were performed both in Tris and in $NaNO_3$ solutions at pH 5, 6.5 and 8. In Fig. 2 the concentration of free cadmium ions, as measured by ISE, is shown as a function of the total amount of cadmium in solution. Most titration curves coincide, indicating that under these conditions no significant complexation with Tris takes place. At pH 8 however, the concentration free cadmium in the Tris environment is distinctly reduced (approximately 25%).

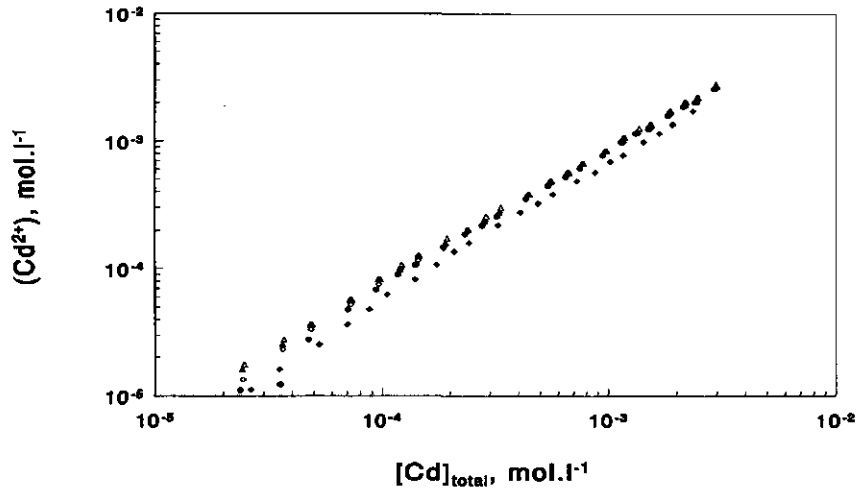


**Fig. 2** Cadmium ion titrations. △ $NaNO_3$, pH 5; ◊ $NaNO_3$, pH 8; ▲ Tris, pH 5; ● Tris, pH 6.5; ♦ Tris, pH 8.

Tris may have an influence on the metabolism of bacteria (4, 5) and may thus influence the metal sorption by the bacterium. Therefore, batch experiments were performed to study the Cd sorption to intact cells at pH 6.5 in 0.01 M Tris, in 0.005 M $NaNO_3$ with 0.005 M Tris, and in 0.01 M $NaNO_3$. The higher cadmium concentrations (50 to 110 µM) are at least one order of magnitude below the concentration at which a sorption maximum is expected to be reached (1). The more complex metal ion binding model that has been used before (1) can be simplified for these conditions to a Freundlich equation (Eq. 1). For each data set parameters of the Freundlich equation are derived by linear regression of the log-transformed data. The obtained equations describe the cadmium sorption by the bacterial cells ($Q_{Cd}$, in µmol.g$^{-1}$) as a function of the total concentration of cadmium in solution ([Cd], in mol.l$^{-1}$):

$$Q_{Cd}=K[Cd]^n \qquad [1]$$

The measured cadmium sorption after 30 minutes exposure and the sorption according to these Freundlich equations are shown in Fig. 3. Between 30 and 90 minutes exposure, sorption has increased slightly (90 minutes sorption data are not shown). Both at low and high cadmium concentrations the sorption measured in the Tris environment is approximately 40% lower than what is expected from the

cadmium experiments in 0.01 M $NaNO_3$. For 0.005 M Tris the average reduction was 32%, for 0.01 M Tris this reduction was 42%. Since at pH 6.5 complexation of cadmium ions with Tris is negligible (< 5%), the reduced cadmium sorption can not be explained by a different speciation of the cadmium in solution.

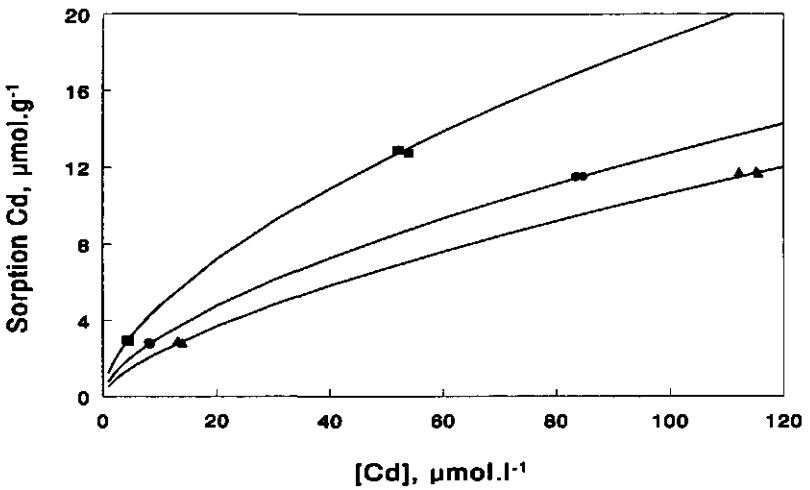


**Fig. 3** Cadmium sorption by intact, living cells of R.A177 at pH 6.5 from different background electrolyte solutions. ■ 0.01 M $NaNO_3$, ● 0.005 M $NaNO_3$ with 0.005 M Tris, ▲ 0.01 M Tris. The solid lines represent the model descriptions.

The effect of the presence of Tris, the exposure time and of the concentration cadmium on the pH during the exposure to cadmium is monitored as well. The pH, as measured after 30 respectively 90 minutes, ranged between 6.5 and 6.2. Main factors determining the pH-reduction were the amount of cadmium added and the exposure time; the presence of Tris seemed to be of minor importance. Therefore, the variation in pH can not explain the different cadmium sorption behavior in Tris environment either.

## Discussion and conclusions

The conditions for the sorption experiments with intact cells will be determined based on the preliminary experiments.
From the $CO_2$ measurements it can be derived that it is not possible with the described procedure to remove all $CO_2$ before the batch experiment starts, and that the $CO_2$-production by the bacterial cells during batch experiments cannot be ignored. Due to the $CO_2$ production, concentrations of carbonate-species were higher than would be expected when the suspension is assumed to be in equilibrium with the $CO_2$ in the atmosphere. In order to avoid complication of speciation (complexation of Cd with carbonate species or proteins, precipitation of $CdCO_3$) and acidification due to $H_2CO_3$ dissociation, the batch experiments will therefore be performed in open systems, and only at pH 5 and 6.5.
Tris-HCl was chosen for the toxicity experiments since the strongly diluted cell suspensions needed a buffer with minimal interactions with metal ions and biological compatibility. However, the metal ion titrations of $NaNO_3$ and Tris solutions showed that Tris does influence speciation of cadmium in solution, although the reduction of the concentration free cadmium due to the presence of Tris is not very large. In addition, the reduction of the free cadmium concentration was not sufficient to explain the sorption behavior in Tris- and in $NaNO_3$ environment. Nevertheless, errors due to the complexation of cadmium are within the accuracy at which the toxicity experiments can be performed (van Beelen, pers. comm).
For the sorption experiments a quite thick cell suspension is used; the amount of proton-dissociating groups present on the cell walls of the bacteria is estimated to be approximately 0.3 $mmol.l^{-1}$ bacterial suspension. Since the proton affinity constant of Tris is $10^{-8.3}$, Tris will not act as a buffer at pH values lower than 7, whereas the bacterial cell walls have a pH-buffering capacity over a wide range of pH-values (11). Therefore, for the sorption experiments at pH 6.5 or lower, an additional buffer is not required, since the buffering capacity of the cells will be sufficient. Because of the rather important reduction of the bacterial cadmium sorption in the presence of Tris, which can not simply be explained by solution speciation, it is preferable to perform the sorption experiments in an indifferent electrolyte. Therefore, the sorption experiments will be performed in a $NaNO_3$ background solution.

## References


1. Plette, A.C.C., Benedetti, M.F., van Riemsdijk, W.H., Competitive binding of protons, calcium, cadmium and zinc to isolated cell walls of a Gram-positive soil bacterium. *Environ. Sci. Technol.*, in press (1996).
2. Xue, H.B., and Sigg, L., Binding of Cu(II) to algae in a metal buffer. *Water Res.* **24**(9), 1129 (1990).
3. van Beelen, P., and Fleuren-Kemilä, A.K., The influence of the pH on the toxic effects of zinc, cadmium and pentachlorophenol on pure cultures of soil micro-organisms. *Environ. Tox. Chem.*, submitted (1996).
4. Good, N.E., Winget, G.D., Winter, W., Connolly, T.N., Izawa, S., and Singh, R.M.M., Hydrogen ion buffers for biological research. *Biochemistry* 5 (2), 467 (1966).
5. Perrin, D.D., and Dempsey, B., Buffers for pH and metal ion control. Chapman and Hall, London, 176p (1974).
6. Rijnaarts, H.H.M., Norde, W., Bouwer, E.J., Zehnder, A.J.B., and Lyklema, J., Bacterial adhesion under static and dynamic conditions. *Appl. Environ. Microbiol.* **59**, 3255 (1993).
7. Schraa, G., Boone, M.L., Jetten, M.S., van Neerven, A.R.W., Goldberg, P.J. and Zehnder, A.J.B., Degradation of 1,4 dichlorobenzene by *Alcaligenes* sp. strain A175. *Appl. Environ. Microbiol.* **52**, 1374 (1986).
8. de Keizer, A., Electrosorption of tetra-alkyl ammonium ion on silveriodide. Ph.D. thesis, chapter 4.4 Wageningen Agricultural University, (1981).
9. Lowry, O.H., Rosebraugh, N.J., Farr, A.L., and Randall, R.J., Protein measurements with the folin phenol reagent. *J. Biol. Chem.* **193**, 265 (1951).
10. Ledin, M., Metal accumulation by microorganisms - Characteristics and implications for soil systems. Ph.D. thesis, Linköping University, Sweden (1994).
11. Plette, A.C.C., Benedetti, M.F., van Riemsdijk, W.H. and van der Wal, A., pH dependent charging behavior of isolated cell walls of a Gram-positive soil bacterium. *J. Colloid Interface Sci.* **173**, 354 (1995).

# Chapter 5

# Sorption of metal ions by intact cells II

Competitive Cd and Zn sorption and uptake related to toxicity for a Gram-positive soil bacterium

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# Sorption of metal ions by intact cells II

In order to quantify the impact of pH and calcium concentration on the sorption of cadmium and zinc, cadmium and zinc sorption experiments are performed with the Gram-positive soil bacterium *Rhodococcus erythropolis* A177 at pH 5 and 6.5, without additional calcium or with 1 mM calcium in solution. The sorption is related to uptake and toxic effects of the metals under similar conditions. Batch sorption experiments showed that cadmium and zinc binding to the intact cells increases with increasing pH, and decreases when additional calcium is applied to the bacterial suspension.
Qualitatively, interactions of cadmium and zinc can be understood quite well by assuming competitive binding to sites on the cell wall, and to specific sites for ion uptake. The influence of protons and other competing ions on the metal ion sorption by intact, living cells can be predicted reasonably well by a model derived for the metal ion binding to isolated cell wall material for the same bacterium. A correlation between the toxicity and the sorption or uptake was expected. The best correlation is found between the sorption to the isolated cell wall material and the effects.

## Introduction

Bioavailability of heavy metals is fully dependent on the metal binding behavior of the abiotic and biotic components of the soil. The metal concentration in solution is mainly determined by sorption equilibria with the major, abiotic soil components.
Metal ion binding to soil is influenced by the pH and the concentration of ions that may compete with metals for binding to the same reactive sites on the soil components (1, 2, 3). In most soil systems, calcium and magnesium are the major bivalent cations. Metal ion sorption by soil biota may thus be influenced by the composition of the soil solution, which can vary depending on the environmental conditions. Apart from the metal ion concentration in solution, the metal ion sorption by the soil organisms depends on the binding characteristics and the uptake mechanism of the organisms themselves. The amount bound to the biomass in soil can be calculated once the binding characteristics of all components separately are known.
In this paper we will describe the impact of the pH and the calcium concentration on the sorption of cadmium and zinc by a Gram-positive soil bacterium. Gram-positive bacteria are convenient model organisms for studying metal sorption to soil biota, due to the relative simplicity of the metal ion interactions with these organisms. It can be assumed that all interactions with the metal take place via the soil solution. Due to the high specific surface of the bacterium, metal sorption studies can be done with rather good accuracy and precision.

Different steps in time of the interaction of metal ions in solution with the bacterium can be distinguished. The first step is the adsorption of the metal ion to the bacterial cell wall. The Gram-positive bacterial cell wall is a very porous structure, built up from large polysaccharide-type molecules, containing acidic groups (mainly carboxylic). These groups may deprotonate with rising pH and thus give rise to a negative charge on the cell wall (4, 5). Apart from protons, these groups may bind metal ions. Thus, protons and metal ions may compete for these binding sites on the cell wall material. This assumption is supported by the work of Fourest and Roux (6), who measured a rapid fall in pH during the first seconds of contact of bacteria with a metal ion solution, which may be interpreted as a release of protons from binding sites due to the binding of the metal ion. Many other authors already observed metal ion adsorption to be pH-dependent (a.o., 7, 8, 9). In addition, other bivalent cations (e.g., calcium) may compete with the metal ion for binding to the reactive sites on the bacterial cell wall (10, 11, 12, 13).

The major part of metal sorbed by a Gram-positive bacterium is bound to the cell wall. Lepp (14) measured the distribution of cadmium in *Bacillus subtilis*. About 90% of the metal was located in the cell wall, 3-4% on the membrane and only 6-7% had passed the membrane and entered the cell. The way a metal is distributed in an organism, may have a large impact on the way a metal passes the food chain. This is closely related to the feeding behavior of the predators of the micro-organisms. E.g., Siepel (15) showed in laboratory experiments that fungivorous grazers (mites ingesting fungal hyphae and digesting all the cell walls) accumulate lead faster than fungivorous browsers (mites ingesting fungal material, but which are unable to digest the fungal cell walls). In an industrial site, contaminated with lead, the most vulnerable group, i.e., the fungivorous grazers, were almost completely absent. Fungivorous grazers also appeared to have a much higher body burden than mites of different feeding strategies present at a lead contaminated site.

The process of adsorption to the cell wall is rapid, reversible, pH dependent and relatively non-specific (16). The second step, the uptake of the metal into the cell, is a much slower process. Uptake involves translocation of the metal across the membrane by a temperature- and energy dependent mechanism that exhibits high specificity for the substrate (16, 17). Metal ions may pass the cytoplasmic membrane by passive uptake, i.e., diffusion through membrane pores, and by active uptake through an energy dependent carrier-mechanism (18). Cadmium has been shown to be transported into cells of *B. subtilis* via an energy-dependent manganese transport system (19).

The uptake may lead to binding to internal functional macro-molecules, causing disfunctioning of the cell. Cytoplasmic concentrations may be reduced by detoxification

mechanisms, for example facilitated precipitation, in which the metal is precipitated with sulphate or phosphate, that is released by the cell, or by complexation with proteins, or by active exclusion via a specific carrier. Even then metal exposure may have an impact on the functioning of the organism in the system, since the maintenance of such detoxifying systems can be energy-consuming.
The toxic effect of metal exposure can be related to the total amount sorbed by the organism (20, 21). Thus, the influence of pH and calcium concentration on heavy metal toxicity will be qualitatively similar to the influence of these parameters on metal ion sorption. Gadd (22) suggested that a reduction in metal toxicity at low pH may result from $H^+$ competition and from pH effects on intracellular uptake. In addition, other cations may affect toxicity of heavy metals by competing for binding sites on cell surfaces including membranes and transport mechanisms.
The Gram-positive bacterium *Rhodococcus erythropolis* A177 is used to asses the influence of the metal ion concentration, the pH, and the calcium concentration on all consecutive steps after exposure to the metal; i.e., on adsorption to the cell wall, on uptake into the cell, and on the response of the bacterium.

## Materials and methods

### The bacterium

The growth conditions for *Rhodococcus erythropolis* A177 (R.A177), and the preparation of the cell suspension are described in detail in chapter 4.

### Conclusions derived from preliminary experiments

Some complications may rise for the study of metal sorption by living bacterial cells. Living cells will respire and produce $CO_2$ from endogenous substrates. Under extreme conditions, the lysis of bacterial cells may lead to an increased concentration of small organic molecules in solution. The use of pH-buffers in batch experiments may influence metal ion speciation and bacterial metabolism. Some experiments (see chapter 4) were performed to study the impact of these processes on the metal sorption by the bacteria. A short outline of the results is given below.
Van Beelen and Fleuren-Kemilä (23) performed toxicity experiments with R.A177 in a Tris-HCl solution, since the strongly diluted cell suspensions needed a buffer with a minimal interaction with metal ions and biological compatibility. The metal ion titrations of $NaNO_3$ and Tris solutions presented in chapter 4 showed that Tris does influence the speciation of cadmium in solution, although the reduction of the concentration free cadmium due to the presence of Tris is not very large. Sorption

experiments were performed in different electrolyte solutions in order to test the effect of Tris on cadmium sorption by living R.A177 cells. The reduction of the free cadmium concentration was not sufficient to explain the reduced sorption (more than 30%) in Tris as compared with the sorption in $NaNO_3$ environment. Errors due to the complexation of cadmium are within the accuracy at which the toxicity experiments can be performed (Van Beelen, personal communication). The thick cell suspensions which were used in the sorption experiments did not need an additional buffer, since the cells themselves show a buffering capacity. Since a higher accuracy is required for the sorption experiments, the sorption experiments will be performed in an unbuffered electrolyte solution.
Due to respiration of endogenous substrates, $CO_2$ was produced by the bacteria during experiments in closed batch systems. The resulting concentrations of carbonate-species in these batches were higher than expected assuming an equilibrium with the atmosphere. In order to avoid complication of speciation (complexation of Cd with carbonate species and precipitation of $CdCO_3$) and acidification due to $H_2CO_3$ dissociation, the batch sorption experiments are performed in open systems, and only at pH 5 and 6.5. The experiments to study the kinetics of metal ion sorption will be performed in a reaction vessel under $CO_2$-free air, and thus can also be performed at pH 7 (zinc) or pH 8 (cadmium).

**Preparation of solutions**
Metal ion solutions were made in the same electrolyte solution as the solution in which the bacterial cells were suspended. All solutions were made of preboiled, double-distilled water. All chemicals used were p.a. quality (Merck).

**Analysis of metal, calcium and proton concentration in solution**
During the kinetic experiments the change in free cadmium concentration was followed by ion selective electrode (ISE, Orion). The calibration of this electrode was performed as described in Plette *et al.* (13). The proton activity was measured with a half cell glass electrode in combination with a double-junction Ag/AgCl sleeve electrode (Orion) of which the outer junction was filled with an equitransferent solution containing 0.125 M $NaNO_3$ and 0.875 M $KNO_3$ (24). To measure the pH in the batches (4 ml Polypropylene (PP) tubes), a micro pH-electrode was used (Orion).
For the determination of the total metal and calcium concentration in solution, the following procedure was used: samples were centrifuged at 6000 rpm (Denley BM402) for 10 minutes. 3 ml of acid (0.1 M $HNO_3$ in 0.01 M $NaNO_3$) was added to 1 ml of the supernatant. The acidified supernatants were kept in PP tubes in the refrigerator until analysis of the metal content of the supernatant. The metal and calcium concentration

were measured either with flame AAS (the kinetics of the metal ion binding), or with ICP-AES (the sorption experiments in batch).

**Kinetics of the metal ion sorption**

Experiments have been performed to study the influence of time on the cadmium ion sorption at pH 5 and 8 and on the zinc sorption at pH 5 and 7, both in the absence and in the presence of calcium. In addition, for both metals, additional metal concentration levels were studied at pH 5 in the presence of calcium ions. The amount of metal ion required to reach a certain equilibrium concentration could be estimated on the basis of the data of Plette *et al.* (13), who used isolated cell walls of the same bacterium for metal sorption experiments. Kinetics of the metal ion sorption were studied in a thermostatic reaction vessel under $CO_2$-free air at 25°C, by following the change in the metal concentration in solution during 3 hours after the metal ion had been added. Duplicate samples were taken from the vessel shortly before the metal ion solution was added, and 5, 15, 30, 60, 90, 120 and 180 minutes after the metal had been added. The calcium and metal ion concentration were measured in the same sample by flame AAS. In order to determine the vitality and purity of the cultures, the number of CFU's (colony forming units; viable cells) was assessed by plate counting on nutrient broth agar, both before addition of the metal ion and at the end of each experiment.

**Sorption of Cd and Zn at two exposure times, batch experiments**

When the bacterial culture was harvested, the cell suspension was split into two equal parts. Half of the total amount was washed three times in a 0.01 M $NaNO_3$ solution, the other half was washed in a solution containing both 0.01 M $NaNO_3$ and 0.001 M $Ca(NO_3)_2$. The suspension with calcium was put into a thermostatted reaction vessel at a constant temperature of 25±1°C, and left over under $CO_2$-free air at a pH of ± 4.7 for 1 hour to promote $CO_2$ removal from the solution. Then, pH was raised to pH 5 by adding NaOH. At the start of the batch experiment, 2 ml of the cell suspension was added to PP tubes of 4 ml, which already contained 2 ml of metal ion solutions of varying concentrations. The tubes were shaken on a vortex and put on a thermostatted shaking machine at 25 ± 1°C. During the exposure time the tubes were shaken every 10 minutes at a vortex. After 30 minutes, the pH in the batches was measured, 2 ml of metal-loaded suspension was taken out of the tubes, and from these samples supernatant was collected. After 120 minutes, the rest of the suspension was taken from the thermostatted water bath, and again pH was measured and supernatant collected.

The pH of the suspension in the reaction vessel was raised to 6.5, in order to repeat the procedure at this pH. Finally, the whole procedure was repeated with the suspension without additional calcium.

## Results

**Kinetics of metal ion binding; determination of two stages in sorption**

The number of viable cells, measured before addition of the metal solution and after 3 hours of exposure to the metal, did not change significantly for most conditions. So, the viability of the bacteria had not changed during the experiment, thus allowing to compare the sorption at the different exposure times.

As an example, the results of a cadmium sorption experiment performed at pH 5 (in the presence of 1 mM calcium, final concentration of cadmium was 50 µM) are shown in Fig. 1. Qualitatively, similar results were obtained for all other experimental conditions. In all cases, two stages could be distinguished. The first stage lead to a strong increase in the metal content of the cells in a relatively short time (usually within the first 15 minutes). After that, the metal content of the bacterial cells was still increasing, but at a much smaller rate than during the first stage. Increase in metal content during this stage became linear in time within approximately 30 minutes of exposure to the metal. Accumulation during the second stage was relatively small.

Zinc behaved quite similar to cadmium, though the net sorption and uptake rate were somewhat smaller than was measured for cadmium.

During the experiments, the concentration of calcium was measured as well. During the first stage, some calcium desorption could be observed, though it was less than expected based on the experiments performed with isolated cell walls (13). Afterwards, in some cases even uptake of calcium seemed to take place. A suggested explanation for the latter phenomenon is given by Kessels *et al.* (25): they observed that $Cd^{2+}$ stimulated $Ca^{2+}$ uptake in yeast by increasing the cation permeability of the cell membrane.

From the results of these experiments, the sampling times for the sorption experiments performed in batch could be derived. The first sampling time should be shortly after the end of the first stage. Both first and second sampling time should be in the linear region of the second stage, in order to be able to calculate uptake rate during the second stage of sorption. In order to correlate sorption and uptake with toxicity, the second sampling time was based on the exposure times used during the toxicity experiments performed by van Beelen en Fleuren-Kemilä (23). Sampling times of 30 and 120 minutes were chosen.

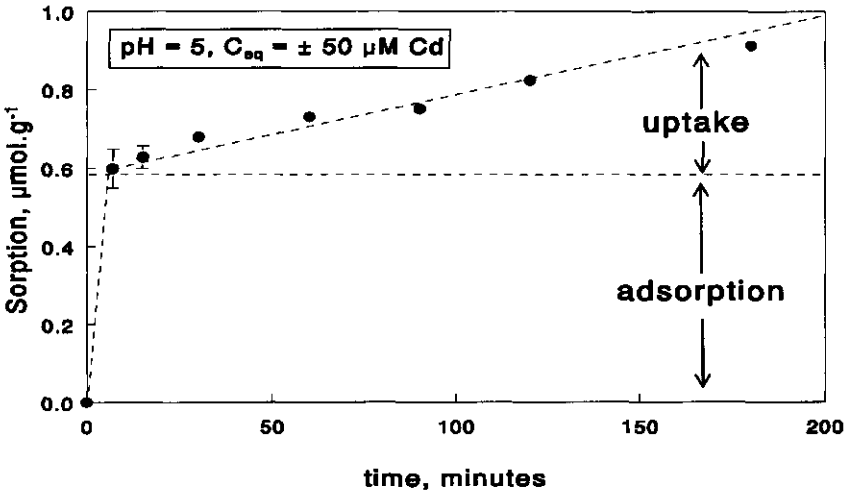



**Fig. 1** Cadmium sorption by living R.A177 cells (µmol.g$^{-1}$ dryweight cells) as a function of time (minutes). This experiment was performed at pH 5 in the presence of 1 mM calcium; cadmium concentration at the end of the experiment was 50µM.

**Sorption after 30 and 120 minutes exposure**

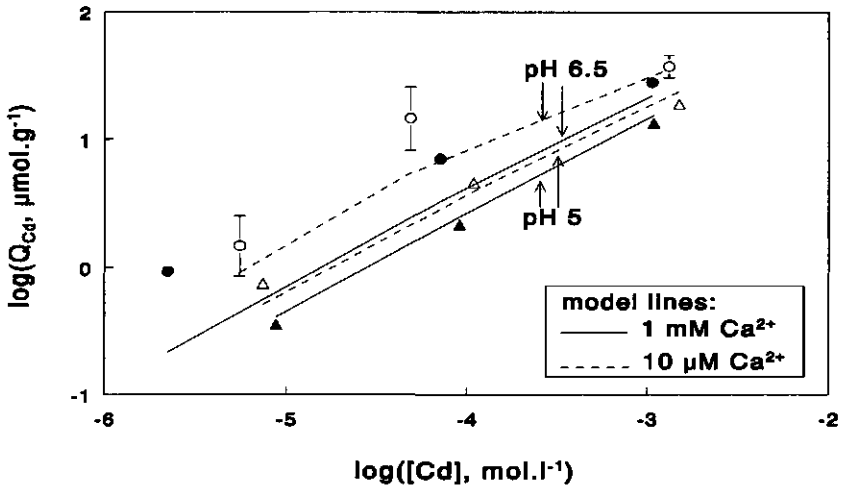
The sorption of cadmium and zinc to intact, living cells was measured at three metal ion concentrations (approximately $10^{-5}$ M, $10^{-4}$ M and $10^{-3}$ M), at two initial pH values (pH 5 and 6.5), without additional calcium or with approximately 1 mM calcium, in 0.01 M $NaNO_3$ as background electrolyte solution. This set up lead to 24 combinations of metal ion, proton and calcium concentration. The experiment was performed three times, for each repetition of the experiment a fresh batch of bacteria was used. Median values of the three repetitions were calculated both for the metal, calcium and proton concentration and for the sorption. During the experiment, apart from the cadmium or zinc concentration, also pH and calcium concentration changed. A slight increase of the calcium concentration (up to at most 20%) was observed after 30 minutes exposure to a metal ion solution. The pH was rather constant (4.9 ± 0.1) when the initial pH was 5; but, e.g., at an initial pH of 6.5 in the presence of 1 mM calcium, the median pH reduced to 6.4 for the low cadmium concentration, and to 6.0 for the highest cadmium concentration applied. Therefore, in the following, when using pH 5 or pH 6.5, the initial pH values are meant.

After 30 minutes exposure, the cadmium and zinc sorption are increased with increasing pH and decreased with increasing concentration calcium in solution (Fig. 2a and 2b). For both metals, the sorption increases in the following order:
pH 5, plus calcium < pH 5, no calcium < pH 6.5, plus calcium < pH 6.5, no calcium.
This is an indication of the competitive nature of the metal ion binding to the cell wall, as already was observed for isolated cell wall material (13). An other indication is the slight increase in calcium and proton concentration during the first stage of the sorption, which is more distinct when higher metal ion concentrations are applied.
After 120 minutes exposure, the metal content of the bacterial cells is slightly increased. The influence of calcium and pH on the metal ion sorption after 120 minutes exposure is similar to the influence after 30 minutes exposure. Therefore, the data of the metal sorption after 120 minutes will not be shown here.

**Prediction of the adsorption to the cell wall of intact cells**

The first stage of the sorption is usually ascribed to adsorption of the metal ion to the cell wall of the organism. We assume that all metal sorbed by the bacterium at 30 minutes is related to cell wall adsorption. Previously (13), cadmium and zinc ion binding to isolated cell walls of R.A177 have been studied. 31% of the dry weight of intact cells is related to the cell wall material (5). It is interesting to compare the cell wall adsorption for intact cells with the cadmium and zinc sorption to isolated cell wall material.
The data of cadmium and zinc binding to isolated cell walls were described by Plette *et al.* (13) with a bi-modal NICA equation (Non-Ideal Competitive Adsorption (26, 27)); a multi-component competitive binding model that accounts for both ion-specific non-ideality and chemical heterogeneity of the sorbent. The reactive groups on the isolated cell wall material were characterized by proton titrations. Three groups were distinguished: carboxylic, phosphatic and amino-type groups (5). For the metal ion binding, the amino type groups were assumed not to be involved in metal ion binding, since this group is positively charged at pH values below 9. Chemical analysis of the isolated cell wall material revealed that not all membrane material had been removed during the purification procedure. Due to this impurity, a phosphate content of the isolated cell walls had been measured (5). The cell wall material itself is not expected to contain phosphatic groups. Predictions of the sorption to cell walls of intact cells are made, assuming that binding to these cell walls takes only place at the carboxylic sites.

a)




b)

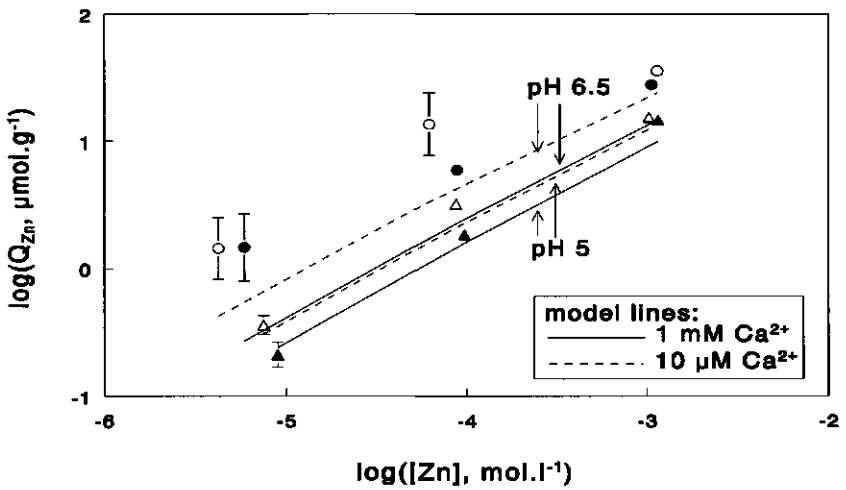



**Fig. 2** Metal ion sorption by living R.A177 cells ($Q_i$, µmol.g$^{-1}$ dryweight cells) after 30 minutes exposure as a function of the concentration cadmium in solution (mol.l$^{-1}$), the pH and the concentration calcium in solution. ▲ pH 5. ● pH 6.5. Closed symbols: in the presence of approximately 1 mM calcium. Open symbols: without additional calcium in solution (approximately 10 µM Ca in solution). The lines represent the model prediction, based on the mono-modal NICA equation (Eq. 2, see text).

**a)** Cadmium.

**b)** Zinc.

Thus, the NICA equation, as given by Plette *et al.* (13), can be simplified to a monomodal equation for the description of competitive metal ion binding to the cell walls of intact, living cells:

$$Q_i = Q_{max} * \frac{(\tilde{K}_i c_i)^{n_i}}{\sum_j (\tilde{K}_j c_j)^{n_j}} * \frac{[\sum_j (\tilde{K}_j c_j)^{n_j}]^p}{1 + [\sum_j (\tilde{K}_j c_j)^{n_j}]^p} \quad [1]$$

in which $Q_{max}$ is the amount of carboxylic sites ($\mu mol.g^{-1}$), $\tilde{K}_i$ is the median affinity constant of the carboxylic group for component i, and $c_i$ is the concentration of component i in solution. The exponent n (component specific non-ideality) accounts for electrostatic effects and other "non-ideal" behavior of component i. The value of p determines the width of the distribution due to the intrinsic chemical heterogeneity of the sorbent and is the same for all components. In case of a cadmium sorption experiment in the presence of calcium, both calcium ions and protons compete with the cadmium for binding to the carboxylic sites:

$$Q_{Cd} = Q_{max} * \frac{(\tilde{K}_{Cd} c_{Cd})^{n_{Cd}}}{(\tilde{K}_{Cd} c_{Cd})^{n_{Cd}} + (\tilde{K}_{Ca} c_{Ca})^{n_{Ca}} + (\tilde{K}_H c_H)^{n_H}} * \frac{[(\tilde{K}_{Cd} c_{Cd})^{n_{Cd}} + (\tilde{K}_{Ca} c_{Ca})^{n_{Ca}} + (\tilde{K}_H c_H)^{n_H}]^p}{1 + [(\tilde{K}_{Cd} c_{Cd})^{n_{Cd}} + (\tilde{K}_{Ca} c_{Ca})^{n_{Ca}} + (\tilde{K}_H c_H)^{n_H}]^p} \quad [2]$$

The isolated cell walls were pretreated by washing at pH 3. This procedure was considered to be too extreme for the intact, living cells, and is omitted. It is therefore likely that the cell walls of the living cells still contain an appreciable amount of calcium. Therefore, for the prediction of the metal ion binding by intact cells, the parameter set derived for binding of cadmium and zinc to isolated cell walls in presence of calcium was used, even for the data set at low calcium concentrations: $Q_{max}$ = 503 $\mu mol.g^{-1}$, $\log\tilde{K}_H$ = 4.7, $\log\tilde{K}_{Cd}$ = 2.3, $\log\tilde{K}_{Zn}$ = 2.0, $\log\tilde{K}_{Ca}$ = 2.3, $n_H$ = 0.75, $n_{Cd}$ = 0.8, $n_{Zn}$ = 0.8, $n_{Ca}$ = 0.3, p = 0.6 (13). This equation is used to predict the sorption at each data point for the measured metal ion, calcium and proton concentrations. Fig. 2a (cadmium) and 2b (zinc) show that, at pH 5, the prediction of the amount sorbed to the living cells is rather good. The predictions at pH 6.5 systematically underestimate the observed sorption of cadmium and zinc. It is remarkable that the binding of metal ions to living bacteria can be derived with a reasonable accuracy based on the detailed study of metal ion binding to isolated cell walls, considering the binding to the phosphate groups as an artefact. This indicates that the detailed metal ion speciation study of dead cell wall material can give important information on metal interactions that take place with living cells as well.

**Sorption during second stage; uptake into the cell**
The uptake rate was calculated from the sorption data using:

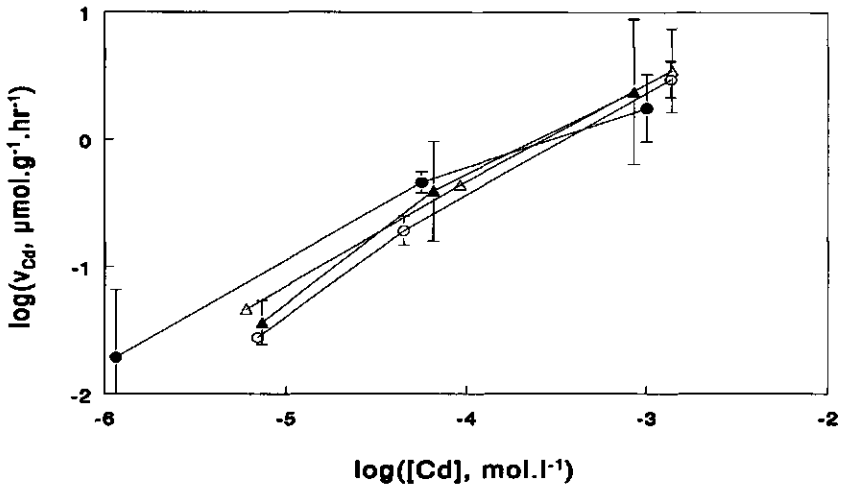
$$v=(S_{120}-S_{30})/1.5 \quad [3]$$

in which v is the uptake rate in $\mu mol.g^{-1}.hr^{-1}$, and $S_{30}$ and $S_{120}$ are the amount of metal sorbed in $\mu mol.g^{-1}$ dry weight cells after 30 and 120 minutes of exposure to the metal respectively. Uptake is expected to take place mainly by an active process via carrier mechanisms. First step for the uptake then will be the binding of the metal ion to a binding site on the carrier. This binding may experience competition with other ions as well, similar to the reactive sites on the cell wall material. Therefore, the metal ion uptake is expected to be reduced when proton or calcium ion concentration is increased. In Fig. 3a (cadmium) and 3b (zinc), the calculated uptake rate is given for all conditions. At high zinc concentrations, the uptake of zinc increased with increasing pH and decreased with increasing calcium concentration, as was expected (Fig. 3b). In all other situations, the influence of the pH and the concentration calcium was different, and no clear relation could be discovered, neither from the separate experiments. Apparently uptake of metal is difficult to asses in this way. The differences between the sorption at 30 minutes and after 120 minutes exposure are rather small. The observed sorption at 120 minutes is the sum of the adsorption to the cell wall, and the uptake into the cell during these two hours. Between 30 and 120 minutes, conditions in the solution change slightly. This will also have an impact on the cell wall sorption, thus making the interpretation of the calculated uptake rates more difficult.

## Comparison with toxicity data; general trends in metal interactions with R.A177

Once the metal is taken up, it may cause toxic effects. The level of these effects strongly depends on the organism, and on the environmental conditions. For R.A177, the influence of the pH on the toxicity of zinc has been determined by van Beelen and Fleuren-Kemilä (23). In addition, measurements were performed with cadmium (unpublished data). They measured acetate respiration as a function of pH and metal ion concentration in solution. The pH was buffered with Tris-HCl, and no other electrolytes were added. From the data $EC_{50}$ values (Effect Concentration; the $EC_{50}$ is the metal ion concentration at which the respiration is reduced with 50% as compared to the situation without metal ions in solution) were calculated (Fig. 4).

a)

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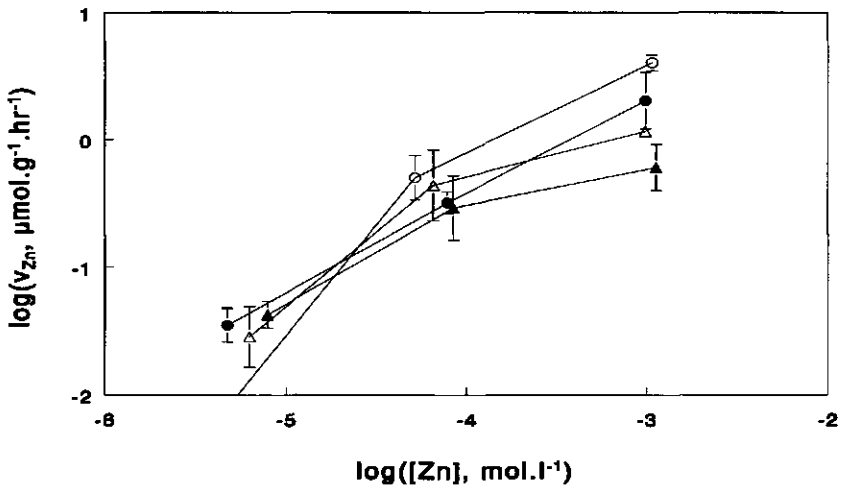


**Fig. 3** Uptake rate (v, µmol.g$^{-1}$.hr$^{-1}$) as calculated from the difference between the sorption at 30 and at 120 minutes exposure. ▲ pH 5. ● pH 6.5. Closed symbols: in the presence of 1 mM calcium. Open symbols: without additional calcium in solution.
a) Cadmium.
b) Zinc.

The bacteria experienced a higher metal toxicity when pH was increased. This is a clear indication that toxicity is not exclusively determined by the free metal ion activity, and that the exposure is also determined by the presence of other ions in solution. Zinc turned out to be more toxic than cadmium at pH 5.9 and 7.9. The influence of calcium on heavy metal toxicity has not been reported yet for this bacterium. However, other authors did already report the influence of calcium on different species. For example, yeast cells were protected by $Ca^{2+}$ from $Cd^{2+}$ toxicity, due to a reduction of the $Cd^{2+}$ uptake in the presence of $Ca^{2+}$ (25).

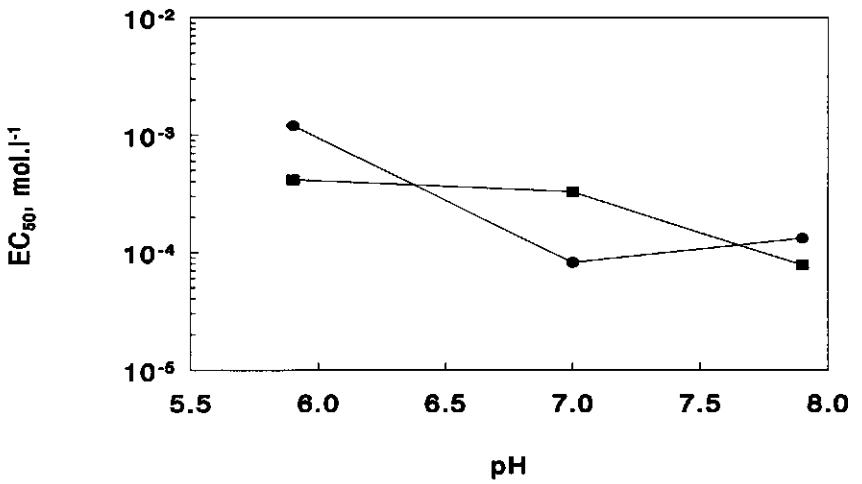


**Fig. 4** $EC_{50}$ (mol.$l^{-1}$) at pH 5.9, 7, and 7.9 for cadmium (●) and zinc (■).

Some authors (20,21) suggest that toxicity is closely related to the total body burden. On the basis of this assumption, one would expect to obtain a similar sorption when the organism shows the same effect, independent of pH. In order to verify this concept, the expected metal sorption by the bacterium at the $EC_{50}$ concentrations is calculated. The influence of the different background electrolyte (Tris-HCl instead of a $NaNO_3$ solution without the buffer) is ignored for these calculations. Sorption to isolated cell wall material was calculated with the bimodal NICA equation derived by Plette *et al.* (13), assuming a low calcium concentration of 10 µM. Metal ions may bind both to carboxylic and to phosphate groups on this material. The sorption is expressed in µmol.$g^{-1}$ dryweight cell wall material.

Sorption to the intact, living cells was calculated with the monomodal NICA equation (Eq. 2), assuming binding to the cell wall of intact cells only takes place at the

carboxylic groups. Like for the isolated cell walls, a low calcium concentration of 10 μM was assumed. Since the amount of cell wall material per gram intact cells is known (31% on a dryweight basis, (5)), the sorption to the intact cells can be expressed in $\mu mol.g^{-1}$ dryweight intact cells. As can be observed in Fig. 2a and 2b, the sorption to intact cells is reasonably predicted at pH 5 using the monomodal NICA equation. However, at pH 6.5 the approach leads to a clear underestimation of the measured sorption. Therefore, in addition, a description of the metal ion sorption by intact cells is made with the help of a three component Freundlich equation, derived for the median cadmium and zinc sorption data after 30 minutes exposure:

$$Q_i = K_i[Me^{2+}]^a[H^+]^b[Ca^{2+}]^c \qquad [4]$$

In which the sorption $Q_i$ ($\mu mol.g^{-1}$) is described as a function of the concentrations metal ion ($Me^{2+}$), proton ($H^+$) and calcium ($Ca^{2+}$), in $mol.l^{-1}$. The Freundlich parameters obtained for both cadmium and zinc are given in Table 1.

**Table 1**

**Freundlich parameters for the metal ion sorption by intact cells and for the uptake rate**

| | sorption cadmium | sorption zinc | uptake rate cadmium | uptake rate zinc |
|---|---|---|---|---|
| log $K_i$ | 0.91 ± 0.34 | 0.58 ± 0.37 | 3.54 ± 0.26 | 3.16 ± 1.66 |
| a | 0.67 ± 0.04 | 0.75 ± 0.05 | 0.82 ± 0.03 | 0.95 ± 0.20 |
| b | -0.39 ± 0.06 | -0.49 ± 0.06 | 0.13 ± 0.05 | -0.03 ± 0.29 |
| c | -0.09 ± 0.04 | -0.09 ± 0.03 | - | - |
| $r^2$ | 0.970 | 0.974 | 0.996 | 0.886 |

On the basis of the derived equations, the sorption of cadmium and zinc can be calculated for the conditions used for the toxicity experiments. As an example, the resulting Freundlich sorption isotherms for cadmium at pH 5.9, 7, and 7.9 are given in Fig. 5. In this graph, the sorption at the measured $EC_{50}$ concentrations is given as well. An overview of all calculations is given in Table 2.
For all ranges, the coefficient of variation (CV) is given in Table 2 as well.

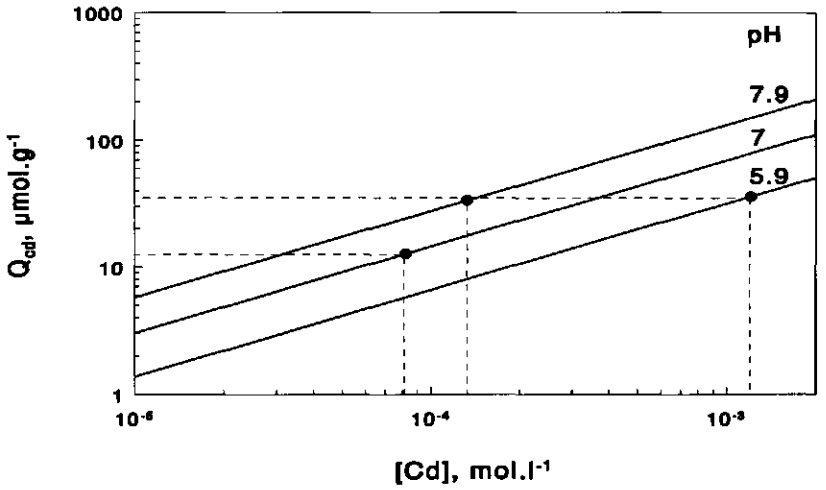


**Fig. 5** Predicted cadmium sorption at the pH values used for the toxicity experiments, based on the three component Freundlich equation for cadmium (Table 1). The closed circles represent the predicted sorption at the EC50 concentrations, as were determined by van Beelen and Fleuren-Kemilä (unpublished data).

One tends to expect that toxic effects are rather the result of the amount of metal taken up, than of the total amount of metal sorbed by the organism, since the major part of the metal is bound to the cell wall, where it probably will not hamper cell functioning. Therefore, in Table 2 also a prediction is given for the uptake rate. The uptake rate for the conditions of the effect concentrations is estimated using a pH-dependent (twocomponent) Freundlich equation, that was derived for the median uptake rates obtained in the environment without additional calcium. The parameters of this Freundlich Equation are given in Table 1.

The $EC_{50}$ concentrations are the concentrations at which, for different pH-conditions, the same effect is observed (50% reduction of acetate respiration).

Very large variations in the $EC_{50}$ are observed for varying pH, especially for cadmium. The variation in the uptake rate or sorption calculated for the metal ion concentration and pH of the $EC_{50}$ should be small if the effect is related to uptake or to the total loading with metal ions. Logically, uptake rate is expected to be best correlated with effect, and sorption to isolated cell walls is expected to correlate less, since the metal ion bound to a site on the cell wall does not necessarily affect the organism.

Surprisingly, the reverse is observed, the sorption to the isolated cell walls at the $EC_{50}$ clearly shows the smallest variation.

**Table 2**

**Cadmium and zinc sorption and uptake rate at the $EC_{50}$**

| | conditions | | cell wall | intact cells | | |
|---|---|---|---|---|---|---|
| | pH | $EC_{50}$ | Sorption | Sorption NICA | Sorption Freundlich | Uptake rate |
| | | $\mu mol.l^{-1}$ | $\mu mol.g^{-1}$ | $\mu mol.g^{-1}$ | $\mu mol.g^{-1}$ | $\mu mol.g^{-1}.hr^{-1}$ |
| Cd | 5.9 | 1210 | 239 | 35.5 | 48.1 | 2.64 |
| | 7.0 | 82 | 111 | 7.8 | 21.1 | 0.21 |
| | 7.9 | 133 | 172 | 11.4 | 65.2 | 0.24 |
| *CV* | | *1.10* | *0.30* | *0.67* | *0.41* | *1.10* |
| Zn | 5.9 | 416 | 110 | 12.1 | 23.4 | 1.39 |
| | 7.0 | 327 | 158 | 12.6 | 67.4 | 1.20 |
| | 7.9 | 78 | 109 | 4.7 | 63.0 | 0.33 |
| *CV* | | *0.52* | *0.18* | *0.37* | *0.39* | *0.47* |

Some remarks have to be made. The Freundlich equations, used to predict the sorption to intact cells and the uptake rate, were calibrated with data between pH 5 and 6.5. Most of the predicted sorption and uptake rates at the conditions of the $EC_{50}$ are thus beyond the measurement window, which may easily lead to erroneous predictions. On the other hand, the NICA model is calibrated with data between pH 5 and 8. Predictions based on these equations thus are all within the measurement window, and will be more accurate than the predictions based on the Freundlich equations.

In spite of the presence of the phosphate impurity in the cell wall material, the best correlation is found between the sorption to the isolated cell wall material and the effects. Two considerations may be made: either the assumption that the phosphatic groups are an impurity in the cell wall has to be rejected, or this impurity reflects the metal ion interactions at the outer site of the cytoplasmic membrane, where it may have an impact on cell metabolism via the disturbance of uptake mechanisms. From the data, a conclusion on this issue can not be drawn.

## Conclusions

Cadmium and zinc sorption to R.A177 intact cells takes place in two stages, which can be distinguished very well when the sorption is studied as a function of time. The major part of the metal sorption is during the first stage, and is ascribed to cell wall adsorption. The duration of the first stage is not influenced by the pH, the metal ion concentration or the calcium concentration. Batch sorption experiments showed that cadmium and zinc binding to the intact cells increases with increasing pH, and decreases when additional calcium is applied to the bacterial suspension. From the difference between the sorption after 30 and 120 minutes exposure the uptake rate could be calculated for all conditions. The influence of pH and calcium could hardly be distinguished for the uptake of cadmium and zinc. This is probably due to the fact that pH and metal ion concentrations could not be controlled during the exposure in batches, thus leading to variations in sorption that could be due to variations in the environmental conditions rather than due to the progressing time.

A correlation between the toxicity and the sorption or uptake was expected. For the uptake rate, as predicted for the conditions at which 50% reduction of the respiration was observed, the calculated coefficient of variation was approximately the same as was found for the $EC_{50}$ values obtained at the three pH-values. Surprisingly, the sorption to isolated cell walls correlated much better with the observed effects; the variation was reduced with a factor 3 (zinc) to 4 (cadmium). Since the major part of the metal ions, sorbed by these bacteria, is bound to the cell wall, this may be an indication that indeed total body burden is a good indicator for the potential effects for this type of organisms.

Qualitatively, results show that on all processes studied, an increase in pH results in an increased effect; both sorption and toxicity increase with increasing pH. The impact of calcium on all processes is smaller, but in general a higher calcium concentration leads to a lower sorption and to a reduction of toxic effects. Like for isolated cell walls (13), under similar conditions more cadmium than zinc was bound to the intact cells. So, the trends for all these processes are similar, and suggest competitive interactions, not only during metal ion binding to the cell wall, but also during the uptake of the metal ion into the cell. The impact of this competitive binding is reflected in the different levels of toxicity experienced for different pH and calcium conditions.

## References


1. Christensen, T.H., Cadmium soil sorption at low concentrations, I. Effect of time, cadmium load, pH and calcium. *Water, Air, and Soil Pollution* **21**, 105 (1984).
2. Chardon, W.J., Mobiliteit van cadmium in de bodem (Mobility of cadmium in soil). Ph.D. thesis, Wageningen Agricultural University, The Netherlands, (1984).
3. Boekhold, A.E., Temminghoff, E.J.M., and van der Zee, S.E.A.T.M., Influence of electrolyte composition and soil pH on Cd sorption by an acid sandy soil. *J. Soil Sci.* **44**, 85 (1993).
4. Carstensen, E.L., and Marquis, R.E., Passive electrical properties of microorganisms, III. Conductivity of isolated bacterial cell walls. *Biophysical Journal* **8**, 536 (1968).
5. Plette, A.C.C., Benedetti, M.F., van Riemsdijk W.H., and van der Wal, A., pH dependent charging behavior of isolated cell walls of a Gram-positive soil bacterium. *J. Colloid Interface Sci.* **173**, 354 (1995). (Chapter 2 of this thesis)
6. Fourest, E. and Roux, J-C., Heavy metal biosorption by fungal mycelial by-products: mechanisms and influence of pH. *Appl. Microbiol. Biotechnol.* **3**, 399 (1992).
7. Ferris, F.G., Schultze, S., Witten, T.C., Fyfe, W.S., and Beveridge, T.J., Metal interactions with microbial biofilms in acidic and neutral pH environments. *Appl. Environ. Microbiol.* **55** (5), 1249 (1989).
8. Wallberg, M., Brynhildsen, L., and Allard, B., Metal binding properties of *Klebsiella oxitoca*. *Water, Air and Soil Pollution* **57-58**, 579 (1991).
9. Xue, H.B, and Sigg, L., Binding of Cu (II) to algae in a metal buffer. *Water Res.* **24** (9), 1129 (1990).
10. Flemming, C.A., Ferris, F.G., Beveridge T.J., and Bailey, G.W., Remobilization of toxic heavy metals adsorbed to bacterial wall-clay composites. *Appl. Environ. Microbiol.* **56** (10), 3191 (1990).
11. Brown, D.H., and Avalos, A., The role of calcium in intracellular cadmium uptake by the lichen Peltigera membranacea. *Annals of Botany* **71**, 467 (1993).
12. Shuttleworth, K.L., and Unz, R.F., Sorption of heavy metals to the filamentous bacterium *Thiotrix* Strain A1. *Appl. Environ. Micrbiol.* **59** (5), 1274 (1993).
13. Plette, A.C.C., Benedetti M.F., and van Riemsdijk, W.H., Competitive binding of protons, calcium, cadmium, and zinc to isolated cell walls of a Gram-positive soil bacterium. *Environ. Sci. Technol.* in press (1996). (Chapter 3 of this thesis).
14. Lepp, N.W., Uptake and accumulation of metals in bacteria and fungi. In: D.C. Adriano (ed.) Biogeochemistry of trace metals. Lewis Publishers, London, 513p (1992).
15. Siepel, H., Are some mites more ecologically exposed to pollution with lead than others? *Exp. Appl. Acarol.* **19**, 391 (1995).
16. Failla, M.L., Zinc: Functions and transport in microorganisms. In: E.D. Weinberg (ed.) Microorganisms and Minerals. Dekker, New York. 492p (1977).
17. Belliveau, B.H., Starodub, M.E., Cotter C., and Trevors, J.T., Metal Resistance and accumulation in bacteria. *Biotech. Adv.* **5**, 101 (1987).
18. Luoma, S.N., Bioavailability of trace metals to aquatic organisms - a review. *Sci. Tot. Environ.* 28, 1 (1983).
19. Laddaga, R.A., Bessen, R., and Silver, S., Cadmium-resistant mutant of *Bacillus subtilis* 168 with reduced cadmium transport. *J. Bacteriol.* **162**, 1106 (1985).
20. Morel, F.F.M. (ed.), Principles of Aquatic Chemistry. Wiley, New York, Chapter 6. 446p (1983).

21. van Wensem, J., Vegter J.J., and van Straalen, N.M., Soil Quality criteria derived from critical body concentrations of metals in soil invertebrates. *Applied Soil Ecology* **1**, 185 (1994).
22. Gadd, G.M., Tansley Review No. 47 Interactions of fungi with toxic metals. *New Phytol.* **124**, 25 (1993).
23. van Beelen, P., and Fleuren-Kemilä, A.K., The influence of the pH on the toxic effects of zinc, cadmium and pentachlorophenol on pure cultures of soil micro-organisms. *Environ. Tox. Chem.*, submitted (1996).
24. de Keizer, A., Electrosorption of tetra-alkyl ammonium ion on silveriodide. Ph.D. thesis, chapter 4.4, Wageningen Agricultural University, (1981).
25. Kessels, B.G.F., Belde P.J.M., and Borst-Pauwels, G.W.F.H., Protection of *Saccharomyces cerevisiae* against $Cd^{2+}$ toxicity by $Ca^{2+}$. *J. General Microbiol.* **131**, 2533 (1985).
26. Koopal, L.K., van Riemsdijk, W.H., de Wit, J.C.M., and Benedetti, M.F., Analytical isotherm equations for multicomponent adsorption to heterogeneous surfaces. *J. Colloid and Interface Sc.* **166**, 51 (1994).
27. Benedetti, M.F., Milne, C.J., Kinniburgh, D.G., van Riemsdijk, W.H., and Koopal, L.K., Metal binding to humic substances; Application of the Non-Ideal Competitive Adsorption model. *Environ. Sci. Technol.* **29**, 446 (1995).

# Chapter 6

# Bioavailability of heavy metals

Bioavailability of heavy metals in terrestrial and aquatic systems; a first step towards quantification

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Erwin J.M. Temminghoff
Willem H. van Riemsdijk

# Bioavailability of heavy metals

A concept is presented that may help to obtain a better insight in the processes that may influence the availability of a metal for an organism in its natural environment. Speciation and bioavailability are the key words in the relation between the total metal content of the soil and the effects of this metal on biota present in soil. The interaction between an organism and metal ions present in soil is considered to be the result of competition for that metal ion by all components (including the organism) present in the system. All interactions with the metal ion are considered to take place via the liquid phase. The concept is explained on the basis of a number of model calculations concerning pH-dependent copper binding to maize root cell walls, fungal mycelia, and yeast cells present in a sandy soil, and concerning cadmium binding to a bacterium present in a clay and a sandy soil, as influenced by pH and calcium concentration. In addition, the concept is applied for calculating the impact of algal bloom on the copper speciation in an aquatic system. The presented approach may be a valuable tool in predicting quantitatively the metal ion sorption to biota present in a complex system, and to predict the relative change in availability due to environmental changes.

## Introduction

In ecotoxicology tests, the effect on biota is usually related to the total metal content in the soil. However, the relationship between the total metal in the soil and its positive or negative effect on soil biota is not straight forward. In natural systems the exposure to metals not only depends on the total metal content of the system, but also on the composition of the system, and on a number of environmental conditions (e.g., pH, concentration competing ions, and concentration complexing ligands in solution). In addition, characteristics of the organism itself may play an important role as well. Therefore, the total metal content is believed not to be a proper tool in risk assessment.

For a certain combination of one soil and one organism, toxicity in relation to total metal content of the soil can be assessed. But what if information is required on effects for this organism in other soils, or for other organisms in the same soil? And what will happen if, due to changes in land use or due to acid precipitation, the pH, and thus the availability of the metal ion, changes?

The potential number of combinations of soils, organisms and environmental conditions is too large to test experimentally. In this paper, a concept is presented that may enable to predict quantitatively the metal sorption to biota present in a

complex system, and to predict the relative change in availability due to environmental changes.

Key words in the relation between the total metal content of the system and effects of this metal on biota present in that system, are speciation and bioavailability.

Speciation of metals in soil can be defined as the distribution of the total metal content over all possible chemical forms (species) either in the solid, the liquid or the biotic phase of the soil. This distribution is influenced by a great number of processes like adsorption, complexation and precipitation, which on their turn depend on the properties of the metal, soil properties, and environmental conditions like pH, salt level, redox potential.

Bioavailability is defined in several ways, and most definitions are related to each other. It can be defined as the amount that is present in the soil solution, the amount that is really taken up, or the amount that has the potential to cause an effect. Since responses on the presence of a metal in the system normally require bioaccumulation as a first step, bioaccumulation of a trace metal will be a more direct measure of metal interactions with organisms (1). According to Morel (2), trace metal interactions with organisms can be understood by considering an organism as an assemblage of reactive ligands. The degree of complexation of these ligands with particular metals is reflected in measurable physiological effects. According to these statements there is a close relationship between the amount that can be bound by an organism, and the amount of metal that potentially may cause an effect. Therefore, in this paper bioavailability will be defined as the amount of metal that can be bound by an organism in a certain environment.

Given these definitions, interaction between a soil organism and metal ions present in soil can be easily understood by considering the organism to be an additional reactive soil component, with its own (pH and other environmental-factor dependent) binding characteristics. Sorption of the metal ion by the organism is thus the result of competition for that metal ion by all reactive components (including the organism) present in the system. All interactions with the metal ion are considered to take place via the liquid phase. Then bioavailability is determined both by the relation between total metal in soil and the free metal ion concentration in soil solution and by the relation between this free metal ion concentration and sorption by the organism. In addition, the total amount of metal in solution is influenced by complexation with dissolved organic and inorganic ligands. This concept is schematically depicted in Fig. 1. The exposure to the metal is expected to take place via the solution, so exposure to organisms that have a more complex uptake route will not be discussed here.

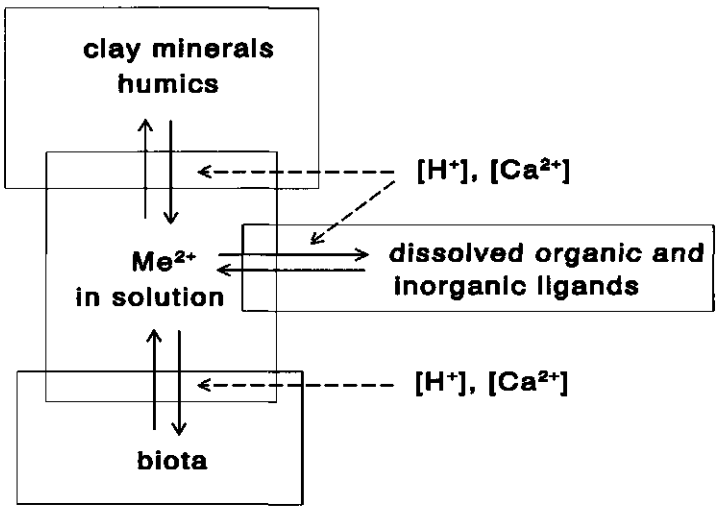



**Fig. 1** Interactions with the metal ion are expected to take place via the soil solution.

In this paper a number of examples will be given to show the impact of different sorption characteristics of organisms (1 soil, 3 organisms) and soils (1 organism, 2 soils) on the metal ion binding by the organism. In soil systems, the free metal ion concentration is (almost) completely determined by the metal ion binding to the soil solid phase (3). In aquatic systems, biota may play an important role in the distribution of the metal. Therefore, apart from examples concerning soil systems, the concept is also applied to an aquatic system. Results from the model calculations will be discussed. On the basis of the examples we will show that there is no such thing as "THE bioavailability".

## Concept in equations

In literature, many models are used to describe metal ion binding to soil components. Binding of metal ions is at least a two component process, since protons (or hydroxyl ions) are inevitably present in aqueous systems (4). Regarding metal ion binding as an exchange reaction with protons, metal ion binding by a site $S_i$ on a soil component can be given by the equation

$$S_iH_x + M \rightleftharpoons S_iM + xH \qquad [1]$$

The fraction of sites i, occupied by a metal ion, may be given by a two-component Langmuir equation:

$$\Theta_i = \frac{S_iM}{S_{i,tot}} = \frac{S_iM}{S_iH_x+S_iM} = \frac{K_i\frac{M}{H^x}}{1+K_i\frac{M}{H^x}} \qquad [2]$$

in which $K_i$ is an exchange constant of site i, $S_{i,tot}$ is the total number of sites i, and M is the concentration of free metal ion in solution. Since every soil component has several reactive groups, each with their own affinity constant for the metal ion, this equation is usually not suitable to describe the binding of metal ions in natural systems.

When the assumption is made that the affinity constants of the groups are pseudo-Gaussian, symmetrically distributed, and that the binding of an ion to one of these groups separately still can be described by a Langmuir equation, the overall adsorption equation can be solved analytically. This overall isotherm for monocomponent adsorption is the Langmuir-Freundlich (LF) equation (5). Extending this equation for proton competition gives a two component Langmuir-Freundlich equation (6, 7, 8):

$$SM = S_{tot} * \frac{(\tilde{K}\frac{M}{H^x})^n}{1+(\tilde{K}\frac{M}{H^x})^n} \qquad [3]$$

in which $\tilde{K}$ is the median exchange constant. At low metal ion concentrations ($\tilde{K}(M/H^x) \ll 1$), this equation can be simplified, resulting in the so-called extended Freundlich equation:

$$SM=K(M)^n(H)^{-nx} \qquad [4]$$

in which K is a composite constant:

$$K = S_{tot}(\tilde{K})^n \qquad [5]$$

The constant K in these equations is a conditional parameter; i.e., these equations are only valid if other environmental factors (e.g., concentration of other competing ions) are kept the same.

With the help of Eq. 4 metal ion binding to soil ($Q_s$, Eq. 6) and to biota ($Q_b$, Eq. 7) can be described separately:

$$Q_s = K_s(M)^{a_s}(H)^{b_s} \quad [6]$$

$$Q_b = K_b(M)^{a_b}(H)^{b_b} \quad [7]$$

in which a = n and b = -nx.
Nederlof and van Riemsdijk (3) calculated the distribution of copper over the abiotic and biotic components in a sandy soil, assuming an organic matter content of 1.6%, a waterfilled porosity of 0.3, and an amount of 0.4 g biota per kg soil. Under these conditions, more than 99% of the total copper is bound to the soil solid phase. The contribution of copper bound to biota and the free metal ions present in solution to the total metal content of the soil can be neglected. This implicates that for practical purposes the amount of metal bound to the abiotic, solid soil components is equal to the total amount of metal present in the soil,

$$(M)_t = Q_s \quad [8]$$

and that the free metal ion concentration is fully determined by the sorption equilibrium with the soil solid phase. The total dissolved metal ion concentration is influenced by complexation with DOC and inorganic ligands, but this does not affect the free metal ion concentration as long as Eq. 8 is approximately valid. Binding to biota can thus be expressed as a function of total metal in soil and pH by combining Eqs. 6 and 7. The resulting equation is given in Eq. 9:

$$Q_b = K_b K_s^{-a_b/a_s}(M)_t^{a_b/a_s}(H)^{b_b - b_s a_b/a_s} \quad [9]$$

The derivation of these latter equations is described in more detail in Nederlof and van Riemsdijk (3), and in Nederlof *et al.* (9).

## 1 Soil, 3 organisms

With the help of the above derived equations three examples are given to show the influence of the binding characteristics of biota. For one and the same soil, copper binding to three different organisms is calculated as a function of the total copper content of the soil and the pH in the soil solution.

**Copper binding by maize root cell walls in soil**

In maize, copper is mainly accumulated in the roots, where it leads to an inhibition of the uptake of essential nutrients in the shoot, thereby reducing growth of both roots and shoot of the plant. Toxicity of copper for maize has been determined both for nutrient solutions (10) and for soils at different pH-values (11). In the experiments performed in nutrient solution a clear rise in copper toxicity was observed with increasing pH. This relationship turned out to be inverted in the experiments performed on the soils; i.e., in soils toxicity was reduced when pH increased. The binding of copper ions to soil (Fig. 2a, data and model description from Lexmond (11)) is described by Lexmond with a two component Freundlich equation (Eq. 6). The binding of copper ions to cell wall material of the maize roots is increased with increasing pH (12) (Fig. 2b).

a)

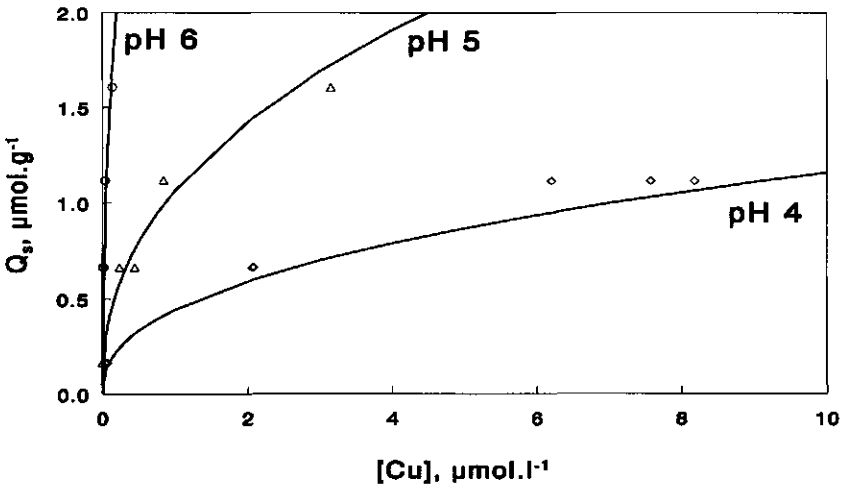
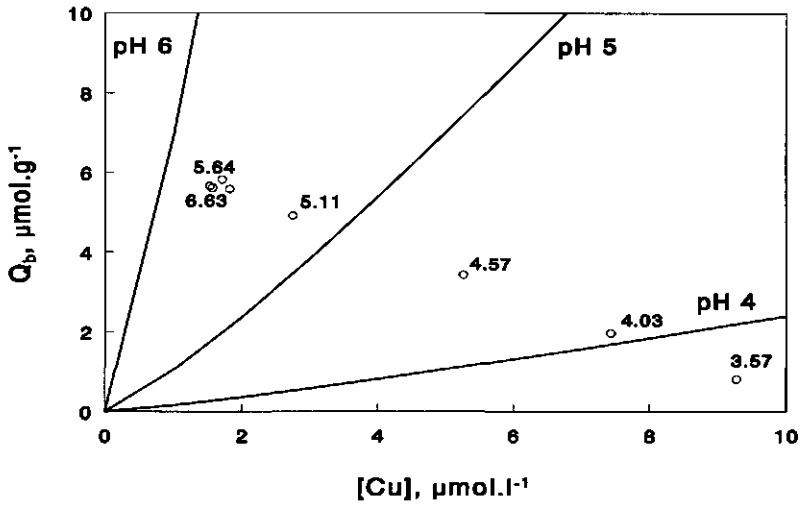



**Fig. 2** a) Copper binding to a sandy soil. Data and model description are derived from Lexmond (11).

b)




c)

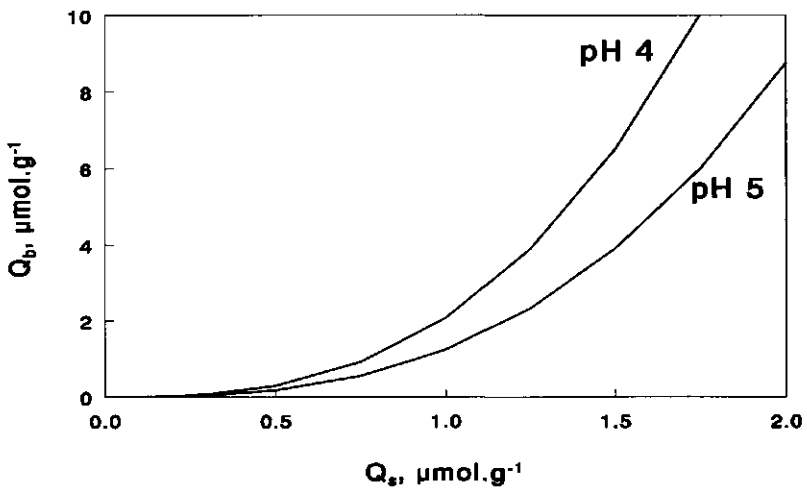



**Fig. 2** b) Copper binding to (4 days old) maize root cell walls. Data of Allan (12), model description derived from Nederlof *et al.* (9).
c) Predicted copper binding to maize root cell walls if the maize were grown on soil.

These data can also be described by a pH-dependent Freundlich equation (9). The parameters used for the description of these data sets (sorption in $\mu mol.g^{-1}$ as a function of proton and metal ion concentration in $mol.l^{-1}$) are given in Table 1.
Binding of copper ions from solution to the soil is increased at higher pH. Thus, at a constant total copper content of the soil, an increase of the soil pH will lead to a reduction of the concentration free copper in soil solution. Sorption to the maize root also depends on the concentration of copper ions in solution and the pH.
In a soil system, the increased pH will increase the affinity of the root cell walls for copper, but on the other hand, the reduced copper ion concentration will lead to a lower amount of copper sorbed by the root. In order to predict the final effect of the increased soil pH, both relationships (Eq. 6 and 7) are combined (Eq. 9). This enables the calculation of the sorption to maize root cell walls in the given soil system. The calculations are only made for pH 4 and 5, since at pH values higher than 5 the model description of the copper sorption to the maize roots is becoming less reliable (see Fig. 2b). The results of the model calculations (Fig. 2c) show, that with increasing pH in soil less copper is bound to the maize root cell walls. This is in full agreement with the observed differences of the influence of pH on toxicity in solution or in soil environment.

**Copper binding by fungal mycelia in soil**

A model description, obtained for the data of copper binding to fungal mycelia (Fig. 3a) (14), is combined with the model for copper binding to the soil. The parameters used for the description of the copper binding data of the mycelia are given in Table 1.
The mycelia have a higher affinity for Cu than the soil; under the same conditions copper sorption to mycelia, expressed in µmol Cu per gram dryweight material, is at least a tenfold higher than for soil. According to the model descriptions the pH-dependency (expressed in the exponent b) was almost equal to the exponent derived for the soil (for soil 0.45, for the fungal mycelia 0.40). When both model equations are combined in order to estimate the Cu binding to mycelia as a function of total copper in soil, the same phenomenon is observed as has been described for the maize roots: Sorption of copper ions from the soil to the mycelia decreases with increasing pH (Fig. 3b), and copper binding increases exponentially with increasing copper content of the soil. This latter relationship is characterized by an exponent over the total copper content ($a_b/a_s$, Eq. 9) that is larger than 1 in both cases.

**Copper binding by yeast in soil**

Data of copper binding by yeast from solution (Fig. 4a, data obtained from Huang *et al.* (13)) reflect a strong pH dependency. The parameters derived for the model description are given in Table 1. Once again, the same soil has been used. For the yeast, combination of the models for copper binding to soil and to the organism gives completely different results. Even at increasing pH the yeast is able to compete with the other soil components for binding of the metal (Fig. 4b). Instead of reduced binding from soil with increasing pH, the copper ion sorption to the yeast cells is still positively correlated with pH.

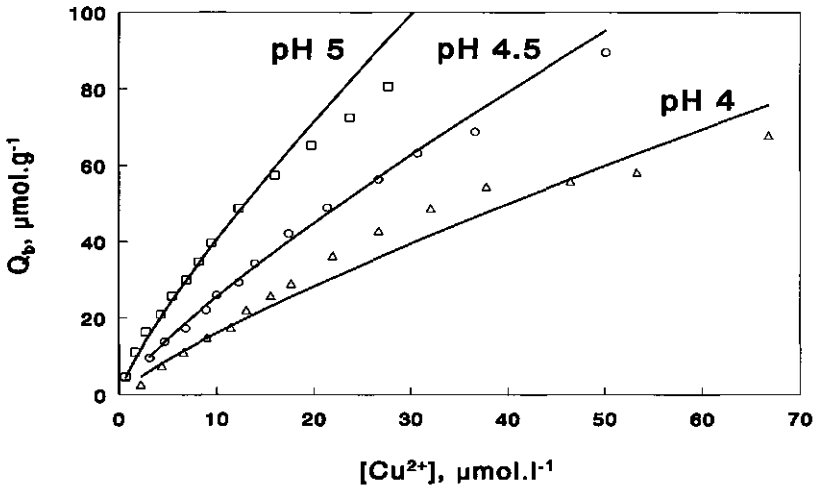
**Table 1**

**Parameters of the two component Freundlich equation**

Parameters for the description of the copper ion binding to a sandy soil (11), maize root cell walls (12), fungal mycelia (14), and yeast cells (13). Parameters for the soil and the maize are given by the authors (11, 12). The factor in the last column is the exponent over the proton concentration, as given in Eq. 9.

| | log K | a | b | $r^2$ | $b_b - b_s a_b / a_s$ |
|---|---|---|---|---|---|
| soil | 0.32 | 0.42 | –0.45 | 0.989 | – |
| maize | 3 | 1.18 | –0.82 | | 0.44 |
| fungi | 3.69 ± 0.12 | 0.82 ± 0.02 | –0.40 ± 0.02 | 0.976 | 0.48 |
| yeast | 0.90 ± 0.12 | 0.27 ± 0.02 | –0.37 ± 0.02 | 0.940 | –0.07 |

a)

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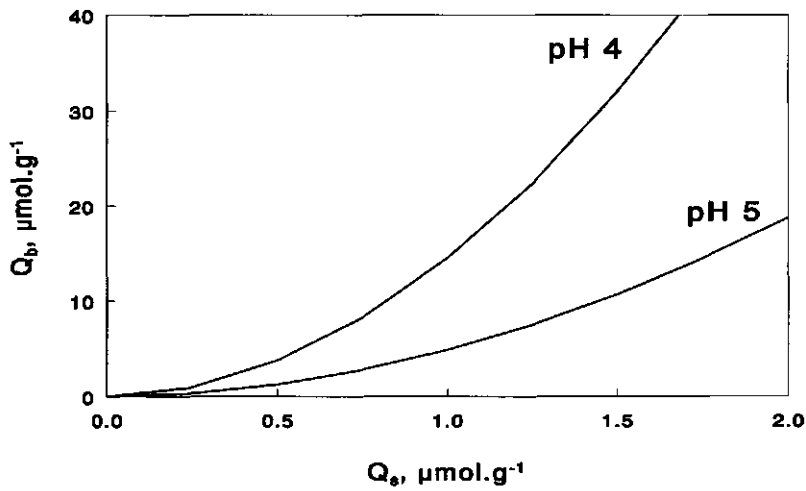
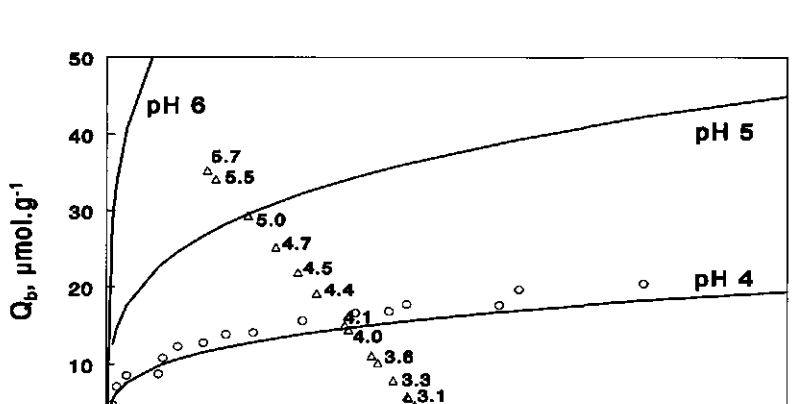



**Fig. 3** **a)** Copper binding to fungal mycelia. Data of Huang *et al.* (14) obtained at pH 4 (△), pH 4,5 (○) and pH 5(□) are used to obtain the model description of the copper binding to fungi from solution.
**b)** Predicted copper binding to fungal mycelia as a function of total copper in the soil system.

a)

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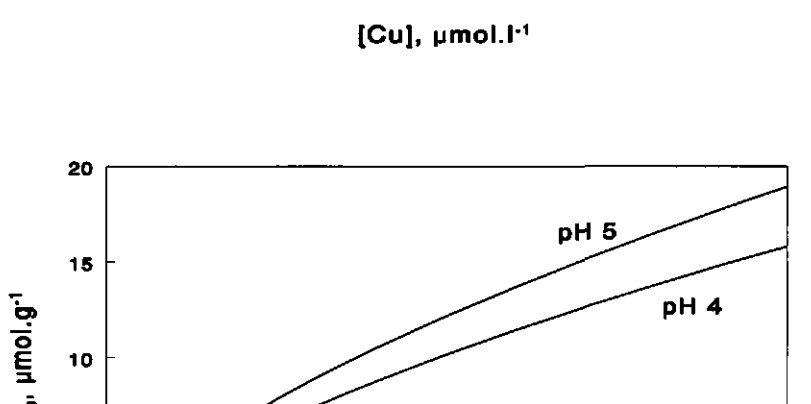



**Fig. 4** a) Copper binding to yeast cells. Data of Huang *et al.* (13) are used to obtain the model description of the copper binding to yeast from solution. ○: data obtained at pH 4, △:data obtained at constant total copper in the system, for various pH values.
b) Predicted copper binding to yeast cells as a function of total copper in the soil system.

The final impact of the pH on the metal ion sorption by organisms present in the soil is expressed in the proton exponent ($b_b$-$b_s a_b/a_s$). This exponent (see Table 1) is positive for the maize root cell walls and the fungal mycelia, leading to reduced metal ion sorption by the organism when the soil pH is increased. For the yeast cells, the resulting proton exponent becomes negative, and the effect of an increase of soil pH will lead to a reverse effect: metal ions will be increasingly sorbed by the yeast when pH is increased. From these examples we may conclude that availability, defined as the amount of metal taken up, differs strongly for different organisms, even if the exposure routes are rather similar (yeast, fungi).

## 1 Organism, different soils

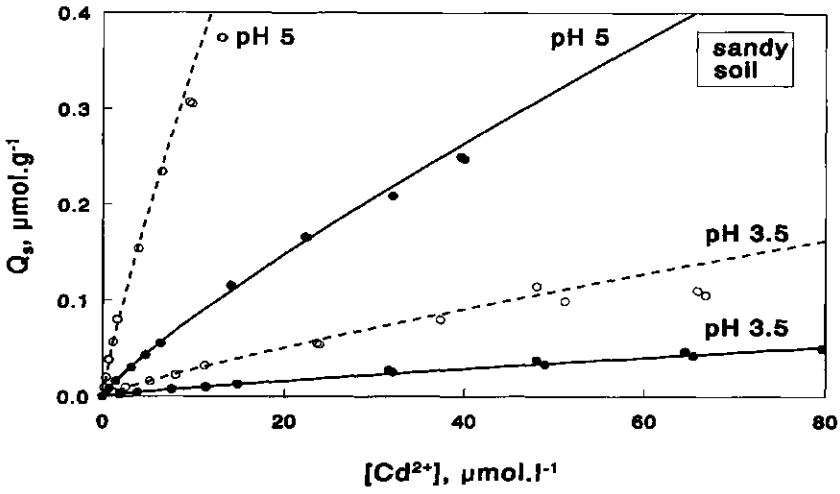
In the following example the influence of soil binding characteristics on the availability for one and the same organism is shown. Apart from the pH, also the concentration of competing ions in solution is taken into account.

**Influence of pH and calcium concentration on cadmium binding to soil**
Temminghoff *et al.* (15) obtained data on binding of Cd to a sandy soil (5% lutum, 1% organic carbon). In addition, cadmium binding to a clay soil (37% lutum, 0.9% organic carbon) was studied (unpublished). The cadmium sorption was studied as a function of the concentration Cd (1 to 100 µM), pH (3.5 to 5.5), and concentration calcium (0.1 to 10 mM). Both calcium ions and protons compete for the binding sites with cadmium. The influence of pH and calcium concentration on the cadmium binding to these two different soils is shown in Fig. 5a and b. Under similar conditions, the clay soil binds twice to three times as much cadmium as the sandy soil. The impact of the pH and the calcium concentration is different for both soils as well. For the sandy soil, the organic matter is the major metal ion binding component. The binding to organic matter is highly pH dependent, therefore the pH is the major factor for the cadmium ion binding to the sandy soil. For the clay soil, where the clay minerals are the main metal ion binding components, the competition with calcium ions turns out to be more important than the pH. For both soils, the data can be described as a function of the concentration cadmium, calcium and protons with a three component Freundlich equation (15, 16):

$$Q = K(Me)^a(H)^b(Ca)^c \qquad [10]$$

Parameters of the Freundlich equation for both soils are given in Table 2.

a)




b)

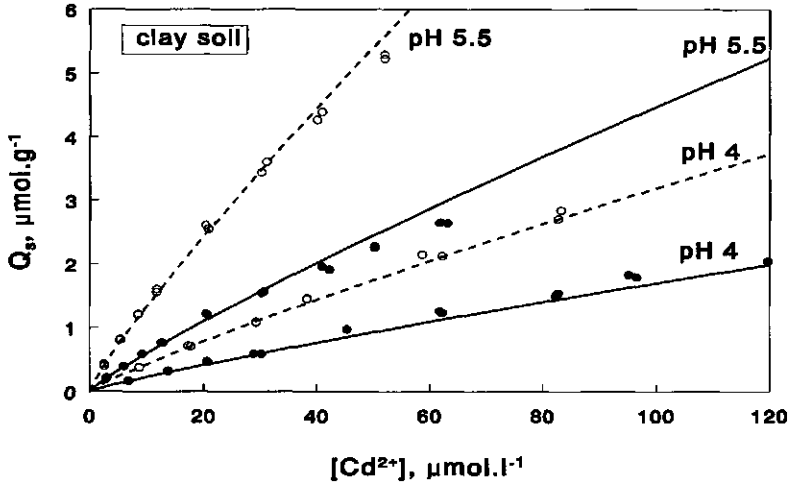



**Fig. 5** Cadmium sorption to soil at various pH values and at a calcium concentration of 1 resp. 5 mM. The lines represent the model description that is obtained on the basis of the data of Temminghoff *et al.* (15). Open symbols and broken lines: low [Ca]. Closed symbols and solid lines: higher [Ca]. **a)** Sandy soil. **b)** Clay soil. For the sandy soil, [Ca] is resp. 0.14 and 8.7 mM at pH 5, and resp. 0.37 and 9.5 mM at pH 3.5. For the clay soil, [Ca] is resp. 2.2 and 8.7 mM at pH 5.5, and resp. 3.9 and 11.4 mM at pH 4.

**Cadmium binding to a soil bacterium**

The major part of the sorption of cadmium by the Gram-positive soil bacterium *Rhodococcus erythropolis* A177 is due to cell wall sorption (17). Binding to these cell walls turns out to depend on the concentration cadmium in solution, the pH, the concentration calcium and the ionic strength. Binding of Cd at a constant ionic strength of 0.01 M can be described as a function of the concentration Cd and Ca and the pH with a model that takes into account competition between the metal ions and the protons (17). Intact, living cells of R.A177 experience a similar impact of pH and concentration calcium on the cadmium ion sorption (18).

In Fig. 6 sorption to intact cells of R.A177 is shown as a function of the concentration free cadmium ions for pH 5 and 6.5. Experiments were performed in the presence of calcium ions (approximately 1 mM) and without additional calcium in solution (in these experiments, the measured calcium concentration was approximately 1 μM). A higher pH value results in increased sorption of Cd, whereas a higher concentration calcium reduces cadmium sorption. So both protons and calcium ions can be regarded as competitors for cadmium in binding to the intact cells. For the description of the data, the same equation is used as for the soils (Eq. 10). The obtained parameters are given in Table 2.

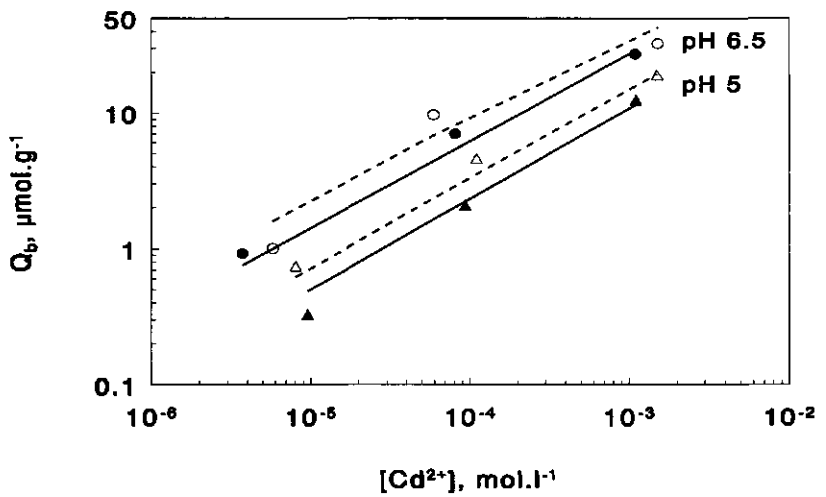



**Fig. 6** Cd sorption to a soil bacterium as a function of the concentration free Cd in solution. △: pH 5, ○: pH 6.5. Open symbols and broken lines: low [Ca], closed symbols and solid lines: 1 mM calcium. The lines represent the model description as is given in Eq. 10.

**Table 2**

**Parameters of the three component Freundlich equation**

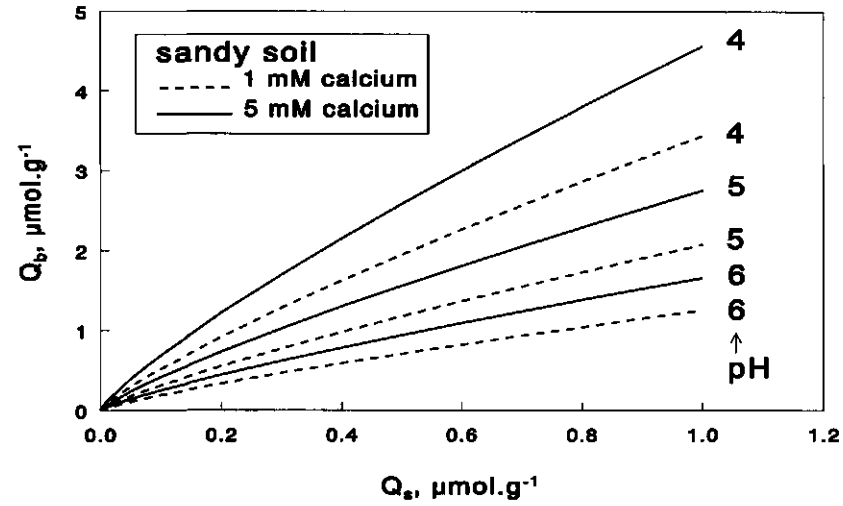
Parameters of the three component Freundlich equation (Eq. 10) for the description of the cadmium ion binding to a sandy soil (15), a clay soil, and the soil bacterium *Rhodococcus erythropolis* A177 (17).

| | log K | a | b | c | $r^2$ |
|---|---|---|---|---|---|
| sand | -0.78 ± 0.03 | 0.84 ± 0.01 | -0.67 ± 0.01 | -0.30 ± 0.01 | 0.992 |
| clay | 1.54 ± 0.04 | 0.87 ± 0.01 | -0.24 ± 0.01 | -0.61 ± 0.02 | 0.984 |
| A177 | 1.31 ± 0.25 | 0.69 ± 0.03 | -0.33 ± 0.04 | -0.07 ± 0.03 | 0.965 |

It is impossible to determine experimentally the cadmium ion sorption to a bacterium when present in soil. However, when the models given above are combined, a prediction of the sorption can be made. Under similar conditions (total cadmium, pH and calcium concentration) the cadmium ion sorption by the bacterium is less in the clay soil than in the sandy soil (Fig. 7a and b). In the sandy soil, more cadmium is bound by the bacterium when pH is decreasing. In the clay soil, the impact of pH is the reverse, i.e., sorption by the bacterium still increases with increasing pH. Since the exponent a is rather similar for both soils, this difference between the two soils can be fully explained by the different pH-dependency (b) of the cadmium sorption by the soils. In both soils, the cadmium sorption increases with increasing pH, but the pH-effect is much stronger for the sandy soil.

In both soils, the cadmium ion sorption by the bacterium is increased with increasing calcium concentration. Due to competition between cadmium and calcium ions for binding to the reactive sites on the soil solid phase, cadmium is desorbed when the calcium concentration is increased. The increased cadmium concentration results in an increased sorption by the bacterium, since the impact of the increased calcium concentration on cadmium sorption is much smaller for the bacterium than for the two soils.

a)




b)

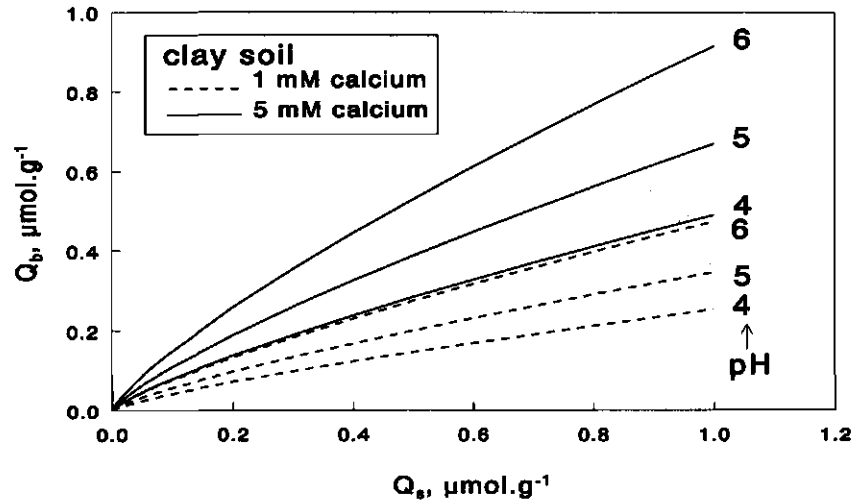
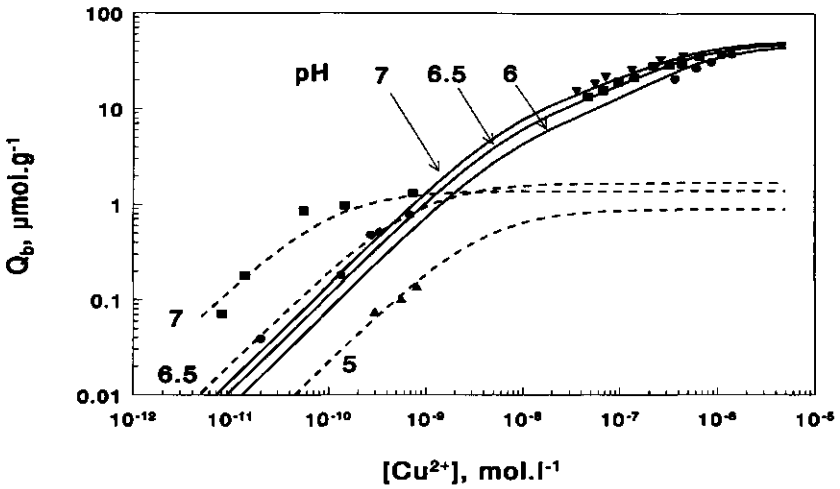



**Fig. 7** Cd sorption to the bacterium as a function of the total amount of cadmium present in soil.
**a)** Predicted bacterial sorption in the sandy soil.
**b)** Predicted bacterial sorption in the clay soil.

## Aquatic systems

For terrestrial systems, the assumption was made that the amount of metal bound to the soil solid phase equals the total amount of metal present in the soil.
In aquatic systems, this assumption can not be made. Uptake in these systems often implicates a redistribution of the metal over the different components; the contribution of metal bound to biota may form a significant part of the total metal present in the system. The metal ion concentration in solution is buffered to some extent by dissolved organic matter (DOM) in solution. In a Swiss lake DOM concentrations were on average 3.5 $mg.l^{-1}$. The total copper concentration ranged between 5 and 28 nM, the concentration free copper ions was much lower, $10^{-16}$ to $10^{-14}$ M, depending on the concentration organic (biotic and abiotic) compounds in solution (19). In case of high (19 $mg.l^{-1}$) DOM concentrations, the complexation of the metal ions by the DOM may even lead to a situation where trace metal ions become a limiting factor for algal growth (20).
The concentration of algae in an aquatic system shows large fluctuations during the year. During algal bloom the total cell density may rise from $10^6$ to $3*10^8$ $cells.l^{-1}$ (21). The algae used by Xue and Sigg (22) had a dry weight of 70 $\mu g/10^6$ cells. Thus, algal concentrations, as expressed in g dryweight per liter, may range from 70 $\mu g.l^{-1}$ to 21 $mg.l^{-1}$, which is in the same order as the amount of DOM present in these systems. It is therefore not unlikely that the algae will also contribute to the buffering of the metal ion concentration. In either case, the major part of the total metal present in the system will be in complexed forms.
Xue and Sigg (23) determined binding of copper to algae at pH 6, 6.5 and 7 for a copper ion concentration of 0.05-1.6 µM. The algae show an increased sorption of copper at increasing pH. They interpreted the results in terms of conditional equilibrium constants, valid at a given pH; for each pH value a two-site monocomponent Langmuir equation was derived. Two years later, Xue and Sigg (22) reported experiments that were performed at lower Cu concentrations, ranging from 0.01 to 1 nM. These data were described with a 1-site monocomponent Langmuir equation. The data and the two models are shown in Fig. 8a. Calculation of the copper sorbed on the basis of the previously derived affinity constants (22) would have given an underestimation of the amount really sorbed under these conditions, especially at higher pH. So, measuring sorption at lower concentrations lead in this case to the discovery of (a group of) sites with an even higher copper affinity.

a)

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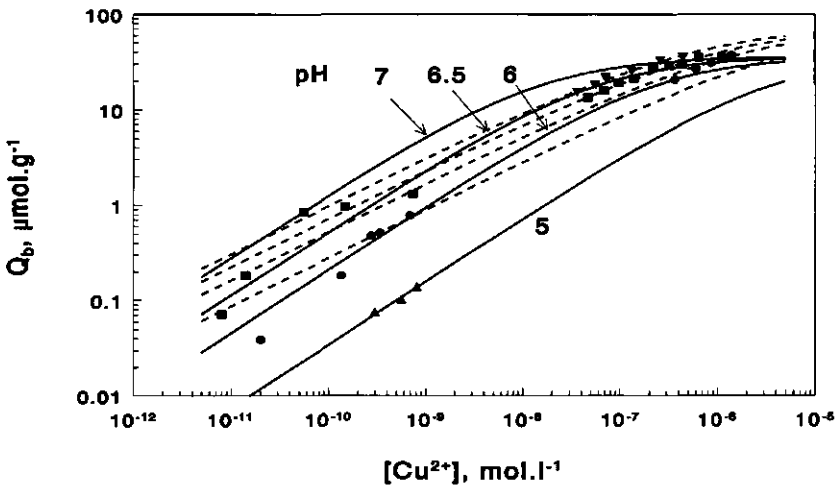


**Fig. 8** a) Copper binding to algae at pH 5 (▲), pH 6 (●), pH 6.5 (■), and pH 7 (▼). Data of Xue and Sigg (22, 23). Solid lines: model description given by Xue and Sigg (23), based on the data obtained at copper concentrations ranging from $10^{-8}$ to $10^{-6}$ M. Broken lines: model description given by Xue and Sigg (22), based on the data obtained at copper concentrations ranging from $10^{-11}$ to $10^{-9}$ M. b) The same data as described for Figure 8a. Broken lines: two-component Langmuir-Freundlich model description as derived for the data obtained at copper concentrations ranging from $10^{-8}$ to $10^{-6}$ M. Solid lines: two-component Langmuir-Freundlich model description as derived for the full data set.

In the lake, free copper ion concentrations were around $10^{-14}$ M, which is several orders of magnitude lower than the conditions of the experiments. In addition, the pH of the lake water is around 8 (19), whereas the experiments were done between pH 5 and 7. The pH may even become higher than 8 during algal bloom in daylight. In order to extrapolate from the experimental data to these more realistic conditions, a model is required that takes into account the pH-dependency, and is valid for the full range of copper ion concentrations used. A two component Langmuir-Freundlich equation (Eq. 3; $\tilde{K}$ = 0.31, $S_{tot}$ = 36 µmol.g$^{-1}$, x = 1.19, n = 0.66, and $r^2$ = 0.986) was used to describe the full data set (Fig. 8b).
For copper binding to the DOM, the data as given by Benedetti *et al.* (24) for copper ion binding to a humic acid are used (Fig. 9). Copper binding to this material has been determined experimentally for a very wide range of conditions; pH ranged from 4 to 8 and the free copper ion concentration ranged from $10^{-14}$ to $10^{-4}$ M. The data can be described reasonably well with a 2 component Freundlich equation (Eq. 6; K = 37.8, x = 0.37, y = -0.532, and $r^2$ = 0.984). Benedetti *et al.* (25) have shown that the copper speciation in the lake, as measured by Xue and Sigg (19), is in very good agreement with predictions, assuming that the DOM in this lake behaves as this humic acid.

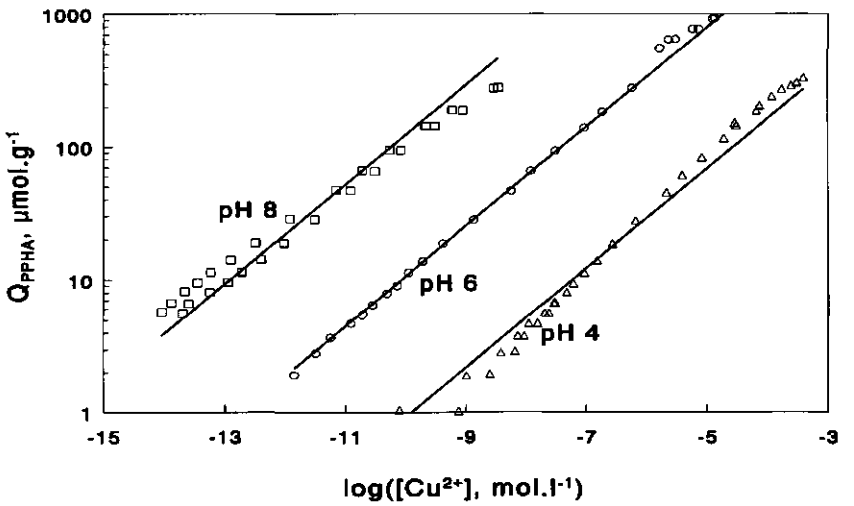


**Fig. 9** Copper binding to a humic acid at pH 4 (△), pH 6 (○), and pH 8 (□). Data obtained from Benedetti *et al.* (23). Solid lines: Two component Freundlich model description of the data.

In the aquatic system no reactive component is present in amounts as were found in the soil solid phase of a terrestrial system. Therefore, calculations were done iteratively in order to calculate the distribution of the metal over the different components in the aquatic system. The model descriptions of copper binding by algae and of copper binding to DOM are used to illustrate the influence of the pH, the presence of DOM and the algal density, on the copper speciation in the model system.

In Fig. 10, copper ion sorption by the algae (the concentration algae is set to 0.1 mg.l$^{-1}$) is shown as a function of the total amount of copper present in solution. When algae are the sole copper binding component in solution, the copper ion binding to the algae increases, as expected, strongly with increasing pH. However, as soon as some DOM is present in the system, the copper ion sorption by the algae is strongly reduced, and a much smaller and even reversed pH-effect is observed. The DOM has a much higher affinity and capacity for copper ion binding; in this model system, with 3.5 mg.l$^{-1}$ DOM and 0.1 mg.l$^{-1}$ algae, at low copper concentrations almost all (> 99.9%) copper present in the system is bound to the DOM. The fraction of copper bound to the algae is less than 1% for all conditions. At pH 5, the DOM is not able to buffer the copper ion concentration; at high total copper concentrations, more than 90% of the total copper is in the free ionic form.

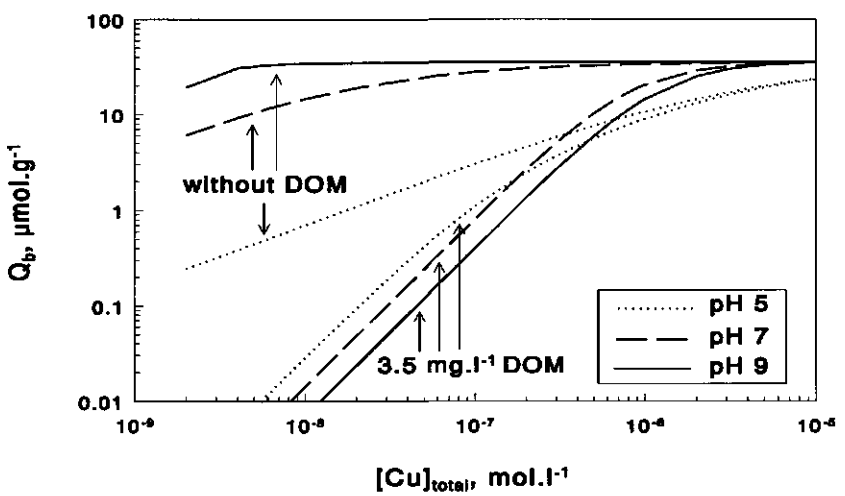


**Fig. 10** Predicted copper binding by algae as a function of the total copper concentration, without DOM or in the presence of 3.5 mg.l$^{-1}$ DOM in the system, and at pH 5, 7 and 9. The concentration algae is 0.1 mg.l$^{-1}$.

The algae concentration, and thus the fraction of the total copper bound to the algae, may vary during the year. The copper ion sorption by algae is given as function of the algae concentration in Fig. 11. The total copper concentration is kept constant at 10 nM. In the absence of DOM, the algae bind more copper at increased pH. The amount of copper bound per gram algae is reduced at increased algae concentration. In the presence of DOM, copper sorption by the algae is reduced with increasing pH. The concentration free copper ions is fully determined by the DOM, and the amount of copper sorbed by the algae (in $\mu mol.g^{-1}$) is not reduced due to the increase of the number of algae in solution.

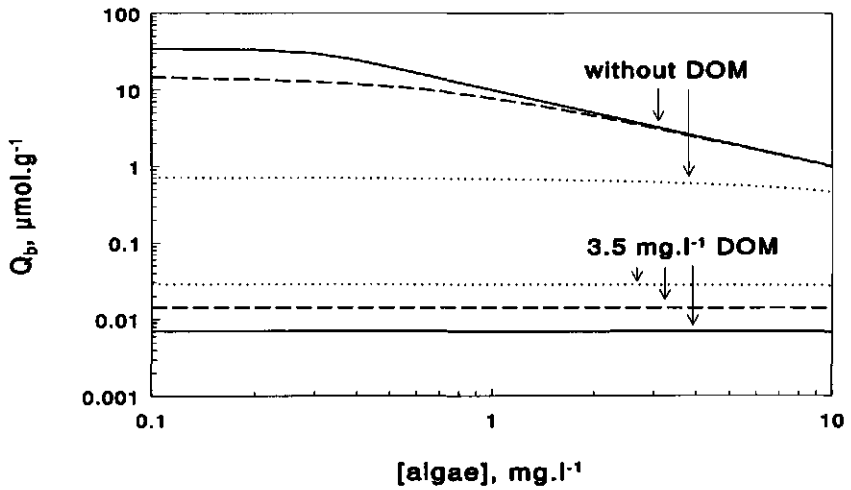


**Fig. 11** Predicted copper binding to algae as a function of the concentration of algae in solution, without DOM or in the presence of 3.5 $mg.l^{-1}$ DOM, and at pH 5, 7 and 9 (see legend Fig. 10). The total copper concentration is 10 nM.

According to Verweij (20), copper is limiting for algal growth below $10^{-12.5}$ M $Cu^{2+}$, and becomes toxic at concentrations higher than $10^{-10.5}$ M. For the Swiss lake with 3.5 $mg.l^{-1}$ DOM, the calculations indicate that at pH 5 and a total copper concentration of 10 nM, the free copper ion concentration is around $10^{-10.1}$ M, and the algae might experience toxic effects from the copper if the pH would be this low. However, at pH-values measured in the lake, the free copper concentration is orders of magnitude lower ($10^{-13}$ at pH 7, and $10^{-16}$ at pH 9). Therefore, according to the model calculations, in this lake the algae will rather experience growth limitation than toxic effects of the copper.

## Discussion

In practice, it will be very difficult or even impossible to measure metal ion sorption by biota in a soil system. The examples have shown that with relatively simple equations effects observed in these complicated systems can be predicted. Nevertheless, some reservations should be made. The choice to use a certain model, to include or exclude an environmental parameter, specific characteristics of the organism, it will all have its impact on the outcome of the calculations.
Therefore, some considerations are given below.
The concept presented in this paper is based on the assumption that exposure only takes place via the solution phase. The uptake route is of great influence; in case of more complex uptake routes (e.g., digestion of soil, herbivorism/carnivorism) the impact of factors like pH can be less distinct. However, even for higher organisms a clear influence of a-biotic factors has been observed. The influence of these factors reaches far into the food chain, since even carnivorous mice (Sorex Araneus, (26)) experience different levels of exposure under different soil conditions. This may be regarded as a plea to take the abiotic environment into account, even if the organism under study is not in direct contact with this environment.
In terrestrial oxic systems the pH and calcium concentration (as a competitor for the same sorption sites as the metal ion) are considered to be two of the most important factors that affect the distribution of the metal over the components present in the system. A model for metal ion binding should in principle be able to describe and to predict binding for a wide range of conditions with respect to pH, solution composition and ionic strength. In the example of copper toxicity for maize a rather simple sorption model could be used to describe metal ion binding to the various components. With the help of pH-dependent Freundlich equations the distribution of the metal over the biotic and a-biotic components in the system under study could be predicted, which explained the observed toxic effects of copper at different pH-values both in soils and in aquatic systems. However, to meet the complexicity of sorption of metal ions to individual soil components with a chemically strongly heterogeneous composition, a more complex ion binding model might be required to describe the data well. As long as the parameters derived for the individual components apply for the same experimental conditions, different models can be combined in order to calculate the distribution of a metal ion over the different components in the system. As long as models are used for interpolation, i.e., prediction of the sorption within the measurement "window", the values obtained will not differ much from one model to another. However, as can

be observed in Fig. 8a and b, the results of extrapolation can differ orders of magnitude. Therefore, one should be aware that model calculations far beyond the measurement "window" used for the model calibration, can easily lead to erroneous predictions.

The model calculations show that pH and composition of the solution may have a large impact on the bioavailability of metals. Impact of pH and calcium ion concentration on the cadmium sorption by the soil bacterium is of the same order as the impact of the different soil types. Unfortunately, the examples also show that the effect of for example the pH is very dependent on the characteristics of the system and the organism, and may be completely reversed from one system to another. In the sandy soil, copper availability for the fungi was strongly reduced when pH increased, whereas for the yeast an increased pH might lead to slightly enhanced toxic effects. Therefore, the speciation approach may be a valuable tool in predicting quantitatively the metal sorption to biota present in a complex system, and to predict the relative change in availability due to environmental changes. As for the effects for biota, this approach will enable at least a qualitative statement on the impact of changing environmental conditions.

## References


1. Tessier, A., Buffle, J. and Campbell, P.G.C., Uptake of Trace Metals by Aquatic Organisms. EERO-cursus 1993, bijdrage J. Buffle (1993).
2. Morel, F.F.M. (ed), Trace Metals and Microorganisms. Principles of Aquatic Chemistry, Wiley Interscience, New York, 300 (1983).
3. Nederlof, M.M. and van Riemsdijk, W.H., Effect of Natural Organic Matter and pH on the Bioavailability of Metal Ions in Soils. In: Environmental impact of soil component interactions, P.M. Huang *et al.* (eds.), CRC Press, Boca Raton (1995), 73-84 (1995).
4. Koopal, L.K., van Riemsdijk, W.H., de Wit, J.C.M. and Benedetti, M.F., Analytical Isotherm Equations for Multicomponent Adsorption to Heterogeneous Surfaces. *J. Colloid Interface Sci.* **166**, 51 (1994).
5. Sips, R., On the Structure of a Catalyst Surface. *J. Chem. Phys.* **16**, 490 (1948).
6. Kinniburgh, D.G., Baker, J.A., and Whitfield, M., A comparison of some simple adsorption isotherms for describing divalent cation adsorption by Ferrihydrite. *J. Colloid Interface Sci.* **95**, 370 (1983).
7. Milne, C.J., Kinniburgh, D.G., de Wit, J.C.M., van Riemsdijk, W.H. and Koopal, L.K., Analysis of Metal-ion Binding by a Peat Humic Acid Using a Simple Electrostatic Model. *J. Colloid Interface Sci.* **175**, 448 (1995).
8. Temminghoff, E.J.M., van der Zee, S.E.A.T.M., and Keizer, M.G., The influence of pH on the desorption and speciation of copper in a sandy soil. *Soil Science* **158**(6), 398 (1994).
9. Nederlof, M.M., van Riemsdijk, W.H. and de Haan, F.A.M., Effect of the pH on the Bioavailability of Metals in Soils. In: H.J.P. Eijsackers and T. Hamers. (eds), Soil and Environment Volume 1): Integrated Soil and Sediment Research: A Basis for Proper Protection, Kluwer Academic Publishers, Dordrecht, 215 (1993).
10. Lexmond, Th.M. and van der Vorm, P.D.J., The Effect of Soil pH on Copper Toxicity to Hydroponically Grown Maize. *Neth. J. Agric. Sci.* **29**, 209 (1981).
11. Lexmond, Th.M., The Effect of Soil pH on Copper Toxicity to Forage Maize Grown under Field Conditions. *Neth. J. Agric. Sci.* **28**, 164 (1980).
12. Allan, D.L., Proton and Copper Adsorption to Maize (Zea mays L.) and Soybean (Glycine max. L. Merr.) Root Cell Walls. Ph.D. thesis, University of California, Riverside (1987).
13. Huang, C.P., Huang, C.P. and Morehart, A.L., The Removal of Cu(II) from Dilute Aqueous Solutions by *Saccharomyces cerevisiae*. *Wat. Res.* **24**, 433 (1990).
14. Huang, C., Huang, C.P. and Morehart, A.L., Proton Competition in Cu(II) Adsorption by Fungal Mycelia. *Wat. Res.* **25**(11), 1365 (1991).
15. Temminghoff, E.J.M., van der Zee, S.E.A.T.M. and de Haan, F.A.M., Speciation and Calcium Competition Effects on Cadmium Sorption by Sandy Soil at Various pHs. *European Journal of Soil Science* **46**, 000 (1995).
16. Chardon, W.J., Mobiliteit van Cadmium in de Bodem. Ph.D. thesis, Wageningen Agricultural University (1984).
17. Plette, A.C.C., van Riemsdijk, W.H., and Benedetti, M.F., Competitive Binding of Protons, Calcium, Cadmium and Zinc to Isolated Cell Walls of a Gram-positive Soil Bacterium. *Environmental Science and Technology*, accepted (1996).

18. Plette, A.C.C., Haanstra, L., van Beelen, P., and van Riemsdijk, W.H., Sorption of metal ions by intact cells, in preparation. (Chapter 4 and 5 of this thesis).
19. Xue, H.B., and Sigg, L., Free Cupric Ion Concentration and Cu(II) Speciation in a Eutrophic Lake. *Limnol. Oceanogr.* **38** (6), 1200 (1993).
20. Verweij, W., Speciation and Bioavailability of Copper in Lake Tjeukemeer. Ph.D. thesis, Agricultural University Wageningen (1991).
21. Sanders, J.G., and Riedel, G.F., Trace Element Transformation During the Development of an Estuarine Algal Bloom. *Estuaries* **16**(3A), 521 (1994).
22. Xue, H.B. and Sigg, L., Binding of Cu(II) to Algae in a Metal Buffer. *Wat. Res.* **24**(9), 1129 (1990).
23. Xue, H.B., Stumm, W. and Sigg, L., The Binding of Heavy Metals to Algal Surfaces. *Wat. Res.* **22**(7), 917 (1988).
24. Benedetti, M.F., Milne, C.J., Kinniburgh, D.G., van Riemsdijk, W.H. and Koopal, L.K., Metal Ion Binding to Humic Substances: Application of the Non-Ideal Competitive Adsorption Model. *Environmental Science and Technology* **29**, 446 (1995).
25. Benedetti, M.F., van Riemsdijk, W.H., Koopal, L.K., Kinniburgh, D.G. and Milne, C. Copperbinding by natural organic matter, from the model tot the field. *Geochimica Cosmochimica Acta* Submitted.(1996).
26. Ma, W.C. and van der Voet, H., A risk-assessment model for toxic exposure of small mammalian carnivors to cadmium in contaminated natural environments. *Sci. Tot. Environ.* Supplement, 1701 (1993).

# Chapter 7

# Epilogue

# Epilogue

In this chapter, some reflections are made on the work presented in this thesis. An indication of practical applications of the obtained knowledge and future challenges is given.

## Proton and metal ion binding to the cell wall

The proton titrations (chapter 2) are a good basis for the metal ion binding studies, and a great help for the interpretation of the metal binding data (chapter 3), since they give insight in the number and type of reactive groups of the cell wall.
For the description of the electrostatic interactions during proton binding a relatively simple model was chosen, assuming a homogeneous potential within the Donnan volume, and a zero potential outside this volume. The Donnan volumes could be estimated from the proton titration data obtained at the different $NaNO_3$ concentrations. For the description of the proton binding to the cell walls this model is detailed enough. Due to the binding of bivalent cations, structural changes apparently took place, which could not be foreseen based on the information derived from the proton titrations. Under natural conditions, structural changes will probably not take place, since calcium is often a dominant cation in soil solution with concentrations of approximately 1 mM. Nevertheless, in order to take the electrostatic interactions into account explicitly, the impact of the sorption of bivalent ions on the Donnan volume must be quantified. The required information can be obtained by performing proton titrations in a calcium environment. In this thesis, the electrostatic interactions were only taken into account implicitly for the description of the metal ion binding.
Prediction of the metal ion binding to the cell wall of intact, living cells on the basis of the model derived for the binding to isolated cell walls was only partly successful (chapter 5). At pH 5, the sorption during the first stage was predicted reasonably well. At pH 6.5 however, this sorption was underestimated systematically. According to the bi-modal NICA equation the contribution of the phosphate groups to the total metal ion binding becomes more important with increasing pH. A suggestion therefore might be, that during the first stage, phosphate groups present on the outside of the cytoplasmic membrane contribute to the metal ion binding as well. This may explain the relatively good correlation that was observed between the metal ion sorption to the isolated (phosphatic groups containing) cell wall material and the toxicity experienced for the same external conditions.

## Application in the treatment of environmental pollution

The biosorption of heavy metal ions by microbial cell material has a potential for industrial use. The metal sorption process could be utilized for the removal of toxic heavy metals from industrial waste waters. An abundant source of metal sorbing biomass are marine algae (1). An other potential source is fungal biomass, which is produced as a waste material from several major industrial fermentation processes (2). Recently, a wide number of studies concerning the use of microorganisms as biosorbents, e.g., fungi, yeast (2,3), algae (1,4), bacterial cell walls (5), have been presented. So far, most biosorption studies have been based on one-metal solutions, ignoring the impact of competition of protons and other (metal) cations on the sorption. Chong and Volesky (6) presented two-metal biosorption equilibria to dead sea-weed material. The competition between the two metals was described by Langmuir type models. Metal binding models that take into account environmental conditions like pH and the concentration of competing ions can be of great help for the determination of the optimal conditions for metal removal from solution.

## Application in risk assessment

For the estimation of maximum acceptable levels of toxic chemicals in soils, external threshold concentrations are used, which are usually estimated by no observed effect concentrations (NOEC) from laboratory experiments. These experiments often do not reflect the bioavailability as experienced in the field. Van Wensem *et al.* (7) assumed that, as soon as the threshold for the internal concentration is exceeded, an effect occurs, irrespective of the way and rate by which this internal concentration has been achieved. The relation between the internal threshold concentration and the field external threshold concentration is given by concentration factors. Knowing the concentration factors both in lab and field experiments, the field external concentration at which the internal threshold concentration, as determined in the lab, will be reached, can be predicted (7). Thus, the concept enables to separate exposure and toxicity. Still, this concept will not enable to predict the impact of environmental factors (composition of the soil, soil pH, redox conditions, composition soil solution) on the exposure to the biota. For all possible combinations of soils and soil biota concentration factors will have to be determined. And even then, the concept is only applicable in a static environment. But what if conditions change, for instance due to acidic precipitation, or due to changes in land use? Van Straalen and Bergema (8) used data from literature concerning the influence of pH on the metal content of

earthworms to adjusted NOEC-values from toxicity experiments. Results of this theoretical analysis clearly showed an increased ecological risk at decreasing pH.
In order to predict the impact of changing environmental conditions on bioavailability and hence toxicity, models need to be developed. In combination with a model description of metal ion binding by the a-biotic soil components, the mechanistic knowledge of metal ion binding to soil microorganisms can serve as a tool in risk assessment, since it leads to a more quantitative insight in the influence of soil conditions on effects of heavy metal contamination on soil microorganisms.

## Extrapolation to field conditions

In this thesis, a concept is presented that enables the extrapolation of results obtained from experiments performed in solution to real world situations in soil (chapter 6). A basic assumption of this "Speciation approach" is that all interactions take place via the solution phase. Model calculations are presented concerning the bioavailability of heavy metals in soils and aquatic systems. For the maize, the outcome of the speciation calculations could be validated based on the toxicity experiments, which were performed both on nutrient solutions and in soil. The speciation approach turned out to give a good indication of the influence of the pH on the bioavailability of copper for maize when grown on soil.
Direct interactions between the different soil components are not taken into account. Some work already has been performed on more-component systems, which showed that the presence of additional components may influence binding behavior of other components. E.g., Flemming *et al.* (9) observed that composites of bacterial cell walls and clay particles sometimes reacted in a manner distinctly different from that of the individual components. Two explanations were given. First of all, the production of the composites will alter the total surface charge and block certain reactive sites of the individual components. On the other hand, at the adhesion zone between the clay particles and the cell wall, new sites may be formed. This type of interactions are ignored in the concept presented in chapter 6. It will be most interesting to perform experiments in multicomponent systems in order to verify the validity of the basic assumptions of the speciation approach, and to validate the model calculations.

## Impact of biodiversity

Van Beelen *et al.* (10) measured the effect of zinc on the mineralization of acetate in acid (pH 3.8) and calcareous (pH 8.2) soils. Effect concentrations (in $mg.kg^{-1}$ soil) obtained from the calcareous soils were much lower than effect concentrations from the acid soils. On the basis of Freundlich adsorption isotherms the zinc concentration in the soil solution was calculated. Even when expressed in $mg.l^{-1}$ soil solution, the effect concentration is two orders of magnitude lower for the calcareous soil. Van Beelen *et al.* (10) mainly ascribed the different sensitivity to differences between the microbial communities in these soils. However, as can be derived from the zinc binding isotherms for R.A177 (chapter 3, figure 4), the observations could, at least partly, be explained on the basis of the influence of the pH on the zinc binding by the microorganisms. Due to the high pH of the calcareous soil, binding to the biota may be similar in both soils, even though the zinc concentration in the calcareous soil is 1 to 2 orders of magnitude lower than in the acid soil.
Nevertheless, for the application in risk assessment, it is of interest whether the variation in exposure due to a variation in environmental parameters is similar for different (groups of) bacteria. For R.A177, a positive correlation was observed between sorption and toxicity. Peptidoglycan, the major component of the cell wall of this bacterium, is a rather common structure for prokaryotic microorganisms. Probably, for groups of microorganisms, rather good estimates of metal binding behavior can be made when the composition of the cell wall is known. As for that, the results of copper binding studies to algae, presented by Sunda and Huntsman (11), are quite promising: 3 out of 4 species of algae showed a similar copper sorption behavior at the pH studied. Pirszel *et al.* (12) determined the CEC of algae and cyanobacteria, using potentiometric proton titration data. First derivatives were calculated from the titration data, two of these curves show remarkable similarity with the proton affinity distribution obtained for *Rhodococcus erythropolis* A177. It would therefore be quite a challenge to repeat the experiments for a wider range of conditions and, most of all, for some other microorganisms.

## Protective function of the cell wall

In Gram-positive bacteria, the cell wall is the outermost cellular structure of the bacterium which must cope with the external environment. In Gram-positive bacteria the cytoplasmic membrane is the site of much enzymatic activity; there is little or no enzymatic activity in the cell wall (13). Major component of the cell wall is

peptidoglycan. When a metal ion is bound to reactive groups of the peptidoglycan, it is rather unlikely that it will affect the organism.
Several studies have shown that the cell wall may protect the cell for harmful effects of heavy metals. The toxicity of Cu and Cd is observed to be much larger for protoplasts (i.e., bacterial cells of which the cell wall has been removed) than for intact cells (14). Recently, Macfie *et al.* (15) studied the influence of the presence of the cell wall on the effects of $Co^{2+}$, $Cu^{2+}$, $Cd^{2+}$, and $Ni^{2+}$ on the growth of a green alga. Growth of a walled and a wall-less mutant of the same alga were compared. The effects were studied at pH 5 and 6.8. The effect of a reduced pH was the same for the walled and the wall-less type. This lead to the conclusion that there must be considerable competition between protons and metal ions for binding sites on the cytoplasmic membrane as well (15). For *Rhodococcus erythropolis* A177, this type of competition is reflected in an increased metal toxicity with increased pH (chapter 5).
The experimental results of Macfie *et al.* (15) demonstrated that the cell wall offers some protection for toxic concentrations of heavy metals, since the growth rates of the wall-less algae were reduced at lower metal ion concentrations than the growth rates of the walled species. This was most clearly observed for $Co^{2+}$ and $Cu^{2+}$; for $Cd^{2+}$ and $Ni^{2+}$ differences in growth between walled and wall-less algae were less distinct. Apparently, the cell wall acts as a barrier for metal ions. Some bacteria developed means to circumvent this wall barrier (16). For example, bivalent cations may enter cells as chelates or as complexes with peptides. The function of the peptides may be to extract the essential bivalent cations from "waiting sites" on the surface of the cells to the transport mechanisms on the cytoplasmic membrane. When complexed, the cation would be effectively neutralized and therefore be able to diffuse through the cell wall (16).
So far, the mechanism behind this barrier effect for toxic metal ions is not well understood. In most bacteria, there is an efflux of protons from the cells during growth. Doyle (16) suggests this efflux leads to a high degree of protonation of the reactive groups of the cell wall. The protonated wall matrix will have little tendency to bind cations. Usually, the cell wall is regarded as a porous structure containing reactive groups that may immobilize toxic metal ions. This concept still cannot explain the observed protective function, since, in a metal-buffering environment like a soil, metal ions may pass through the cell wall once the reactive sites are occupied.
The protective effect of the cell wall can however be understood if one considers the variable charge/variable potential character of the cell walls. In natural soil systems, the reactive cell wall sites will be mainly occupied by bivalent (calcium) cations, causing a net positive charge in the cell wall. Due to the positive electrostatic potential, resulting from this charge, cations will be excluded from the aqueous part of the cell

wall. Thus, due to the presence of the cell wall, the metal ion concentration in the proximity of the cytoplasmic membrane will be lower than the concentration in the bulk solution.

At some metal ion concentration level, Macfie *et al.* (15) observed that the presence of the cell wall offers no additional advantage to the cell. The authors suggest this simply reflects saturation of metal binding sites on the cell wall and subsequent lack of further protection of the cytoplasmic membrane. In line with the reasoning given above, this observation could also be explained by assuming that, in spite of the repulsive potential, the concentration at the membrane becomes sufficiently high to result in an effect.

Under normal conditions, calcium concentrations will be high enough to maintain the metal ion repulsive potential on the cell wall. However, due to acidic deposition, depletion of calcium in surface soils takes place. Directly, due to exchange with protons, or, as suggested by Lawrence *et al.* (17), indirectly, via the exchange with mobilized aluminum. In these acidic soils, a low pH (3 to 4.5) is combined with a low calcium concentration in the soil solution (10 to 100 μM) (17,18). Lawrence *et al.* (17) even measured calcium concentrations as low as 2 μM. For these conditions, calcium sorption by the bacterial cell wall will be low, leading to a reduced net cell wall charge. Thus, repulsion of metal ions will be reduced, and, in case the net charge of the cell wall becomes negative, metal ions even can be accumulated in the Donnan volume of the cell wall. This leads to a relatively increased metal ion concentration at the cytoplasmic membrane as compared to the bulk concentration. Thus, for these conditions, the cell wall is rather enhancing exposure to metal ions than protecting the organism for harmful effects.

Once more, these latest considerations stress that for a proper estimation of ecological risks, not only the concentration of the toxicant, but also other environmental conditions can be of great importance.

## References


1 Volesky, B., and Prasetyo, I., Cadmium removal in a biosorption column. *Biotechnol. Bioengin.*, **43**(11), 1010 (1994).

2 Mchale, A.P., and Mchale, S., Microbial biosorption of metals - potential in the treatment of metal pollution, Review. *Biotechnology Advances*, **12**(4), 647 (1994).

3 Tobin, J.M., White, C., and Gadd, G.M., Metal accumulation by fungi - applications in environmental biotechnology. *J. Ind. Microbiol.*, **13**(2), 126 (1994).

4 Wehrheim, B., and Wettern, M., Biosorption of cadmium, copper and lead by isolated mother cell walls and whole cells of chlorella fusca. *Appl. Microbiol. Biotechnol.* **41**(6), 725 (1994).

5 Mclean, R.J.C., Campbell, A.M., Khu, P.T., Persaud, A.T., Bickerton, L.E., and Beauchemin, D.. Repeated use of bacillus-subtilis cell walls for copper binding. *World J. Microbiol. Biotechnol.*, **10**(4), 472 (1994).

6 Chong, K.H., and Volesky, B., Description of two-metal biosorption equilibria by Langmuir-type models. *Biotechnol. Bioengin.* **47**(4), 451 (1995).

7 van Wensem, J., Vegter J.J., and van Straalen, N.M., Soil Quality criteria derived from critical body concentrations of metals in soil invertebrates. *Applied Soil Ecology* **1**, 185 (1994).

8 van Straalen, N.M., and Bergema, W.F., Ecological risks of increased bioavailability of metals under soil acidification. *Pedobiologia* **39**, 1 (1995).

9 Flemming, C.A., Ferris, F.G., Beveridge, T.J., and Bailey, G.W., Remobilization of heavy metals adsorbed to bacterial wall-clay composites. *Appl. Environ. Microbiol.* **56**(10), 3191 (1990).

10 van Beelen, P., Fleuren-Kemilä, A.K., and van Mil, C.H.A.M., Stimulatory and toxic effects of acid, pentachlorphenol or zinc on the mineralization of acetate in acid or calcareous soils and subsoils. *J. Environ. Sci. Health*, **A29**(7), 1391 (1994).

11 Sunda, W.G., and Huntsman, S.A. Regulation of copper concentration in the oceanic nutricline by phytoplankton uptake and regeneration cycles. *Limnol. Oceanogr.* **40**(1), 132 (1995).

12 Pirszel, J., Pawlik, B., and Skowronski, T., Cation-exchange capacity of algae and cyanobacteria: a parameter of their metal sorption abilities. *J. Industrial Microbiol.*, **14**, 319 (1995).

13 James, A.M., The electrical properties and topochemistry of bacterial cells. *Adv. Colloid Interface Sci.* **15**, 171 (1982).

14 Gadd, G.M., White, C., and Mowl, J.L., Heavy metal uptake by intact cells and protoplasts of *Aureobasidium pullulans*. *FEMS Microbiology Ecology*, **45**, 261 (1987).

15 Macfie, S.M., Tarmohamed Y., and Welbourn, P.M., Effects of cadmium, cobalt, copper, and nickel on growth of the green alga *Chlamydomonas reinhardtii* - the influences of the cell wall and pH. *Arch. Environ. Contam. Toxicol.* **27**(4), 454 (1994).

16 Doyle, R.J., How cell walls of Gram-positive bacteria interact with metal ions. In: Beveridge, T.J., and Doyle, R.J., Metal ions and bacteria. Wiley, New York, 461p (1989).

17 Lawrence, G.B., David, M.B., and Shortie, W.C., A new mechanism for calcium loss in forest-floor soils. *Nature* **378**, 162 (1995).

18 Goody, D.C., Shand, P., Kinniburgh, D.G., and van Riemsdijk, W.H., Field-based partition coefficients for trace elements in soil solutions. *European J. Soil Sci.* **46**, 265 (1995).

# Summary

Soil bacteria play an important role in the decomposition of organic matter and thus in the cycling of nutrients in natural systems. Heavy metal contamination may inhibit microbial activity. In addition, heavy metals may accumulate in the microorganism, which may result in an increased exposure for their predators as well. The impact of heavy metal contamination for an organism can be expressed in terms of availability of the metal for the organism under study. In the soil system, availability is affected by the distribution of the heavy metal ions over the reactive groups of the abiotic components (humic and fulvic acids, clay minerals and (hydr-)oxides) on the one hand, and the reactive groups of the biotic components on the other hand. The degree of binding to the biotic reactive groups is an indication of the availability of the metal. The distribution of the metal over all soil components may change due to changes in the environment (e.g., changes in the pH or the concentration competing ions ($Ca^{2+}$, $Mn^{2+}$, etc.)). In order to predict effects of changing pH and composition of the soil solution, all interactions with abiotic soil components as well as with the organism are assumed to take place via the soil solution. Then, binding to each component can be studied separately and binding models can be combined afterwards to predict environmental risks.
The influence of pH and [Ca] on metal interactions with the Gram-positive soil bacterium *Rhodococcus erythropolis* A177 are studied at different levels: adsorption to isolated cell walls, sorption by intact cells, and toxic effects. Metal ion binding to the bacterial cell wall is the first step in the interactions of the metal with the bacterium. In addition, the major part of the metal, sorbed by the bacterium, is bound to the cell wall. Therefore, the metal ion binding to the cell wall is studied in detail.

Cell walls of Gram-positive bacteria are highly porous structures. Peptidoglycan, the main component of these cell walls, contains many acidic groups, leading to a pH- and salt dependent charge. Peptidoglycan is a very common structure, since it is a cell wall component of almost all prokaryotic organisms. To characterize the reactive sites on the isolated cell wall material, proton titrations are performed at three salt levels. The influence of the salt level can be described using the Donnan model, an electrostatic model that assumes the charge to be distributed homogeneously over the volume of the cell wall. The specific volume of the cell walls is derived from the data by using the so-called "master-curve approach", an approach that is based on the principle that if electrostatic effects are eliminated, charging curves obtained at different salt levels coincide. The calculated volumes indicate that swelling of the cell walls becomes significant for salt levels below 0.1 M, and that above pH 5 the volume is rather

independent of the particle charge. The obtained Donnan potentials are close to potentials given in literature.
After elimination of the influence of the salt level the intrinsic proton affinity distribution is obtained. The result indicates the presence of strong chemical heterogeneity. At least three groups of sites are distinguished: carboxylic-type groups with an average log $\bar{K}_H$ of 4.6, amino-type groups with a log $\bar{K}_H$ of 10.0 and in between a small number of groups with a log $\bar{K}_H$ of 7.8. This latter group is related to phosphate originating from membrane phospholipids, which in fact is an impurity. On the basis of this distribution a binding model, the Langmuir Freundlich (LF) equation, is selected. The combination of a three modal LF-equation in combination with the Donnan model gives a very close reproduction of the observed charging behavior.

Cadmium and zinc titrations are performed with the isolated cell walls for three pH values (5, 6.5 and 8 for cadmium; 5, 6, 7 for zinc) in the presence of approximately 1 mM calcium. In addition, titrations are performed at high pH in the absence of calcium. The results show an important influence of the pH and of the presence of calcium. Sorption of cadmium is increased at increased pH. In addition, during the adsorption of cadmium, a strong release of calcium ions and protons is observed which almost equals the amount of cadmium sorbed. This is a clear indication of the competitive nature of the metal ion binding. At low calcium concentrations, a cooperative effect of the presence of calcium on the cadmium and zinc binding is observed; e.g., at pH 8 more cadmium is bound in the presence of 1 mM calcium than without calcium in solution. This lead to the conclusion that bivalent ions alter the cell wall structure.
On the basis of the metal binding data and the pH-dependent charging behavior, the net charge present on the cell wall material is calculated for all conditions. At high coverage with bivalent ions, charge reversal takes place. According to these calculations, cadmium and zinc binding takes place in spite of the positive charge present on the cell walls due to the adsorption of calcium, which is a strong support of the assumption that most of the metal ion binding to the cell walls is related to specific binding.
For the description of the data, the NICA (non ideal competitive adsorption) model is selected. Two types of sites (the carboxylic and the phosphatic sites) are assumed to bind metal ions under the experimental conditions. The amino type groups are not expected to be involved in metal ion binding, since these sites are positively charged at pH values below 9. The developed bimodal NICA equation gives a close description of the data. With the help of the NICA-equations for the binding of cadmium and zinc separately, the cadmium sorption in the presence of an excess of zinc is predicted

reasonably well. In addition, the model is able to discriminate between the binding of metal ions to both the carboxylic and the phosphatic sites, and therefore can be used to extrapolate the experimental results to metal ion binding to the cell walls of intact, living cells.

The study of metal ion sorption to intact, living cells is complicated by metabolic processes. In order to avoid complication of speciation (complexation of cadmium with carbonate species or proteins, precipitation of $CdCO_3$) and acidification due to $H_2CO_3$ dissociation, the conditions for the batch sorption experiments with intact cells are restricted to pH 5 and 6.5, either in the presence of approximately 1 mM calcium, or without additional calcium in solution.
Two stages can be distinguished for metal ion sorption by intact cells. During the first stage, relatively large amounts of metal ions are sorbed within a few minutes. Sorption during this stage generally is ascribed to adsorption of the metal ion to the cell wall. The metal content of the bacteria is still slowly increasing during the second stage, which may last hours to days and is considered to be the uptake into the cell. In order to be able to distinguish both stages, batch sorption experiments are performed at two exposure times: 30 minutes and two hours. The experiments were performed at three metal ion concentrations, at pH 5 and 6.5, and in the presence of approximately 1 mM calcium or without additional calcium in solution. From these data both cell wall sorption and uptake into the cell are derived for all conditions. It turned out that, like the adsorption to the isolated cell walls, sorption and uptake by intact cells increases with increasing pH and decreases with increasing concentration calcium. The uptake contributes for about 10% to the total amount sorbed by the intact cells in two hours. Like with the isolated cell walls, total sorption is somewhat higher for Cd than for Zn, under similar conditions. In the first stage, desorption of calcium is observed.
The sorption after 30 minutes exposure is predicted reasonably well with the NICA-model derived for the metal ion binding to isolated cell walls, provided the assumption that the phosphate groups are not involved in metal ion binding to cell walls of intact cells. At pH 6.5 however, sorption is systematically underestimated by this monomodal NICA equation.
The metal ion uptake is expected to be reduced when proton or calcium concentration is raised. However, with the exception of the zinc uptake at high zinc concentrations, no clear relation between uptake rate, pH and calcium concentration can be derived from the data. This is probably related to the experimental setup.

For *Rhodococcus erytropolis* A177 the influence of pH on toxicity of cadmium and zinc has been determined by measuring the acetate respiration as a function of metal

concentration and pH in solution. The respiration is reduced at a lower Me-concentration when pH increased. Zinc turns out to be more toxic than cadmium. The influence of calcium has not been investigated for this bacterium yet.
Since toxic effects are closely related to the amount taken up, a correlation is expected between the toxicity and the sorption or uptake rate. Surprisingly, the best correlation is found between the sorption to the isolated cell wall material and the effects. Since the major part of the metal ions, sorbed by these bacteria, is bound to the cell wall, this may be an indication that indeed total body burden is a good indicator for the potential effects for this type of organism. The trends for all processes (from cell wall sorption to toxic effects) suggest competitive interactions, not only during metal ion binding to the cell wall, but also during the uptake of the metal ion in the cell. The impact of this competitive binding is reflected in the different levels of toxicity experienced for different pH and calcium conditions.

The interaction between an organism and metal ions present in soil is considered to be the result of competition for that metal ion by all components (including the organism) present in the system. All interactions with the metal ion are considered to take place via the liquid phase. The concept is explained on the basis of a number of model calculations concerning cadmium binding to *Rhodococcus erythropolis* A177 from a clay and a sandy soil, as influenced by pH and calcium concentration, and pH dependent copper binding to maize root cell walls, fungal mycelia, and yeast cells from a sandy soil. In addition, the concept is applied for calculating the copper speciation in an aquatic system.
In soil systems, the free metal ion concentration is (almost) completely determined by the metal ion binding to the soil solid phase. In aquatic systems, biota may play an important role in the distribution of the metal.
Under similar conditions (total cadmium, pH and calcium concentration) the cadmium ion sorption by the bacterium is less in the clay soil than in the sandy soil. In the sandy soil, less cadmium is bound by the bacterium when pH is increasing. In the clay soil, the impact of pH is the reverse, i.e., sorption by the bacterium still increases with increasing pH. The difference between the two soils can be fully explained by the different pH dependency of the cadmium sorption by the soils. In both soils, the cadmium sorption increases with increasing pH, but the pH effect is much stronger for the sandy soil.
Due to competition between cadmium and calcium ions for binding to the reactive sites on the soil solid phase, cadmium is desorbed from the soil when the calcium concentration is increased. The increased cadmium concentration results in an increased sorption by the bacterium, since the impact of the increased calcium

concentration on cadmium sorption is for this bacterium much smaller than for the two soils.

The model calculations show that availability, defined as the amount of metal taken up, differs strongly for different organisms, even if the exposure routes are rather similar. The pH and the composition of the soil solution may have a large impact on the bioavailability of metals. The impact of pH and calcium ion concentration on the cadmium sorption by R.A177 is of the same order as the impact of the different soil types. The examples also show that the effect of the pH is very dependent on the characteristics of the system and the organism, and may be completely reversed from one system to another. E.g., in the sandy soil, copper availability for the fungi was strongly reduced when pH increased, whereas for the yeast an increased pH might lead to slightly enhanced toxic effects.

One should be aware that model calculations far beyond the measurement "window" used for the model calibration, can easily lead to erroneous predictions. Within the measurement window, however, the speciation approach may be a valuable tool in predicting quantitatively the metal sorption to biota present in a complex system, and to predict the relative change in availability due to environmental changes. As for the effects for biota, this approach will enable at least a qualitative statement on the impact of changing environmental conditions.

# Samenvatting

Bodembacteriën spelen een grote rol in de afbraak van organisch materiaal. De uiteindelijke mineralisatie is vrijwel uitsluitend een microbieel proces. De bodemvruchtbaarheid in natuurlijke ecosystemen is volledig afhankelijk van deze processen. De aanwezigheid van zware metalen kan sterk remmend werken op de activiteit van microorganismen, hetgeen indirect dus het hele ecosysteem kan beïnvloeden. Daarnaast kunnen de zware metalen in deze organismen accumuleren, hetgeen tot een grotere blootstelling voor hun predatoren kan leiden.
De beschikbaarheid van zware metalen in de bodem voor bodemorganismen hangt sterk samen met de speciatie van het metaal in de bodem, d.w.z. de verdeling van het metaal over alle fasen en componenten (zowel de a-biotische als de biotische) in de bodem. De mate van binding door biota is een goede maat voor de beschikbaarheid van het metaal in die bodem. De verdeling van het metaal over de diverse bodemcomponenten kan veranderen als milieufactoren (zoals de bodem pH) veranderen. Als wordt aangenomen dat alle interacties met het metaal alleen via de oplossing plaats vinden, kan de invloed van de verandering van dergelijke factoren op de beschikbaarheid van het metaal voor de bodemorganismen voorspeld worden. Dan moet echter wel de invloed van deze factoren op de binding van het metaal voor alle componenten afzonderlijk bekend zijn. Ten aanzien van de binding van zware metalen door abiotische bodemcomponenten als kleimineralen, oxiden en organische stof is al veel bekend. Er is echter nog weinig onderzoek gedaan naar de invloed van factoren als de pH op de binding van metalen door bodemorganismen. Deze invloed moet gekwantificeerd worden om, in combinatie met modellen die de metaal binding aan de andere bodemcomponenten beschrijven, een voorspelling te kunnen geven van de hoeveelheid metaal die door een organisme in de grond gebonden kan worden.
Daarom is de invloed van de pH en de concentratie calcium op de interacties van cadmium en zink met de Gram-positieve bodembacterie *Rhodococcus erythropolis* A177 bestudeerd. Naast een gedetailleerde studie van de adsorptie door geïsoleerde celwanden is de sorptie en opname door intacte cellen bestudeerd. Een relatie wordt gelegd met toxiciteitsgegevens voor dezelfde bacterie.

Celwanden van Gram-positieve bacteriën zijn sterk poreuze structuren, die meer dan 80% water kunnen bevatten. De belangrijkste component van de celwand is peptidoglycaan. Peptidoglycaan is een heel algemeen voorkomende structuur, het is namelijk een component in de celwand van vrijwel alle prokaryotische organismen. Peptidoglycaan bevat een groot aantal zure groepen, die bij toenemende pH deprotoneren, hetgeen leidt tot een pH- en ionsterkte-afhankelijke lading. Om de

reactieve groepen op de celwand te kunnen identificeren en kwantificeren, zijn zuur/base titraties uitgevoerd bij drie zoutsterktes (0.01, 0.1 en 1 M $NaNO_3$). De invloed van de zoutsterkte op het ladingsgedrag kan beschreven worden met behulp van het Donnan model. De belangrijkste aanname die bij dit model wordt gedaan, is dat de lading homogeen over het hele volume van de celwand is verdeeld. Om dit model te kunnen gebruiken, is dus kennis over het celwand volume nodig. Met behulp van de "Mastercurve approach" (vrij vertaald de "basiscurve benadering") kan het Donnan volume worden geschat. Er wordt hiervoor aangenomen dat de titratie curven, die verkregen zijn bij verschillende zoutsterktes, samenvallen als op de juiste wijze de invloed van de zoutsterkte uit de data is geëlimineerd. Binnen een zekere marge kan het volume bij de hoogste zoutsterkte gekozen worden, de volumina bij de lagere zoutsterktes liggen dan vast. De op deze wijze berekende volumina laten zien dat de celwanden zwellen als de zoutsterkte lager wordt dan 0.1M, en dat boven pH 5 de invloed van de lading op de celwand op het celwand volume verwaarloosbaar is. Met behulp van het Donnan model en de berekende volumina kan de electrostatische potentiaal, zoals die bij een bepaalde pH en zoutsterkte aanwezig is, berekend worden. Het blijkt dat deze berekende potentialen goed overeenkomen met gemeten potentialen, zoals die gegeven zijn in de literatuur.
Nadat de data gecorrigeerd zijn voor de invloed van de zoutsterkte kan de intrinsieke proton affiniteit verkregen worden. De resultaten tonen aan dat de celwand chemisch een sterk heterogene samenstelling heeft. Op basis van de proton affiniteit distributie kunnen tenminste drie soorten reactieve groepen worden onderscheiden: carboxyl-achtige groepen met een gemiddelde log $\tilde{K}_H$ van 4.6, amino-achtige groepen met een gemiddelde log $\tilde{K}_H$ van 10.0, en daartussen een kleine groep met een gemiddelde log $\tilde{K}_H$ van 7.8. Deze laatste groep is waarschijnlijk fosfaat, afkomstig van fosfolipiden van het cytoplasmatisch membraan. Door onvolledige zuivering van het celwand materiaal zijn vermoedelijk membraanresten in het materiaal achtergebleven.
Op basis van de affiniteitsdistributie is een proton-bindingsmodel, de Langmuir-Freundlich vergelijking, geselecteerd voor de beschrijving van de titratie data. De combinatie van de drie-modale Langmuir-Freundlich vergelijking met het Donnan model geeft een uitstekende beschrijving van de oorspronkelijke titratie data.

Cadmium en zink binding aan de geïsoleerde celwanden is bestudeerd door metaal ion titraties uit te voeren bij drie pH-waarden (pH 5, 6.5 en 8 voor cadmium; pH 5, 6 en 7 voor zink), in aanwezigheid van 1 mM calcium in de oplossing. Daarnaast zijn titraties uitgevoerd bij hoge pH in een $NaNO_3$ milieu zonder calcium in de oplossing. De binding van cadmium en zink neemt sterk toe bij toenemende pH. Tevens wordt tijdens de titraties een behoorlijke desorptie van calcium ionen en protonen gemeten.

De hoeveelheid protonen en calcium ionen die vrijkomt bij sorptie van cadmium is vrijwel gelijk aan de hoeveelheid cadmium die gebonden wordt. Dit is een duidelijke aanwijzing dat bij sorptie van de metaalionen competitie tussen de diverse ionen voor de bindingsplaatsen op de celwand een grote rol speelt. Bij lage calcium concentratie in de oplossing lijkt de aanwezigheid van calcium de binding van cadmium en zink echter te bevorderen. Zo is bijvoorbeeld de sorptie van cadmium bij pH 8 in aanwezigheid van 1 mM calcium sterker dan in afwezigheid van calcium. Deze waarnemingen kunnen alleen verklaard worden door aan te nemen dat in aanwezigheid van tweewaardige kationen de celwandstructuur verandert.
Op basis van de metaal sorptie data en het eerder beschreven ladingsgedrag is de netto lading van de celwand berekend voor alle bestudeerde condities. Het blijkt dat, bij een hoge bezetting van de reactieve groepen van de celwand met tweewaardige kationen, omlading van de celwand plaats vindt. Dit betekent dat cadmium en zink ionen nog kunnen binden, ondanks de aanwezigheid van een repulsieve potentiaal in de celwand. Dit is een heel duidelijke aanwijzing voor het chemische karakter van de metaal binding aan dit type materiaal. Voor de beschrijving van de sorptie data is het NICA model (NICA is het acroniem voor niet-ideaal competitief adsorptie model) geselecteerd. Aangenomen is, dat de aminogroepen niet bijdragen aan de binding van cadmium en zink, omdat deze groepen pas gaan deprotoneren bij pH-waarden groter dan 9, en dus, voor de condities gehanteerd tijdens de sorptie experimenten, positief geladen zijn. Binding van cadmium, calcium en zink vindt dan alleen plaats aan de carboxyl en fosfaatgroepen.
Daar de structuur van de celwand bij een lage concentratie tweewaardige ionen anders is dan bij een hoge concentratie, is voor beide condities een aparte parameterset afgeleid. Het verkregen model beschrijft niet alleen de metaalbinding zelf; ook de desorptie van protonen en calcium als gevolg van de metaalsorptie wordt redelijk beschreven. Het model is gebruikt om de invloed van een overmaat zink (de concentratie zink is hierbij ongeveer honderd keer zo hoog als de cadmium concentratie in oplossing) op de binding van cadmium te voorspellen. Bij lage cadmium concentraties wordt de invloed onderschat, maar bij een cadmium concentratie van 1 µM wordt een redelijk goede schatting van de reductie van de cadmium sorptie verkregen.

Een volgende stap is de sorptie van cadmium en zink door intacte, levende cellen. Hierbij kunnen complicaties optreden als gevolg van metabolische processen. Door de verademing van endogene reserves kan de $CO_2$ concentratie in de suspensie sterk toenemen, en onder relatief extreme condities voor de bacterie (bijvoorbeeld hoge metaalconcentraties of hoge pH), kan lysis optreden, waarbij eiwitten vrij komen in de

oplossing. Uit de voorbereidende experimenten bleek dat deze problemen met name optreden bij hogere pH. Om de speciatie in oplossing zo eenvoudig mogelijk te houden, is er voor gekozen de sorptie experimenten met intacte cellen alleen uit te voeren bij pH 5 en 6.5. Op deze manier wordt voorkomen dat complexatie van cadmium en zink door vrijkomende eiwitten en verzuring door dissociatie van $H_2CO_3$ een grote rol kunnen gaan spelen. Tijdens de sorptie van metaal ionen door intacte cellen kunnen twee stadia onderscheiden worden. Tijdens het eerste stadium wordt in een korte periode van enkele minuten een relatief grote hoeveelheid metaal gebonden. In het algemeen wordt aangenomen dat het hier om adsorptie aan de celwand gaat. Gedurende het tweede stadium, dat uren tot dagen kan voortduren, neemt de hoeveelheid gesorbeerd metaal nog langzaam toe. Deze verdere toename wordt toegeschreven aan opname in de cel. Om onderscheid te kunnen maken tussen celwand sorptie en opname door de intacte cellen, zijn de sorptie proeven in batch uitgevoerd, waarbij bemonsterd is na twee blootstellingstijden: 30 minuten en twee uur. De sorptie experimenten zijn uitgevoerd bij pH 5 en 6.5, en zowel met 1 mM calcium in oplossing als zonder extra calcium in de oplossing. De metaal concentraties zijn dusdanig gekozen dat ze overeenkomen met de concentratie range waarin een effect voor deze bacterie is waargenomen. Aan de hand van deze data is zowel de adsorptie aan de celwanden van de intacte cellen als de snelheid van de opname in de cel berekend.

Het blijkt dat de invloed van de pH en de concentratie calcium op de sorptie en de opname van cadmium en zink door intacte cellen goed te vergelijken is met de invloed hiervan op de sorptie door geïsoleerde celwanden. Zowel de sorptie als de opname snelheid nemen toe met toenemende pH, en nemen af met toenemende calcium concentratie. De opname in de cel bedraagt gemiddeld ongeveer 10% van de totale hoeveelheid gesorbeerde metaal ionen. Net zoals bij de geïsoleerde celwanden, is de sorptie van cadmium bij verder gelijke omstandigheden iets hoger dan van zink. Tijdens het eerste stadium van de metaalsorptie aan de intacte cellen is desorptie van calcium gemeten.

Het fosfaat, dat aanwezig is in de geïsoleerde celwanden, wordt beschouwd als een artefact als gevolg van een onvolledige zuivering van het celwandmateriaal. Het is waarschijnlijk, dat de celwanden van intacte cellen geen fosfaat bevatten. Daarom wordt aangenomen dat de fosfaatgroepen niet betrokken zijn bij de metaalbinding aan de celwand van intacte cellen. Als gevolg van deze aanname reduceert de bimodale NICA vergelijking die gebruikt is voor de beschrijving van de metaalsorptie door geïsoleerde celwanden tot een monomodale NICA, waarin alleen de carboxylgroepen nog metaalionen kunnen binden. De sorptie na 30 minuten blootstelling bij pH 5 kan

goed voorspeld worden met behulp van deze NICA vergelijking. De sorptie bij pH 6.5 wordt door dit model echter systematisch onderschat.
De verwachting is, dat een hogere proton of calcium concentratie in de oplossing tot een reductie van de opnamesnelheid leidt. Met uitzondering van de metingen bij hoge zink concentratie is dit echter niet eenduidig waargenomen. Vermoedelijk is de opzet van de experimenten niet voldoende geschikt om deze verschillen waar te nemen.

De invloed van de pH op de toxiciteit van cadmium en zink voor *Rhodococcus erythropolis* A177 is bepaald door het meten van de acetaat verademing als functie van de concentratie metaal en de pH in oplossing. De ademhaling wordt bij hogere pH al bij een lagere metaalconcentratie geremd dan bij lagere pH, hetgeen aangeeft dat de bacterie een hogere toxiciteit ervaart bij hogere pH. Daarnaast bleek voor de bacterie zink iets toxischer te zijn dan cadmium. De invloed van de calcium concentratie op de toxiciteit van cadmium en zink is nog niet bepaald voor deze bacterie.
Het is niet onlogisch te veronderstellen dat er een duidelijke relatie is tussen de effecten en de hoeveelheid metaal die gesorbeerd of opgenomen is. Daarom is voor de condities waarbij de acetaat verademing met 50% gereduceerd was de sorptie aan de celwand, de totale sorptie en de opname snelheid geschat. De beste correlatie werd gevonden voor de sorptie aan geïsoleerde celwanden. Dit kan opgevat worden als een indicatie dat de totale hoeveelheid gesorbeerd metaal een goede indicator is voor potentiële effecten voor dit organisme, omdat het grootste deel van het door intacte cellen gesorbeerde metaal gebonden is in de celwand.

Tot hier hebben alle bestudeerde interacties alleen maar plaats gevonden vanuit de vloeistoffase. In de bodem wordt de uiteindelijke blootstelling niet alleen door eigenschappen van het organisme zelf bepaald, maar ook door sorptie evenwichten met de andere, a-biotische componenten in de bodem. Aangenomen wordt dat alle interacties plaats vinden via de vloeistoffase. De blootstelling wordt dan bepaald door de competitie voor binding van het metaal ion tussen het organisme en de andere bodemcomponenten. De biobeschikbaarheid van zware metalen wordt gedefinieerd als de totale hoeveelheid metaal die gesorbeerd wordt door het organisme. Dit concept is uitgelegd aan de hand van een aantal modelmatige berekeningen. De invloed van de pH en de calcium concentratie op de binding van cadmium door *Rhodococcus erythropolis* A177 in een zand en een klei grond is berekend. Daarnaast is gekeken naar de verschillen die zijn waar te nemen tussen een aantal organismen die via dezelfde zandgrond bij meerdere pH-waarden worden blootgesteld aan koper,

en is het concept gebruikt om voorspellingen te doen t.a.v. de invloed van de pH op de koperspeciatie in een aquatisch systeem.
In een terrestrisch systeem wordt de concentratie vrij metaal in oplossing vrijwel volledig bepaald door het sorptie evenwicht met de vaste fase van de bodem. In aquatische systemen kunnen de biota een grote rol spelen bij de distributie van het metaal in het systeem.
Onder verder vergelijkbare omstandigheden (totaal cadmium, pH en calcium concentratie) wordt door de bacterie minder cadmium gebonden in de kleigrond dan in de zandgrond. Het effect van de pH op de binding van cadmium door de bacterie is heel verschillend voor de twee gronden. In de zandgrond neemt de sorptie door de bacterie toe als de pH in de bodem daalt. In de kleigrond neemt de binding door de bacterie af bij verlaging van de bodem-pH. Dit verschil kan volledig verklaard worden aan de hand van het verschil in pH-afhankelijkheid van de binding van cadmium door de twee gronden. Hoewel voor beide gronden de binding van cadmium toeneemt met toenemende pH, is dit effect voor de kleigrond veel kleiner. Een verlaging van de bodem-pH zal dus in het geval van de zandgrond tot een veel sterkere toename van de concentratie cadmium in de bodemoplossing zorgen dan in het geval van de kleigrond. Daar de binding door de bacterie zowel afhangt van de concentratie cadmium als van de pH, is het goed mogelijk dat in het geval van de kleigrond het effect van de lagere pH sterker is dan het effect van de toegenomen concentratie cadmium, met als uiteindelijk resultaat een lagere hoeveelheid gebonden cadmium.
Als gevolg van competitie tussen calcium en cadmium voor binding aan de reactieve bindingsplaatsen op de vaste fase van de bodem, wordt cadmium gedesorbeerd als de calcium concentratie in de oplossing toeneemt. Daar de invloed van de calcium concentratie op de cadmium binding door de bacterie relatief gering is, leidt de toegenomen cadmium concentratie in oplossing voor beide gronden tot een hogere cadmium sorptie door de bacterie.
De modelberekeningen ten aanzien van de koper beschikbaarheid voor verschillende organismen laten duidelijk zien, dat de biobeschikbaarheid van zware metalen verschilt voor verschillende organismen, zelfs als de blootstellingsroutes vrijwel identiek zijn. De pH en de samenstelling van de bodemoplossing kunnen een grote invloed hebben op de biobeschikbaarheid van zware metalen. Zo is de invloed van de pH en de concentratie calcium op de cadmium sorptie door R.A177 van dezelfde grootte als de invloed van de verschillende bodemsoorten. De voorbeelden laten ook zien, dat het uiteindelijke effect van de pH sterk afhangt van de eigenschappen van het systeem en van het organisme, en dat het effect in het ene systeem compleet omgekeerd kan zijn vergeleken met een ander systeem. Zo is berekend dat in de zandgrond de beschikbaarheid van koper voor een schimmel sterk wordt gereduceerd bij een

stijgende bodem-pH, terwijl onder die omstandigheden de beschikbaarheid voor een gist zelfs heel licht toeneemt.
Een kleine kanttekening bij deze modelberekeningen is op zijn plaats. Modelberekeningen, ver buiten de range aan experimentele condities waarop het model geijkt is, kunnen al snel tot onjuiste voorspellingen leiden. Binnen die range echter, is dit concept een waardevol stuk gereedschap bij de kwantitatieve voorspelling van de metaalsorptie door organismen die aanwezig zijn in een complex systeem als de bodem. Tevens is het hiermee mogelijk veranderingen in de biobeschikbaarheid als gevolg van bijvoorbeeld een verlaging van de bodem-pH te berekenen. Ten aanzien van de effecten voor de biota, zal deze benadering in ieder geval een kwalitatieve uitspraak over de te verwachten effecten van dergelijke veranderingen mogelijk maken.

# Levensloop

Alexandra Christine Caroline Plette is geboren in Oss op 28 september 1966. In haar tiende levensjaar verhuisde zij, met haar ouders en twee broers, naar Zwolle. Daar behaalde zij in 1984 het VWO-diploma aan de Thorbecke Scholengemeenschap.
Van 1984 tot 1991 heeft zij in Wageningen bodemkunde gestudeerd (specialisatie bodemscheikunde). In het studiejaar 1987/1988 heeft zij een bestuursfunctie vervuld bij de dat jaar lustrerende studenten roeivereniging, de W.S.R. Argo. Daarna is, tijdens het afstudeervak bodemscheikunde onder begeleiding van Tjisse Hiemstra, aan twee onderwerpen gewerkt: het ladingsgedrag van kaoliniet en de adsorptie van aluminium en citraat aan goethiet. Daarnaast heeft zij een afstudeervak microbiologie uitgevoerd; begeleid door Huub Rijnaarts is onderzoek gedaan naar de afbraak van alcoholen door gehechte bacteriën. Haar, toch al latent aanwezige, francofilie is nog eens fors aangescherpt tijdens de vijf maanden stage bij het Station de Science du Sol van het INRA (Institute de Recherche Agronomique) in Avignon. Onder begeleiding van Mme A.M. de Cockborne zijn veldmetingen uitgevoerd naar de uitspoeling van nitraat in een perzik boomgaard onder traditionele irrigatie.
Van februari 1991 tot juli 1995 werkte zij als Assistent in Opleiding bij de vakgroep Bodemkunde en Plantevoeding van de Landbouwuniversiteit Wageningen in het kader van het Speerpuntprogramma Bodemonderzoek, voor een project in de cluster "Speciatie en biologische beschikbaarheid van anorganische stoffen". Een groot deel van die tijd is zij gedetacheerd geweest bij de afdeling Ecotoxicologie van het Instituut voor Bos- en Natuuronderzoek (IBN-DLO) in Arnhem (het voormalige RIN). Tussentijds is zij twee maanden aangesteld in het kader van een "Topping-up" project van het Speerpuntprogramma Bodemonderzoek.
De belangrijkste resultaten van het promotieonderzoek en het tussentijdse project zijn weergegeven in dit proefschrift.