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Bacterial growth yields on EDTA, NTA, and their biodegradation intermediates

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Abstract Ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) are widely used anthropogenic chelating agents for control of metal speciation and are ubiquitous in natural waters and wastewaters. This is the first report of systematic measurement of the growth yields of a mixed culture (BNC1-BNC2) on EDTA and its biodegradation intermediates, and of *Aminobacter aminovorans* (aka *Chelatobacter heintzii*) ATCC 29600 on NTA and its biodegradation intermediates. The yields measured for BNC1-BNC2 co-culture were 75.0 g of cell dry weight (CDW) (mole of EDTA)$^{-1}$, 68.6 g of CDW (mole of ED3 A)$^{-1}$, 51.2 g of CDW (mole of N,N′-EDDA)$^{-1}$, 34.5 g of CDW (mole of ED)$^{-1}$, 26.3 g of CDW (mole of IDA)$^{-1}$, 12.2 g of CDW (mole of glycine)$^{-1}$, and 9.7 g of CDW (mole of glyoxylate)$^{-1}$. The yields measured for *A. aminovorans* were 44.3 g of CDW (mole of NTA)$^{-1}$, 37.9 g of CDW (mole of IDA)$^{-1}$, 15.2 g of CDW (mole of glycine)$^{-1}$, and 10.4 g of CDW (mole of glyoxylate)$^{-1}$. The biodegradation pathways of EDTA, NTA, and several of their metabolic intermediates include reactions catalyzed by oxygenase enzymes, which may reduce energy available for cell synthesis. Comparison of measured yields with predicted yields indicates that the effect of oxygenase reaction on cell yield can be quantified experimentally as well as modeled based on thermodynamics.

Keywords Yield · EDTA · NTA · Intermediate · BNC1-BNC2 · ATCC 29600

Abbreviations

EDTA	Ethylenediaminetetraacetic acid
NTA	Nitrilotriacetic acid
ED3A	Ethylenediaminetriacetic acid
3KP	3-ketopiperazine-N,N-diacetate
N,N′-EDDA	N,N′-ethylenediaminediacetic acid
EDMA	Ethylenediaminemonoacetic acid
ED	Ethylenediamine
IDA	Iminodiacetic acid
C. heintzii	*Chelatobacter heintzii* ATCC 29600

Introduction

Ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid (NTA) are two important

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anthropogenic chelating agents that form stable, water-soluble complexes with various metal cations. They are widely used in many industrial processes and consumer products such as detergents, photo processing, the paper and pulp industry, and the nuclear industry, to control the chemistry of metals. The total worldwide use of EDTA, NTA, and diethylenetriaminepentaacetic acid (DTPA) was 200,000 tons in 2000 (Schmidt et al. 2004). The widespread use and slow transformation of these chelating agents has led to concerns regarding the possibility of their persistence in the environment. For example, EDTA is one of the highest concentration anthropogenic compounds in European surface waters: reported at 10-100 μg/l in rivers and 1–10 μg/l in lakes (Bucheli-Witschel and Egli 2001; Frimmel 1997; Frimmel et al. 1989; Sacher et al. 1998; Schmidt et al. 2004; Sillanpaa 1997; Sillanpaa et al. 1997; Wolf and Gilbert 1992). EDTA has been found in surface and groundwaters (Barber et al. 1997; Barber et al. 1999) as well as wastewaters (Alder et al. 1990) in the United States. NTA has also been detected in surface waters of Europe (Bucheli-Witschel and Egli 2001; Frimmel et al. 1989) and Canada (Wooddiwiss et al. 1979). EDTA is recalcitrant in many environmental systems (including most wastewaters) while NTA can be biodegraded under aerobic and anaerobic conditions by several different organisms (Bucheli-Witschel and Egli 2001).

The major environmental concern related to EDTA and NTA is their ability to enhance the mobility and transport of radionuclides and heavy metals once released into the environment (Baik and Lee 1994; Cleveland and Rees 1981; Killey et al. 1984; Means et al. 1978; Riley et al. 1992). Chelates have also been implicated in enhanced eutrophication in natural systems (Nowack 2002). Biodegradation of EDTA and NTA in wastewaters before their release into the environment may prevent these effects.

The biodegradation of EDTA and NTA in waste streams and the environment is dependent on activation reactions catalyzed by oxygenase enzymes. The biodegradation pathways of EDTA (Bohuslavek et al. 2001; Kluner et al. 1998; Liu et al. 2001; Nörtemann 1999; Payne et al. 1998) and NTA (Bucheli-Witschel and Egli 2001; Firestone and Tiedje 1975, 1978; Uetz and Egli 1993; Uetz et al. 1992; Xu et al. 1997) are shown in Fig. 1. As shown in the figure, the metabolic intermediates of EDTA biodegradation include ethylenediaminetriacetic acid (ED3A), N,N′-ethylenediaminediacetic acid (N,N′-EDDA), ethylenediaminemonoacetic acid (EDMA), ethylenediamine (ED), and glyoxylate. The metabolic intermediates of NTA biodegradation are iminodiacetic acid (IDA), glycine, and glyoxylate. Several of these metabolic intermediates retain chelation ability. Accumulation and persistence of these intermediates could lead to continued environmental effects even after removal of the primary substrate. Also, dependence on oxygenase activation reactions and oxidase-catalyzed reactions in chelate biodegradation may lead to reduced cell yields and slower growth of chelate-degrading microorganisms, increasing the time for complete removal of the chelate (Madigan et al. 2000; Yuan and VanBriesen 2002, Yuan and VanBriesen 2006).

In order to predict and model biodegradation of chelates, the yields of microorganisms using them as primary substrates for growth must be known. Yields have been previously reported by Henneken et al. (1998) for mixed culture EDTA biodegradation, Weilenmann et al. (2004) for pure culture (DSM 9103) EDTA biodegradation, and Bally et al. (1994) for pure culture NTA biodegradation. Yields for some of the common downstream intermediates (glyoxylate and glycine) have been previously reported by several sources for different bacterial species (Engelbrecht and McKinney 1957; Rutgers et al. 1989). Weilenmann et al. (2004) reported the yields of DSM 9103 on IDA and N,N′-EDDA. This work presents the yields for biodegradation of EDTA and its intermediates (ED3A, N,N′-EDDA, ED, IDA, glycine, and glyoxylate) by a mixed culture BNC1-BNC2 and for biodegradation of NTA and its intermediates (IDA, glycine, and glyoxylate) by ATCC 29600 *Aminobacter aminovorans* (aka *Chelatobacter heintzii).*

Further, comparison of experimentally-determined yields with model-predicted yields demonstrates that the effect of oxygenase activation reactions and oxidase reactions on cell yield during

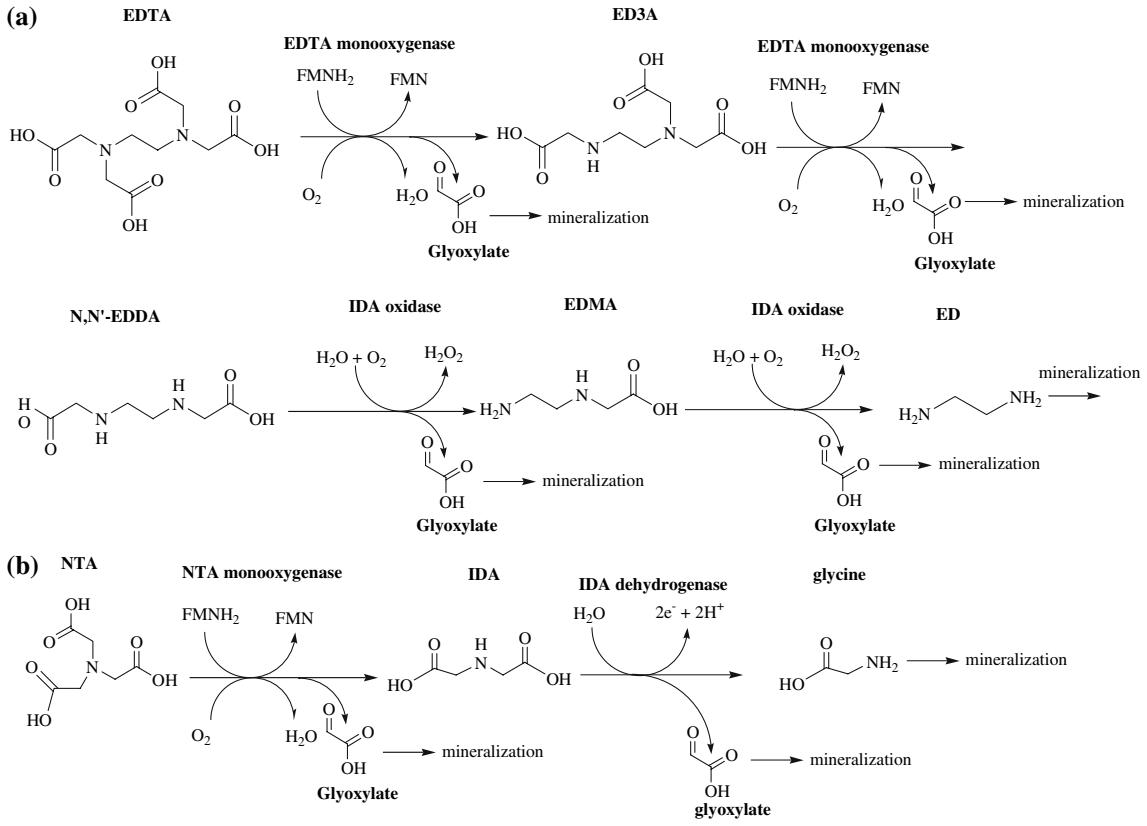

Fig. 1 (**a**) Proposed biodegradation pathways of EDTA (Bohuslavek et al. 2001; Kluner et al. 1998; Liu et al. 2001; Nörtemann 1999; Payne et al. 1998). (**b**) NTA biodegradation pathway by *A. aminovorans* (Bucheli-Witschel and Egli 2001; Firestone and Tiedje 1975; Firestone and Tiedje 1978; Uetz and Egli 1993; Uetz et al. 1992; Xu et al. 1997)

chelate biodegradation can be thermodynamically modeled as well as observed.

Materials and methods

Bacterial strains and stock cultures

The EDTA degrading co-culture (hereafter BNC1-BNC2) used in this work was a gift from Dr. Harvey Bolton, Jr. at Pacific Northwest National Laboratory, USA who originally received the culture from Dr. Bernd Nörtemann at the Technical University of Braunschweig in Germany. The mixed culture has not been classified or further characterized in our laboratory; however, Gschwind (1992) and Witschel et al. (1995) isolated an EDTA degrader, DSM 9103 that they report is closely related to BNC1. Weilenmann et al. (2004) published extensive characteristics of DSM 9103, indicating it is in the α-subclass of *Proteobacteria* with similarity to the *Mesorhizobium* and *Aminobacter* genera. ATCC 29600 used in this work was purchased from ATCC, and was expected to be *Chelatobacter heintzii*, a previously characterized and well-studied NTA degrading organism (Bucheli-Witschel and Egli 2001; Firestone and Tiedje 1975, 1978; Uetz and Egli 1993; Uetz et al. 1992; Xu et al. 1997). Kampfer et al. (2002) reported that *Chelatobacter heintzii* is a synonym of *Aminobacter aminovorans*, which was described previously.

The focus on a mixed culture rather than the pure culture BNC1 or BNC2 for EDTA biodegradation was designed to enable collection of yields of relevance in natural systems. While BNC1 is a critical organism for biodegradation of

EDTA, it has been suggested that many different organisms might be capable of taking the place of BNC2 and enhancing the rate of EDTA biodegradation by removing the downstream products of EDTA transformation and/or making available additional substrates or vitamins to BNC1 (Nörtemann 1992). For NTA, *A. aminovorans* is capable of the complete transformation of NTA and the rate of this transformation is not enhanced by the presence of co-species. Thus, the pure culture was considered for determination of yields relevant to environmental conditions.

A stock culture of the BNC1-BNC2 co-culture was maintained in Erlenmeyer flasks with growth on the optimal EDTA mineral medium (see below) at room temperature. Similarly a stock culture of *A. aminovorans* was maintained in Erlenmeyer flasks on the optimal NTA mineral medium (see below) at room temperature. Periodically, a small portion of stock culture from exponential growth phase was transferred into fresh optimal culture medium. All transfers and sampling were conducted under sterile conditions, and all media and equipment were autoclave-sterilized. To check the purity of these stock cultures, periodically cells of *A. aminovorans* were plated onto NTA mineral agar plates, and cells of BNC1-BNC2 co-culture were plated onto nutrient broth EDTA mineral agar plates. The reactors were seeded from these stock solutions by sterile pipette.

Media and growth conditions

Cells were cultured in batch in four 2 l fermentors (BioFlo 2000, New Brunswick Scientific, USA) under their optimal growth conditions—for the BNC1-BNC2 co-culture: temperature 35°C, aeration flowrate 2 l/min, agitation speed 750 rpm, and pH 7.8–8.2 (Henneken et al. 1998; Kluner 1996); and for *A. aminovorans*: temperature 30°C, aeration flowrate 2 l/min, agitation speed 750 rpm, and pH 6.8–7.0 (Bally et al. 1994; Egli et al. 1988).

The culture medium for EDTA and ED3A biodegradation was modified from Nörtemann (1992). It contained (per liter): different concentrations of $Na_2EDTA{\cdot}2H_2O$ or $Na_3{\cdot}ED3A$ as the sole source of carbon, nitrogen and energy, 2.75 g Na_2HPO_4, 1.0 g KH_2PO_4, 1.0 g $MgSO_4{\cdot}7H_2O$, 0.11 g $CaCl_2$, 0.0005 g $FeCl_3{\cdot}6H_2O$, 1 ml trace element solution and 1 ml vitamin solution. The trace element solution contained (per liter): 0.3 g H_3BO_3, 0.11 g $CoCl_2{\cdot}6H_2O$, 0.01 g $CuCl_2{\cdot}2H_2O$, 0.0136 g $MnSO_4$, 0.0304 g $Na_2MoO_4{\cdot}2H_2O$, 0.011 g $NiSO_4{\cdot}6H_2O$, and 0.056 g $ZnSO_4{\cdot}7H_2O$. The vitamin solution contained (per liter): 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine·HCl, 50 mg riboflavin, 50 mg thiamine, 50 mg nicotinic acid, 50 mg d-pantothenic acid, 1 mg B12, 50 mg p-aminobenzoic acid, and 50 mg thiotic acid (DL-6,8). Vitamin solution was filter-sterilized with a 0.2 μm membrane filter and added after autoclaving. Henneken et al. (1995) reported that EDTA was only degraded if divalent metal ions were present in a stoichiometric excess and the viability of cells was drastically reduced by uncomplexed EDTA. EDTA can remove functional Ca^{2+} and Mg^{2+} from the cell membrane and cause rapid lysis of cells because of its strong chelating ability (Wilkinson 1970). Thus Ca^{2+} and Mg^{2+} were always present in excess in the EDTA culture medium.

The culture medium for other substrates (N,N′-EDDA, ED, NTA, IDA, glycine, and glyoxylate) contained (per liter): 1.6 g Na_2HPO_4, 0.4 g KH_2PO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 25 mg $CaCl_2$, 2.5 mg $FeCl_3{\cdot}6H_2O$, 1 ml trace element solution, 1 ml vitamin solution, and the investigated substrate as the sole source of carbon, energy and nitrogen. 0.5 g NH_4Cl was added as the nitrogen source when measuring the yields on glyoxylate.

The initial substrate concentration for these batch experiments was selected to balance (1) the need for significant growth to improve the accuracy of the measured yield with (2) the need for optical density (OD) measurements that could be directly correlated with cell dry weight (CDW). High initial substrate concentration results in higher biomass production (higher OD), leading to a lower error when measuring differences in biomass and substrate concentrations. Higher substrate concentrations in the reactor also ensure that bacteria spend significant time in exponential growth, thus reducing any error associated with the lag phase. However, the assumption of a linear relationship between CDW and OD is best suited for low bacterial concentrations (low OD) and its error increases

with high OD values (Gerhardt et al. 1994). Thus, in our experiment, the initial substrate concentration was chosen high enough for sufficient biomass growth and the growth experiment was stopped once OD was too high for reliable unit conversions (when OD > 0.7).

Biomass measurement

The specific growth rate of cells was close to their maximum growth rate under above described culture conditions until the substrate was nearly completely consumed. To monitor cell growth, two samples were taken from the reactor for each sampling point and the results were averaged. The sampling points were evenly distributed across the growth curve to better represent growth course.

Cell concentrations were monitored by OD using a Spectronic $20D^+$ spectrophotometer (Milton Roy, USA). 0.3 ml 1 M HCl solution was added to 3 ml samples to dissolve precipitates formed during growth on metal-chelates and after 5 min, the OD was measured at 546 nm against deionized water. The final OD was obtained by multiplying the measured OD by a coefficient of 1.1 to correct for the added HCl solution.

The measured OD was converted to CDW using a relationship determined through a separate experiment. In this experiment, cells were grown on their primary substrate (EDTA or NTA) and harvested in late exponential phase. These cells were treated with HCl (at a 1:10 volume ratio) and harvested by centrifugation. The cell pellets were resuspended into ultrapure water to remove the impurities. After washing for three times, the OD of the resulting cell suspension solution was then measured using the same procedure as described above; the CDW was measured following standard methods (Gerhardt et al. 1994). A linear relationship between CDW and OD was determined from 7 replicates for the EDTA degraders as 0.366 $^{gCDW}/_{L}/_{Unit\ OD}$ and from 8 replicates for *A. aminovorans* as 0.364 $^{gCDW}/_{L}/_{Unit\ OD}$

Chemical reagents and chemical analyses

Na_3·NTA was purchased from Acros Organics, USA. Iminodiacetic acid was from Sigma Chemicals, USA. Na_3·ED3A was a gift from Hampshire Chemicals, USA. N,N′-EDDA was from Tokyo Kasei Kogyo, Japan. Glyoxylic acid monohydrate was from ICN Biomedicals, Germany. All other reagents were reagent grade or better and purchased from Fisher Scientific, USA.

For each sample from the reactor, an aliquot was filtered by 0.2 μm syringe membrane filter and the concentrations of investigated primary substrate (EDTA, NTA or their biodegradation intermediates) were measured. EDTA was measured by HPLC after complexation with iron (Bergers and DeGroot 1994). NTA was also measured by HPLC (Parkes et al. 1981). ED3A and N,N′-EDDA were measured by normal phase HPLC after complexation with Cu^{2+} (Yuan and VanBriesen 2005). IDA and glycine were measured as amino acids after derivatization with 9-fluorenylmethyl chloroformate (Yuan and VanBriesen 2005). ED was measured by HPLC after derivatization with 1-naphthylisothiocyanate (Yuan and VanBriesen, unplublished). Glyoxylate was measured by spectrophotometry (Trijbels and Vogles 1966).

Data treatment

When the specific growth rate (μ) is much lower than the maximum growth rate (μ_{max}), the observed yield is much lower than the true yield. Thus, to measure the maximum yield for organisms on a substrate, growth at μ close to μ_{max} is needed. In this work, at least three observed yields measured with μ close to μ_{max} were obtained for each substrate, and the average value was reported as the final yield. To reduce lag phase, cells in exponential growth phase were inoculated into fresh media. If significant lag phase was observed in a batch run, its results were rejected and are not reported here.

The observed cell yield was calculated as:

$$Y_{obs} = \frac{(X_n - X_0)}{(S_0 - S_n)} \quad (1)$$

where Y_{obs} is the observed cell yield (g of CDW (mole of substrate)$^{-1}$), X_0 is the initial cell concentration (g of CDW l^{-1}), X_n is the final cell concentration (g of CDW l^{-1}), S_0 is the initial

substrate concentration (mole of substrate l^{-1}) and S_n is the final substrate concentration (mole of substrate l^{-1}). Thus for this work, Y_{obs} is defined as the ratio of biomass increase to substrate consumption during exponential growth.

During growth, cells must also invest a part of the primary substrate for maintenance purposes (Pirt 1975). The diversion of energy and electrons to these non-growth activities leads to observed yields that are lower than true yields. Y_{obs} can be converted to the true maximum yield by estimating cell maintenance or decay effects:

$$Y_{true} = \frac{(\mu + b)}{\mu} \times Y_{obs} \quad (2)$$

where Y_{true} is the true cell yield (g of CDW (mole of substrate)$^{-1}$), μ is the specific growth rate (h^{-1}), and b is the cell decay constant (h^{-1}). When μ is much higher than b, the effect of cell decay on the observed yield is not significant. However if μ is close or lower than b, the observed yield will be much lower than the true yields. Henneken et al. (1998) reported that μ_{max} of the co-culture BNC1-BNC2 on EDTA varied between 0.03–0.07 h^{-1} and the cell decay constant was 0.0042 h^{-1}. Bally et al. (1994) reported that μ_{max} of *C. heintzii* (*A. aminovorans*) on NTA was 0.18 h^{-1} and the cell decay constant was 0.0048 h^{-1}. Thus, when growing optimally, the co-culture BNC1-BNC2 and *A. aminovorans* yields show little effect of maintenance costs. In this work, previously reported cell decay constants were used to convert the observed yields to true yields for comparison with model-predicted theoretical maximum yields.

Results and discussion

The BNC1-BNC2 co-culture precultured with EDTA degraded ED3A, N,N′-EDDA, ED, IDA, glycine, and glyoxylate immediately but sometimes was inhibited by ED3A even though sufficient Ca^{2+} and Mg^{2+} were present in solution to fully complex the ED3A. Kluner et al. (1998) reported that a pure culture of the BNC1 species precultured with EDTA could degrade ED3A, IDA, glycine and glyoxylate, but not N,N′-EDDA and ED. This was consistent with our experimental results where the co-culture BNC1-BNC2 was needed to degrade N,N′-EDDA and ED. *A. aminovorans* precultured with NTA degraded IDA, glycine, and glyoxylate without a time lag as previously reported for *C. heintzii* by many investigators (Kampfer et al. 2002; Bally et al. 1994; Bolton et al. 1996).

Yields for EDTA and its biodegradation intermediates

A typical growth curve from our experiments for the BNC1-BNC2 co-culture growing on EDTA as the sole source of carbon and energy is shown in Fig. 2. Five sampling points were approximately evenly distributed across the growth curves for each investigated substrate (EDTA, NTA, or their biodegradation intermediates). The error between the duplicate samples at each time point was always less than 1% for all experiments. Triplicate batch experiments were conducted for each substrate. The specific growth rate was estimated from these data as 0.0675 h^{-1} (R^2 = 0.9997). The observed yield and true yield were calculated following Eqs. (1) and (2) for each batch run. The average observed yield of triplicate measurements was 75.0 ± 1.5 g of CDW (mole of EDTA)$^{-1}$ and the average true yield was 80.0 ± 1.5 g of CDW (mole of EDTA)$^{-1}$. Henneken et al. (1998) previously reported the average observed yield of BNC1-BNC2 co-culture on

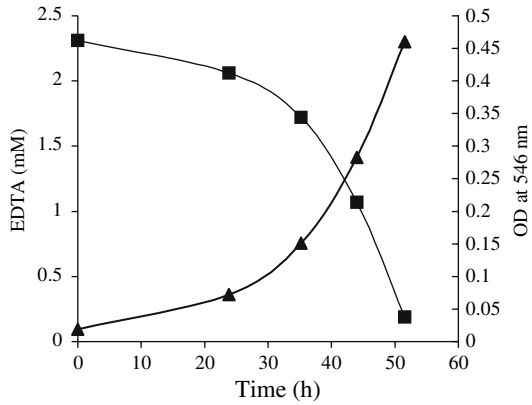

Fig. 2 Typical growth curve of BNC1-BNC2 co-colture on EDTA. Solids squares are EDTA in mM. Solids triangles are OD measured at 546 nm against deionized water

EDTA was 0.243 g of CDW (g of EDTA·H_4)$^{-1}$ (measured in batch reactors), i.e., 71.0 g of CDW (mole of EDTA)$^{-1}$, and the true yield was 0.271 g of CDW (g of EDTA·H_4)$^{-1}$ (measured in chemostat), i.e., 79.1 g of CDW (mole of EDTA)$^{-1}$. Thus our results are consistent with previous work for growth of the co-culture BNC1-BNC2 on the primary substrate EDTA. Table 1 presents the measured yields of BNC1-BNC2 co-culture on EDTA and its biodegradation intermediates (ED3A, N,N′-EDDA, IDA, ED, glycine, and glyoxylate). As expected, yields for downstream intermediates are all *less* than the yield on the primary substrate, EDTA. From the pathway for EDTA biodegradation (see Fig. 1a), assuming the oxygenase-catalyzed transformations are not energy-generating and result in no cell growth, the yield for ED3A might be expected to be ''1 glyoxylate'' less than the yield on EDTA and the yield on N,N′-EDDA might be expected to be ''2 glyoxylates'' less than the yield on EDTA. This is not precisely accurate; however, as the oxygenase-catalyzed steps require the addition of electrons (delivered by the electron carrier, FMN or FAD). Of the four electrons invested in reducing molecular oxygen in the oxygenase-catalyzed reaction, two are from an electron carrier and two are from the reduction of one of the carbon atoms in the substrate. The electron carrier must ultimately be regenerated, thus utilizing electrons released during the subsequent biodegradation of the glyoxylate and reducing the bacterial yield on this substrate.

The oxidase-catalyzed steps, likewise, affect the yield. IDA oxidase is a soluble flavin-containing oxidase that likely removes two electrons from the substrate with reduction of the flavin (FAD to $FADH_2$). The reduced flavin is then re-oxidized by molecular oxygen in the cytoplasm (Liu et al, 2001). This re-oxidation, however, is not tied to the electron transport system and is not expected to generate energy for cell synthesis. Thus, these electrons are lost to the cell, much as the electrons invested in the oxygenation reaction are lost.

The general pattern of observed yields moving down the pathway suggests the yields for substrates are approximately additive, confirming that although significant energy is liberated in the reaction that inserts oxygen into the organic compound and energy could be liberated when the flavin in the oxidase is re-oxidized, this energy is NOT captured by the organisms or available for cell growth. The yield for growth of the co-culture on IDA is also given in Table 1. IDA is not an intermediate in the EDTA biodegradation pathway, but the utilization of IDA oxidase for transformation of IDA in the co-culture is in direct contrast to the use of IDA dehydrogenase by *A. aminovorans*. Modeling these different pathways and the effect on predicted yield is discussed below.

Yields for NTA and its biodegradation intermediates

A typical growth curve from our experiments for *A. aminovorans* growing on NTA as the sole source of carbon and energy is shown in Fig. 3. Sampling points were distributed across the growth curve, and duplicate measurements had less than 1% difference for all experiments. The specific growth rate was estimated from these data as 0.122 h^{-1} (R^2 = 0.9998). The average

Table 1 Measured yields of BNC1-BNC2 co-culture on EDTA and its degradation intermediates

Substrate	μ (h^{-1})	Average observed yield (g of CDW/mole of substrate)	True yield computed from observed yield (g of CDW/mole of substrate)
EDTA	0.053 ~ 0.068	75.0 ± 1.5	80.0 ± 1.5
ED3A	0.032 ~ 0.048	68.6 ± 4.2	76.1 ± 3.3
N,N′-EDDA	0.034 ~ 0.050	51.2 ± 1.9	56.7 ± 1.4
ED	0.032 ~ 0.038	34.5 ± 1.1	38.6 ± 1.4
IDA	0.060 ~ 0.063	26.3 ± 0.4	28.2 ± 0.4
Glycine	0.106 ~ 0.129	12.2 ± 0.1	12.7 ± 0.1
Glyoxylate	0.102 ~ 0.121	9.7 ± 0. 4	10.1 ± 0.4

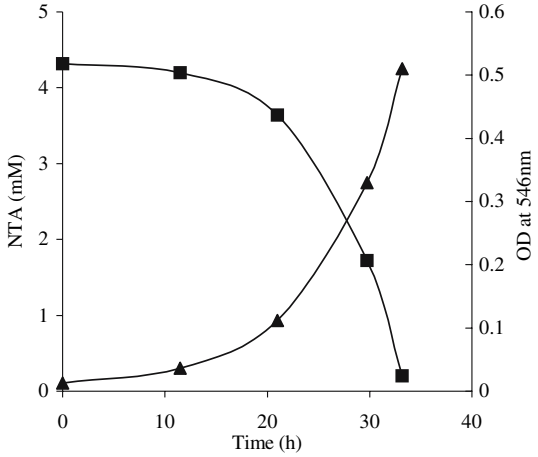

Fig. 3 Typical growth curve of *A. aminovorans* on NTA. Solids squares are NTA in mM. Solids triangles are OD measured at 546 nm against deionized water

observed yield of triplicate measurements was 44.3 ± 1.1 g of CDW (mole of NTA)$^{-1}$ and the average true yield was 46.0 ± 1.1 g of CDW (mole of NTA)$^{-1}$. Bally et al. (1994) previously reported the true yield of *C. heintzii* (*A. aminovorans*) on NTA was 0.24 g of CDW/g of NTA, i.e., 45.8 g of CDW (mole of NTA)$^{-1}$, at dilution rate greater than 0.11 h^{-1} (measured in chemostat). Thus our results are consistent with previous report for the primary substrate NTA. Weilenmann et al. (2004) reported the average observed yield for DSM 9103 was 0.11 g of CDW (g of NTA), which is much lower than *C. heintzii* (*A. aminovorans*). The possible reason is that NTA is not the preferred substrate for DSM 9103. Table 2 presents the measured yields of *A. aminovorans* on NTA and its biodegradation intermediates (IDA, glycine, and glyoxylate). Again, the downstream intermediates have lower yields than the primary chelate as expected. The pathway for NTA biodegradation in *A. aminovorans* (see Fig. 1b) suggests that the yield for IDA might be ''1 glyoxylate'' less than the yield for NTA assuming again that the oxygenase-catalyzed reaction in the first step is not energy-generating. This effect is observed. However, considering the second step, a dehydrogenase is used for transformation of IDA to glycine and glyoxylate. Two reducing equivalents are produced in this step and would be used for energy generation for growth as they are transferred to a common electron carrier that can be coupled to the electron transport system. Thus, the yield on IDA would *not* be expected to equal the yield on glycine plus the yield on glyoxylate. Again, this expectation is observed in the data where the IDA yield is 32% higher than the sum of the yields of glyoxylate and glycine.

Different yields for N,N′-EDDA, IDA, glycine and glyoxylate

N,N′-EDDA, IDA, glycine and glyoxylate yields are given in Tables 1 and 2 for the different bacterial strains. Glycine and glyoxylate are simple carbon sources, and yields have been previously measured with other microorganisms. Previously reported yields on glycine and glyoxylate are 0.198 g of CDW (g of glycine)$^{-1}$ (Engelbrecht and McKinney 1957), i.e., 14.9 g of CDW (mole of glycine)$^{-1}$, and 0.146 g of CDW (g of glyoxylate)$^{-1}$ (Rutgers et al. 1989), i.e., 10.8 g of CDW (mole of glyoxylate)$^{-1}$, respectively. N,N′-EDDA and IDA are less common substrates. However, Weilenmann et al. (2004) reported that the average observed yields for DSM 9103 (measured in batch) are 0.31 g CDW (g of IDA)$^{-1}$, i.e., 41.2 g CDW (mole of IDA)$^{-1}$, and of 0.29 g CDW (g of N,N′-EDDA)$^{-1}$, i.e., 51.0 g of CDW (mole of N,N′-EDDA). As shown in Table 2, yields for

Table 2 Measured yields of *A. aminovorans* on NTA and its degradation intermediates

Substrate	μ (h^{-1})	Average observed yield (g of CDW/mole of substrate)	True yield computed from observed yield (g of CDW/mole of substrate)
NTA	0.120 ~ 0.125	44.3 ± 1.1	46.0 ± 1.1
IDA	0.154 ~ 0.160	37.9 ± 0.5	39.1 ± 0. 5
Glycine	0.053 ~ 0.076	15.2 ± 0.2	16.2 ± 0.4
Glyoxylate	0.073 ~ 0.102	10.4 ± 1.1	11.0 ± 1.1

Table 3 Comparison of measured true yields and model predicted true yields for substrates degraded via oxygenase-catalyzed and oxidase-catalyzed reactions

Compound	Number of oxygenase reactions	Number of oxidase reactions	True yield computed from observed yield (g of CDW/mole of substrate)	True yield predicted by oxygenase-modified model (g of CDW/mole of substrate)	
BNC1-BNC2 co-culture					
EDTA	2	2	80.0	79.2	–1%
ED3A	1	1	76.1	69.0	–9.2%
N,N′-EDDA	0	2	56.7	58.8	+3.6%
ED	0	0	38.6	32.4	–16%
IDA	0	1	28.2	31.1	+10.5%
A. *aminovorans*					
NTA	1	0	46.0	45.5	–1.2%
IDA	0	0	39.1	35.4	–9.5%

(Yield prediction is based on an efficiency of energy capture of 0.41 (Xiao and VanBriesen 2005) and a cell formula of $CH_2O_{0.6}N_{0.2}$ (Andrews 1989) representing 90% of CDW. Error calculated as: $\text{Error} = \frac{\text{Predicted Yield–Measured True Yield}}{\text{Measured True Yield}} \times 100\%$)

A. aminovorans measured here are very close to these previously reported values for IDA, glyoxylate and glycine (less than 8% different). The average observed yield for BNC1-BNC2 co-culture measured here was 51.2 g of CDW (mole of N,N′-EDDA), very close to that of DSM 9103. However, the BNC1-BNC2 co-culture showed lower yields for the other substrates (Table 1). For IDA, the co-culture showed a 32% lower yield than *A. aminovorans* and a 44% lower yield than DSM 9103. Further, the co-culture showed a 6% lower yield on glyoxylate and a 20% lower yield on glycine when compared with *A. aminovorans*. The reason for the differences in glyoxylate and glycine yields is not known; however, for the case of IDA, enzymatic differences may play a role.

Degradation pathway effect on cell yield for IDA

The results in Tables 1 and 2 show significant yield difference for IDA for the two bacterial strains. The measured yield of the BNC1-BNC2 co-culture on IDA was 26.3 g of CDW (mole of IDA)$^{-1}$ while the measured yield of *A. aminovorans* on IDA was 37.9 of CDW (mole of IDA)$^{-1}$, a difference of 36%.

Uetz and Egli (1993) reported *C. heintzii* (*A. aminovorans*) utilizes a membrane-bound IDA dehydrogenase to catalyze the cleavage of IDA into glycine and glyoxylate. This reaction does not require the investment of electrons and energy, and releases 2 electrons to intracellular carriers. However, Liu et al. (2001) reported the isolation of a soluble IDA oxidase from BNC1. The oxidase, as discussed above, involves the reduction of the flavin-moiety with subsequent re-oxidation in the cytoplasm by molecular oxygen. This reaction removes two electrons from the mineralization pathway and the oxidation of the flavin is not coupled to the electron transport chain and thus does not produce energy for growth. Since microorganisms utilize reducing equivalents to produce energy to drive cell synthesis reactions, the loss of the electrons in the oxidase reaction results in fewer electrons available for cell growth reactions when BNC1 grows on IDA compared with growth of *A. aminovorans* on the same substrate. Thus, the same compound degraded through different pathways by different bacterial species is expected to produce different cell yields, and this was observed.

Comparison of measured yields with model predicted yields

Previously, Yuan and VanBriesen (2002) published a model that considers the effect of oxygenase reactions on cell yield by modifying a thermodynamic yield prediction model originally

developed by McCarty (1975) and still widely used in environmental engineering (Rittmann and McCarty 2001). Xiao and VanBriesen (2005) further updated the model of Yuan and VanBriesen (2002) to incorporate a more complete carbon and nitrogen balance and to consider oxidase-catalyzed reactions. The original model (as described in Rittmann and McCarty 2001) is ''black box'' and no pathway information is used to predict the yield for a given substrate. The updated model (as described most recently by Xiao and VanBriesen, 2005) considers the diversion of energy and reducing equivalents in enzymatic reactions, when they are known. Thus, as shown in the degradation pathways (Fig. 1), EDTA requires two oxygenase-catalyzed steps and two oxidase-catalyzed steps and NTA requires only one oxygenase-catalyzed step. For each substrate evaluated, the yield was predicted using putative pathway information and is shown in Table 3.

It is critical to understand that while the pathway and enzymes involved in NTA biodegradation by *A. aminovorans* are well characterized, there are still uncertainties in the EDTA pathway by pure cultures (BNC1 and DSM9103) and there are even more uncertainties in the pathway and enzymes for the mixed culture. Thus, the yield predictions here are based on the assumption that the initial steps are catalyzed by EDTA-monooxygenase (with loss of four reducing equivalents per step) and the subsequent steps are catalyzed by IDA oxidase (with loss of 2 reducing equivalents per step). If this pathway is subsequently proven to be incorrect, the yield predictions would need to be updated based on the new information

Yield prediction is based on an efficiency of energy capture of 0.41 (Xiao and VanBriesen 2005) and a cell formula of $CH_2O_{0.6}N_{0.2}$ (Andrews 1989) representing 90% of CDW. The average error of yield prediction for substrates degraded via oxygenase and oxidase reactions in the EDTA biodegradation pathway is 4.6% and in the NTA biodegradation pathway is 1.2 % for substrates in the NTA biodegradation pathway. These predictions show significant improvement over previous prediction models that were ''black box'' (see for example, VanBriesen 2001; Yuan and VanBriesen 2002).

Conclusions

This work presents a systematic measurement of growth yields for EDTA, NTA, and their biodegradation intermediates by chelate-degrading bacteria. The yields reported here for the primary substrates (EDTA and NTA) were consistent with previous work. This is the first measurement of yields for ED3A and ED by any bacterial species and the first measurement of yields for glycine and glyoxylate by chelate-degrading microorganisms. Further, this is the first report of yields for a mixed-culture on N,N′-EDDA and IDA. The yields for intermediates in the biodegradation pathways of EDTA and NTA indicate oxygenase- and oxidase- catalyzed reactions divert electrons from energy generation or cell synthesis pathways. The dehydrogenase-catalyzed cleavage of IDA in the NTA pathway results in a higher yield than the same transformation when catalyzed by an oxidase enzyme in the EDTA pathway. The comparison of measured yields with model yields demonstrates that the effect of oxygenase- and oxidase-catalyzed reactions in biodegradation pathways can be predicted using thermodynamics.

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