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ORIGINAL PAPER

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Effect of metal complexation on the bioavailability of nitrilotriacetic acid to *Chelatobacter heintzii* ATCC 29600

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Abstract Many polluted sites contain a mixture of organics and heavy metals. Nitrilotriacetic acid has been chosen as a model organic compound to study the effect of metal binding on organic bioavailability and degradation of organics. The effect of varying the ratio of metal to nitrilotriacetic acid on its utilisation has been examined using the gram-negative bacterium *Chelatobacter heintzii* ATCC 29600. The following parameters of substrate utilisation were examined: growth, degradation, respiration, mineralisation and nitrilotriacetic acid uptake. Complexation of nitrilotriacetic acid by Cu(II), Ni(II), Co(II) and Zn(II) prevented utilisation of nitrilotriacetic acid by *C. heintzii*; complexation to Fe(III) or Mn(II) did not. The pattern of inhibition was consistent with a 1:1 stoichiometry of metal binding to nitrilotriacetic acid. Inhibition was not due to metal ion toxicity, but was a result of metal–nitrilotriacetic acid complexes being recalcitrant to degradation. In addition, the effect of complexing (phosphate) and non-complexing (PIPES) buffers on bioavailability was examined; Co and Zn prevented degradation of nitrilotriacetic acid in PIPES buffer, but not in phosphate buffer. This was due to the removal of Co and Zn from solution by phosphate precipitation, leaving nitrilotriacetic acid uncomplexed. The results demonstrated that metal–organic complexation can alter the bioavailability of organic pollutants and may also modulate the toxicity of heavy metals.

Key words Bioavailability · Complexation · Chelation · Speciation · Nitrilotriacetic acid · Metals · Biodegradation

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Abbreviation *NTA* Nitrilotriacetic acid

Introduction

There has been a great deal of research over the last two decades aimed at exploiting microorganisms for the remediation of contaminated sites and the purification of industrial effluents. This research has largely focused on the treatment of organic pollution. In more recent years, attention has turned also to the remediation of metal-polluted sites. However, many sites contain more than one type of pollutant; they are co-contaminated with both organics and metals. It has been estimated that approximately 37% of sites in the US which are contaminated with organic pollutants are also contaminated with metals (Roane et al. 1996).

The remediation of mixed wastes is much more complex than dealing with single pollutants. A microorganism which is able to degrade one component of the waste may be totally inhibited by another, more toxic component. Furthermore, organic compounds and inorganic elements may react with each other to varying degrees. The reactions can include organic-inorganic complex formation and precipitation reactions (Francis 1990). It has long been acknowledged that the bioavailability of pollutants to microorganisms is a critical factor in bioremediation. However, this has generally been taken to refer to the physical access of a microorganism to the pollutant molecule, and the importance of chemical speciation has not been widely recognised. The issue of metal speciation, in other words the particular chemical form in which a metal is present, and its effect on bioavailability has rarely been addressed (Roane et al. 1996), although awareness of the subject is growing. In addition, the question of organic speciation in mixed wastes, and the effect this has on the bioavailability, and hence the biodegradability of the organic component, has received scant attention.

There is a need to overcome the unpredictability of bioremediation and a desire to develop methods to enable the success of a remediation strategy to be predicted be-

fore a costly treatment process is initiated (Head 1998). A greater understanding of the factors involved is vital to achieving this goal. In addition, an understanding of the mechanisms involved in the transformation of highly complex mixed wastes will enable more accurate modelling of the long-term fate and transport of pollutants in waste sites (Francis 1990).

A number of studies have been carried out on the biodegradability of metal complexing agents. These include EDTA (Lauff et al. 1990; Henneken et al. 1995; Kluner et al. 1998; Thomas et al. 1998), nitrilotriacetic acid (NTA) (Firestone and Tiedje 1975; Uetz et al. 1992; Bolton et al. 1996; Xun et al. 1996; Witschel 1999), ethylenediaminedisuccinate (EDDS) (Witschel and Egli 1998) and citrate (Madsen and Alexander 1985; Francis et al. 1992; Francis and Dodge 1993). In each case it was found that metal complexation had a significant effect on biodegradation of the complex, and that complexes of certain metals were degraded, while others were not.

NTA is a xenobiotic-chelating agent whose metal-binding properties are exploited in a number of applications including detergent formulation, nuclear decontamination, and agriculture (Bolton et al. 1996; Egli 1994; Firestone and Tiedje 1975; Tiedje 1975). It is readily biodegradable in a variety of environments including river water, sewage sludge and soils (Bally et al. 1994; Firestone and Tiedje 1975; Tiedje 1975; Swisher et al. 1973; Tiedje et al. 1973). However, concern has been expressed that NTA may mobilise toxic metals in the environment through the formation of soluble metal chelates (Banat et al. 1974; Samanidou and Fytianos 1990). Thus, any decrease in the biodegradability of NTA resulting from metal chelation could have a significant negative effect on the environment. For these reasons, NTA was chosen as a model compound to study the effects of metal complexation on organic degradation.

The bacterial strain used in this study was *Chelatobacter heintzii* ATCC 29600. This was isolated as a NTA-degrading strain (Tiedje et al. 1973) and the mechanisms of NTA degradation have been well characterised for this organism (Firestone and Tiedje 1978; Uetz et al. 1992; Egli 1994). Previous studies have examined its ability to degrade metal NTA chelates (Firestone and Tiedje 1975; Uetz et al. 1992; Bolton et al. 1996; Xun et al. 1996), but have only considered the effect of mixing the chelator and metal in equimolar amounts. In this study, the effect of speciation on NTA degradation was investigated in more detail. In parallel experiments, varying concentrations of metals were added with a fixed concentration of NTA. The effect of differing proportions of free and complexed NTA in the experimental medium was observed on bacterial growth and NTA degradation, respiration and mineralisation. In this way, the effects of complexation were examined, as well as the influence of the chemical environment. In addition the effects of speciation on NTA uptake were investigated.

Materials and methods

Growth of bacteria

The mineral salts medium used was based on that of Miller (1972) and consisted of phosphate buffer (Na_2HPO_4, 6 g l^{-1}; KH_2PO_4, 3 g l^{-1}; NaCl, 0.5 g l^{-1}), mineral salts solution ($CaCl_2{\cdot}2H_2O$, 14.7 mg l^{-1}; $MgSO_4{\cdot}7H_2O$, 246 mg l^{-1}) and trace elements solution (MgO, 10.75 mg l^{-1}; $CaCO_3$, 2 mg l^{-1}; $FeSO_4{\cdot}7H_2O$, 4.5 mg l^{-1}; $CuSO_4{\cdot}5H_2O$, 0.25 mg l^{-1}; $CoSO_4{\cdot}7H_2O$, 0.28 mg l^{-1}; H_3BO_3, 0.06 mg l^{-1}; $ZnSO_4{\cdot}4H_2O$, 1.44 mg l^{-1}; HCl (conc.) 51.3 ml l^{-1}) in deionised water. Concentrations quoted are the final concentrations in the prepared medium. The medium was prepared as follows: phosphate buffer, mineral salts solution and carbon and nitrogen sources (see below) were mixed and sterilised by autoclaving at 121 °C for 15 min. Trace element solution was autoclaved separately and added to sterile medium. This method of preparation avoided the formation of metal precipitates. When metals were included above trace amounts they were added to autoclaved media as their chloride salt (filter-sterilised) and the pH adjusted to 7.0±0.1 with HCl or NaOH. In experiments in which PIPES buffer was used, phosphate buffer was replaced with 50 mM PIPES and 10 μM DL-α-glycerophosphate.

A nutrient agar slope of *C. heintzii* ATCC 29600 was resuspended with 1 ml starter medium (mineral salts medium plus 10 mM NTA) and used as an inoculum into 50 ml starter medium in a 250-ml Erlenmeyer flask, which was incubated for 3 days. The resulting stationary phase culture was used as a 2% (v/v) inoculum into 25 ml mineral salts medium plus 2 mM NTA in a 125-ml conical flask. NTA was used as both a sole carbon and a sole nitrogen source. Where acetate was used as an alternative carbon source, the starter culture in this case contained 20 mM acetate and 2 mM $(NH_4)_2SO_4$ instead of the NTA. This was inoculated into medium containing 6 mM acetate and 1 mM $(NH_4)_2SO_4$. All cultures were incubated at 30 °C with orbital shaking at 180 rpm.

Estimation of growth

Growth was estimated by recording the optical density at 600 nm using a Pye Unicam PU 8600 UV/visible spectrophotometer. Samples were acidified with 50% (w/v) 2 M HCl prior to reading the optical density, to dissolve any metal precipitates which may have formed, to prevent interference. Growth typically took 2–3 days, although in cases in which growth was slow or did not occur flasks were incubated for up to 1 week. Initial optical density was <0.05. Direct comparisons were only made between flasks inoculated at the same time to the same cell density. Measurements of optical density were converted to protein from a calibration curve prepared using the method of Bradford (1976). Cells were lysed prior to protein determination by freezing then thawing, followed by the addition of 10% (v/v) 5 M NaOH.

Analysis of NTA

Samples were taken from the culture medium and centrifuged at 24,000×*g* for 4 min in a MSE Microcentaur microcentrifuge to remove the bacteria. The supernatant was mixed with 10 mM $Cu(NO_3)_2$ to chelate any NTA and centrifuged for a further 4 min to remove particulates. NTA was analysed by high pressure liquid chromatography using a Techsphere 5ODS column (HPLC Technology) with a mobile phase of 0.4% tetrabutylammonium hydroxide, 90% methanol, pH 7.5. The flow rate was 1.5 ml min^{-1}, the column temperature was 50 °C, and detection was at 254 nm. This was a modification of the method of Parkes et al. (1981). When Co or Ni were present, it was necessary to further modify the method. With Co–NTA the above method was used, but with the addition of 2 mM $CoCl_2$ as well as 10 mM $Cu(NO_3)_2$ in the standards, as it was found that the presence of Co resulted in smaller peak areas. In the case of Ni–NTA, chelation with Cu was ineffective. Instead an excess of Ni was added (10 mM $NiCl_2$).

Ni–NTA does not absorb at 254 nm, instead it was found (using a PU 8720 UV/visible scanning spectrophotometer) that 220 nm was the optimal wavelength for its detection.

Oxygen-uptake experiments

Bacterial cell suspensions were prepared by growing *C. heintzii* cells in 200 ml phosphate-buffered media containing 10 mM NTA. The bacteria were harvested in late exponential phase by centrifugation at 4 °C, 15,300×*g* for 10 min. Cells were washed twice in 50 mM PIPES buffer, pH 7.0, and resuspended in 2 ml PIPES buffer. To prepare cell-free extracts, washed cells were resuspended in 2 ml PIPES buffer containing 0.5 mM PMSF (phenylmethylsulfonyl fluoride), 0.5 μg leupeptin ml^{-1}, 1 μg pepstatin A ml^{-1} and 2 mM dithiothreitol. The bacteria were sonicated for 30 min using a Braun Labsonic U sonicator at 250 W with a cycle of 0.3 s sonication followed by 0.3 s rest. The sonicate was centrifuged for 4 min at 24,000×*g* in a MSE Microcentaur microfuge to remove any cell debris. The protein concentrations of both whole cells and cell-free extracts were determined using the method of Bradford (1976). Cell suspension (50 μl) was added to 2.9 ml PIPES buffer, pH 7.0, at 30 °C in a Rank Digital Oxygen System model 10 and allowed to equilibrate. NTA stock solution (50 μl of 3 mM) was added to give a final volume of 3 ml and an NTA concentration of 50 μM. The rate of respiration was calculated as the difference between the NTA-stimulated and the endogenous rates of O_2 uptake. Metal chlorides were mixed with NTA at equimolar concentrations before addition to the reaction chamber. Cell-free extract (10 μl) was added to 2.84 ml PIPES buffer containing 0.2 mM NADH and 1 μM FMN, and 50 μl 50 mM NTA was added following equilibration to give a final NTA concentration of 0.833 mM and a total volume of 3 ml.

Mineralisation of NTA

Mineralisation of NTA by whole cell suspensions was followed using universally labelled [^{14}C]-NTA which was a gift from Unilever plc. *C. heintzii* cells were grown in 400 ml 10 mM NTA in PIPES medium at 30 °C and harvested in the late exponential phase by centrifugation at 15,300×*g*, 4 °C for 10 min. Cells were washed twice in 50 mM PIPES buffer, pH 7.0, and resuspended in the same buffer to an approximate OD_{600nm} of 1.0. A cell suspension of 1.25 ml was added to duplicate serum-capped Erlenmeyer flasks which were fitted with a centre well containing 4 ml 1 M KOH to trap CO_2. The reaction was started by addition of 0.1 mM [^{14}C]-NTA, 1.79 μCi. Flasks were incubated at 30 °C with orbital shaking at 180 rpm. At timed intervals the KOH was removed and replaced with fresh KOH. A KOH sample (1 ml) and 1 ml of culture medium were added to vials containing 3 ml scintillation cocktail (Beckman ReadyValue) and read for 5 min on a Wallac 1217 RackBeta Liquid Scintillation Counter. Where metals were included, the metal chloride and NTA were mixed prior to addition to the cell suspension. Experiments with different metals were carried out on different days.

Modelling of metal speciation

Metal speciation in culture media was predicted for the experimental systems employed using the chemical equilibrium computer program MINEQL$^+$ (Schecher 1994). The thermodynamic database of MINEQL+ is identical to that of MINTEQA2 (Brown and Allison 1987) and produces results compatible with EPA specifications. Trace elements were excluded from the calculations for simplification, as it was found that they had no significant effect on the overall speciation. Calculations were carried out for 30 °C and pH 7.0.

Uptake of NTA into bacterial cells

Chelatobacter heintzii cells were grown in 400 ml 20 mM NTA in PIPES medium at 30 °C and harvested in late exponential phase by centrifugation at 15,300×*g*, 4 °C for 10 min. Cells were washed twice in 50 mM PIPES buffer, pH 7.0, and resuspended in the same buffer to an approximate $OD_{600\ nm}$ of 5.0. Cell suspension was kept at 4 °C and placed in a 30 °C water bath for 10 min immediately prior to use. Cell suspension (10 ml) was aliquoted into a 50 ml-Erlenmeyer flask which was serum-capped. The reaction was started by the addition of [^{14}C]-NTA,1.01 μmol, 0.894 nCi. Where metal–NTA complexes were added, metal chloride (1.1 μmol) was mixed with NTA (1.01 μmol) to ensure that the metal was in a slight molar excess. At intervals, 1 ml of cell suspension was removed and filtered through a 0.2-μm cellulose nitrate filter. The filter and 0.5 ml filtrate were each immediately placed in a vial containing 5 ml scintillation cocktail. Scintillation counting was carried out for 5 min. NTA was not adsorbed to the filter, as demonstrated by a cell-free control.

Metal binding by bacterial cells

Cells were grown in 400 ml PIPES medium with 10 mM NTA in a 2-l Erlenmeyer flask, harvested at late exponential phase by centrifugation at 15,300×*g*, 4 °C, for 10 min and washed twice with 50 mM PIPES buffer, pH 7.0. Cells were resuspended with 200 ml PIPES buffer and 50-ml aliquots placed in 250-ml flasks. Metal chloride (1 mM) or metal–NTA complex (1 mM, prepared by mixing Na_2NTA and metal chloride) was added to duplicate flasks. Flasks were serum-capped to prevent evaporation and incubated at 30 °C with rotary shaking at 180 rpm. After 1 h or 24 h incubation the cell suspensions were treated as follows: 5 ml was filtered through a 0.2-μm cellulose nitrate filter and the filtrate used to determine soluble metal. Two portions of 10 ml were centrifuged at 27,200×*g* for 10 min; one portion was washed twice with PIPES buffer, and the other was washed twice with 10 mM EDTA (dissolved in 20 mM NaOH). It was noticed that metal precipitates were centrifuged with the cell pellet, and the EDTA wash was carried out in order to solubilise these precipitates. Cell pellets were resuspended with 10 ml deionised water. Aliquots (2 ml) of each of the cell pellets and filtrates, and also of the untreated suspension and the original metal-free suspension, were diluted with 10 ml deionised water and acid-digested with 6 ml conc. HCl and 2 ml conc. HNO_3. Metal analysis was carried out using a Perkin Elmer A Analyst 100 atomic absorption spectrophotometer.

Results

The effect of varying the ratio of metal to NTA was studied for a number of parameters of bacterial activity. These were growth and degradation, mineralisation, respiration and uptake of NTA. The metals studied were the first-row transition elements Mn, Fe(III), Co, Ni, Cu and Zn. Of these metals, copper was studied in the most depth. The results for each metal are presented below. For the mineralisation and uptake studies metals were selected which had shown contrasting effects on growth, degradation and respiration, as it was not possible to conduct these experiments with every metal.

Copper

Studies to determine the effect of copper complexation on bacterial growth were carried out using shake-flask cultures. Growth and NTA degradation were recorded with a

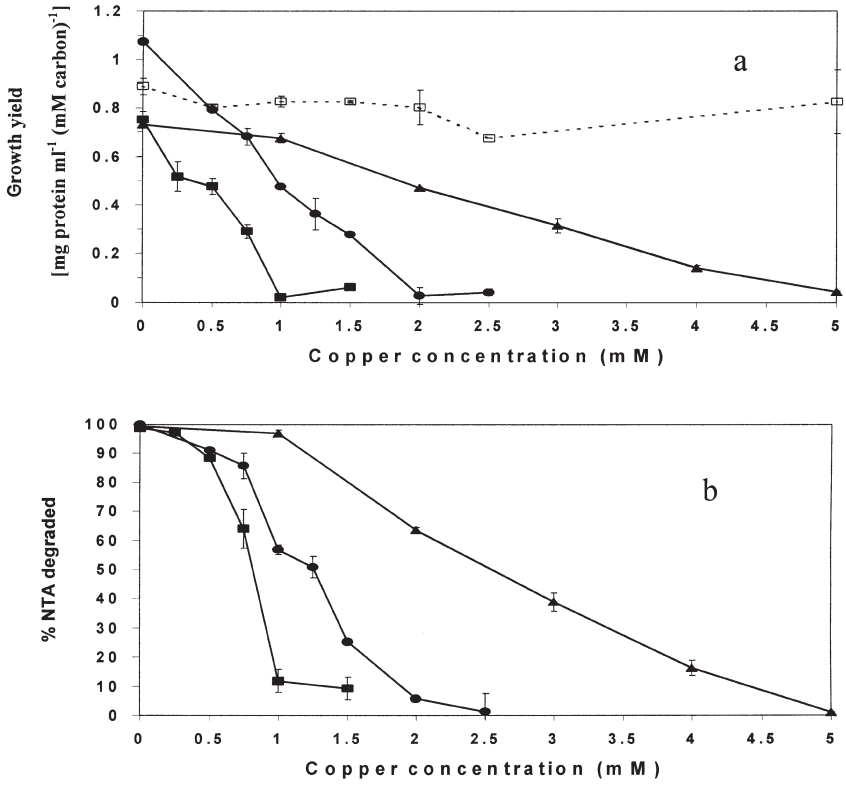

Fig. 1a,b Effect of Cu on growth and nitrilotriacetic acid (NTA) degradation by *Chelatobacter heintzii* in phosphate-buffered medium (pH 7.0) with: ■ 1 mM NTA, ● 2 mM NTA, ▲ 4 mM NTA, or ❒ 6 mM acetate plus 4 mM NH_4^+ as carbon and nitrogen source. **a** Maximum final growth yield, **b** percentage NTA degraded after 48 h growth. *Error bars* represent the standard error of duplicate cultures

fixed concentration of NTA and varying copper concentration. Increasing the copper concentration resulted in the same pattern of growth inhibition with three different fixed NTA concentrations (1 mM, 2 mM and 4 mM; Fig. 1a). As the concentration of copper in the growth medium was increased, there was a decrease in the growth yield of *C. heintzii*. There was also a corresponding decrease in the proportion of NTA that was degraded (Fig. 1b). No growth occurred when copper was present in an equimolar or greater concentration relative to NTA, and degradation either did not occur or was minimal.

NTA complexes metal ions stoichiometrically, i.e. in a 1:1 molar ratio (Swisher et al. 1967), and this was predicted by MINEQL+ when it was used to model the speciation of Cu and NTA in phosphate-buffered medium. Figure 2 compares the experimental results for degradation of 2 mM NTA with the predicted speciation of NTA as the copper concentration was increased. The proportion of NTA that was degraded corresponds to the proportion of NTA that was not bound to copper. This suggests that the copper complex of NTA is not bioavailable to *C. heintzii*. The observed decrease in growth yield can be attributed to the decrease in available growth substrate, i.e. the bacteria utilised only the NTA which was not copper-complexed.

The growth inhibition was not due to copper toxicity. In a similar experiment with acetate as the carbon source, copper concentrations up to 5 mM had no effect on the final growth of *C. heintzii* (Fig. 1a). Acetate has little capacity to complex metal ions, and therefore it can be assumed that if a metal caused any inhibition towards growth on acetate it would be due to copper toxicity and not to complex formation by the growth substrate.

The buffer used did not influence the effect of copper on growth or NTA degradation. With both phosphate and PIPES (50 mM, pH 7.0) buffers the same pattern of inhibition was seen – only the free NTA (i.e. the NTA in stoichiometric excess relative to copper) was degraded. Growth was directly related to the concentration of free (degradable) NTA (data not shown). The choice of buffer did, however, affect copper toxicity towards *C. heintzii*. In PIPES buffer, copper completely inhibited growth on acetate at concentrations as low as 0.1 mM. PIPES buffer does not complex metal ions (Good et al. 1966), and therefore copper remains in solution. Phosphate, however, readily precipitates cations out of solution (Good et al.

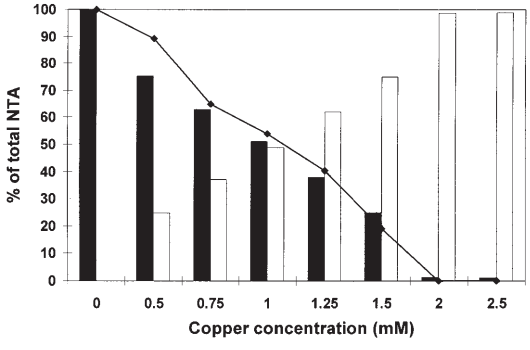

Fig. 2 Comparison of experimental degradation data with predicted NTA speciation, for 2 mM NTA and increasing Cu concentration. *Filled bars* show the portion of NTA predicted to be unbound, *empty bars* show the portion of NTA predicted to be copper complexed. The *line* shows the percentage of 2 mM NTA degraded experimentally

Table 1 Initial rate of nitrilotriacetic acid (*NTA*)-stimulated oxygen uptake [nmol min^{-1} (mg protein)$^{-1}$] with varying Cu:NTA ratios. NTA concentration was 0.33 mM in each case. Data shown are the mean values of five replicates±standard error. A negative value indicates a decrease in the respiration rate following the addition of NTA

Cu concentration (mM)	Cu:NTA ratio	O_2 uptake rate [nmol min^{-1} (mg protein)$^{-1}$]
0	0	0.120±0.043
0.165	0.5	0.102±0.036
0.231	0.7	0.105±0.025
0.33	1.0	0.001±0.021
0.66	2.0	–0.023±0.011

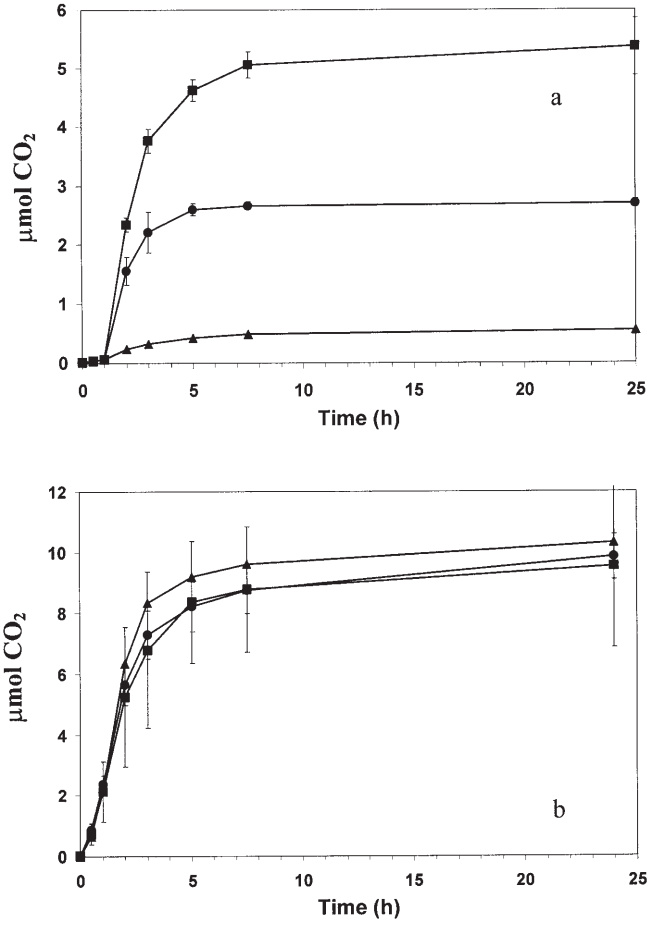

Fig. 3a,b Mineralisation of 0.1 mM NTA by non-growing *C. heintzii* cell suspensions in PIPES buffer (pH 7.0) with **a** copper, **b** manganese added at: ■ 0 mM, ● 0.05 mM, ▲ 0.1 mM. Total carbon=15 µmol

1966; Hughes and Poole 1991) and hence the copper is not available to exert any toxic effect. The effect of copper on NTA utilisation in both buffers can be attributed to the formation of a Cu–NTA complex, which is not bioavailable, and not to copper toxicity, as complexed copper did not exert toxic effects.

Copper had the same effect on the respiration of NTA as on degradation. When the concentration of NTA was kept constant (50 µM) and the concentration of copper was varied, it was found that NTA-stimulated oxygen uptake only occurred when the Cu:NTA ratio was less than 1, i.e. when NTA was in a molar excess, and there was uncomplexed NTA available (Table 1). When the Cu:NTA ratio was 1 or greater, all the NTA was copper complexed and respiration was not stimulated. This was not due to copper toxicity, as the subsequent addition of additional NTA (so that NTA was again in a molar excess) stimulated oxygen uptake. The inhibition was instead due to the NTA–copper complex not being available for respiration.

A similar effect was observed on the mineralisation of NTA (Fig. 3a). Copper inhibited mineralisation of NTA in a ratio-dependent manner. When no copper was present, 35.7% of the NTA was mineralised. When the Cu:NTA ratio was 1:2 (0.05 mM Cu, 0.1 mM NTA), only 18.0% of the NTA was mineralised, representing a 49.6% decrease in mineralisation relative to the copper-free control. When the ratio of Cu to NTA was 1:1, minimal mineralisation occurred.

Nickel

Nickel, like copper, prevented growth of *C. heintzii* on NTA in both PIPES and phosphate, showing a 1:1 inhibition. MINEQL+ predicted that, in phosphate buffer, all the NTA was complexed in the presence of equimolar nickel, and the formation of this complex prevented NTA utilisation. Nickel was more toxic to *C. heintzii* than the other metals tested. A concentration of 0.4 mM nickel did not affect final growth yield on acetate in phosphate buffer, but did result in a greatly increased lag phase of 45 h compared to no lag phase in the absence of Ni. In PIPES buffer a similar effect was observed. Respiration of NTA was prevented by the addition of equimolar Ni. This agrees with the formation of a 1:1 Ni–NTA complex, which, like Cu–NTA is unable to be degraded by *C. heintzii*.

Iron (III) and manganese (II)

Iron and manganese, in contrast to copper and nickel, did not affect bacterial growth or NTA degradation in either phosphate or PIPES buffer (Table 2), and neither metal inhibited growth on acetate. However, MINEQL+ predicts that, in phosphate buffer, these metals do not fully complex to NTA (Table 3), with 81.9% of the Fe forming insoluble $FePO_4$ and all the Mn precipitating as $MnHPO_4$. Therefore the observed growth and degradation may have been due to NTA being in an unbound, and hence bioavailable, form, rather than because the Fe(III)- and Mn(II)–NTA complexes can be degraded. In PIPES buffer, MINEQL+ predicts that 4.9% of the total NTA complexes with Fe, while 91.5% of NTA complexes with Mn when NTA and metal ions are both added at 2 mM. It appears that, in the presence of Fe(III), it was free NTA that was degraded. It is possible that *C. heintzii* is able to degrade the Mn–NTA complex; alternatively the free NTA was degraded, and the changing equilibrium caused

Table 2 Summary of the effects of metals on the growth of *Chelatobacter heintzii* on NTA and on NTA degradation in phosphate and PIPES buffers. + Indicates that growth occurred (as measured by an increase in optical density), – indicates inhibition of growth

	Phosphate buffer (complexing)		PIPES buffer (non-complexing)	
	Growth on 2 mM NTA+2 mM metal	Percentage of 2 mM NTA degraded in the presence of 2 mM metal ion	Growth on 2 mM NTA+2 mM metal	Percentage of 2 mM NTA degraded in the presence of 2 mM metal ion
Mn(II)	+	100	+	99.6
Fe(III)	+	100	+	92.1
Co(II)	+	90.4	–	0
Zn(II)	+	100	–	0
Ni(II)	–	9.3	–	0
Cu(II)	–	0	–	0
Cd(II)	–	0	–	0

Table 3 Predicted speciation of 2 mM NTA in phosphate-buffered growth media in the presence of 2 mM metal chloride, calculated by MINEQL+. Figures quoted are percentage of total NTA

Metal	H–NTA	Ca–NTA	Mg–NTA	Na–NTA	Metal–OH–NTA	Metal–NTA	Metal–$(NTA)_2$
Phosphate buffer							
None	84.7	4.4	8.8	1.6			
Mn^{2+}	84.9	4.5	8.9	1.7			
Fe^{3+}	59.1	4.3	14.9	1.2	14.7		5.1
Co^{2+}						99.9	
Ni^{2+}						100	
Cu^{2+}	1.1				5.9	93.2	
Zn^{2+}	3.4					96	
PIPES buffer							
Mn^{2+}		2.6	5.1			91.5	
Fe^{3+}	50.5		43.6	1.0			4.9
Co^{2+}						99.7	
Ni^{2+}						99.9	
Cu^{2+}					5.9	93.3	
Zn^{2+}						99.7	

Table 4 Respiration of metal–NTA complexes by whole cells and cell-free extract. Data shown as mean O_2 uptake rate [nmol min^{-1} (mg protein)$^{-1}$]±standard error of 3 replicates

	Whole cells	Cell-free extract
No metal	0.108±0.029	0±0
Mn(II)	0.106±0.007	0.179±0.034
Fe(III)	0.109±0.022	0.046±0.019
Co(II)	0±0	0.128±0.008
Ni(II)	0±0	0±0
Cu(II)	0±0	0±0
Zn(II)	0±0	0±0

by NTA consumption ensured that there was always a small amount of uncomplexed NTA available to the bacteria.

Neither iron nor manganese had any effect on the respiration of NTA (Table 4), and manganese was additionally shown to have no effect on NTA mineralisation (Fig. 3b). This was in contrast to copper, which caused an inhibition related to the metal to NTA ratio.

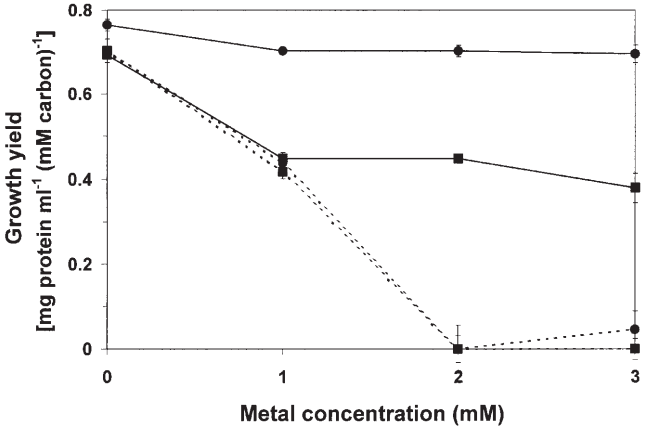

Fig. 4 Effect of cobalt ■ and zinc ● on the maximum final growth yield of *C. heintzii* on 2 mM NTA. *Solid lines* show results in phosphate-buffered medium, *dotted lines* results in PIPES-buffered medium

Cobalt and zinc

Up to 2 mM Zn(II) did not significantly inhibit growth on acetate in either phosphate or PIPES buffers, showing that it was not toxic at the concentrations used nor did it cause phosphate limitation by complexing the low amount of phosphate present in the PIPES medium. Cobalt caused a decrease in yield on acetate at concentrations above 1 mM but there was not a strong correlation between decrease in yield and cobalt concentration (data not shown).

In phosphate buffer, NTA was degraded and utilised for growth in the presence of either equimolar Co or Zn (Fig. 4). Cobalt caused a decrease in growth yield, resulting in a pattern of inhibition similar to that seen for growth on acetate, suggesting that metal toxicity, and not complex formation, was the cause. In PIPES buffer, however, growth and degradation of 2 mM NTA showed inhibition by both Co(II) and Zn(II) and was directly related to the metal to NTA molar ratio (Fig. 4). NTA degradation mirrored the growth of *C. heintzii* in all cases. In addition, neither complex was respired by *C. heintzii* cells in PIPES buffer, and this was not due to metal toxicity as the subsequent addition of an excess of free NTA stimulated oxygen uptake. This is similar to the findings with Cu–NTA.

Speciation modelling predicts that, in phosphate-buffered medium, NTA is completely complexed to Co and 96% complexed to Zn, and that in PIPES buffer NTA is completely complexed by both metals. The results in phosphate buffer suggest that *C. heintzii* is able to degrade Co–NTA and Zn–NTA, but the results in PIPES buffer are consistent with a 1:1 complex forming between Co or Zn and NTA, which is not able to be degraded. This apparent contradiction can be explained by the metal-complexing capacity of the two different buffers. In phosphate buffer, there is an equilibrium between the NTA complex of the metal and the insoluble metal phosphate. It could be expected that a proportion of the metal will form insoluble metal precipitates, and, as these are removed from solution, the equilibria will favour further formation of phosphate salts. The availability of NTA in the presence of cobalt or zinc will be determined not only by the overall speciation, but also by the rate of exchange of metal ions between species and is significantly affected by the inorganic ions present. In PIPES buffer, no such equilibrium exists, and the NTA remains in a stable, soluble complex which is not available for degradation.

Respiration of metal–NTA complexes by cell-free extract

To investigate whether the lack of respiration was due to a lack of transport of the metal–NTA complex into the cell, rather than the inability of intracellular enzymes to degrade the complex, the respiration of metal–NTA complexes by cell-free extracts was examined (Table 4). Experiments were carried out using PIPES buffer. NTA was not respired by cell-free extract in the absence of metal ions, suggesting that the free form of NTA is not the normal substrate for metabolism. This agrees with the findings of Uetz et al. (1992) who found that oxidation of NTA by NTA monooxygenase was strictly dependent on the presence of metal ions. The Mn(II) and Fe(III) complexes were, however, respired by cell-free extract as well as by whole cells, although the rate of O_2 uptake by cell-free extract was significantly less for Fe–NTA than for Mn–NTA. The complexes with Cu(II), Ni(II) and Zn(II) were not respired, which is in agreement with the results obtained with whole cells. In contrast to the whole cell studies, the NTA complex of Co(II) was respired by cell-free extract but not by whole cells, suggesting that complexation in this case could have prevented NTA utilisation by inhibiting its transport into the cell.

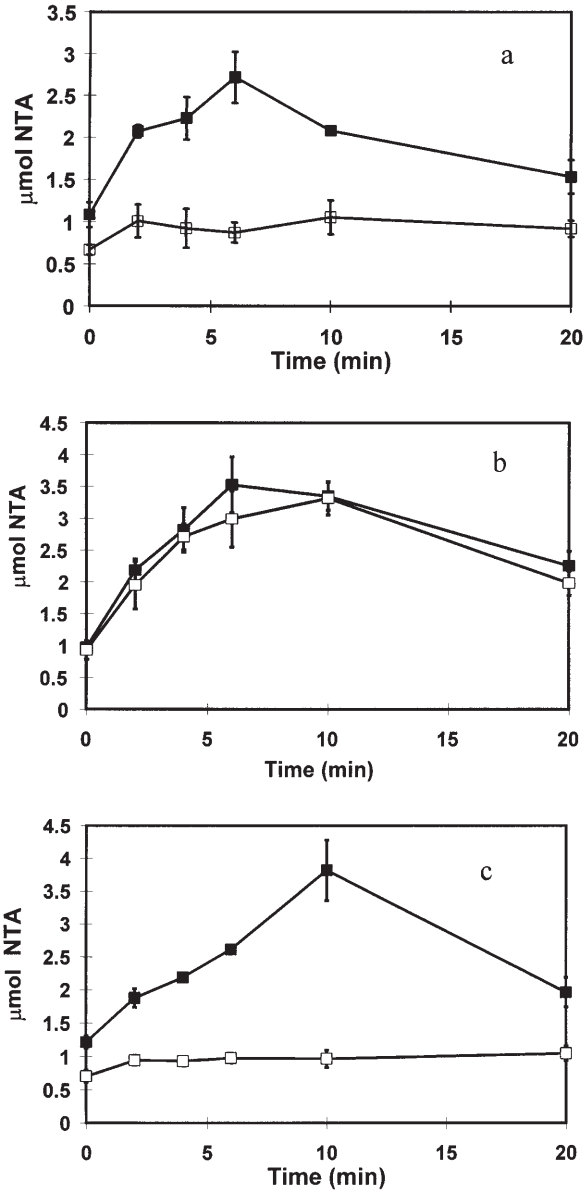

Fig. 5a–c Uptake of NTA by *C. heintzii* cells. *Solid symbols* depict free NTA; *open symbols* depict **a** Cu–NTA, **b** Mn–NTA, **c** Co–NTA

Uptake of NTA into bacterial cells

The uptake of [^{14}C]-NTA was studied in the presence of Cu, Mn and Co. These three metals were chosen because Cu–NTA was respired by neither whole cells nor cell-free extract, Mn–NTA was respired by both, while Co–NTA was respired by cell-free extract but not whole cells. Studies of the uptake of ^{14}C-labelled NTA into cells of *C. heintzii* revealed that NTA was taken up from the manganese complex, but not from the copper or cobalt complexes (Fig. 5). This agrees with data on NTA utilisation;

the Mn–NTA complex was utilised by whole cells, whilst the Cu and Co complexes were not. It also gives weight to the hypothesis that Co–NTA is not bioavailable because the NTA is not transported into the cell.

Fate of metals

Metal-binding studies were carried out with manganese and copper. It is not known whether NTA enters the bacterial cell as the free acid or the metal complex. In order to deduce this, it is necessary to establish the fate of the metal ion. There was no evidence that either metal was associated with the bacterial cells after 24 h incubation either in the presence or absence of NTA. Less than 0.3% of the metal was found to be associated with either PIPES-washed or EDTA-washed cells when NTA was present. When the metals were added as the chloride salts alone, Mn was not associated with the cells. Cu was largely insoluble (less than 10% in the filterable fraction) and a blue layer of copper precipitate was deposited with the cell pellet during centrifugation. Washing with EDTA removed this, and again, little Cu was associated with the cells.

Discussion

The results of this study, which are summarised in Table 5, indicate that certain metals prevent the biodegradation of NTA by *C. heintzii* by forming a metal chelate which is not bioavailable. Previous studies have only examined the effect of adding equimolar amounts of metal and NTA (Bolton et al. 1996; Firestone and Tiedje 1975). However, this study examines the effect of varying the ratio of metal to NTA and thus alters the proportions of NTA species present. The results indicate that where a metal (for example copper) forms a 1:1 chelate with NTA, only the uncomplexed portion of the NTA is utilised by the bacteria.

The results are in agreement with those of Firestone and Tiedje (1975), who found that Cu completely inhibited NTA respiration, while Ni and Zn caused inhibition to a lesser extent. Bolton et al. (1996) found that the rate and extent of NTA degradation was much lower with Ni and Cu than Co or Fe. They found no significant difference in the extent of degradation of free NTA or its complexes with Co, Fe, Zn or Al. However, they only considered the effect of adding equimolar metal and NTA, and did not vary the ratio of free to complexed NTA. The maximum NTA degradation they recorded was 65% and it is not clear why degradation was incomplete. Also in their study, the aqueous speciation calculations predict a maximum 95% of NTA complexed by Cu, Ni, Zn or Fe, with only 84% of NTA forming a Co complex, compared to 99.9% calculated in this study. This increased amount of free NTA may explain why there was apparent degradation of these complexes; it is possible that the only NTA degraded was the free acid present in equilibrium with the complex.

The results showed that different metals have different effects on the bioavailability of NTA to *C. heintzii*. Those complexes which were degraded were also respired by whole cells and utilised as a source of carbon, nitrogen and energy for growth.

The inhibition of NTA utilisation was not due to the toxicity of the metal, and it was found that the ability of metals to inhibit growth on a non-chelating substrate (acetate) depended on the buffer used. In PIPES buffer, the metals remained in solution, whereas in phosphate buffer they formed insoluble phosphates. This demonstrates that, not only do metals affect the degradation of organics such as NTA by forming complexes that are not bioavailable, but also that metals themselves may be complexed by other species (inorganic or organic), making them non-bioavailable. The abatement of metal toxicity, either by precipitation by inorganic anions or complexation with an organic molecule, has been well documented in the literature (Gadd and Griffiths 1978; Hughes and Poole 1991; Shuttleworth and Unz 1991) and is in fact one mechanism of microbial resistance towards heavy metals (Gadd and Griffiths 1978; Morrison et al. 1989). Hughes and Poole (1991) cite instances in which high metal tolerance has been falsely reported for some organisms when in fact the concentration of toxic cation in solution had been negligible. This illustrates the point that metal speciation is both complicated and an important factor in determining the fate of both organic and metal pollutants in the environment. It is therefore important when considering the treatment (biological or otherwise) of a polluted site or effluent to have an understanding of the chemical species present and the effects of complexation and chelation within the system.

Various suggestions have been made about the factors that determine whether or not a particular organic–metal complex will be degraded. Madsen and Alexander (1985) found that the only NTA species mineralised by sewage populations was the calcium complex. Firestone and Tiedje (1975) suggested that the particular type of complex formed, i.e. whether it is tridentate or tetradentate, will determine whether the NTA is degraded. Joshi-Tope

Table 5 Summary of results. All results refer to 2 mM NTA+2 mM metal. + Indicates a positive result, – indicates a negative result, *ND* denotes not determined. Respiration, mineralisation and uptake experiments used PIPES buffer

	Phosphate buffer		PIPES buffer		Respiration whole cells	Respiration cell-free extracts	Mineralisation	Uptake
	Growth	Degradation	Growth	Degradation				
Fe(III)	+	+	+	+	+	+	ND	ND
Mn(II)	+	+	+	+	+	+	+	+
Co(II)	+	+	–	–	–	+	ND	–
Ni(II)	–	–	–	–	–	–	ND	ND
Cu(II)	–	–	–	–	–	–	–	–
Zn(II)	+	+	–	–	–	–	ND	ND

and Francis (1995) also claimed that the nature of the complex formed will determine whether metal citrate complexes are degraded. They reported that bidentate metal citrate complexes were degraded by *Pseudomonas fluorescens* whereas tridentate complexes were not. Witschel et al. (1997) reported that there was no clear relationship between the stability constant and degradability of EDTA complexes by a bacterium and speculated that the structure of the complex somehow determines degradability. Such models do not necessarily take into account the dynamic equilibria in solution, and the rate of exchange of metal ions between different species may determine whether there is a small, transient, amount of free NTA available for biodegradation.

Fe(III) or Mn(II) did not prevent the degradation of NTA; however, speciation modelling showed that in phosphate buffer the majority of the NTA was present as the free acid, and therefore the species degraded was free NTA. In PIPES buffer, Mn was predicted as complexing to NTA, and the results suggest that Mn–NTA is able to be degraded by *C. heintzii*. Cu(II) and Ni(II) both inhibited the degradation of NTA by forming a stoichiometric complex with NTA, i.e. one molecule of NTA bound one metal ion. The proportion of NTA that was degraded corresponded to the portion of NTA which was predicted to be available and not complexed to Cu or Ni. The effect of either Co(II) or Zn(II) was dependent on the complexing capacity of the buffer; in a complexing buffer (phosphate) NTA degradation occurred, whereas in a non-complexing buffer (PIPES), degradation was inhibited by Co and Zn in a ratio-dependent manner. Speciation modelling predicted that in each case a stoichiometric metal–NTA complex was formed.

The results can be explained as follows. Where NTA does not form a metal complex, or where it forms a weak complex with a relatively low stability constant (e.g. Mn–NTA, Mg–NTA, Ca–NTA), it is degraded. Those complexes with high stability constants (Cu–NTA, Ni–NTA) are not degraded. Co and Zn form complexes with NTA which are stronger than Mn–NTA, but not as strong as Cu–NTA and Ni–NTA. These complexes could be degraded in a complexing buffer, where there was competition between NTA and phosphate for metal binding, but they were not degraded in PIPES buffer. It seems that the bioavailability of NTA in these complexes of intermediate strength is modulated by the inorganic ions present and the dynamic equilibria between the species present. This interpretation of the results is similar to that of Kluner et al. (1998) who found that metal-EDTA complexes with a log stability constant below 12 were degraded by a bacterial isolate, but stronger complexes were not. Similarly, Witschel and Egli (1998) found that the biodegradability of metal complexes of [*S,S*]-EDDS was related to their stability constants; only the weaker complexes were degraded. Bolton et al. (1996) proposed that the lability of the complex (i.e. the rate of dissociation of the complex to a degradable species of NTA) determines the rate of degradation and stated that it is not related to the stability constant of the complex, but is dependent on the kinetics of the equilibrium between free and bound NTA. There is as yet no general rule to allow prediction of the biodegradabilty of metal–organic complexes (Witschel and Egli 1998). Despite this, similarities are emerging between different compounds, and further studies in this area with more chelators may well allow more general conclusions to be reached.

One factor which may prevent the biodegradation of a metal–NTA complex is its transport into the bacterial cell. Experiments with cell-free extract were carried out to investigate this possibility. It was found that the cell-free extract was unable to respire free NTA. This is in agreement with the findings of Cripps and Noble (1973), Uetz et al. (1992) and Xun et al. (1996), who found that the metabolism of NTA by cell-free extract or purified NTA monooxygenase required the addition of metal cations. Similarly, Witschel et al. (1997) found that cell-free extract from an EDTA-degrading bacterium only transformed EDTA in the presence of metal cations, although the range of activating metals was different. The results agree in part with the findings of Uetz et al. (1992), who found that NTA monooxygenase activity was strictly dependent on the addition of Mg^{2+} or Co^{2+} ions, while there was no activity with Ca^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+} or Ni^{2+}. Co was of especial interest in that it prevented respiration of NTA by whole cells, but not cell-free extract, suggesting that in this case NTA was prevented from entering the bacterial cell.

The transport of NTA into *C. heintzii* was studied for free NTA, Mn–NTA, Cu–NTA and Co–NTA. This is, to our knowledge, the first report of NTA uptake by *C. heintzii*, and the first investigation of the uptake of metal–NTA complexes; previous work by Wong et al. (1973) studied transport of free NTA into an unnamed bacterial mutant. NTA complexed to Mn was utilised by *C. heintzii*, and the NTA was taken into the cells, although whether this was as free NTA or the Mn complex is unknown. In contrast, the NTA complexes of Cu and Co, which were not utilised, were not taken up into the cells. This agrees with the findings of the respiratory studies with Co–NTA, namely that cell-free extract but not whole cells was able to respire Co–NTA, suggesting that complexation by Co inhibits bacterial transport. Further investigations with a wider selection of metals than was possible here would be of value in determining the effect of metal complexation on the uptake of NTA. In addition, more thorough examination of the fate of the metal would help to determine the form in which NTA enters the bacterial cell. Metal-binding studies showed that the Mn and Cu were not associated with *C. heintzii* cells either in the presence or absence of NTA. This agrees with the findings of Bolton et al. (1996) who found that almost all the Co or Ni incubated with cells was found in the aqueous phase and not associated with the cells. It is possible that the small amount of metal associated with the cells in both this study and that of Bolton et al. (1996) was due to metal efflux mechanisms, although the inclusion of NTA had no effect on the amount of metal associated with the cells, compared to metal chloride alone. The results do not support the up-

take of a metal–NTA complex by the cells; however, more work is needed to draw definite conclusions.

A full understanding of the mechanisms involved in the degradation of metal–organic complexes is still to be attained. There is no general rule to describe the effect of complexation on the degradation of organic chelators, although similarities have been noted between different compounds. Further studies in this areas with a wider range of chelators may allow more general conclusions to be reached, which would enable the biodegradability of metal–organic complexes to be more accurately predicted. In addition, an increased knowledge of the effects of speciation on biodegradation is essential to the development of effective solutions to the problem of co-contaminated sites and effluents.

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