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Nickel removal from nickel plating waste water using a biologically active moving-bed sand filter

Thomas Pümpel[1], Lynne E. Macaskie[2], John A. Finlay[2], Ludo Diels[3] & Marios Tsezos[4]
[1]*Institut für Mikrobiologie, Universität Innsbruck, Technikerstrasse 25, A-6020 Innsbruck, Austria;* [2]*School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK;* [3]*Vlaamse Instelling voor Technologisch Onderzoek (VITO), Boeretang 200, B-2400 Mol, Belgium;* [4]*National Technical University of Athens, Heroon Polytechniou 9, GR-15780 Zografou, Greece*

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Abstract

Efficient removal of dissolved nickel was observed in a biologically active moving-bed 'MERESAFIN' sand filter treating rinsing water from an electroless nickel plating plant. Although nickel is fully soluble in this waste water, its passage through the sand filter promoted rapid removal of approximately 1 mg Ni/l. The speciation of Ni in the waste water was modelled; the most probable precipitates forming under the conditions in the filter were predicted using *PHREEQC*. Analyses of the Ni-containing biosludge using chemical, electron microscopical and X-ray spectroscopic techniques confirmed crystallisation of nickel phosphate as arupite ($Ni_3(PO_4)_2.8H_2O$), together with hydroxyapatite within the bacterial biofilm on the filter sand grains. Biosorption contributed less than 1% of the overall sequestered nickel. Metabolising bacteria are essential for the process; the definitive role of specific components of the mixed population is undefined but the increase in pH promoted by metabolic activity of some microbial components is likely to promote nickel desolubilisation by others.

Introduction

Microbial nickel uptake and bioaccumulation

Nickel is an essential trace element which serves as a co-factor for several enzymes such as those involved in the metabolism of molecular hydrogen, urea and methane. The state-of-the-art of bacterial transport systems for the controlled uptake of nickel was recently reviewed by Eitinger & Mandrand-Berthelot (2000). A nickel resistant, hyper-accumulating strain of the fungus *Neurospora crassa* was described by Kumar *et al.* (1992), but there is no information about its application in bioremediation technology. Hyper-accumulating plants are more common and are of major interest for biomining applications (Robinson *et al.* 1997; Anderson *et al.* 1999) and soil bioremediation, e.g., *Alyssum lesbiacum* accumulates more than 30 mg Ni/g dry weight when grown on contaminated soil (Kramer *et al.* 1996). Recently, Ni accumulation by an *Escherichia coli* strain was augmented four-fold by inserting the *nixA* gene (coding for the Ni transport system HpNixA; Eitinger & Mandrand-Berthelot 2000) from *Helicobacter pylori* (Krishnaswamy & Wilson 2000). However, genetically modified microorganisms will probably be unacceptable in an open waste water treatment system and attention is focused on the use of naturally occurring microbial strains for nickel removal.

Physico-chemical nickel biosorption

The biosorptive capacity for nickel has frequently been shown to be low in contrast to other transition metals, e.g., Cu^{2+}, Pb^{2+} or Ag^{+}, in accordance with the Irving-Williams series, an empirically determined series of stability constants of organo-metal complexes (Irving & Williams 1953). The biosorptive capacities of several microorganisms and chemical properties

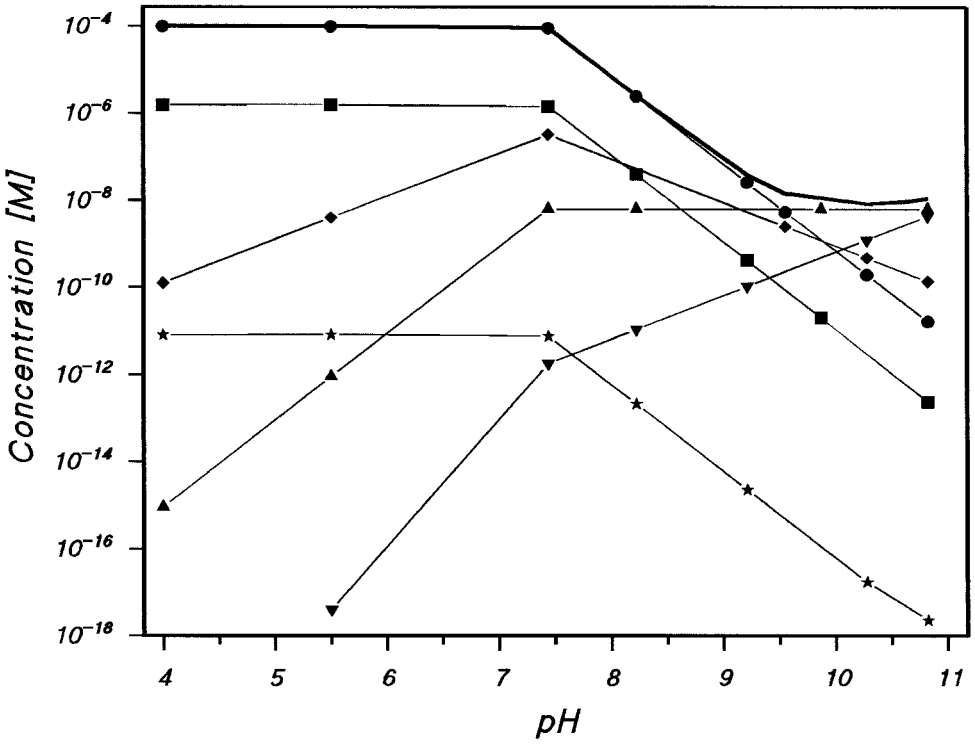

Fig. 1. Major soluble Ni-species of 0.1 mM $NiSO_4$ added to pure water at 25 °C; pH adjustment with H_2SO_4/NaOH calculated with *PHREEQC*. Total soluble Ni (bold line), Ni^{2+} (●), $NiOH^+$ (♦), $Ni(OH)_2$ (▲), $Ni(OH)_3^-$ (▼), $NiSO_4$ (■), $Ni(SO_4)_2^{2-}$ (★).

of nickel in that context have been summarised and discussed by Tsezos *et al.* (1995).

Without obvious differences between the major groups of microorganisms, the biosorptive capacities of algae, bacteria and fungi range between 5 and 50 mg Ni/g dry matter, corresponding to high equilibrium concentrations of 100 mg/l and above, and neutral to slightly acidic pH values (Lu *et al.* 1998; Sag & Kutsal, 1995, 1997, 1999; Lau *et al.* 1999; Ceribasi & Yetis 2001; Dönmez & Aksu 2001; Klimmek & Stan 2001). In the concentration range of practical interest for bioremediation studies (typically only a few mg/l) nickel biosorption only reaches 0.5 to 10 mg/g (Galun & Galun 1988; Traxler & Wood 1990; Cabral 1992; Ramelow *et al.* 1992; Wong & Pak 1992; Holan & Volesky 1994; Asthana *et al.* 1995; Tsezos *et al.* 1995; Ivanitsa *et al.* 1999). However, some of the higher published values were obtained at pH values >7.5 (e.g., Wnorowski 1991; Wong & Fung 1997) and must be disregarded in this context, as modelling of nickel speciation suggests the formation of nickel hydroxides (Figure 1) and hence the capacity of biomass to trap precipitates rather than sorb nickel ions is measured. In the low mg/l concentration range the Ni^{2+} cation is the dominant species up to pH 7.5 (Figure 1; Baes & Mesmer 1976). Precipitation starts to occur at 2–3 pH units below the pK_a of the aqua complex; the pK_a value of Ni^{2+} is 9.9 (Hughes & Poole 1991).

A pH value of 6 and above has been reported as optimal for Ni biosorption by bacteria. Below pH 7 the competition for binding sites by protons becomes evident, and biosorption decreases markedly with decreasing pH (Figure 2). For algae and fungi lower pH optima are reported, pointing to bonds with higher affinity (Kambe-Honjoh *et al.* 1997; Klimmek & Stan 2001), and reflecting different cell surface compositions between microbial groups.

The data summarised above were obtained under laboratory conditions, either in distilled water or in 'non-complexing' buffers like PIPES or HEPES. In real solutions with more complex ionic matrices competition by other cations plays an important role, especially due to the low affinity of nickel for biomass. Lead (25 mg/l), for example, reduced Ni biosorption of *Rhizopus arrhizus* by 50% (Sag & Kutsal 1997), calcium, chromium(III) and iron(II) affected Ni biosorption by *Aspergillus niger* (Natarajan *et al.* 1999), and sodium, present in high concentrations

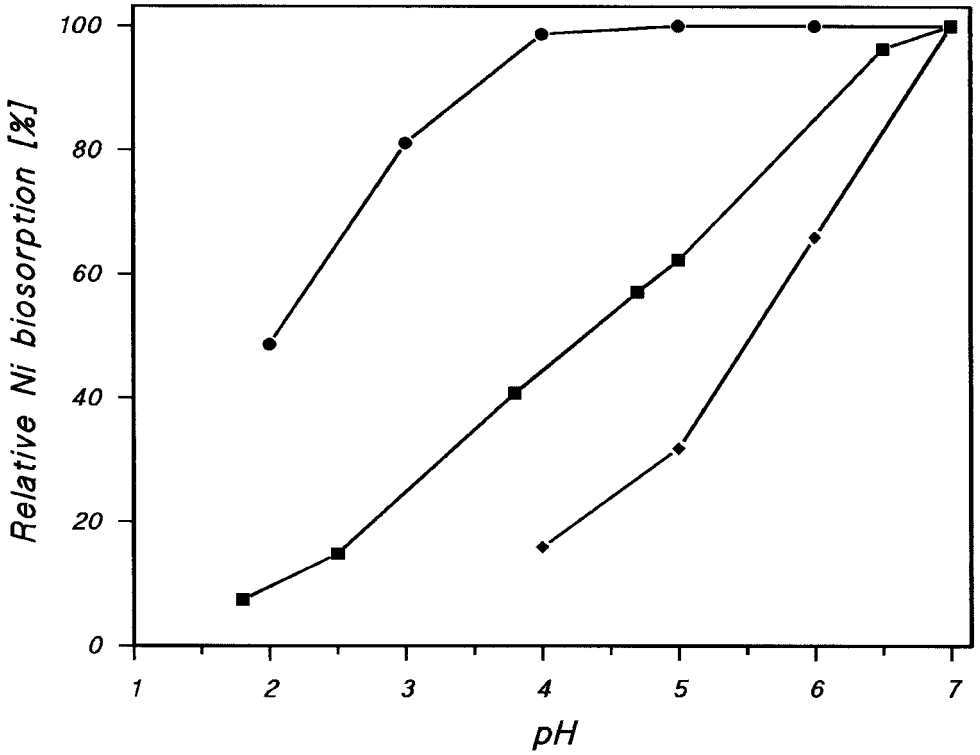

Fig. 2. Normalised pH-dependence of Ni biosorption by bacteria (♦, Lu *et al.* 1998), yeast (●, Kambe-Honjoh *et al.* 1997) and algae (■, Ramelow *et al.* 1992).

in a Ni-electroplating water, completely inhibited Ni biosorption by cyanobacteria (Corder & Reeves 1994). Hence, biosorption of nickel alone could not become an efficient treatment technology for actual waste waters with complex ionic matrices and low pollutant concentration levels. In contrast, other processes leading to a much higher loading of biomass (e.g., bioprecipitation) make use of biosorbed nickel on nucleation sites which facilitate overall nickel accumulation. In this respect biosorption would become important as an initial step in a successful bioremediation process for nickel sequestration.

Nickel bioprecipitation and biocrystallisation processes

Nickel can be bioprecipitated onto cell surfaces with a number of biogenic precipitant ligands, as described in the literature for other metals, for example Ni^{2+} could be removed as its phosphate precipitate since metal phosphates are highly insoluble. Accordingly, heavy metals were removed as their phosphates via the activity of cell-bound phosphatase (PhoN) of *Citrobacter* sp. N14 or an *E. coli* strain containing a homologous *phoN* gene (Basnakova *et al.* 1998b). However, Ni^{2+} was not removed by this technique (Bonthrone *et al.* 1996).

In contrast to metal phosphates, the formation of metal sulphides is the most prominent mechanism for biologically mediated heavy metal precipitation. Many heavy metals and some metalloids form sulphides with very high stability constants, for example 10^{-18}, $10^{-19..-26}$ and 10^{-53}, for FeS, NiS and HgS, respectively (Morel 1983). The reaction is widely used in conventional waste water treatment, when complex forming substances like short-chain organic acids, NTA or EDTA, hinder the usual neutralisation precipitation with lime or caustic soda. However sulphide itself (applied as H_2S or Na_2S) is harmful, underlying statutory emission regulations. For effective precipitation it must be added in excess, and residual sulphide must then be removed. Furthermore, most metal sulphides form only small particles or colloids with poor settling characteristics, usually necessitating a polishing filter. Synthetic organosulphides have recently become available, offering easier dosage, better settling sludge, and easy removal of excess. However,

they are expensive and can be used economically only under special conditions (Mühlbacher 1994).

Sulphide can also be made available by sulphate-reducing bacteria (SRB) which transfer electrons anoxically to oxidised inorganic sulphur species as terminal electron acceptors; H_2S (and HS^-, respectively, depending on pH; $pK_a = 6.99$) is the final product of this dissimilatory sulphate reduction. Organic acids (e.g., lactate, butyrate, acetate, propionate, formate), alcohols and molecular hydrogen are typical electron donors (Schlegel 1992).

This biological route offers several advantages compared to conventional chemical precipitation. Several pilot and full-scale applications using sulphidic bioprecipitation are now in operation for the treatment of mining water, industrial waste water and groundwater (White *et al.* 1997; Saunders 1998; Pümpel & Paknikar 2001). Although it is possible to grow SRB in medium in the absence of agents to poise the E_h, production of excess H_2S is required for this purpose (Postgate 1979). Since excess H_2S production is problematic (above) an aerobic route may be preferable, particularly in oxic wastewaters.

In the presence of sodium carbonate, the formation of mixed precipitates of nickel carbonate and hydroxide ($xNiCO_3.yNi(OH)_2.zH_2O$) can be observed in the moderately alkaline pH range. The higher the pH, the higher the proportion of the hydroxide (Mühlbacher, 1994). This was exploited using a BICMER (**B**acteria **I**mmobilised **C**omposite **ME**mbrane **R**eactor) reactor (Peys *et al.* 1997) for the removal of Ni from wastewater. Here, the Ni-resistant bacterium *Ralstonia metallidurans* CH34 (Mergeay *et al.* 1985; Siddiqui *et al.* 1989; Diels *et al.* 1995a) and *Alcaligenes xylosoxidans* 31A (Schmidt *et al.* 1991) were employed in the BICMER system, comprising a composite membrane (polysulfone and zirconium oxide) supporting a bacterial biofilm challenged under a tangential flow (Diels *et al.* 1995b, c). Nutrients were provided from the other side of the membrane and diffused to the biofilm. Ni was removed by precipitation of nickel carbonates by both microorganisms. In the case of CH34 the Ni-precipitation could only be induced by the addition of Cd or Zn ions at the nutrient side of the membrane but the Ni precipitation process was self-induced in the case of *A. xylosoxidans*. Both bacteria harbour a large plasmid, encoding for Ni-resistance, by the *cnr*- or *ncc*-operon, respectively (Nies 1992; Tibazarwa *et al.* 2000). These operons encode a chemiosmotic Ni-hydrogen efflux system (antiport), which resulted in high Ni concentrations exterior to the outer cell membrane with a subsequent metal precipitation at local Ni-binding sites (various functional groups) on extracellular polysaccharides. The initial Ni-binding was used as a nucleation site for the further crystallisation of, in this case, Ni-carbonates. Ni concentrations could be reduced from 14.8 mg Ni/l to below 1 mg Ni/l.

The above suggests that nickel bioprecipitation is promoted by the presence of nucleation sites. This concept was illustrated previously, where removal of thorium as its phosphate was promoted by the predeposition of nucleation foci of a dissimilar metal phosphate, in this case $LaPO_4$, giving a co-crystal of lanthanum and thorium phosphate (Yong & Macaskie 1998). A co-crystal was also obtained with Ni^{2+} in the presence of uranyl ion, (Bonthrone *et al.* 1996) which was subsequently attributed to intercalation of Ni^{2+} within the crystalline lattice of cell surface-bound $HUO_2PO_4.4H_2O$ (Basnakova & Macaskie 1997; Basnakova *et al.* 1998a), in accordance with the known intercalative ion exchange properties of this 'host' crystal (Clearfield 1988). A bioinorganic ion-exchanger was developed using $HUO_2PO_4.4H_2O$ pre-deposited on the biomass, which functioned in repeated deposition and washing cycles for removal and recovery of Ni using desorbents such as citrate (Basnakova & Macaskie 1997), which requires a secondary treatment step to degrade the citrate to release Ni^{2+} (Thomas *et al.* 2000), or seawater, yielding the soluble nickel-chloride complex (Basnakova & Macaskie 2001). However this approach is limited by the range of host crystals that can be used as ion-exchangers for Ni^{2+}; the use of uranium is unattractive due to its long-lived radioactivity. Zirconium (IV) phosphate has similar ion-exchange properties (Clearfield 1988) but attempts to intercalate Ni^{2+} into biologically-manufactured $Zr(HPO_4)_2$ were unsuccessful; only amorphous zirconium phosphate was found on the cells, with no interpretable X-ray powder diffraction pattern (Basnakova & Macaskie 1999) and not the required crystalline material (Clearfield 1988). Attempts to use biogenic Ti(IV) or Sn(IV) phosphates as hosts for Ni^{2+} were, similarly, unsuccessful (Basnakova & Macaskie 1999) and, given the problems inherent in the use of U(VI) (above), this approach is unattractive for a routine Ni-removing bioprocess.

The MERESAFIN concept

The MERESAFIN process (**ME**tal **RE**moval by **SA**nd **F**ilter **IN**oculation; Diels *et al.* 1998; MERESAFIN

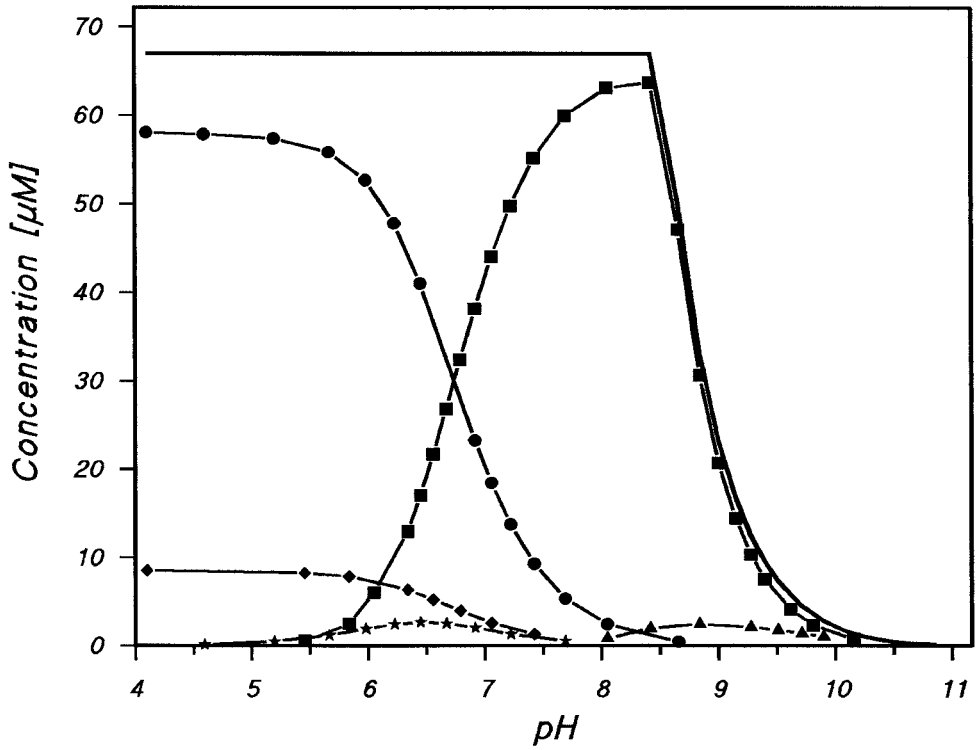

Fig. 3. Major soluble species of Ni in a typical filter feed water sample. Calculated with *PHREEQC* using analytical data presented in Table 1, with theoretical addition of H_2SO_4 and NaOH, respectively. Total soluble Ni (bold line), Ni^{2+} (●), $NiCO_3$ (■), $NiHCO_3^+$ (★), $Ni(CO_3)_2^{2-}$ (▲), $NiSO_4$ (♦).

1999) was designed to combine the optimal conditions for more than one of the above processes of metal immobilisation in a single-stage treatment system for industrial waste water. The approach uses a continuously operated moving-bed Astrasand® filter (Assen 1995) which is inoculated with a mixed population of selected metal biosorbing, bioprecipitating and biodegrading bacteria (Diels *et al.* 1999; Pernfuß *et al.* 1999; Pümpel *et al.* 2001). Active biofilms form on the sand grains by continuous supply of nutrients. Parts of the biofilm, including the bound metals, are detached from the sand grains by attrition in the internal airlift and in the sand washer of the filter and are continuously removed from the device. Base layers of biofilms remain on the sand grains, and biofilms are replenished in the next cycle (Diels *et al.* 1999).

One of four pilot plants based on this concept was commissioned at a metal plating company in Vienna, Austria, to treat waste water from an electroless nickel plating line. In the course of optimisation of the metal removal process the metabolic activity of bacteria in the filter was increased by increasing the dosage of carbon source and electron acceptors during several months of operation (Pümpel *et al.* 2001). A correlation between carbon consumption and nickel removal was found ($r = 0.75$).

The purpose of this study was to elucidate the role of the microorganisms and the mechanisms responsible for Ni removal in the MERESAFIN process, allowing for better process steering and optimisation. The metal accumulative properties of the filter microorganisms as well as the speciation of the feed water nickel and the form of the nickel on the biomass were investigated.

Materials and methods

Biofilm

Samples of biofilm-laden sand grains were collected from a pilot-scale MERESAFIN moving-bed sand filter plant treating waste water from an electroless nickel plating line (details of operation and data on metal removal were published by Pernfuß *et al.* (1999) and Pümpel *et al.* (2001)). Sand grains covered with

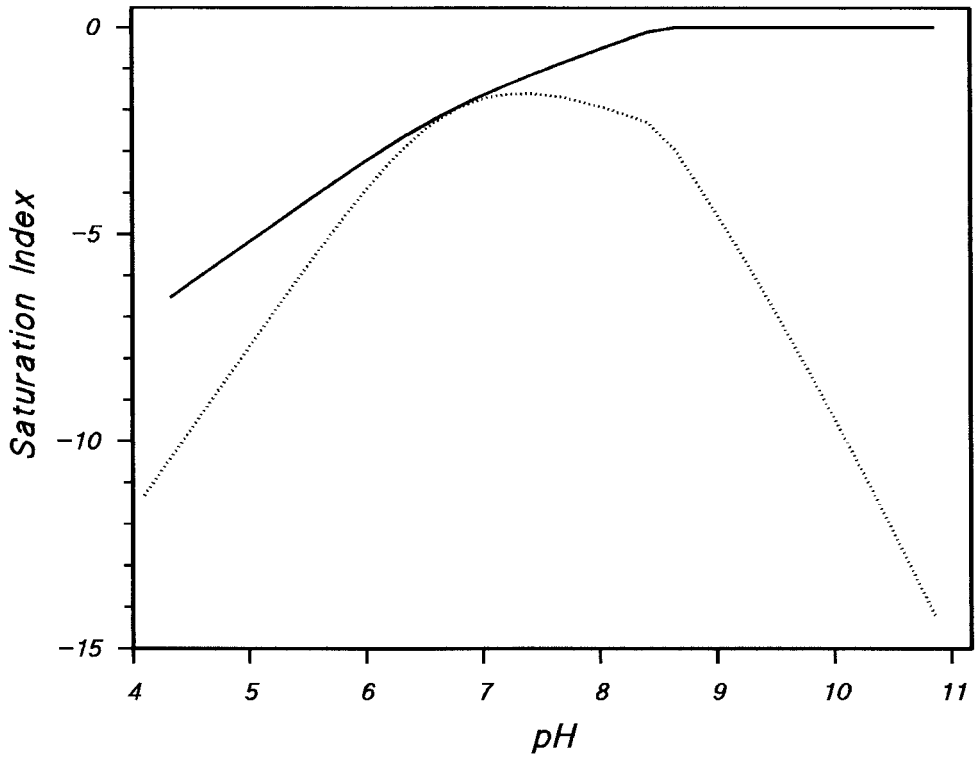

Fig. 4. Saturation indices of nickel hydroxide (solid line) and nickel phosphate (dashed line) in the filter feed water. Calculated with *PHREEQC* using analytical data presented in Table 1, with theoretical addition of H_2SO_4 and NaOH, respectively.

biofilm and associated precipitated metals were periodically taken from the top of the filter bed and analysed using scanning and transmission electron microscopy (SEM, TEM) with energy dispersive X-ray microanalysis (EDAX). Excess biomass was continuously sloughed off from the sand grains during normal operation in the filter, collected in a lamella separator and thickened in bag filters. This material (biosludge) contained a metal burden (see later) accumulated during the sand filter operation.

Water analyses

Total element concentrations in feed water, extracts and digests were analysed by ICP-Atomic Emission Spectrometry (Perkin-Elmer Plasma 400): Ca, Fe, Mg, P, Ni and Zn, Flame-Atomic Absorption Spectrometry (Perkin-Elmer 2100): Ni in biosorption experiments, and by Flame- Atomic Emission Spectrometry (Perkin-Elmer 2380): K and Na.

Concentrations of organic acids originally present in the waste water or added as nutrient were determined by HPLC: Aminex HPX-87H column (Biorad), 0.5 ml/min 2 mM H_2SO_4, 40 °C, UV-detection at 213 nm.

Biofilm and biosludge analyses

Sand grains were periodically removed from the filter and stored in the waste water (see Table 1) at 4 °C. For analysis samples were washed gently in distilled water and examined using scanning electron microscopy as described by Finlay *et al.* (1999). For examination of metal uptake biofilm was dislodged by vortex-mixing, air dried on formvar-coated titanium grids and carbon coated prior to examination by transmission electron microscopy using EDAX as described previously (Basnakova *et al.* 1998a; Finlay *et al.* 1999).

Nickel biosorption by biosludge

Biosludge was dried at 40 °C for 24 h and pulverised. In order to estimate the biosorption capacity of the biosludge and exclude any interference from previously-bioprecipitated metals it was necessary to equilibrate the biosludge to a fixed pH prior to the

Table 1. Typical analysis of the filter feed water.

Parameter	Conc. [mg/l]	Parameter	Conc. [mg/l]	Parameter	Value
Ca	34	O_2	6.1	pH	7.5
Na	18	Carbonate-C	14	E_h	+400 mV
K	9.1	Sulphate-S	8	T	25 °C
Mg	7.9	Phosphate-P	14.5		
Ni	3.9[a]	Ammonium-N	5.3		
Fe	0.08	Lactate	25.6		
Zn	0.02	Acetate	25		

[a]For modelling purposes the equivalent value of 66 μM was used (Mol. wt. = 58.7).

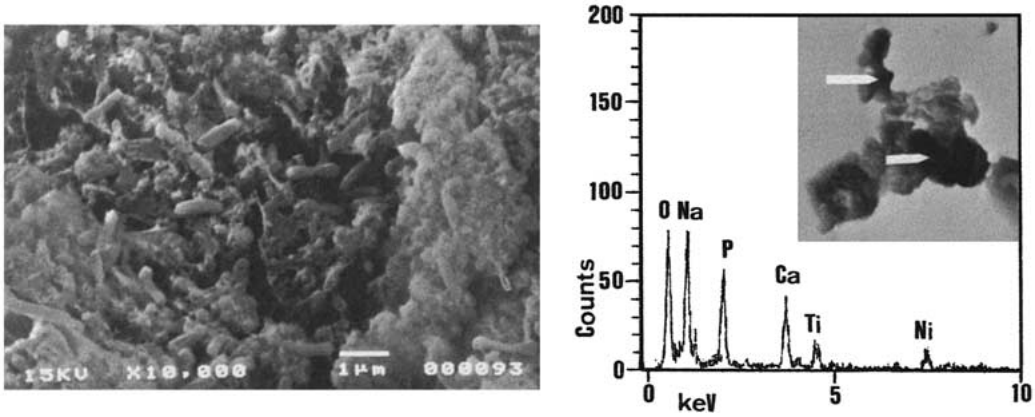

Fig. 5. SEM image of biofilm on a sand grain from the pilot filter (left), and TEM close-up with EDAX-scan of the arrowed precipitate (right).

contact experiments: A slurry of 35 g dry material in 500 ml of water was stirred at 250 rpm. The pH was maintained at a value of 3.5 by periodic addition of HNO_3 as the pH of the slurry drifted to alkaline, reaching equilibrium after ~24 h. The solid phase was collected by centrifugation (30 min at 5000 *g*) and dried as before.

Biosorption equilibrium isotherms were determined by contacting Ni-containing waste water (Table 1), spiked with nickel nitrate ($Ni(NO_3)_2.6H_2O$) up to 100 mg Ni/l, with 0.5 g of dry biosludge. The contact volume was 100 ml in stoppered Erlenmeyer flasks of 250 ml, agitated on an orbital shaker at 250 rpm at a constant temperature of 25 °C for 24 h, and the initial pH was adjusted to 3.5 by dropwise addition of 0.1 M HNO_3. (Nickel, in the range of the concentrations used in the biosorption experiments, is present in solution predominantly in the soluble ionic form Ni^{2+} at the chosen pH value; Figure 1).

Following the 24 h of contact the solutions were separated from the biomass by centrifugation as above and filtered through 0.45 μm preweighed Millipore membrane filters in a glass vacuum filtration apparatus. Approximately 20–25 ml of each solution were first filtered and discarded to bring the filter to adsorption equilibrium with the solution. The subsequent filtrate was then collected for pH and nickel (C_e in equation 1) analyses. The membrane filters were dried and weighed, and the difference of weight before and after filtration was used as the dry weight basis (M in equation 1) for calculation of sorption/desorption equilibrium uptake capacities.

Due to the high nickel pre-loading of the biosludge a fraction of the metal was leached into solution (C_{BG} in equation 2) and analysed before spiking with additional nickel (C_S in equation 2). The biosorption equilibrium uptake capacity for each sample was calculated according to the following mass balance on the metal ion expressed by equations 1 and 2:

$$q = V(C_0 - C_e)/M \quad (1)$$

$$C_0 = C_{BG} + C_S, \quad (2)$$

where q is the biosorption equilibrium metal uptake capacity in [mg/g], V is the sample volume in [l], C_0 is the initial metal ion concentration in [mg/l], C_e is

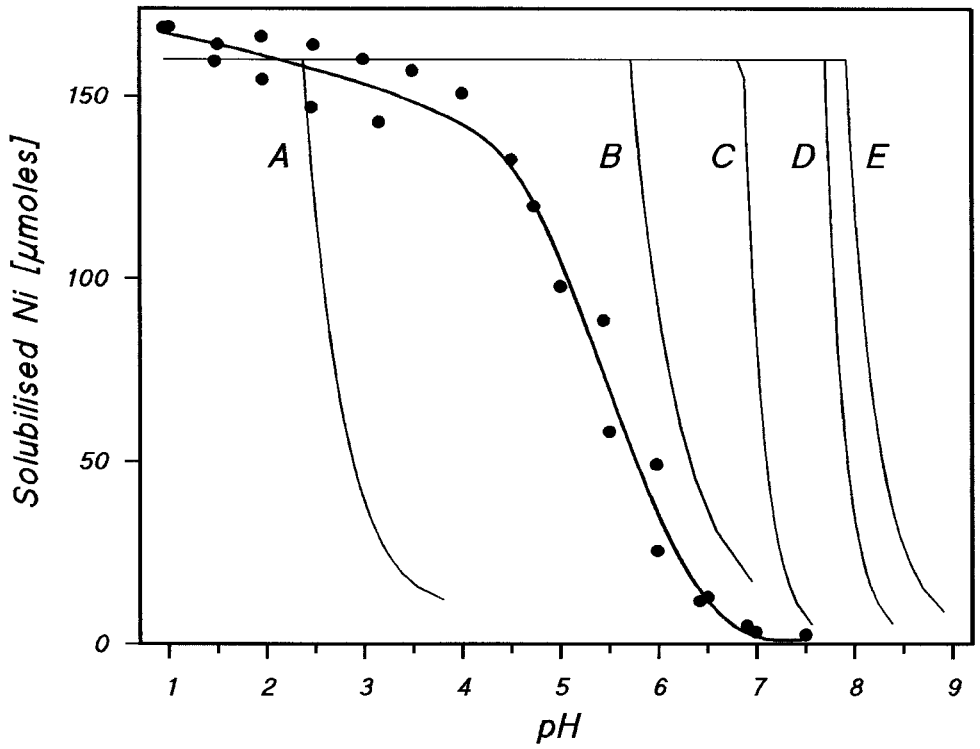

Fig. 6. Leaching of nickel from biofilm with HCl (2 independent experiments: ● with fitted line), and data calculated with *PHREEQC* for NiS (A), $Ni_3(PO_4)_2$ (B), $Ni(OH)_2$ (C), NiO (D), and $Ni_4(OH)_6SO_4$ (E).

the equilibrium metal ion concentration in [mg/l], M is the dry weight of the biomass in [g], C_{BG} is the leached background metal ion concentration in [mg/l], C_S is the spiked metal ion concentration in [mg/l].

Glassware and other materials were carefully cleaned in order to avoid sample contamination according to the following protocol: Rinsed with tap water, soaked (24 h) in a 2% detergent solution (Decon Prolabo), rinsed with tap water, soaked (3 d) in 0.1 M HNO_3 solution, and finally rinsed with deionized water. Mean relative errors of metals analysis were less than 10%.

Extraction of nickel and other elements from biosludge

Biofilm-loaded filter sand (10 g wet weight) and KCl (100 mg for adjustment of ionic strength) were added to 100 ml distilled water in an Erlenmeyer flask (250 ml). The pH of the slowly stirred suspension was controlled by the automatic addition of 100 mM HCl (Metrohm titrator). The pH setpoint was decreased by 0.5 pH units every 24 h to pH 1. Samples (5 ml) were filtered through 0.2 μm syringe membrane filters and stabilised with 1 drop of 65% HNO_3 for metals analysis. The efficiency of extraction was related to a wet digest: 3 aliquots of 1 g of the same fresh sand, and sand after the extraction, respectively, were boiled in 5 ml 65% HNO_3 to near dryness in volumetric flasks of 50 ml, and made up to volume with 1% HNO_3. In extracts and digests, the concentrations of Ca, Fe, K, Mg, Na, P, Ni and Zn were analysed as described above.

X-ray powder diffraction analysis (XRD) of precipitates

Precipitates were extracted from the biofilm due to interference by biomass on the XRD signal. In all extraction steps the original slightly alkaline pH and ambient temperatures was retained in order to avoid any chemical alteration of the Ni-phase. 30 g of filter sand carrying biofilms were shaken by tilting in a neutral detergent solution (1% RBS Neutral Konzentrat, Carl Roth GmbH) for 24 h. The dense precipitates were then separated from biomass by centrifugation through a 40% sucrose solution (1000 *g*, 10 min). The pellet free of biomass by microscopic exami-

nation, was dried at room temperature and analysed with a Bruker-AXS D-8 Powder X-ray diffractometer (Cu-target 40 V, 40 mA, scintillation-counter stepsize 0.01°, counting time 10 s).

Speciation modelling

The computer code *PHREEQC* (MS-DOS version 1.6, U.S. Geological Survey) with database *Minteq* and additional thermodynamic constants of Ni-complexes (Morel 1983; Shuttleworth & Unz 1993) was used to model (i) Ni-speciation in pure water and filter feed water, (ii) influence of microbial metabolism on Ni-speciation, and (iii) acid-resolubilisation of Ni from appropriate solid Ni-phases for evaluation of the experimental data of Ni-extraction with HCl.

Results

Characterisation of the filter feed water

The sand filter was fed with rinsing water from an electroless nickel plating line containing nickel sulphate, phosphates, and organic acids at a neutral pH. A typical analysis is shown in Table 1. It was found by experiment that chemical precipitation of nickel hydroxide with caustic soda started at pH 9, which is in agreement with the model predictions for loss of soluble Ni species presented in Figure 3.

Speciation modelling of the feed water with *PHREEQC* confirmed the pattern of removal of soluble nickel and also suggested that at pH 7 to 8 most of the soluble nickel should be present as Ni^{2+} and $NiCO_3$ (Figure 3), with carbonate being the key determinant of nickel speciation. Neither the organic acids present (for control of nickel speciation in the concentrated, more acidic plating bath) nor ammonium ion markedly affect the fate of soluble nickel. Amongst the solid nickel phases only nickel hydroxide ($Ni(OH)_2$) and nickel phosphate ($Ni_3(PO_4)_2$) were taken into account by *PHREEQC*. Nickel phosphate reaches the maximal concentration in the pH range from 7 to 8, and only exceeds the saturation level (saturation index = 0), if inorganic carbon is below 0.3 mM. $Ni(OH)_2$ never touches the saturation level under the conditions and the pH range regarded (Figure 4). From this theoretical approach, which is based on analytical data of the feed water, nickel phosphate and nickel hydroxide are the thermodynamically favoured nickel phases, but these should not precipitate spontaneously without alteration of the water composition. Indeed, preliminary experiments using uninoculated sandfilters showed no retention of Ni by the sandfilter itself (unpublished work).

Biofilm and biosludge analysis

The sand filter was inoculated with a mixed population of five bacterial strains (*Pseudomonas mendocina* AS302, *Arthrobacter* sp. BP7/26, *R. metallidurans* CH34, *P. fluorescens* K1/8a, *Methylobacillus* sp. MB127, all with potent biosorptive and bioprecipitative mechanisms of metals removal (Pümpel *et al.* 2001). After eight months of operation *R. metallidurans* CH34 was a major component of the biofilm, none of the other added strains could be identified with certainty (Pernfuß *et al.* 1999) and the proportion of unculturable organisms introduced from the waste water could not be determined. Examination of the metal-loaded sand grains by scanning electron microscopy showed clear colonisation by microbial biofilm (Figure 5, left panel). Using TEM electron opaque areas were visible (Figure 5, right panel; arrowed in inset). Analysis by EDAX (Figure 5, right panel) confirmed the presence of nickel in the deposits, apparently co-precipitating with Na, Ca, and P. Quantitative analysis was not done at this stage (see later) but the data indicate a deposit comprising a mixture of $Ca(NaPO_4)_2$, and $Ni(NaPO_4)_2$ and/or $Ni_3(PO_4)_2$.

Biosorption of Ni by biosludge

In biosorption experiments with the nickel loaded filter biosludge (composition in Table 3) at pH 3.5 a portion of the biofilm-bound nickel was leached from the solid to the liquid phase (leaching pattern according to Figure 6) before spiking the solution with additional nickel, giving equilibrium concentrations of 76 to 92 mg/l Ni (C_{BG} in Table 2). Therefore, biosorptive capacities could not be analysed for nickel equilibrium concentrations below 76 mg/l. Although the pH drifted from 3.5 to 5.7–5.8 during the biosorption experiments due to the high buffering capacity of the material, only biosorptive nickel binding could account for the observed nickel depletion of the solution; according to model predictions for the respective water matrix (Figures 3 & 4) and precipitation experiments, nickel precipitation only occurs above pH 7. The amount of the metal sorbed per unit weight of dry material and unit weight of biomass, respectively, was calculated (Table 2). (Biomass is assumed to equal the volatile fraction of 350–400 mg/g dry material, determined as loss on ignition; Table 3.)

Table 2. Biosorption results from equilibrated biosludge.

C_S	C_{BG}	C_0	C_e	pH		Biosorption capacity	
[mg Ni/l]	[mg Ni/l]	[mg Ni/l]	[mg Ni/l]	Start	End	[mg Ni/g dry material]	[mg Ni/ g biomass]
0	76	76	72	3.5	5.7	0.8	2.0
5	79	84	76	3.5	5.7	1.5	4.0
10	80	90	77	3.5	5.7	2.5	6.6
20	84	104	81	3.5	5.7	4.6	12.0
30	86	116	90	3.5	5.8	5.1	13.4
50	88	138	107	3.5	5.8	6.1	16.0
100	92	192	152	3.5	5.7	7.9	20.7

(C_S spiked concentration; C_{BG} leached background; C_0 initial concentration; C_e equilibrium concentration)

Table 3. Major components of continuously produced biosludge (proposed typical composition).

Component	Content in dry material [mg/g]	Proposed compounds	Methods
Biomass	350–400	bacteria and exopolymers	loss on ignition at 800–900 °C
Ca	100–200	hydroxyapatite; gypsum	total: acid digestion – ICP species: X-ray diffraction, *PHREEQC*
Si	70–80	quartz (from carrier)	total: acid digestion – ICP species: X-ray diffraction
Ni	50–60	arupite	total: acid digestion – ICP *localisation*: EDAX *species*: X-ray diffraction
Fe	20–40	FeOOH, Fe_2O_3, Fe_3O_4	total: acid digestion – ICP *species*: *PHREEQC*
Al, As, Co, Cu, Mg, Zn	10–20	Not investigated	total: acid digestion - ICP

Due to of the complexity of the biosludge, which contained a high content of extant nickel precipitates and other inorganic material, the results, although not quantitative, provide an indication of the contribution of biosorption to the overall nickel removal process. Since the sand filter operates continuously each cycle will include newly-divided cells available for a new biosorption and precipitation cycle. The biosludge was generated in the sand filter challenged with an equilibrium concentration of 2–3 mg Ni/l waste water, with a respective nickel loading of 50–60 mg Ni/g total solids (Table 3) and 130–160 mg Ni/g biomass (calculated with the volatile fraction as above). Under the same conditions as the ones prevailing in the filter, biosorption alone would contribute only much less than 1 mg Ni/g biomass at the appropriate low equilibrium concentration of 2–3 mg Ni/l (Table 2), which is less than 1% of the overall sequestered nickel. From the biosludge analysis (above) a bioprecipitation mechanism was implicated, which formed the focus of subsequent tests.

Chemical analysis and modelling

The biosludge had a relatively high content of inorganic material, ranging from 60 to 65% of the dry material. Nickel, the target heavy metal of the removal process, reached a 6% content at the end of the pilot operation. Other main elements of the sludge were silicon (from small quartz particles chipped from the carrier sand material), phosphorus, iron and calcium (Table 3).

From preliminary biosorption experiments conducted using the bacteria selected for filter inoculation, in addition to biosludge from the filter (see above), and also from literature data (Tsezos *et al.* 1995), it is clear that processes other than biosorption contributed to the observed high nickel content of the biofilm. EDAX analysis of sections of biofilm bacteria failed to detect intracellularly bioaccumulated nickel but showed the metal, together with phosphorus, calcium and sodium in extracellular particles (Figure 5), pointing to an extracellular biomineralisation process.

Acid extraction was used to estimate the stability of the Ni-bonds involved and to narrow the spectrum of possible solid phases by comparison of stability constants and calculated dissolution behaviours. The initial pH stabilised at pH 8 in the extraction experiment with biofilm bearing sand from the filter (c.f. the original value of the filter effluent was 7.5; Table 1). No nickel dissolved at this pH during the first 24 h, whereas the solubilisation of Ni occurred mainly in the range of pH 6.5 to 4, under addition of HCl (Figure 6), with total leaching at pH 1. Nickel extractions from several possible nickel solid phases were modelled in parallel using *PHREEQC* and compared to the experimental data (Figure 6). In the concentration range of interest, $Ni(OH)_2$, $Ni_4(OH)_6SO_4$ and NiO (bunsenite) are fully soluble at pH 7 and could therefore be excluded. The dissolution pattern of $Ni_3(PO_4)_2$ between pH 7–5.5 partially overlaps with the experimental data and could explain approximately 50% of the Ni-extraction. NiS (millerite) dissolves between pH 4–2.5; the small increase in the experimental curve in this pH range could be attributed to a minor content of millerite in the sample. However, no sulphur was detected by examination with EDAX (Figure 5) and the oxic conditions of the sand filter and washer would have precluded growth of sulphate-reducing bacteria. Since no other Ni-phase included in the *PHREEQC* database has stability constants between those of NiS and $Ni_3(PO_4)_2$, a major part of the extraction curve still requires explanation; this was not attributable to desorption of biosorbed material since biosorption comprised less than 1% of the biosludge-bound Ni (above).

Further analysis using X-ray powder diffraction (XRD) of isolated particles (Figure 7) confirmed that Ni was present as crystalline $Ni_3(PO_4)_2.8H_2O$ (arupite) which was converted to NiO by heat treatment of the precipitate at 200 °C, showing that Ni is present as a separate phase. For modelling purposes (Figure 6) the thermodynamic data of only amorphous $Ni_3(PO_4)_2$ were available; taking into consideration the usually higher stability of crystalline phases, it is likely that the arupite phase was more stable to acid dissolution. In addition to the diagnostic peak of arupite (Figure 7, arrowed), the XRD spectrum (Figure 7) was completely characteristic of poorly crystallised hydroxyapatite, in accordance with the X-ray emission energies characteristic of Ca and P (Figure 5) and was identical to the spectrum of biogenic hydroxyapatite obtained using metal phosphate precipitating *Serratia* sp. (P. Yong and L.E. Macaskie, unpublished; the *Serratia* was previous classified as a *Citrobacter* sp: Pattanapipitpaisal *et al.* 2002).

Discussion

Although nickel is fully soluble in the plating waste water, its passage through the sand filter promoted removal of ~1 mg Ni/l within the few minutes retention time. Increases in microbial metabolic activity gave correspondingly enhanced nickel removal from solution ($r = 0.75$; Pümpel *et al.* 2001), suggesting a microbially-assisted process promoted by *R. metallidurans* CH34 (the predominant organism), together with the naturally-developed population, which probably included a proportion of non-culturable organisms, the specific contributions of which cannot be quantified.

Nickel is stable in the natural, slightly alkaline pH range (7.5 to 8) of the dilute waste water. The modelling software *PHREEQC* suggested nickel cation – Ni^{2+} and nickel carbonate – $NiCO_3$ as the major soluble species (Figure 3). The organic acids present in the water apparently had no impact on nickel speciation here, although they are needed to stabilise the thousand-fold higher metal concentration in the more acidic plating bath. Experimental and theoretical investigations showed nickel precipitation to start near pH 9 (Figure 3), with $Ni(OH)_2$ predominant at high pH (Figure 4). According to the calculations, only one additional solid phase, nickel phosphate – $Ni_3(PO_4)_2$, should be considered in the relevant pH and concentration range. Calculated with *PHREEQC*, but not shown here in detail, the saturation index of this nickel phosphate is very sensitive to changes in carbonate concentration (itself pH-dependent), but nickel phosphate *per se* would be soluble in the actual water matrix (Figure 4); it is likely that additional carbonate is being contributed via bacterial metabolism (see below).

As expected from earlier investigations with the bacteria used for filter inoculation, in a simple water matrix (Tsezos *et al.* 1995), and using the respective waste water (Pümpel *et al.* 2001), the biosorption of nickel ions to functional groups of biomass was shown to contribute <1% to the mass of sequestered nickel. Nevertheless, by the formation of nucleation sites for subsequent crystallisation biosorption may play a paramount role in the overall removal process, as previously shown with the precipitation of other metal phosphates by immobilised *Citrobacter* sp. (Bon-

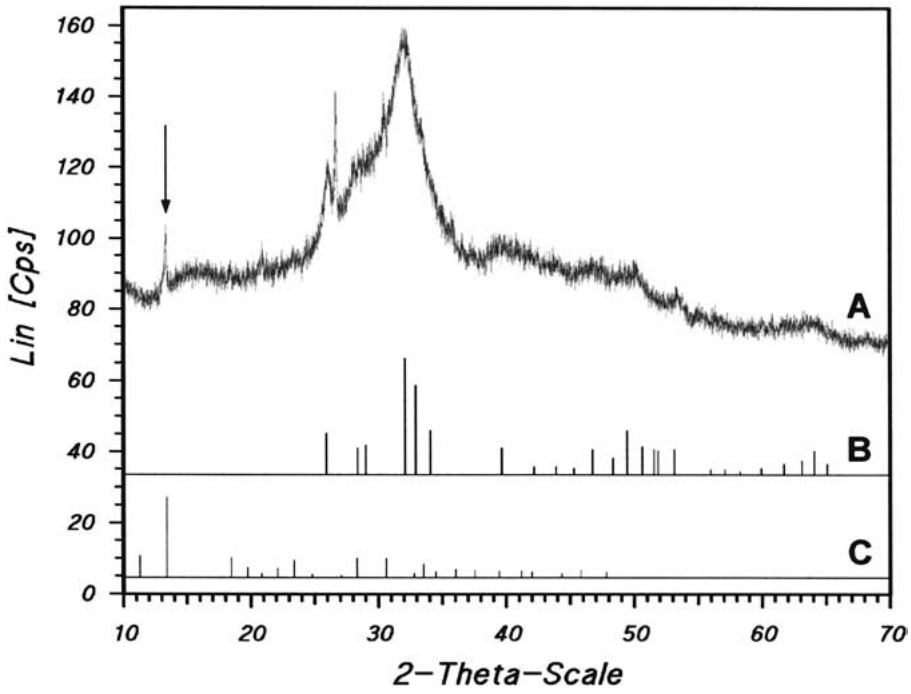

Fig. 7. X-ray powder diffractogram of precipitates isolated from biofilm from the pilot sand filter (A), and reference spectra of hydroxyapatite (B) and arupite (C). Diagnostic arupite peak arrowed.

throne *et al.* 2000; Macaskie *et al.* 2000). Here, it was shown using ^{31}P NMR, that nucleation onto phosphate groups of bacterial lipopolysaccharide preceded more sustained metal phosphate biomineralisation. In the present case transmission electron microscopy showed electron opaque particles within the microbial biofilm, but outside the cells (Figure 5), containing Ni, Ca, P and Na, pointing to possible co-precipitation or crystallisation processes. With the EDAX data no stoichiometric calculation was done, but $Ca(NaPO_4)_2$, $Ni(NaPO_4)_2$, $Ni_3(PO_4)_2$ or mixtures thereof are likely; in the previous case substantial Na was also found and the formation of a mixed metal/sodium phosphate concluded (Bonthrone *et al.* 2000; Macaskie *et al.* 2000). The comparison of experimental and theoretical dissolution studies of the precipitates with acid (Figure 6) also pointed to nickel phosphate, and X-ray powder diffraction analysis (XRD; Figure 7) confirmed the presence of crystalline arupite, $Ni_3(PO_4)_2.8H_2O$. It is likely that the precipitate comprised a mixture of $Ni(NaPO_4)_2$ and $Ni_3(PO_4)_2$. A clear XRD spectrum was obtained also for (poorly crystallised) hydroxyapatite – $Ca_{10}(PO_4)_6(OH)_2$ (Figure 7). It is likely that the calcium mineral was, similarly, a mixture of hydroxyapatite and $Ca(NaPO_4)_2$; the latter would probably be an amorphous solid, not detectable by XRD.

From the above it is concluded that phosphorus is a key element in microbial nickel immobilisation. Fuhrmann and Rothstein (1968) reported a 5-20-fold increase in nickel uptake by bakers' yeast, which was pre-treated with phosphate. Sar *et al.* (2001), using EDAX and XRD, suggested that nickel, deposited in the membrane and periplasm of *P. aeruginosa* cells, was in the form of crystalline nickel phosphides – Ni_5P_4, NiP_2, $Ni_{12}P_5$ and nickel carbide – Ni_3C. Itoh *et al.* (1998) found intracellular particles containing mainly Fe, Cr, Ni and P in *Acidiphilium rubrum*, and concluded the presence of a Fe–Cr–Ni alloy from XRD spectra. Klimmek & Stan (2001) increased the biosorption capacity of algae four-fold by phosphorylation of the biomass, suggesting the participation of cell surface phosphate groups in metal binding, as was also found using ^{31}P NMR (Bonthrone *et al.* 2000; Macaskie *et al.* 2000). However, precipitation of nickel phosphate in the current study contradicts the negative results for Ni bioprecipitation reported previously for the metal phosphate accumulating *Citrobacter* sp. (Bonthrone *et al.* 1996). Other experiments (Pattanapipitpaisal *et al.* 2002) have shown that this strain is unable to bioprecipitate Cr^{3+} as $CrPO_4$ under conditions where $LaPO_4$ was precipitated extensively. However, Cr^{3+} was co-deposited with phos-

phorus by a strain of *Bacillus pumilis* suggesting that differences in cell surface nucleation sites between Gram positive and Gram negative bacteria may be contributory. *R. metallidurans* is Gram negative but the mixed sand filter population also contained members of the Gram positive *Nocardiaceae* (Pernfuß *et al.* 1999); it is possible that Ni biomineralisation requires contributions from these organisms (biosorption and nucleation foci) and also *R. metallidurans* (see below). The precipitation-supporting surface characteristics of bacteria (and also exopolymers, which form a major part of the organic material in biofilms) are only one aspect; alteration of the chemical matrix by microbial metabolism may also be necessary. An alkaline pH drift is often associated with microbially mediated metals removal. The sand filter bacteria shifted the pH to 9 or above in batch growth within 2 days, using the organic acids present in the waste water as carbon and energy sources (Ebner 2001); in the filter bulk fluid the pH never increased to >8.2, due to the low water retention time of 12 to 30 min.

The biofilms on the sand grains are thin because of the regular cleaning passage through the airlift (by SEM: Figure 5). The development of a steep pH gradient above the biofilm is therefore unlikely; furthermore, pH values above pH 8.5 would favour the formation of nickel hydroxide rather than of phosphate (Figure 4).

The modelling study unmasked carbonate as a very strong regulator of nickel speciation in the waste water matrix. About 1 mM of carbonate is sufficient to shift nickel precipitation from pH 7.5 (Figure 1) to near 9 (Figure 3). The biofilm bacteria grow on organic acids using oxygen and then nitrate as electron acceptors and thereby add further carbon from their metabolism to the inorganic carbon (CO_2) pool. Carbonate precipitation could be a key factor controlling the inorganic carbon speciation. Reliable analytical data was not available from within the biofilm micro-environment; the EDAX microprobe technique cannot measure carbon reliably, while the use of proton induced X-ray emission analysis (PIXE: see Bonthrone *et al.* 2000 for references), which has a greater sensitivity and can also measure the light elements, has insufficient resolution to probe at the sub-micron level. However in this case nickel removal was strictly correlated with microbial substrate turnover ($r = 0.75$; Pümpel *et al.* 2001), and nickel biosorption by the continuously produced biomass was negligible.

One of the inoculated bacteria, *R. metallidurans* CH34 (former name *Alcaligenes eutrophus* CH34), recovered in high numbers (50% of the culturable organisms) from the sand filter, carries a well understood mechanism of metal resistance, which leads to the bioprecipitation of metal carbonates via local alkalinisation of the medium (Diels *et al.* 1995b). In the BICMER reactor system, with immobilised bacteria (Diels *et al.* 2000), CH34 alone was able to bioprecipitate nickel carbonate after induction of the resistance mechanism with cadmium or zinc (Diels *et al.* 1995b); a contribution of this mechanism to nickel removal in the MERESAFIN sand filter is therefore very likely, especially since traces of zinc are present in the waste water. In the present mixed culture system it is likely that this strain is responsible for the observed increase in pH which would also promote metal phosphate deposition following nucleation onto the appropriate members of the consortium.

Conclusions

Nickel-phosphorus interactions have been shown to contribute the largest portion of microbially mediated nickel sequestration. The speciation model predicted the preferred formation of nickel phosphate, which was confirmed by XRD and which was localised in extracellular deposits with TEM and EDAX. Metabolising bacteria are required for the process, the definitive role of individual bacteria is not clear but nickel removal is correlated with the substrate consumption. The following processes contribute:

- Biosorption of nickel ions to functional groups at cell envelopes and exopolymers, forming nucleation foci;
- Entrapment of micro-precipitates and colloids in the gel-like biofilm, forming crystallisation templates;
- Metal resistance mechanism of *R. metallidurans* CH34, creating high local carbonate concentrations due to the chemiosmotic efflux system (metal-proton antiport: Nies & Silver 1989) of metal ions and ensuing high exocellular pH which may also promote arupite deposition onto extant precipitation foci.

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References

Anderson CWN, Brooks RR, Chiarucci A *et al.* 1999 Phytomining for nickel, thallium and gold. *J Geochem Explor* **67**, 407–415.

Assen H. 1995 Filter device. Patent EP 730895B1.

Asthana RK, Chatterjee S, Singh SP. 1995 Investigations on nickel biosorption and its remobilization. *Process Biochem* **30**, 729–734.

Baes CF, Mesmer RE. 1976 The hydrolysis of cations. New York: Wiley.

Basnakova G, Macaskie LE. 1997 Microbially enhanced chemisorption of nickel into biologically synthesized hydrogen uranyl phosphate: A novel system for the removal and recovery of metals from aqueous solutions. *Biotechnol Bioeng* **54**, 319–328.

Basnakova G, Macaskie LE. 1999 Accumulation of zirconium and nickel by Citrobacter sp. *J Chem Technol Biotechnol* **74**, 509–514.

Basnakova G, and Macaskie LE. 2001 Microbially-enhanced chemisorption of Ni^{2+} ions into biologically-synthesised hydrogen uranyl phosphate (HUP) and selective recovery of concentrated Ni^{2+} using citrate or chloride ion. *Biotechnol Lett* **23**, 67–70.

Basnakova G, Spencer AJ, Palsgard E, Grime GW, Macaskie LE. 1998a Identification of the nickel uranyl phosphate deposits on Citrobacter cells by electron microscopy with electron probe X-ray microanalysis and by proton-induced X-ray emission analysis. *Environ Sci Technol* **32**, 760–765.

Basnakova G, Stephens ER, Thaller MC, Rossolini GM, Macaskie LE. 1998b The use of Escherichia coli bearing a phoN gene for the removal of uranium and nickel from aqueous flows. *Appl Microbiol Biot* **50**, 266–272.

Bonthrone KM, Basnakova G, Lin F, Macaskie LE. 1996 Bioaccumulation of nickel by intercalation into polycrystalline hydrogen uranyl phosphate deposited via an enzymatic mechanism. *Nature Biotechnol* **14**, 635–638.

Bonthrone KM, Quarmby J, Hewitt CJ *et al.* 2000 The effect of the growth medium on the composition and metal binding behaviour of the extracellular polymeric material of a metal-accumulating Citrobacter sp. *Environ Technol* **21**, 123–134.

Cabral JPS. 1992 Selective binding of metal ions by Pseudomonas syringae cells. *Microbios* **71**, 47–53.

Ceribasi IH, Yetis U. 2001 Biosorption of Ni(II) and Pb(II) by Phanerochaete chrysosporium from a binary metal system – Kinetics. *Water SA* **27**, 15–20.

Clearfield A. 1988 Role of ion exchange in solid-state chemistry. *Chem Rev* **88**, 125–148.

Corder SL, Reeves M. 1994 Biosorption of nickel in complex aqueous waste streams by Cyanobacteria. *Appl Biochem Biotech* **45–46**, 847–859.

Diels L, Dong Q, van der Lelie D, Baeyens W, Mergeay M. 1995a The czc operon of Alcaligenes eutrophus CH34: from resistance mechanisms to the removal of heavy metals. *J Ind Microbiol Biotechnol* **14**, 142–153.

Diels L, Van Roy S, Doyen W, Mergeay M, Leysen R. 1995b The use of bacteria immobilized in tubular membrane reactors for heavy metal recovery. In: Jerez CA, Vargas T, Toledo H, and Wiertz JV, eds. *Biohydrometallurgical processing. Vol. II.* Proceedings of the International Biohydrometallurgy Symposium IBS-95, Nov. 1995, Chile. Santiago: University of Chile; 201–209.

Diels L, Van Roy S, Somers K *et al.* 1995c The use of bacteria immobilised in tubular membrane reactors for heavy metal recovery and degradation of chlorinated aromatics. *J Membrane Sci* **100**, 249–258.

Diels L, Van Roy S, Spaans PH, Wouters H, Kramer A. (1998) Method and plant for purification of metal containing water. Patent EP 0952120

Diels L, Spaans PH, Van Roy S *et al.* 1999 Heavy metals removal by sand filters inoculated with metal sorbing and precipitating bacteria. In: Amils R and Ballester A, eds. *Biohydrometallurgy and the Environment toward the Mining of the 21st Century – Part B.* Amsterdam: Elsevier; 607–616.

Diels L, Leysen R, van Roy S, Doyen W, Mergeay M. 2000 Membranes with immobilized microorganisms thereon and therein, process for obtaining such membranes, reactor comprising said membranes and process involving the use of said membranes, in particular for the elimination of metals or the degradation of xenobiotic organic compounds. Patent EP 579630B1.

Dönmez G, Aksu Z. 2001 Bioaccumulation of copper(II) and nickel(II) by the non-adapted and adapted growing Candida sp. *Water Res* **35**, 1425–1434.

Ebner C. 2001 Schwermetallentfernung aus Industrieabwässern mit einem mikrobiologisch aktiven, kontinuierlich betriebenen Sandfilter. Thesis, University of Innsbruck.

Eitinger T, Mandrand-Berthelot MA. 2000 Nickel transport systems in microorganisms. *Arch Microbiol* **173**, 1–9.

Finlay JA, Allan VJM, Conner A, Callow ME, Basnakova G, Macaskie LE. 1999 Phosphate release and heavy metal accumulation by biofilm-immobilized and chemically-coupled cells of a Citrobacter sp. pregrown in continuous culture. *Biotechnol Bioeng* **63**, 87–97.

Fuhrmann GF, Rothstein A. 1968 The transport of Zn, Co and Ni into yeast cells. *Biochim Biophys Acta* **163**, 325–330.

Galun M, Galun E. 1988 Removal of contaminants. Patent US4732681.

Holan ZR, Volesky B. 1994 Biosorption of lead and nickel by biomass of marine algae. *Biotechnol Bioeng* **43**, 1001–1009.

Hughes MN, Poole RK. 1991 Metal speciation and microbial growth – the hard (and soft) facts. *J Gen Microbiol* **137**, 725–734.

Irving H, Williams RJP. 1953 The stability of transition-metal complexes. *J Chem Soc* 3192–3210.

Itoh S, Iwaki M, Wakao N, Yoshizu K, Aoki A, Tazaki K. 1998 Accumulation of Fe, Cr and Ni metals inside cells of acidophilic bacterium *Acidiphilium rubrum* that produces bacteriochlorophyll a. *Plant Cell Physiol* **39**, 740–744.

Ivanitsa VO, Vasilyeva TV, Buchtiyarov AE, Lindström EB, McEldowney S. 1999 Interactions between marine bacteria and heavy metals. In: Amils R and Ballester A, eds. *Biohydrometallurgy and the Environment toward the Mining of the 21st Century – Part B.* Amsterdam: Elsevier; 317–325.

Kambe-Honjoh H, Sugawara A, Yoda K, Kitamoto K, Yamasaki M. 1997 Isolation and characterization of nickel-accumulating yeasts. *Appl Microbiol Biot* **48**, 373–378.

Klimmek S, Stan HJ. 2001 Comparative analysis of the biosorption of cadmium, lead, nickel, and zinc by algae. *Environ Sci Technol* **35**, 4283–4288.

Kramer U, Cotterhowells JD, Charnock JM, Baker AJM, Smith JAC. 1996 Free histidine as a metal chelator in plants that accumulate nickel. *Nature* **379**, 635–638.

Krishnaswamy R, Wilson DB. 2000 Construction and characterization of an *Escherichia coli* strain genetically engineered for Ni(II) bioaccumulation. *Appl Environ Microb* **66**, 5383–5386.

Kumar SC, Sastry SK, Mohan MP. 1992 Use of wild type and nickel resistant *Neurospora crassa* for removal of Ni^{2+} from aqueous medium. *Biotechnol Lett* **14**, 1099–1102.

Lau PS, Lee HY, Tsang CCK, Tam NFY, Wong YS. 1999 Effect of metal interference, pH and temperature on Cu and Ni biosorption by *Chlorella vulgaris* and *Chlorella miniata*. *Environ Technol* **20**, 953–961.

Lu YJ, Chua H, Wong PK. 1998 Changes in cell surface dielectric constant in biosorption of nickel ion (Ni^{2+}) by *Enterobacter* sp. 4-2. *Enzyme Microbial Technol* **23**, 403–407.

Macaskie LE, Bonthrone KM, Yong P, Goddard D. 2000 Enzymatically-mediated bioprecipitation of uranium by a *Citrobacter* sp.: A concerted role for exocellular lipopolysaccharide and associated phosphatase in biomineral formation. *Microbiology* **146**, 1855–1867.

MERESAFIN (1999) Removal and recovery of heavy metals from waste water by sand filters inoculated with metal biosorbing or bioprecipitating bacteria. European Union Project BE95–1610.

Mergeay M, Nies D, Schlegel HG, Gerits JP, van Gijsegem F. 1985 Alcaligenes eutrophus CH34, a facultative chemolithotroph with plasmid-bound resistance to heavy metals. *J Bacteriol* **162**, 328–334.

Morel FMM. 1983 Principles of aquatic chemistry. Wiley & Sons.

Mühlbacher R. 1994 Abtrennung von Schwermetallen aus Abwässern. Thesis, University of Graz.

Natarajan KA, Subramanian S, Modak JM. 1999 Biosorption of heavy metal ions from aqueous and cyanide solutions using fungal biomass. In: Amils R and Ballester A, eds. *Biohydrometallurgy and the Environment toward the Mining of the 21st Century – Part B.* Amsterdam: Elsevier; 351–361.

Nies D. 1992 CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium czc system of *Alcaligenes eutrophus*. *J Bacteriol* **174**, 8102–8110.

Nies DH, Silver S. 1989 Plasmid-determined inducible efflux is responsible for resistance to cadmium, zinc, and cobalt in *Alcaligenes eutrophus*. *J Bacteriol* **171**, 896–900.

Pattanapipitpaisal P, Mabbett AN, Finlay JA *et al.* 2002 Reduction of Cr(VI) and bioaccumulation of chromium by Gram positive and Gram negative microorganisms not previously exposed to Cr-stress. *Environ Technol*, **23**, 731–745.

Pernfuß B, Ebner C, Pümpel T *et al.* 1999 The behaviour of five metal biosorbing and bioprecipitating bacterial strains, inoculated in a moving-bed sand filter. In: Amils R, Ballester A, eds. *Biohydrometallurgy and the Environment toward the Mining of the 21st Century - Part B.* Amsterdam: Elsevier; 373–382.

Peys K, Diels L, Leysen R, Vandecasteele C. 1997 Development of a membrane biofilm reactor for the degradation of chlorinated aromatics. *Water Sci Technol* **36**, 205–214.

Postgate JR. 1979 The Sulphate-reducing Bacteria, Cambridge: Cambridge University Press.

Pümpel T, Paknikar KM. 2001 Bioremediation technologies for metal-containing waste waters using metabolically active microorganisms. *Adv Appl Microbiol* **48**, 135–169.

Pümpel T, Ebner C, Pernfuß B, *et al.* 2001 Treatment of rinsing water from electroless nickel plating with a biologically active moving-bed sand filter. *Hydrometallurgy* **59**, 383–393.

Ramelow GJ, Fralick D, Zhao Y. 1992 Factors affecting the uptake of aqueous metal ions by dried seaweed biomass. *Microbios* **72**, 81–93.

Robinson BH, Chiarucci A, Brooks RR *et al.* 1997 The nickel hyperaccumulator plant *Alyssum bertolonii* as a potential agent for phytoremediation and phytomining of nickel. *J Geochem Explor* **59**, 75–86.

Sag Y, Kutsal T. 1995 Copper(II) and nickel(II) adsorption by Rhizopus arrhizus in batch stirred reactors in series. *Chem Eng J 58*, 265–273.

Sag Y, Kutsal T. 1997 The simultaneous biosorption process of lead(II) and nickel(II) on *Rhizopus arrhizus*. *Process Biochem* **32**, 591–597.

Sag Y, Kutsal T. 1999 An overview of the studies about heavy metal adsorption process by microorganisms on the lab scale in Turkey. In: Amils R, Ballester A, eds. *Biohydrometallurgy and the Environment toward the Mining of the 21st Century – Part B*. Amsterdam: Elsevier; 307–316.

Sar P, Kazy SK, Singh SP. 2001 Intracellular nickel accumulation by *Pseudomonas aeruginosa* and its chemical nature. *Lett Appl Microbiol* **32**, 257–261.

Saunders JA. 1998 *In situ* bioremediation of contaminated groundwater. Patent US 5833855.

Schlegel HG. 1992 Allgemeine Mikrobiologie. Stuttgart: Thieme.

Schmidt T, Stoppel RD, Schlegel HG. 1991 High-level nickel resistance in *Alcaligenes xylosoxydans* 31A and *Alcaligenes eutrophus* KTO2. *Appl Environ Microb* **57**, 3301–3309.

Shuttleworth KL, Unz RF. 1993 Sorption of heavy metals to the filamentous bacterium *Thiothrix* strain A1. *Appl Environ Microb* **59**, 1274–1282.

Siddiqui RA, Benthin K, Schlegel HG. 1989 Cloning of pMOL28-encoded nickel resistance genes and expression of the genes in *Alcaligenes eutrophus* and *Pseudomonas* spp. *J Bacteriol* **171**, 5071–5078.

Thomas RA, Beswick AJ, Basnakova G, Moller R, Macaskie LE. 2000 Growth of naturally occurring microbial isolates in metal-citrate medium and bioremediation of metal-citrate wastes. *J Chem Technol Biotechnol* **75**, 187–195.

Tibazarwa C, Wuertz S, Mergeay M, Wyns L, van der Lelie N. 2000 Regulation of the cnr cobalt and nickel resistance determinant of *Ralstonia eutropha* (*Alcaligenes eutrophus*) CH34. *J Bacteriol* **182**, 1399–1409.

Traxler RW, Wood EM. 1990 Bioaccumulation of metals by a *Coryneform* SL-1. *J Ind Microbiol Biotechnol* **6**, 249–252.

Tsezos M, Remoudaki E, Angelatou V. 1995 A systematic study on equilibrium and kinetics of biosorptive accumulation. The case of Ag and Ni. *Int Biodet Biodegrad* **1995**, 129–153.

White C, Sayer JA, Gadd GM. 1997 Microbial solubilization and immobilization of toxic metals: key biogeochemical processes for treatment of contamination. *FEMS Microbiol* Rev **20**, 503–516.

Wnorowski AU. 1991 Selection of bacterial and fungal strains for bioaccumulation of heavy metals from aqueous solutions. *Water Sci Technol* **23**, 309–318.

Wong MH, Pak DCH. 1992 Removal of Cu and Ni by free and immobilized microalgae. *Biomed Environ Sci* **5**, 99–108.

Wong PK, Fung KY. 1997 Removal and recovery of nickel ion (Ni^{2+}) from aqueous solution by magnetite-immobilized cells of *Enterobacter* sp. 4-2. *Enzyme Microbiol Technol* **20**, 116–121.

Yong P, Macaskie LE. 1998 Bioaccumulation of lanthanum, uranium and thorium, and use of a model system to develop a method for the biologically-mediated removal of plutonium from solution. *J Chem Technol Biotechnol* **71**, 15–26.