Biotransformation of micropollutants: kinetics, threshold and residual concentrations

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Stellingen

1. Het gebruik van de term 'threshold' ('drempel') van een organische verbinding waar de drempelconcentratie voor groei van een bacteriepopulatie op deze verbinding wordt bedoeld, wekt ten onrechte de indruk dat deze organische verbinding beneden deze concentratie niet door de betreffende bacteriën kan worden omgezet.
   Alexander, M. (1994). Biodegradation and Bioremediation. Academic Press Inc., San Diego, USA.

2. De ruime zendtijd die is toebedeeld aan weerberichten op radio en televisie staat in geen verhouding tot de geringe voorspellingskracht van weersvooruitzichten.

3. Het verdient de voorkeur om voor de gekke-koeienziekte BSE - naar analogie met het Duits - de term 'runderwaanzin' in te voeren, aangezien men door deze term meer gestimuleerd wordt zich af te vragen waar en wanneer de waanzin in de (intensieve) veehouderij nu eigenlijk is begonnen.

4. De versoepeling van de kledingvoorschriften voor promovendae, die in de laatste decennia heeft plaatsgevonden aan de meeste Nederlandse universiteiten, doet niet alleen recht aan de voortgaande ontwikkelingen in het kleedgedrag van vrouwen, maar ook aan het al eeuwenoude spreekwoord: 'Een zotte vrouw kent men aan haar rokje'.

5. Het gemiddelde menselijke bioritme van ongeveer 25 uur manifesteert zich het duidelijkst in het gevoel van de meeste mensen iedere dag weer veel te vroeg te moeten opstaan.
   Intermediair, 11 oktober 1996.

6. De maatschappelijk omstreden opsporing van genetische factoren die medebepalend zijn voor homoseksueel gedrag bij de mens (M/V), zou de wereldwijde acceptatie van homoseksualiteit als natuurlijk menselijk verschijnsel als enig doel moeten hebben.

7. Schaarste aan de genotmiddelen koffie en thee in een omgeving waar men toch al kampt met voedsel- en waterschaarste is een geluk bij een ongeluk: van thee krijg je honger en van koffie krijg je dorst.

8. Het meest opvallende gevolg van het toenemende aantal commerciële zenders op het televisienet is de tijdverspilling. Deze betreft zowel de tijd die door de zenders wordt gebruikt om over elkaar te praten, als de tijd die het kost om erachter te komen dat er niets nieuws geboden wordt.

9. Wanneer de visie dat klassieke muziek alleen uitgevoerd dient te worden door musici uit het land van herkomst van de componist(e), werkelijkheid zou worden, zou dit het einde betekenen van de meeste Nederlandse muziekgezelschappen.

10. De opvallend vaak voorkomende verwisseling van Nederland, Kopenhagen, Denemarken en Amsterdam door inwoners van de Verenigde Staten doet eerder een fout vermoeden in de gebruikte schoolboeken of leermethoden dan een grote overeenkomst tussen de beide Europese landen en bevolkingsgroepen.

11. De boudste stellingen worden nooit gedrukt.

Stellingen behorende bij het proefschrift "Biotransformation of micropollutants: kinetics, threshold and residual concentrations" van Marijke E. Tros.

Wageningen, 4 december 1996.

Ik wil graag iedereen, zowel binnen als buiten de werkkring, bedanken die mij op welke wijze dan ook heeft geholpen bij het tot stand brengen van dit proefschrift.

I would like to thank everyone in both private and working environment, who supported me during the realization of this thesis.

Gerne möchte ich mich bei allen die mich - innerhalb und ausserhalb meines Arbeitkreises - bei dieser Dissertation unterstützt haben, bedanken.

Contents

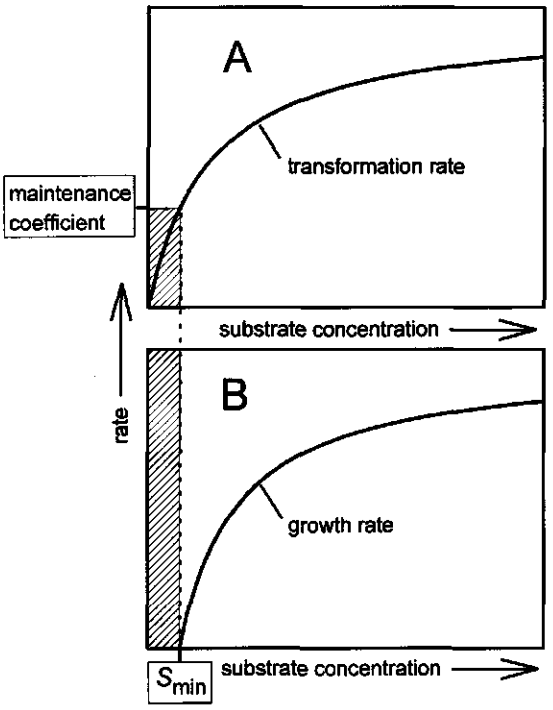
Chapter 1

General Introduction

This thesis describes the results of a research project entitled "Microbial transformation of xenobiotic compounds at low concentrations in the soil: role of the threshold concentration". The study started in 1988 and was financed for four years and four months by The Netherlands Integrated Soil Research Programme. The aim of this project was twofold: (i) examination of the biotransformation kinetics of xenobiotic compounds in the low concentration range, and (ii) search for the lowest attainable concentration, and study of the factors which determine such residual concentrations.

The research project arose from previous joint studies by the Department of Microbiology in Wageningen and the Municipal Water Works of Amsterdam. These studies evaluated the fate and biodegradation of a range of chlorinated organic contaminants during infiltration and passage through soil. These chemicals occur as

Figure 1 Saturation kinetics of substrate transformation (A) and bacterial growth (B). For reasons of clarity, the figure exaggerates the differences between the curves at low substrate concentrations. The transformation rate just satisfies the maintenance requirement of the biomass at concentration S_{min}. Net bacterial growth is then zero, and the transformation rate equals the maintenance coefficient.



micropollutants in water of the river Rhine (Table 1) and are undesirable since this water is used as a source for drinking water. Percolated soil columns with the indigenous organisms demonstrated the ability to degrade several of these xenobiotics under different operating conditions. Degradation of many compounds was only partial, however, and residual concentrations could still be detected (16). This gave rise to questions about the cause of such residuals, and about transformation kinetics at low, environmentally relevant concentrations. Knowledge about transformation processes at low concentration levels is important in the setting of standards for the highest allowed pollutant concentrations in soil, groundwater and waste water. Furthermore, this information is essential in the decision about the feasibility of certain bioremediation techniques.

Several other studies reported low concentration levels below which biodegradation did not occur or was much slower than predicted. Some of these studies focussed on the role of the threshold concentration for growth, S_{min}, at which the transformation rate just satisfies the maintenance energy demand of the bacterial population (Fig. 1; 1, 13, 95, 103). Others suggested involvement of a threshold for induction of the necessary enzymes for uptake or transformation, the presence of easily degradable substrates or a limiting bioavailability (16, 106, 120, 135). Most of these studies were done with mixed microbial populations in undefined soil or freshwater samples, however, precluding the possibility to provide conclusive evidence on the factors that determined and influenced the observed residual concentrations.

We therefore decided to investigate residual pollutant concentrations and transformation kinetics using a pure culture in a defined medium. We used *Pseudomonas* sp. strain B13 as our model organism, and 3-chlorobenzoate (3CB) and acetate as model pollutant and easily degradable substrate, respectively. Our approach was to use experimental systems with different levels of complexity (Fig. 2). This would give us the possibility to distinguish the influence of intrinsic microbial properties and system-linked factors on the kinetics and residual concentrations.

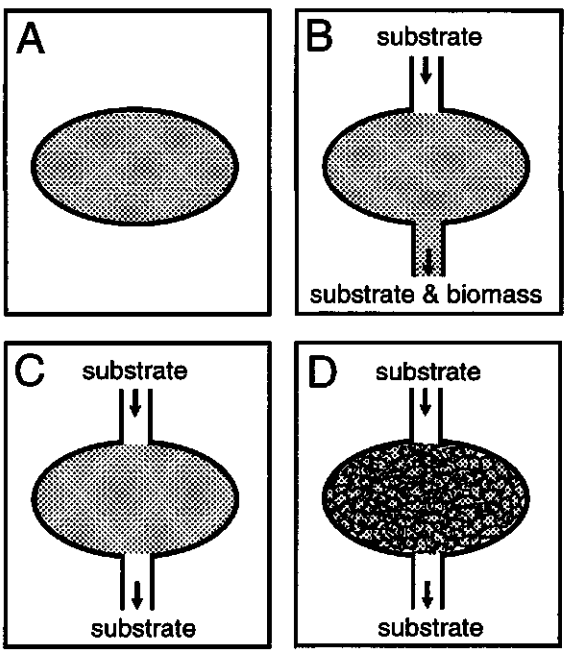
The outline of this thesis is as follows. Chapter 2 reports on the transformation of 3CB and acetate in batch systems with resting cell suspensions of strain B13 (Fig. 2A). Two different systems for 3CB uptake or transformation were found to be active in the nanomolar and low micromolar range. The simultaneous transformation of acetate did not affect the 3CB conversion kinetics and no residual concentrations of either substrate were detected in batches with resting cells. Chapter 3 deals with consumption of 3CB and acetate in well-mixed fermentor systems with continuous substrate replenishment (Fig. 2B and C). The lowest attainable residual concentration in fermentors with 100% biomass retention was S_{min}, in a stationary situation of net zero growth. The S_{min} values of both 3CB and acetate turned out to be lower during simultaneous utilization than in single substrate use. Measured S_{min}

Table 1 Maximum concentrations of a number of (chlorinated) aromatic compounds in Dutch surface waters (the rivers Rhine and Meuse and lake IJsselmeer), and in the drinking water that is prepared from these surface waters. The data were collected in the years 1987 to 1991 (136).

Pollutant	Surface water[a]	Drinking water[a]
anilines		
aniline	xx	x
2-chloroaniline	xx	x
3-chloroaniline	x	n.d.
4-chloroaniline	x	<
2,3-dichloroaniline	xx	<
2,4-dichloroaniline	x	<
3,4-dichloroaniline	xx	<
3,5-dichloroaniline	xx	<
2,4,5-trichloroaniline	xx	x
benzenes		
benzene	xxx	<
chlorobenzene	x	<
1,2-dichlorobenzene	xx	xx
1,3-dichlorobenzene	x	n.d.
1,4-dichlorobenzene	xx	n.d.
trichlorobenzene	x	<
tetrachlorobenzene	x	<
benzoates		
benzoate	xx	xxx
3-chlorobenzoate	xx	n.d.
2,4-dichlorobenzoate	xx	n.d.
tetrachlorobenzoate	x	x
phenols		
phenol	x	n.d.
chlorophenol	x	n.d.
2,4- or 2,5-dichlorophenol	xx	<
3,4-dichlorophenol	x	<
3,5-dichlorophenol	xx	<
2,4,5-trichlorophenol	x	<
2,4,6-trichlorophenol	x	<
3,4,5-trichlorophenol	x	<
2,3,4,5-tetrachlorophenol	x	<
2,3,4,6-tetrachlorophenol	x	<
2,3,5,6-tetrachlorophenol	x	<
pentachlorophenol	x	<

[a] < : below detection limit; x : below 0,1 $\mu g \cdot liter^{-1}$; xx : 0,1 to 1 $\mu g \cdot liter^{-1}$; xxx : 1 to 10 $\mu g \cdot liter^{-1}$; n.d.: not determined.

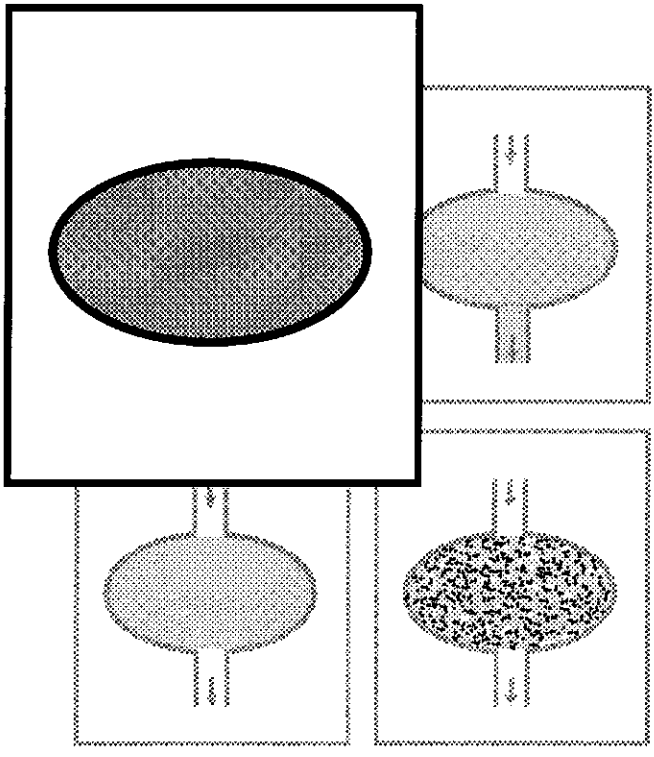
Figure 2 Schematic representation of the used experimental systems. The batch systems (A) contained a resting cell suspension and an initial amount of substrate. Chemostat cultures (B) were continuously fed with substrate and other nutrients. The produced biomass and the residual substrate were continuously removed. All biomass was retained in the recycling fermentor (C). This biomass was continuously fed with substrate and other nutrients. The used medium with residual substrate was continuously removed. Resting bacterial cells were retained within percolation columns (D) by attachment to sand particles. The columns were operated with continuous inflow of fresh substrate, and outflow of residual substrate.



values were compared with the values predicted from kinetic parameters obtained with steady-state chemostats (Chapter 3) and resting cell batches (Chapter 2). The knowledge on intrinsic microbial kinetics was used in spreadsheet models predicting the transformation activity of strain B13, attached to soil particles in percolation columns (Fig. 2D; Chapter 4). Mass transfer of substrate and oxygen are discussed as the processes limiting biodegradation and causing much higher experimental effluent concentrations than predicted (Chapter 4). Finally, the results presented in this thesis are discussed in a framework of relevant literature on residual and threshold concentrations, and transformation kinetics at low substrate concentrations (Chapter 5).

Chapter 2

Transformation of low concentrations of 3-chlorobenzoate by Pseudomonas sp. strain B13: kinetics and residual concentrations



Marijke E. Tros, Gosse Schraa and Alexander J.B. Zehnder
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Abstract

The transformation of 3-chlorobenzoate (3CB) and acetate at initial concentrations in the wide range of 10 nM to 16 mM was studied in batch experiments with *Pseudomonas* sp. strain B13. Transformation rates of 3CB at millimolar concentrations could be described by Michaelis-Menten kinetics (K_m 0.13 mM; V_{max} 24 nmol·mg of protein^{-1}·min^{-1}). Experiments with nanomolar and low micromolar concentrations of 3CB indicated the possible existence of two different transformation systems for 3CB. The first transformation system operated above 1 μM 3CB, with an apparent threshold concentration of 0.50 ± 0.11 μM. A second transformation system operated below 1 μM 3CB and showed first order kinetics (rate constant 0.076 l·g of protein^{-1}·min^{-1}) with no threshold concentration in the nanolmolar range. A residual substrate concentration, as has been reported for some other *Pseudomonas* strains, could not be detected for 3CB (detection limit 1.0 nM) in batch incubations with *Pseudomonas* sp. strain B13. The addition of various concentrations of acetate as a second, easily degradable substrate neither affected the transformation kinetics of 3CB nor induced a detectable residual substrate concentration. Acetate alone also showed no residual concentration (detection limit, 0.5 nM). The results presented indicate that the concentration limits for substrate conversion obtained by extrapolation from kinetic data at higher substrate concentrations may underestimate the true conversion capacity of a microbial culture.

Introduction

The production and use of chlorinated organic chemicals has increased dramatically in the last few decades, and concomitantly, large amounts of these chemicals have been discharged into the environment. Numerous chlorinated organic contaminants are almost ubiquitously present at low concentrations in soil, groundwater, and wastewater (nanomoles to micromoles per liter), though some are intrinsically biodegradable. Obviously, there are factors which prevent a complete degradation of these pollutants. These factors also set limits to the applicability of bioremediation techniques for the cleanup of contaminated sites.

The biodegradation kinetics at low, environmentally relevant concentrations can differ significantly from the kinetics at higher concentrations. Some individual bacterial strains seem to cause multiple degradation kinetics for different concentration ranges. Transformation of methyl parathion by a *Flavobacterium* sp. involved at least two transformation systems, one operating below a concentration of 20 μg·l^{-1} and another one operating below a concentration of 4 mg·l^{-1} (67). According to several studies, the presence of a second, easily degradable organic substrate can have both inhibiting and enhancing effects on the transformation rate. In slurries of aquifer solids, the mineralization of toluene, *p*-nitrophenol or ethylene

dibromide at a 100-$\mu g \cdot kg^{-1}$ concentration was inhibited when glucose or amino acids (at 0.1 and 2 $mg \cdot kg^{-1}$, respectively) were added (120). Batch studies with a *Pseudomonas* sp. strain showed an increase in the utilization rate of methylene chloride (0.01 to 1 $mg \cdot l^{-1}$) when 1 mg of acetate $liter^{-1}$ was amended (61).

Residual concentrations in the aqueous phase (i.e., concentrations that remain after biodegradation has stopped) have been observed for some xenobiotic compounds under various experimental conditions (61, 80, 106, 135). But for more common substrates, such as acetate or benzoate, residual concentrations have also been found (45, 52, 95). After the initial degradation of 40 μg of 1,3- and 1,4-dichlorobenzene and 1,2,4-trichlorobenzene $liter^{-1}$ by a *Pseudomonas* strain in batch incubations, residual concenrations of 10 to 20 $\mu g \cdot l^{-1}$ could still be observed (135). In batch studies with a *Pseudomonas* strain degrading 10 μg of methylene chloride $liter^{-1}$, a residual concentration of ca. 2.5 $\mu g \cdot l^{-1}$ could be reduced significantly in the presence of 1 mg of acetate $liter^{-1}$ (61). Initial concentrations below the measured residuals were not tested in these studies. Up to now, little about the processes causing a residual concentration in aerobic batch systems has been outlined.

This paper presents data on residual substrate concentrations and transformation kinetics in a batch system using *Pseudomonas* sp. strain B13 to degrade 3-chlorobenzoate (3CB). It is shown that below a concentration of about 1 μM 3CB, kinetic parameters which are different from the parameters that govern the degradation of 3CB at higher concentrations are valid. Residual concentrations of 3CB in the batches could not be observed within the range of detection. The presence of acetate as an additional, easily degradable substrate neither affected the transformation kinetics nor induced a detectable residual 3CB concentration.

Materials and methods

Medium and culture conditions

Pseudomonas sp. strain B13, which can use 3-chlorobenzoate as a sole energy and carbon source, was previously isolated from a wastewater treatment plant (28). The cells were pregrown aerobically at 20°C on a rotary shaker in 2.3 l Erlenmeyer flasks (culture volume, 500 ml). The mineral salts medium consisted of (per liter of demineralized water) 2.86 g of $Na_2HPO_4 \cdot 2H_2O$, 1.46 g of KH_2PO_4, 1 g of NH_4NO_3, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.05 g of $Ca(NO_3)_2$ and 1 ml of a trace-element solution (as modified from reference 152). The solution contained (per liter of demineralized water) 2.0 g of $FeCl_3 \cdot 6H_2O$, 0.05 g of H_3BO_3, 0.05 g of $ZnCl_2$, 0.05 g of $MnCl_2 \cdot 4H_2O$, 0.05 g of $CuSO_4 \cdot 5H_2O$, 0.05 g of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.05 g of $AlCl_3$, 0.05 g of $CoCl_2 \cdot 6H_2O$, 0.05 g of $NiCl_2$, 0.5 g of Na-EDTA, 0.05 g of $Na_2SeO_3 \cdot 5H_2O$, 0.05 g of Na_2WO_4, 0.05 g of $Na_2MoO_4 \cdot 2H_2O$ and 1 ml of concentrated HCl. For growth, 3CB was added from a 0.5 M stock solution of both 3CB and NaOH (final concentration, 5 mM). The pH in the growth medium was about 6.9. For the experiments with acetate alone, cells were pregrown on 6 mM acetate. In the late exponential growth phase, cells were harvested aseptically by centrifugation (10,200 × *g*), washed twice in mineral

medium, and resuspended in the same medium. Purity checks were done by plating bacteria on nutrient agar.

Before use, all glassware was cleaned with a 5% solution of $K_2Cr_2O_7$ in 50% H_2SO_4. The mineral salts medium was prepared with highly purified water (Milli-Q Systems; Millipore Co., Bedford, Mass.).

Transformation kinetics experiments

Transformation rates of 3CB at three different initial concentration ranges - 1.5 μM to 16 mM 3CB, 1 to 5 μM 3CB, and 100 nM to 3 μM 3CB - were measured. In this last study, 100 nM 3CB of the total 3CB applied was added as ^{14}C-labeled 3CB, giving a total activity of 2,300 dpm/ml in each bottle. To the middle range, acetate at 0, 50, 500 and 5,000 μM was added, whereas the lowest range received 0 or 500 μM acetate.

A series of 100-ml serum bottles with 20 ml of sterile mineral medium was inoculated with washed cell suspension, giving a final cell concentration of up to 10^9 cells·ml^{-1}. The bottles were sealed with a viton septum and incubated stationary for 20 h at 20°C. This incubation was done to allow the residual intracellular substrate as well as residual carbon in the medium to be degraded.

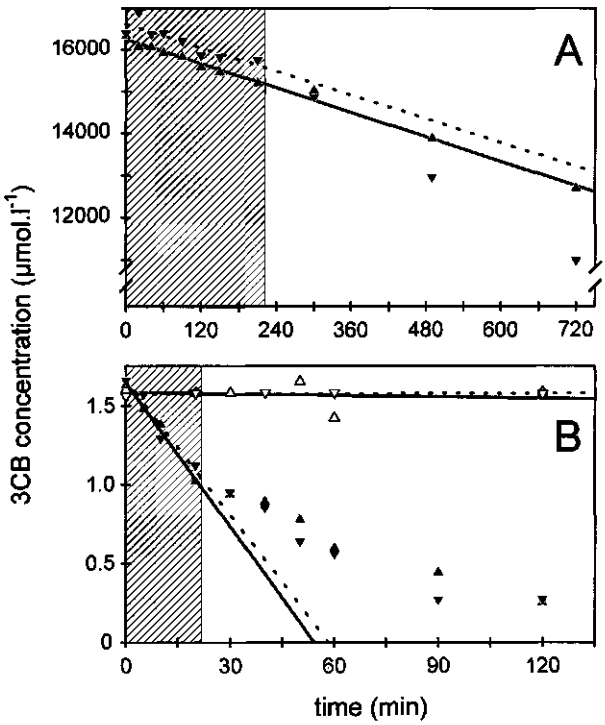

Figure 1 3CB depletion curves in the millimolar (A) and micromolar (B) ranges. Regression through the linear part of the curves (shaded) gives the initial transformation rates. Cells were incubated in NaCl-phosphate buffer (▲, solid regression line) and mineral medium (▼, dashed regression line). Open triangles represent the values obtained with sterile controls.

Starvation experiments had demonstrated that cells remained fully active for at least 24 h after harvesting. After starvation, each bottle was sampled to measure protein (initial biomass concentration). A few bottles were checked for purity. The substrate was provided as a solution of 3CB-NaOH or a mixture of acetate and 3CB-NaOH in mineral medium. Bottles with medium and substrate but without cells were used as controls. At the start of the experiment, and after all the manipulations, the total volume of each culture was 24 ml. Cultures were incubated on a rotary shaker at 20°C. Each bottle was regularly sampled (1-ml aliquots) during periods varying from 20 minutes to 12 h, depending on the initial substrate concentration. The data in the linear part of the depletion curve (four to eight datum points) were used for calculating the initial transformation rates (Fig. 1). Incubations in NaCl-phosphate buffer were compared with incubations in mineral medium, and this comparison showed that in this linear part of the depletion curve, transformation rates were not yet affected by growth of the bacteria (Fig. 1). Only in the mineral medium at the high concentration range was an effect of growth seen after 300 min of incubation. Conversion of the 3CB in the samples was stopped by acidification with HCl (final pH, 1.5).

Adsorption of 3CB to the cell material did not affect the disappearance of the compound, even at the lowest concentration range. At the start of the experiments, cell suspensions and controls without cells contained equal 3CB concentrations. Adsorption of 3CB to the cell material could not be observed in a test in which cells were inactivated with Na-azide.

Residual concentration experiments

The occurrence of a residual substrate concentration was investigated in three different batch experiments. Each experiment consisted of about 60 independent batch incubations in which 3CB, acetate, or a mixture of 3CB and acetate was tested. In the first experiment, 3CB was added at an initial concentration of 1.2 μM or 11 nM. The second set of batches was incubated with 8.6 μM or 85 nM acetate. In the third experiment, 1.5 μM 3CB was added in combination with 23 μM acetate. To be able to detect a low residual concentration, ^{14}C-labeled substrates were used. The third experiment consisted of two series: one with [^{14}C]3CB combined with cold acetate and a second with cold 3CB combined with [^{14}C]acetate. To test whether any residual concentration was associated with the radiolabel alone (e.g., nondegradable impurities), a set of batches was spiked for a second time with the same amount of labeled substrate after 4 h of incubation. Sterile controls with chemical concentrations in both the micromolar and nanomolar ranges as well as unlabeled controls were included in all experiments to check for abiotic disappearance of the compounds and for contamination with the radiolabel during the experimental procedure.

A series of Hungate tubes (Bellco, Vineland, N.J.) containing 4 ml of sterile mineral medium and sealed with butyl rubber stoppers was inoculated with washed cell suspension to give a final cell concentration of about 10^9 cells·ml^{-1} in the experiments with 3CB. In the experiment with acetate alone, the concentration of bacteria was 10^7 cells·ml^{-1}. After a 20-h starvation period, four of the batches were sacrificed to verify the purity and the number of cells. The experiment was started by adding the substrate to the incubations. The total final volume was 5 ml. All tubes were incubated stationary at 20°C. Substrate degradation was followed for 3 days. Samples were taken in duplicate by sacrificing two tubes at a time. Microbial activity was stopped by injecting sodium azide solution through the septum (final concentration, 25 mM).

Analytical procedures

(i) Transformation kinetics. Samples from the experiments done with 3CB concentrations in the millimolar and micromolar ranges were centrifuged at 10,000 × *g* with a table centrifuge (Beun de Ronde, Amsterdam, The Netherlands). The supernatant was analyzed for 3CB by

high-performance liquid chromatography (HPLC) (LKB 2150 pump and LKB 2152 controller; LKB, Woerden, The Netherlands). A total of 20 μl was injected into a ChromSphere C_8 reversed-phase column (Chrompack, Middelburg, The Netherlands). 3CB was detected by UV A_{206} (LKB 2158 Uvicord SD), and quantified by an SP 4290 computing integrator (Spectra Physics Inc., San Jose, Calif.). The mobile phase was a mixture of acetonitrile and 5 mM H_2SO_4 in water with a volume ratio of 40:60 and a flow rate of 0.6 ml·min^{-1}. Acetate concentrations in these experiments were determined with the same HPLC equipment and under the same conditions. The column, an RT300-6,5 PolySphere OAHY organic acids column, was run at 60°C (Merck, Darmstadt, Germany) with 5 mM H_2SO_4 in water as the mobile phase at 0.6 ml·min^{-1}.

Experiments with concentratins in the nanomolar range were done with ^{14}C-labeled 3CB. Acidified samples (final pH, 1.5) were left open for 4.5 h in a safety hood to release all labeled CO_2. Thereafter, no $^{14}CO_2$ was detectable in the medium. 3CB concentrations were not influenced by this $^{14}CO_2$ removal. Cells were removed by centrifugation with a table centrifuge (10,000 $\times$ g). Unlabeled 3CB (100 μM) was added to the supernatant. The cold 3CB allowed UV detection and prevented loss of labeled 3CB. Supernatant (200 μl) was injected into a ChromSphere C_8 reversed-phase column. The mobile phase was a mixture of acetonitrile and 5 mM H_2SO_4 (30:70) at a flow rate of 0.5 ml·min^{-1}. Fractions (1 ml each) were captured and counted in 4.5 ml of Aqualuma scintillation cocktail (Lumac, Olden, Belgium) in an LKB Wallac scintillation counter (LKB). Quench corrections were made by the external standard-channel ratio method.

Control experiments showed that the different analytical methods used fro the separate concentration ranges gave the same results. The data could therefore be directly compared.

(ii) Residual concentrations. Before analysis, a known amount of unlabeled 3CB or acetate was added to each batch to minimize losses of the radiolabel during the analytical procedure. After they were mixed, the batches were brought to pH 1.5 with HCl and $^{14}CO_2$ was stripped from the solution by purging it for 12 min with 30 ml of air min^{-1}. Longer purging did not result in higher $^{14}CO_2$ recovery. Controls showed no loss of either acetic acid or 3-chlorobenzoic acid. $^{14}CO_2$ was trapped in a 1 M NaOH solution. After the suspensions were purged, the pHs of the suspensions with 3CB were increased to 6.9 to dissolve the 3-chlorobenzoic acid precipitate that had been formed at pH 1.5.

The amount of the radiolabel incorporated in the biomass was determined by filtering 0.5-ml suspensions through 0.45-μm-pore-size filters. Filters were washed with 5 ml of a 10 mM solution of unlabeled substrate in mineral medium. The filters were dissolved in 4.5 ml of scintillation fluid, and the radioactivity was counted.

For the analysis of 3CB and acetate, each of the remaining suspensions was filtered (filter pore size, 0.45 μm), brought to pH 11.5 with NaOH, freeze-dried, and dissolved again in 100 μl of 0.8 M H_2SO_4 solution. This procedure concentrated the 3CB or acetate by a factor of about 50. Acetate and 3CB were separated by HPLC, and the fractions containing them were analyzed for radioactivity.

Biomass measurements

Protein measurements in the transformation kinetics experiments were done according to the method of Lowry et al. (70), with bovine serum albumin being used as the standard protein (Boehringer, Mannheim, Germany). In the residual concentration experiments, biomass was determined by direct microscopic counting with a Bürker Türk counting chamber.

Chemicals and radiochemical purity

[1-^{14}C]sodium acetate (53 mCi·mmol^{-1}) was obtained from Amersham International plc (Buckinghamshire, England). The radiochemical purity reported by the manufacturer was 99.0%. 3-Chlorobenzoic acid as well as 3-chlorobenzoic acid-ring-UL-^{14}C (10.8 mCi·mmol^{-1}) were purchased from Sigma Chemical Company (St. Louis, Mo.). The radiochemical purity reported by the manufacturer was >98%.

3-Chlorobenzoate was contaminated with about 1% 4-chlorobenzoate, which cannot be utilized by strain B13. This contamination was positively identified with a 4-chlorobenzoate standerd after 3CB biodegradation. The radiolabeled 3CB was contaminated with 0.9 ± 0.4% of a nondegradable (by strain B13) compound which comigrated with 3CB in the HPLC analysis. The compound is presumably also 4-chlorobenzoate. The contamination was quantified by repeatedly adding [^{14}C]3CB to a B13 culture. Each addition increased the radioactive, nondegradable residual by about 0.9% of the [^{14}C]3CB supplied. [^{14}C]acetate was tested in the same way for purity. It contained 0.6 ± 0.3% of compound which can be very slowly degraded by strain B13 and which comigrated with acetate.

All other chemicals were of analytical grade and were used without further purification.

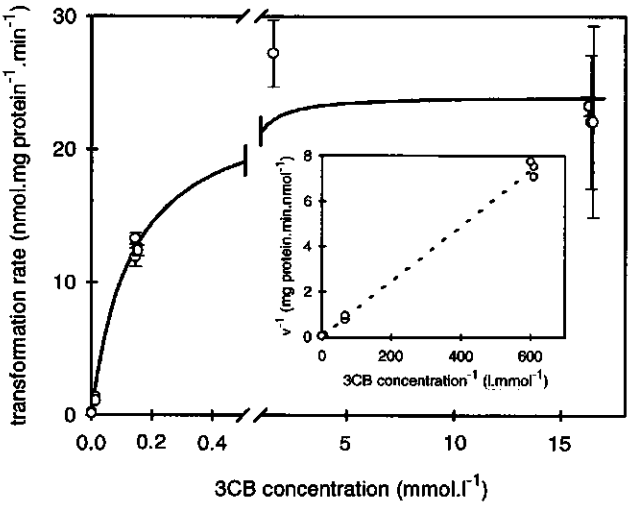

Figure 2 Kinetics of 3CB transformation in the 3CB concentration range from 1.5 μM to 15 mM. The data are presented as a direct plot and as a Lineweaver-Burk linearization (inset). Error bars show standard deviations. v^{-1} is the inverse 3CB transformation rate.

Results

Transformation kinetics

3CB is converted by *Pseudomonas* sp. strain B13 following Michaelis-Menten kinetics over a wide concentration range from 1.5 μM to 16 mM (Fig. 2). Each of the initial transformation rates presented in Fig. 2 was based on at least four datum

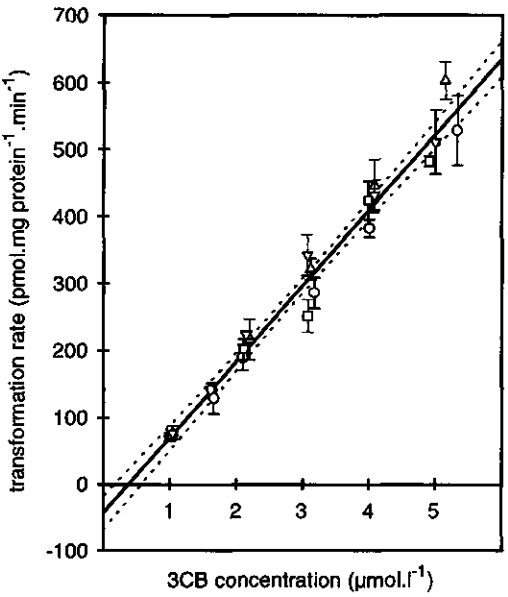

Figure 3
Kinetics of 3CB transformation in the 3CB concentration range from 1 to 5 μM in the presence of 0 (○), 50 (▽), 500 (△) and 5,000 (□) μM acetate. Regression through all the data is presented with a 95% confidence interval. Error bars show standard deviations.

points in the linear part of the substrate depletion curve. Nonlinear regression analysis according to the Michaelis-Menten model yielded an apparent half saturation constant K_m of 0.13 mM and a maximum specific transformation velocity V_{max} of 24 nmol·mg of protein^{-1}·min^{-1}.

At 3CB concentrations far below the half-saturation constant, the Michaelis-Menten model approaches a first-order relationship with V_{max}/K_m as the first-order rate constant. Experiments in this lower concentration range were done to investigate whether this pattern of the Michaelis-Menten model was valid for the transformation rates at initial concentrations of a few micromoles per liter. A linear relationship at the concentration range of 1 to 5 μM was indeed observed, with a rate constant 0.113 l·g of protein^{-1}·min^{-1} (Fig. 3). Each of the initial transformation rates presented in Fig. 3 was based on four or five datum points in the linear part of the substrate depletion curve. Extrapolation of these data gives an intercept on the x axis, with a threshold concentration for transformation of about 0.5 μM. This value differs significantly from zero, as was calculated with a 95% confidence interval. In the third, independent experiment, which used a 3CB concentration range from 100 nM to 3μM, a threshold of ca. 0.5 μM can also be calculated by linear regression through the transformation data for the range from 1 to 3 μM 3CB (Fig. 4). This regression results in the same first order rate constant as that for Fig. 3. Below 1 μM, however, conversion followed a linear relationship, with a first order rate constant of only 0.076 l·g of protein^{-1}·min^{-1}.

The influence of an easily degradable additional substrate was studied by incubating the cells with different concentrations of acetate in addition to micromolar concentrations of 3CB. Acetate and 3CB analyses showed that simultaneous uptake of acetate and 3CB occurred in these incubations. The transformation rate of 3CB was not affected by the presence and simultaneous uptake of acetate, as is demonstrated in Fig. 3. At the lowest 3CB concentration range from 100 to 500 nM, transformation rates were also not affected by the presence of acetate (Fig. 4).

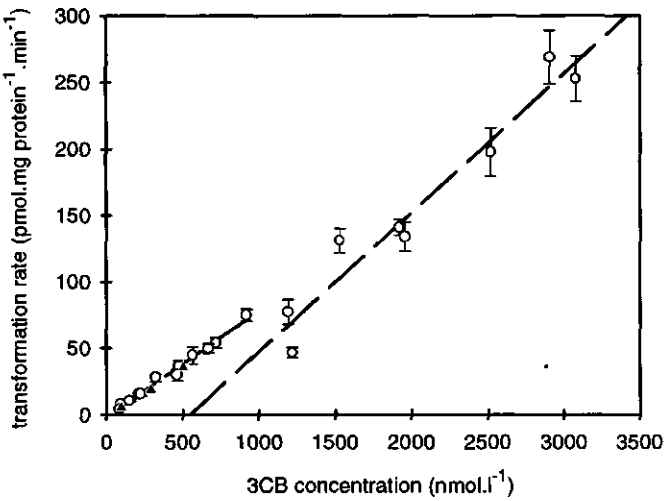

Figure 4 Kinetics of 3CB transformation in the 3CB concentration range from 0.1 to 3 μM in the presence of 0 (○) and 500 (▲) μM acetate. Symbols without error bars indicate datum points with standard deviations that are so small that they fall within the size of the symbols used.

Residual concentrations

As part of the search for a lower limit for the degradation of 3CB, residual concentration experiments were carried out down to the lower nanomoles-per-liter range. Figure 5 shows the disappearance of radiolabeled 3CB over time, expressed as 3CB equivalents of the radiolabel. Each datum point represents the measurement of one individual tube sacrificed at a given incubation time.

In the batches with an initial concentration of 1.2 μM 3CB, the residual detected was about 1.2% of the 3CB added; thus, the residual represented the contamination. After 48 h, the cells were still active. They degraded a second 3CB spike at a rate comparable to that for the first addition (12 nmol·l^{-1}·min^{-1} after the first spike and 13.3 nmol·l^{-1}·min^{-1} after the second spike) (Fig. 5).

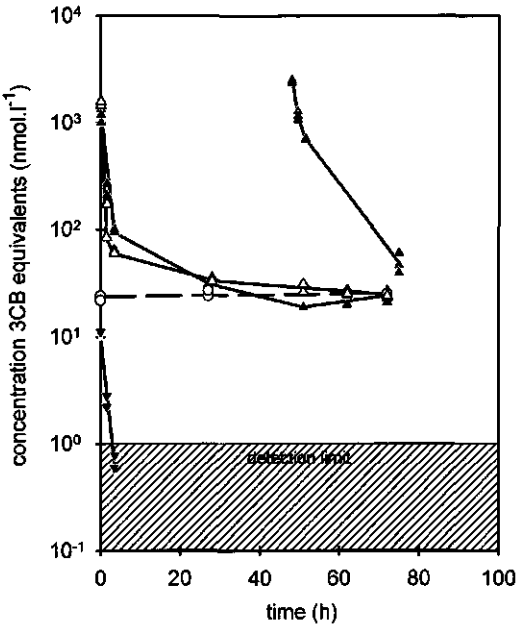

Figure 5
Transformation of 3CB over time, expressed as 3CB equivalents of radiolabel that were measured in the HPLC fraction with 3CB. Cells were incubated with [^{14}C]3CB alone at two different substrate concentration levels (solid triangles). The activity of the cells was tested with a second spike after 48 h. Open triangles correspond to the radiolabel in the incubations with [^{14}C]3CB and 23 μM cold acetate. Open circles represent the values obtained with sterile controls.

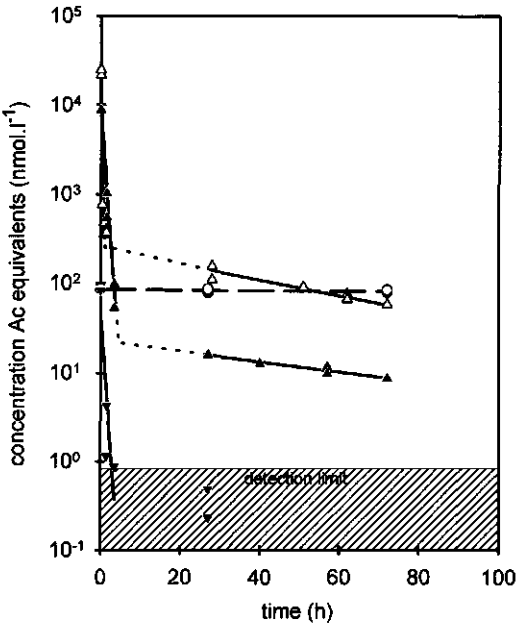

Figure 6
Transformation of acetate over time, expressed as acetate (Ac) equivalents of radiolabel that were measured in the HPLC fraction with acetate. Cells were incubated with [^{14}C]acetate alone at two different concentration levels (solid triangles). Open triangles correspond to the radiolabel in the incubations with [^{14}C]acetate and 1.5 μM cold 3CB. Open circles represent the values obtained with sterile controls.

When the incubations were started with 11 nM 3CB, 3CB disappeared immediately. After a few hours, the label was below the detection limit of about 1.0 nmol·l^{-1}. Table 1 shows that the total amount of radiolabel that had been added in this low-concentration series was recovered as $^{14}CO_2$, as biomass, or as dissolved compounds. In the batches in which all 3CB had been degraded (after 27 to 72 h of incubation), 10% of the ^{14}C was recovered as biomass and 8% of the ^{14}C was collected in HPLC fractions other than the 3CB fraction. The amount of $^{14}CO_2$ produced was 82%.

Similar results were obtained in batch experiments with acetate as the only substrate (Fig. 6). An initial concentration of 8.6 μM acetate was rapidly transformed to about 0.3% of the radiolabel added, which was followed by a very slow disappearance of this residual label. This residual corresponded to the contamination. Sterile controls at the nanomolar level did not show any loss of label. A 100-times-lower initial acetate concentration showed an analogous pattern but with a 50- to 100-times-lower residual concentration, which was around the detection limit (about 0.5 nmoles of acetate equivalents per liter [Fig. 6]).

In the third experiment, in which both 3CB and acetate were added, both substrates were transformed simultaneously (Fig. 5 and 6). The observed residual radiolabel was ca. 1.3% of the initial label in the incubations with [^{14}C]3CB and cold acetate. In the suspensions with [^{14}C]acetate and cold 3CB, the acetate was transformed to about 0.9% of the initial radiolabel. This residual label subsequently disappeared at a very slow rate. Both residuals represented the contaminations. It can therefore be concluded that the presence of an additional substrate did not markedly influence the residual radioactivity.

Table 1 Mass balance of radiolabel in the incubation series at an initial concentration of 11 nM.

Label category	Amt of label ± S.D. (dpm)	Percentage
Total label added in 14 incubations	40,700 ± 2,100	100 ± 5
Total label recovered in solution or as biomass	10,700 ± 3,200	26 ± 8
Label cumulatively trapped as $^{14}CO_2$[a]	32,900 ± 6,900	81 ± 17
Total label recovered	43,600 ± 10,100	107 ± 25

[a] The trapped $^{14}CO_2$ was measured after all 14 incubations of this series, which were sacrificed at different time intervals, were purged.

Discussion

The data presented here suggest that *Pseudomonas* sp. strain B13 possesses two different uptake or transformation systems for 3CB. Our experimental setup did not allow us to distinguish the transport and transformation kinetics. Only experiments with membrane vesicles could give the necessary insights. Therefore, in the following text, transformation system for strain B13 should be read as combined uptake and transformation systems. Multiphasic kinetics of uptake or conversion of substrate have been reported for a number of organisms (33, 47, 67, 138, 148). In most of these studies, the individual kinetic systems can be described by separate sets of Michaelis-Menten parameters. The systems working for the high-concentration range are often found have high capacities with regard to their maximal uptake and transformation rates but with a low affinities for the substrate. Systems active at low concentrations can be categorized as high-affinity, low-capacity systems.

At 3CB concentrations in the millimolar range, the transformation kinetics of strain B13 can be described by a Michaelis-Menten type model. However, for predicting transformation rates at concentrations below 0.1 mM 3CB, an extended Michaelis-Menten model should be used: $V = V_{max} \times S/(K_m+S) - a$, where V is the transformation rate, S is the substrate concentration, and a is the virtual negative conversion rate. This equation is analogous to the growth model of Monod, extended with a term for maintenance (137). The virtual negative conversion rate a at a zero substrate concentration does not have a practical meaning, but the essence of this equation is to describe transformation kinetics at low concentrations. Far below the K_m value, this model approaches the linear relationship $V = S \times V_{max}/K_m - a$, and predicts a substrate threshold concentration of $a \times K_m/V_{max}$. Combination of all the data for the 3CB concentration range of 1 to 5 μM (Fig. 3 and 4) gives an apparent threshold concentration of 0.50 $\pm$ 0.11 μM for *Pseudomonas* sp. strain B13 and 3CB.

Interestingly, *Pseudomonas* sp. strain B13 could convert 3CB at the concentration range of 0.1 to 0.9 $\mu mol \cdot l^{-1}$ apparently by using another transformation system. The kinetics of this system are first order, which is not in agreement with the high-affinity, low-capacity systems described for other organisms (47, 67). Possibly, only the first part of a Michaelis-Menten relationship was observed, and at concentrations higher than 1 μM 3CB, the second transformation system was taking over. The data suggest that only one of these systems was operating at a time. The kinetic data around 1 μM 3CB do not show the curvature that would indicate the summation of two transformation systems operating simultaneously. A similar conclusion was drawn for conversion of methyl parathion by a *Flavobacterium* species (67).

No effects of the presence and simultaneous uptake of acetate on the transformation kinetics of 3CB could be demonstrated. This result is in disagreement with those of other studies in which both enhancing and adverse effects of readily degradable substrates on the transformation rate of specific xenobiotic compounds was seen (10, 61, 102, 120, 126).

Residual substrate concentrations above the detection limits for 3CB and acetate (1.0 and 0.5 nmol·l^{-1}, respectively) could not be detected for *Pseudomonas* sp. strain B13 in batch studies. This observation contradicts observations from batch studies with other organisms (52, 106, 135). In aerobic liquid batch cultures, a residual concentration in the lower nanomoles-per-liter or picomoles-per-liter range is not likely when adsorption or diffusion processes are not limiting the availability of the substrate. At 3CB and acetate concentrations around our detection limits, $\Delta G'$ values of -3,134 kJ per mole of 3CB and -815 kJ per mole of acetate can be calculated for their total oxidation (at around 10 mM 3CB and acetate, concentrations at which aerobic cultures are often grown, the $\Delta G'$ values are -3,175 kJ·mol^{-1} for 3CB and 858 kJ·mol^{-1} for acetate). This outcome is in contrast with those of acetate conversions in methanogenic systems, in which the limits for the change in free energy are clearly the cause for the observed residual concentration (52).

A bacterial cell has to meet a substrate molecule to enable biodegradation. The number of collisions between particles A and B (Z_{AB}) is given by the formula $Z_{AB} = N_A N_B d_{AB}^2 [8\pi kT(m_A+m_B)/m_A m_B]^{1/2}$, in which N is the concentration, d_{AB} is the average diameter, k is the Boltzmann constant, T is the absolute temperature, and m the mass of the particles. At a substrate concentration of around 0.1 nmol·l^{-1} (0.1 pmol·ml^{-1}) at 20 °C and a cell concentration of 10^9 cells·ml^{-1}, each cell collides around 10^7 times per s with substrate molecules. This frequency is more than sufficient for an active metabolism if we consider the 24-h starvation period, after which cells of strain B13 are still fully active. As long as we assume the Michaelis-Menten concept to be correct, namely, that for a reaction to occur one substrate molecule has to meet one enzyme, two factors will determine a possible residual concentration for a given time period. First, there is the frequency with which substrate molecules make successful contacts with the enzymes converting them. Obviously, the time period between two successful contacts should be shorter than the average lifetimes of the enzymes. Unfortunately, data on enzyme decay rates are extremely scarce, and no relevant data on the enzymes of the 3CB degradation system exist. Second, the change in free energy must be sufficiently negative for the overall conversion to allow the cell to maintain a necessary proton motive force. In our case, the second prerequisite is certainly fulfilled, and on the basis of the fact that we could not detect a residual concentration in this study, it can be assumed that the first one is fulfilled too.

A residual concentration in a resting cell suspension is something entirely different from the residual concentration or minimum substrate concentration for growth (S_{min}) in a continuous system or a system with a growing cell population. A residual substrate concentration may be related to the substrate concentration required for the induction of the responsible enzymes. In chemostats, the residual concentrations at steady state are determined by the growth kinetic parameters of the organism. For mixtures of glucose, fructose and galactose Lendenmann (64) could show that in case of *Escherichia coli* growing in a chemostat at 0.3 h^{-1}, the residual concentrations of each sugar added up to the residual substrate concentration, which was obtained with only one of the sugars as the single substrate. S_{min} has been defined as the substrate concentration at which the substrate flux into the cells equals the maintenance requirement of the entire bacterial population, i.e., the substrate concentration at which the cell population stays constant. For chemostats, S_{min} values can be obtained by extrapolation of the data at low specific growth rates (22). In a continuous biofilm reactor, Rittmann and McCarty could measure no significant biofilm activity once the steady-state substrate concentration equaled the predicted value for S_{min} (95). Continuously fed soil columns, repeatedly inoculated with a *Pseudomonas* strain, showed residual effluent concentrations of about 70 nM 1,2-dichlorobenzene, independent of the feeding concentration. Subsequent batch incubation of the effluent, however, could reduce this residual to the detection limit of 0.7 nM (135). This result illustrates that residual substrate concentrations obtained in batches under nongrowth conditions are generally lower than residual concentrations obtained in continuous systems.

For *in situ* bioremediation of polluted aquifers the treatment has to be, at least partly, continuous. A regular supply of limiting nutrients or primary substrates is necessary to maintain a microbial population which (co)metabolically or after primary substrate depletion degrades a pollutant. A feasible method to bring the residual contaminant concentration below the S_{min} of the bacterial population is a pulsing substrate addition. Systems with pulses of the limiting substrates can be regarded as a series of batch incubations between the successive pulses. A well-studied example of a pulsing system is the one involving the degradation of chlorinated ethenes in an aquifer to which methane (electron donor) or oxygen (electron acceptor) were added as a growth stimulator at specific time intervals (113). On the basis of the data available up to now, fed batch or pulsing systems are superior to continuously fed systems for the attainment of low pollutant concentrations, providing that adsorption and diffusion do not limit the availability of the substrate.

Acknowledgments

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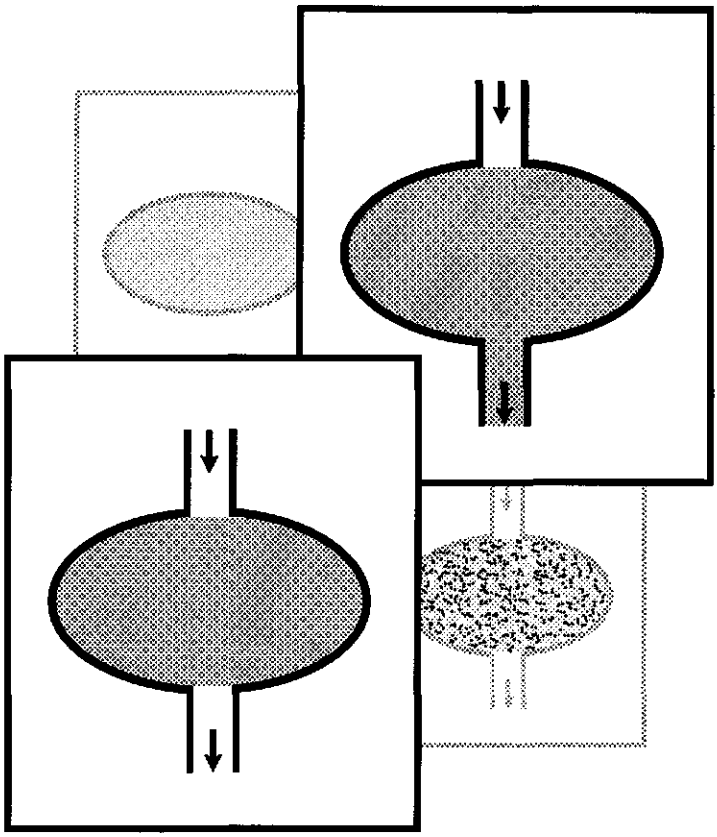
We acknowledge Mike S.M. Jetten for technical advice and Tom N.P. Bosma for helpful discussions.

Chapter 3

Measurement of minimum substrate concentration (S_{min}) in a recycling fermentor and its prediction from the kinetic parameters of *Pseudomonas* sp. strain B13 from batch and chemostat cultures



Marijke E. Tros, Tom N.P. Bosma, Gosse Schraa and Alexander J.B. Zehnder

Applied and Environmental Microbiology 62: 3655-3661 (1996)

Abstract

The minimum substrate concentration required for growth, S_{min}, was measured for *Pseudomonas* sp. strain B13 with 3-chlorobenzoate (3CB) and acetate in a recycling fermentor. The substrates were provided alone or in a mixture. Predicted S_{min} values using kinetic parameters from resting cell batches and chemostat differed clearly from the measured values. When fed as a single substrate, the measured S_{min} value of both 3CB and acetate was higher than the individual S_{min} values in the mixture. S_{min} in the mixture reflected the relative energy contribution of the two substrates in the fermentor feed. The energy based maintenance coefficients during zero growth in the recycling fermentor were comparable for all influent compositions (0.34 ± 0.07 J·mg $dw^{-1}·h^{-1}$). Maintenance coefficient values for acetate were significantly higher in chemostat than in recycling fermentor experiments. 3CB maintenance coefficients were comparable in both experimental systems. The parameters for 3CB consumption kinetics varied remarkably between the experimental growth conditions in batch, chemostat and recycling fermentor. The results demonstrate that the determination of kinetic parameters in the laboratory for prediction of microbial activity in complex natural systems, should be done under conditions which mimic best the system under consideration.

Introduction

The widespread production and use of chlorinated organic chemicals in the last few decades has raised serious concern about their occurrence and persistence in soil, groundwater and wastewater. For numerous chlorinated organic contaminants, biodegradation is the only way to eliminate them from the environment. Although many chlorinated organic compounds are intrinsically biodegradable, they are widespread in soil, groundwater and natural waters at low concentrations (nanomoles to micromoles per liter) (1). Obviously, there are factors which prevent a complete degradation of these pollutants. The phenomenon of these low, persisting concentrations is one of the factors which limits the application of bioremediation techniques for the clean-up of contaminated sites.

Residual concentrations in the water phase (i.e. concentrations that remain after initial biodegradation) have been observed for both xenobiotic and natural substrates under various experimental conditions (16, 52, 61, 95, 135). In methanogenic systems, the observed residual concentration can often be explained by limitations in the free energy change of the degradation reaction (52). Aerobic degradation reactions are usually sufficiently exergonic and need other explanations for the observed residual concentrations. Several authors have suggested that residual concentrations are due to limiting diffusion kinetics (15, 103), the maintenance energy demand of the organisms (15, 103), the minimum concentration required for

enzyme induction (63, 106) and environmental factors like the presence of additional substrates (102).

It is generally accepted that the residual substrate concentrations in carbon-limited chemostats at steady state are directly related to the dilution rate (i.e. the growth rate) and growth kinetic parameters of the microorganisms. The most widely used model for this relation is the one originally proposed by Monod (51, 73). This one is occasionally extended with a negative term, which represents endogenous respiration for maintenance purposes (12, 137). Rittmann incorporated the concept of van Uden in a model for steady-state biofilm kinetics. This model successfully predicted a steady-state substrate concentration S_{min} below which the biofilm could not be maintained (95, 96). S_{min} is the substrate concentration at which the net bacterial growth is zero, and is generally calculated by extrapolating chemostat growth data to zero growth (22, 59).

A direct measurement of S_{min} requires a continuous system with zero net growth. Such a situation would develop in a dialysis culture of bacteria when they are retained and the medium would be refreshed continuously (108). The maintenance energy requirement would then theoretically prevent unlimited growth of the biomass. This concept has been used for the design of a recycling fermentor (26). A recycling fermentor is continuously fed with substrate, but the biomass is retained for 100% in the reactor. Growth in a recycling fermentor was found to proceed discontinuously, and three growth phases with progressively lower growth rates could be distinguished (25, 139). A fourth growth phase with zero growth was described in later recycling fermentor studies (75, 128).

The objective of this study was to achieve a situation of zero growth for *Pseudomonas* sp. B13, and to verify the S_{min} predicted from kinetic parameters obtained from chemostat and batch experiments. Growth substrates were 3-chlorobenzoate (3CB) and acetate and were provided as single substrates or as a mixture. The kinetic parameters for 3CB consumption (i.e. the combination of uptake and conversion) from recycling fermentor, chemostat and batch systems were compared.

Materials and methods

Medium and preculture conditions

Pseudomonas sp. B13 has been isolated from a sewage treatment plant using a chemostat (28). This organism is able to grow on 3-chlorobenzoic acid as a sole carbon and energy source. The cells were pregrown aerobically in 100 ml serum bottles containing 25 ml of mineral medium (129) amended with acetate (15 mM final concentration) or 3CB (5 mM final concentration) on a rotary shaker at 20°C. Cells were pregrown on acetate for the experiments with acetate as the only test compound. The pH of the growth medium was about 6.9.

The medium used for the continuous cultures and the recycling fermentors was prepared in 8 liter quantities, and contained in addition 25 $mg.l^{-1}$ yeast extract and 1 $ml.l^{-1}$ vitamin solution (107). The substrate concentration in this medium was 15 mM of acetate or 5 mM of 3CB. For the experiments with mixed substrates, the growth medium contained 7.5 mM of acetate plus 2.5 mM of 3CB. Cells from the mid to late exponential growth phase were used for the inoculation (ca. 1%) of batch, chemostat and recycling fermentor experiments. The inoculum was plated on nutrient agar to check for purity.

Batch experiments

For batch-wise determination of the apparent growth yield (Y) and μ_{max}, cells were grown in 2.3 l erlenmeyer flasks (500 ml culture volume) on a rotary shaker at 20°C. Either 3CB or acetate was used as growth substrate. Growth was monitored by optical density measurements at 623 nm (A_{623}) in at least two independent cultures. For growth yield determination, the initial conditions were fixed by addition of HCl to samples of the cultures immediately after seeding. The final pH of 1.5 stopped substrate conversion effectively. Samples from the late exponential phase were used to determine the final substrate and biomass concentrations.

Chemostat experiments

Two liter bioreactors (Applikon Dependable Instruments b.v., Schiedam, The Netherlands), with a culture volume of about 1 liter, were used for the carbon- and energy-limited continuous culture experiments. The cultures were thermostated at 20°C, continuously aerated and stirred at 1000 rpm. The pH in cultures on acetate was kept at 6.9 with HCl. Correction of the pH of cultures growing on 3CB was not necessary since the pH never dropped below 6.7. In all chemostat experiments pH checks were regularly done outside the fermentor. The purity of the culture was monitored by plating on nutrient agar.

Each run at a specific dilution rate (D, h^{-1}) was started with a newly sterilized fermentor, containing 1 liter of medium with the organic substrate. The fermentor was run batch-wise after inoculation with strain B13. When the onset of growth was clearly visible, the medium supply was started and a D was set. When steady state was reached, the culture was either harvested or μ_{max} was determined with a wash-out experiment. In none of the chemostat experiments, the number of volume changes exceeded 30. This to prevent mutant selection. Samples of the culture fluid and influent medium were brought to pH 1.5 to stop substrate conversion. The rest of the harvested culture was immediately used for biomass determination.

Recycling fermentor experiments

Three liter bioreactors (Applikon Dependable Instruments b.v., Schiedam, The Netherlands) were used for the recycling fermentor experiments under carbon- and energy-limitation. The cultures were thermostated at 20°C, continuously aerated and stirred at 1000 rpm. Influent medium was pumped into the fermentor and the working volume was kept at 1 liter by means of a liquid-level indicator, controlling an effluent pump. The effluent was withdrawn from the culture liquid through a 0.22 μm Durapore filter (Millipore, Etten-Leur, The Netherlands) that was fixed around a unit of sintered stainless steel. Both, liquid-level controller and recycling unit were constructed by the electronics and mechanics workshop of the Faculty of Biology, Free University of Amsterdam, The Netherlands (140). The pH in cultures on acetate was kept at 6.9 with HCl. No pH correction was necessary in cultures on 3CB. Purity and pH of the culture were checked routinely outside the reactor.

Each run was started with a newly sterilized fermentor, containing 1 liter of mineral medium. The fermentor was seeded with strain B13, the substrate supply was started and samples were taken regularly. The substrate provision rates (SPRs) were about 0.5 $mmol \cdot l^{-1} \cdot h^{-1}$ for acetate, 0.15 $mmol \cdot l^{-1} \cdot h^{-1}$ for 3CB, and about 50% of these values in the experiment with both substrates present. Less than 4% of the culture was used per sample to minimize disturbance of the culture. An aliquot was immediately brought to pH 1.5 to stop substrate conversion. The rest of the sample was immediately used to determine the biomass concentration (A_{623}) and the respiration activity (in runs with 3CB or acetate alone).

Activity measurements

Oxygen uptake rates were measured polarographically, with an oxygen electrode at 20°C (YSI Inc., Yellow Springs, Ohio). Initially, an 0.4 ml sample from the recycling fermentor was added to 4.5 ml of oxygen saturated mineral medium and the endogenous oxygen uptake was measured for 5 to 10 min. After addition of 0.1 ml substrate solution (initial concentrations, 0.33 mM 3CB or 1.0 mM acetate), oxygen uptake was followed for another 10 to 20 min.

Analytical methods

All samples for substrate analysis were centrifuged at 10,000 × *g*. The supernatant was analyzed for 3CB by High Performance Liquid Chromatography, as described previously (129). Different from (129), the volume ratio of the mobile phase was set at 30:70, acetonitrile:water. The water contained 5 mM H_2SO_4. The concentrations were determined using calibration curves generated with primary standards. The detection limit was about 1 μM 3CB.

Acetate concentrations in samples from chemostats with a $D > 0.1$ were determined with HPLC, as described previously (129). In all other cases acetate was analyzed with a Chrompack CP9001 gas chromatograph (Chrompack, Middelburg, The Netherlands). Samples (10 μl) were injected on a Chromosorb 101 column (130°C; molecular sieve, 80-100 mesh) and acetate was detected with FID. Carrier gas was N_2, saturated with formic acid (flow rate, 20 $ml \cdot min^{-1}$). The concentrations were determined using primary standard calibration curves. The detection limit was about 10 μM acetate.

Biomass concentrations in batch and chemostat samples were determined directly by dry weight measurements. Duplicate samples (100 to 250 ml) were centrifuged at 16,000 × *g* and washed with highly purified water (model D4700 Nanopure System; Barnstead, Dubuque, Iowa) to remove residual salts. The pellet was brought quantitatively on a dried and preweighed aluminum dish (about 1.5 g) and dried overnight (105°C). Each biomass sample contained 25 to 60 mg of biomass dry weight.

Biomass concentrations in the recycling fermentor experiments were calculated using calibration curves of biomass dry weight against A_{623}. Calibration curves for batch-grown biomass did not differ significantly from the calibration curves for biomass from different growth phases in a recycling fermentor. In addition, the different growth substrates 3CB and acetate yielded the same calibration curves.

Chemicals

All chemicals used were of analytical grade and were used without further purification.

Calculation of kinetic parameters

Maximum specific growth rates were determined with three independent methods. Direct measurements of μ_{max} were performed in batch experiments. In chemostat experiments μ_{max} was calculated from the rate of cell wash-out at $D > \mu_{max}$. With the steady-state substrate concentrations in chemostat experiments at different dilution rates, μ_{max} was estimated by non-linear parameter estimation (NPE) according to the Monod growth model. The values of the maximum or true growth yield Y_{max} [milligram of dry weight per millimole (mg dw·mmol^{-1})] and the maintenance coefficient m (mmol·mg dw^{-1}·h^{-1}) were obtained from the regression parameters of the relationship (86):

$$1/Y = 1/Y_{max} + m/\mu \tag{1}$$

with Y the apparent growth yield at different dilution rates ($\mu = D$) in a chemostat. The half-saturation constant K_s was estimated with NPE on chemostat data, according to the extended Monod model as proposed by van Uden (137):

$$\mu = \mu_{max}^{true} S/(K_s+S) - b \tag{2}$$

In this model μ_{max}^{true} represents the true maximum specific growth rate, which cannot be reached because of the maintenance costs:

$$\mu_{max}^{true} = \mu_{max} + b \tag{3}$$

in which b (h^{-1}) represents the endogenous biomass consumption for maintenance purposes. The maintenance coefficient m relates to b via Y_{max} (86):

$$b = mY_{max} \tag{4}$$

K_s was estimated according to eq. (2) with fixed values of μ_{max} and b.

The specific growth rate in the recycling fermentor was obtained from the growth curve of biomass (X, mg dw·l^{-1}):

$$\mu = dX/dt/X \tag{5}$$

and the consumption rate of substrate used for growth q_g (mmol·mg dw^{-1}·h^{-1}) is

$$q_g = \mu/Y_{max} \tag{6}$$

The overall substrate consumption rate q was calculated as

$$q = F(S_{in}-S)/(V_f X) \tag{7}$$

with F the flow rate of the influent (l·h^{-1}), S_{in} the substrate concentration of the influent (mmol·l^{-1}), and V_f the liquid volume in the fermentor (l). Thus, the maintenance coefficient in the recycling fermentor was calculated as

$$m = q - q_g \tag{8}$$

The maintenance coefficients were normalized to energy equivalents based on the work of Tijhuis and Heijnen (39, 124). The Gibbs energy for maintenance m_e (J·mg dw^{-1}·h^{-1}) was derived as:

$$m_e = m\Delta G' \tag{9}$$

with $\Delta G'$ the Gibbs energy of the substrate mineralization reaction with oxygen as the terminal electron acceptor. $\Delta G'$ values were calculated as -858 J·mmol^{-1} for acetate and -3,175 J·mmol^{-1} for 3CB.

The model for substrate consumption kinetics that is consistent with eq. (2), is of the Michaelis-Menten type (12):

$$q = q_{max}S/(K_s+S) \tag{10}$$

in which q_{max} is the maximum substrate consumption rate. The specific affinity a^0 for the substrate is

$$a^0 = q_{max}/K_s \tag{11}$$

Chemostat growth parameters were transformed into parameters of substrate consumption via

$$q_{max} = \mu_{max}^{true}/Y_{max} \tag{12}$$

Recycling fermentor data of independent duplicate experiments were pooled. The maximum growth rates measured in the first, exponential growth phase of a recycling fermentor, as well as the μ_{max} determined in batch were transformed similarly:

$$q_{max} = \mu_{max}/Y \tag{13}$$

with Y the apparent growth yield determined from batch experiments. Consumption rates determined in later growth phases of the recyling fermentor (eq. (7)) were used for kinetic parameter estimation with NPE according to eq. (10). Consumption kinetic parameters in resting cell batches with 3CB were derived from a study by Tros et al. (129).

Theoretical values of S_{min}, the minimum substrate concentration for growth, were calculated using either eqs. (2-4), with $\mu = 0$:

$$S_{min} = K_s m Y_{max}/\mu_{max} \tag{14}$$

or eq. (10), with $q = m$:

$$S_{min} = K_s m/(q_{max}-m) \tag{15}$$

For a situation with mixed substrates, the S_{min} of each substrate i was calculated analogous to Lendenmann (29, 64):

$$S_{min,i} = (S_{min})_{100\%,i} \times S_{in,i}\Delta G'_i/\Sigma(S_{in,i}\Delta G'_i) \tag{16}$$

in which $(S_{min})_{100\%,i}$ was calculated with the kinetic parameters of the individual substrates (eq. (14) or (15)) and $S_{in,i}\Delta G'_i/\Sigma(S_{in,i}\Delta G'_i)$ is the relative energy contribution of substrate i in the fermentor feed. Thus, 3CB and acetate were assumed to be perfectly substitutable, i.e. both substrates were assumed to satisfy the same essential needs of the cells (66).

Results

Batch and chemostat experiments

The kinetic parameters for growth of *Pseudomonas* sp. B13 on 3CB and acetate are listed in Table 1. Batch, chemostat wash-out experiments and NPE on chemostat data yielded about the same value for μ_{max}. The generally higher standard deviation for the μ_{max} value obtained with NPE is based on the high dependency of the two parameters μ_{max} and K_s. An accurate NPE of μ_{max} and K_s requires 8 to 10 data pairs (98). Since we had only 5 available, μ_{max} values from batch cultures were used for further calculations.

Recycling fermentor experiments

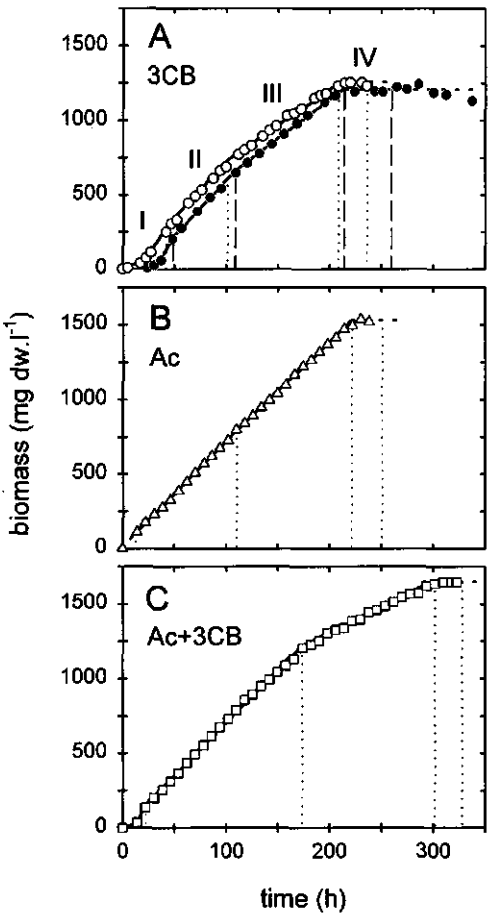
Growth in a recycling fermentor followed the same pattern independent of the substrate (Fig. 1). The first three growth phases (I, II and III) correspond closely to those found for *Escherichia coli* and *Paracoccus denitrificans* (26, 139). In the first phase (I), growth is exponential until the SPR becomes rate limiting. The second (II) and third (III) growth phases are both linear, which means that in each growth phase

Table 1 Kinetic parameters of *Pseudomonas* sp. B13 on 3CB and acetate.

Growth substrates	μ_{max} (h^{-1})	K_s (mmol·l^{-1})	Y (mg dw·mmol^{-1})	Y_{max} (mg dw·mmol^{-1})	m (μmol·mg dw^{-1}·h^{-1})
3CB	0.130 ± 0.001[A]	0.05 ± 0.01	54.1 ± 1.3[A]	61.9 ± 3.0	0.126 ± 0.013
	0.127 ± 0.011[B]				
	0.13 ± 0.04[C]				
acetate	0.295 ± 0.001[A]	1.56 ± 0.53	---[D]	20.3 ± 2.7	1.02 ± 0.24
	0.296 ± 0.008[B]				
	0.25 ± 0.03[C]				

[A] from batch experiments; [B] from chemostat wash-out experiments;
[C] estimated with NPE on chemostat data; [D] not determined

Figure 1 Growth curves of *Pseudomonas* sp. B13 with various substrates in a recycling fermentor. Fermentors with 3CB were run in duplicate. (A) Growth on 3CB; the four growth phases are indicated with roman numbers, (B) growth on acetate and (C) growth on a mixture of acetate and 3CB.



dX/dt and the apparent growth yield *Y* are constant. At the end of the third growth phase the growth curve is abruptly cut off and enters phase IV in which net growth is zero. In this stage, all ingoing substrate serves the maintenance energy demand of the biomass present. After a period of about 1 day, the growth curves started to oscillate and the optical density often increased again in a more irregular way as before. From this, we concluded that cell death and cryptic growth had started to interfere with growth on the incoming substrate, and the run was stopped. The independent duplicate fermentor runs with 3CB showed that the growth curves were well reproducible.

Figure 2 exemplifies the behavior of some parameters during the various growth phases using one of the runs from Fig. 1A. The residual 3CB concentration in the culture fluid was in the micromolar range and decreased slowly during the linear growth phases, down to an S_{min} value of about 7 μmol 3CB·l^{-1} in the phase of net zero growth (Fig. 2B). During phases II-IV the respiration activity of the cells on 0.33 mM 3CB was constant (Fig. 2C). Every change of growth phase is marked by a discontinuous step in the otherwise continuous decrease of the specific growth rate (Fig. 2D) and a stepwise increase of the maintenance coefficient (Fig. 2E). The same pattern as given in Fig. 2 was observed for the other substrate combinations.

Maintenance coefficients

The values of the maintenance coefficients calculated in the last growth phase (zero growth) in the recycling fermentor are listed in Table 2, together with the maintenance values calculated from chemostat data. The two separate maintenance coefficients in the recycling fermentor with mixed substrates reflect the consumption rates of the individual substrates, which both contribute to maintenance. The maintenance coefficients for 3CB have about the same value in both experimental systems. The acetate maintenance coefficient in the recycling fermentor at zero growth is about three times lower than the one calculated from steady-state chemostat data. The normalization of the maintenance coefficients to energy equivalents gives a value between 0.3 and 0.4 J·mg dw^{-1}·h^{-1} for all substrate combinations in the recycling fermentors. In chemostats, the energy based maintenance coefficient is two times higher for acetate than for 3CB.

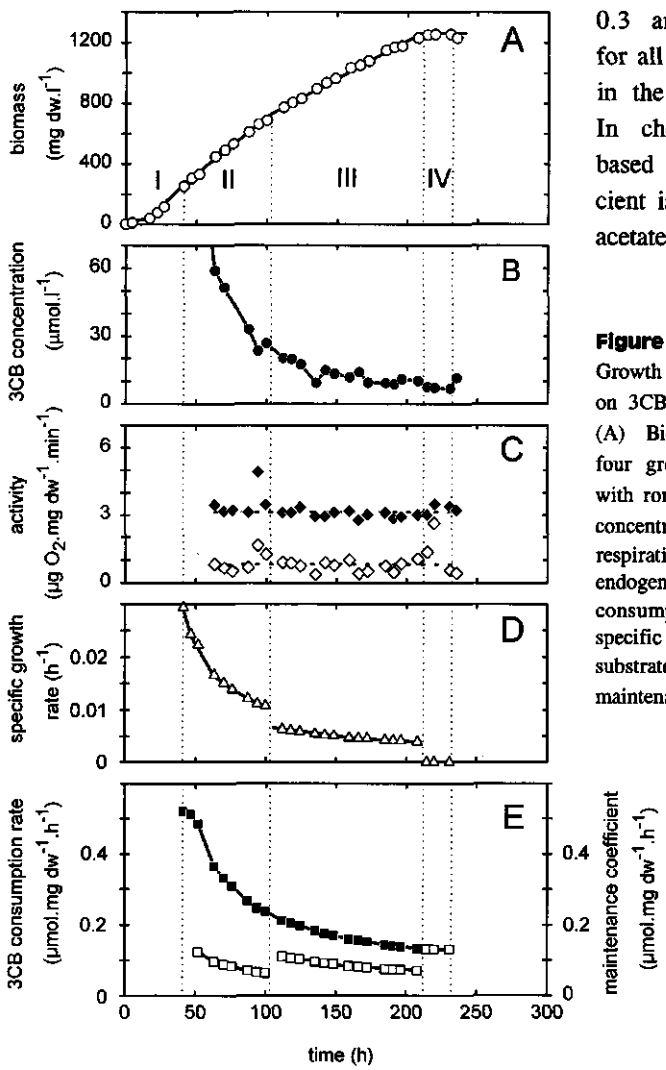


Figure 2
Growth of *Pseudomonas* sp. B13 on 3CB in a recycling fermentor. (A) Biomass concentration; the four growth phases are indicated with roman numbers, (B) substrate concentration, (C) endogenous respiration rate (◇) and sum of endogenous and substrate O_2-consumption rate (◆), (D) specific growth rate, (E) overall substrate consumption rate (■) and maintenance coefficient (□).

Table 2 Maintenance coefficients, determined in chemostat and recycling fermentor (zero growth).

Growth substrates	Recycling fermentor		Chemostat	
	(μmol·mg dw^{-1}·h^{-1})	(J·mg dw^{-1}·h^{-1})	(μmol·mg dw^{-1}·h^{-1})	(J·mg dw^{-1}·h^{-1})
3CB	0.11 ± 0.01[A] 0.13 ± 0.02[A]	0.36 ± 0.04 0.42 ± 0.07	0.13 ± 0.01	0.40 ± 0.04
3CB + acetate:		0.28 ± 0.04	---[B]	---[B]
3CB	0.05 ± 0.01	0.15 ± 0.02		
acetate	0.14 ± 0.02	0.12 ± 0.01		
acetate	0.34 ± 0.04	0.29 ± 0.04	1.0 ± 0.2	0.88 ± 0.20

[A] Parameters for 3CB were determined in two independent recycling fermentor runs;
[B] Not determined

3CB consumption kinetics

The kinetic parameters for 3CB consumption in batch, chemostat and recycling fermentor systems were calculated and are listed in Table 3. Kinetics are graphically presented in Fig. 3. Table 3 and Fig. 3 show that the 3CB consumption kinetics can vary significantly between the different experimental conditions. The

Figure 3
Kinetics of 3CB consumption in batch (resting cells, ◇; exponential growth, ◆), recycling fermentor (linear growth phases, △; exponential growth, ▲) and chemostat (○).

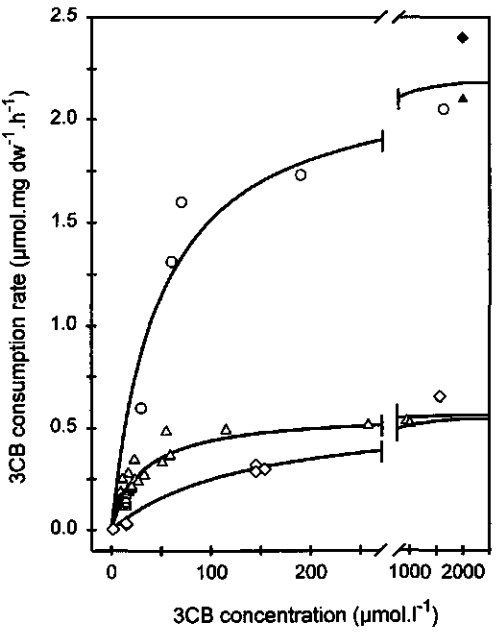


Table 3 Kinetic parameters of 3CB consumption under different experimental conditions.

Experimental conditions	q_{max} (μmol·mg dw^{-1}·h^{-1})	K_s or K_m (μmol·l^{-1})	a^0 (10^{-3} l·mg dw^{-1}·h^{-1})
batch, resting cells[A]	0.58 ± 0.08	133 ± 27	4.4 ± 1.5
batch, exponential growth	2.4 ± 0.1	---[B]	---[B]
chemostat, exponential growth	2.2 ± 0.1	47 ± 10	47 ± 13
recycling fermentor, exponential growth	2.1 ± 0.2	---[B]	---[B]
recycling fermentor, phases II-IV	0.57 ± 0.02	30 ± 3	19 ± 3

[A] Derived from (129); [B] Not determined

q_{max} values calculated for exponential growth were comparable in all three experimental systems, but they exceeded the q_{max} of resting cells in batch by a factor of about 4. The q_{max} achieved in the linear growth phases of the recycling fermentor equaled the q_{max} of resting cell batches. Resting cells in batch showed a significantly higher K_m and a much lower specific affinity as compared to both continuous systems.

Measured and predicted S_{min}

Measured residual substrate concentrations in the recycling fermentor at μ = 0 (S_{min}) are listed in Table 4. The residual concentrations in the fermentor run with both acetate and 3CB were about 50% of the residual concentrations in the runs with the individual substrate. This ratio corresponds to the relative energy contribution of both substrates to the SPR, which was 44% for acetate and 56% for 3CB. This supports the assumption of perfectly substitutable substrates that was made for the calculation of S_{min} values of mixed substrates. S_{min} values were calculated with the separate kinetic parameter sets from the three different experimental systems and are listed in Table 4. The S_{min} reflects the concentration at which the substrate consumption rate equals the maintenance coefficient. Thus, the S_{min} values predicted from resting cells in batch are fundamentally different from the apparent threshold concentration of the active transformation system of strain B13 (129). No S_{min} values could be predicted in the recycling fermentor with acetate, since the concentrations in the samples analyzed for acetate covered a too narrow concentration range to estimate the kinetic parameters. The S_{min} values predicted from chemostat and resting cell system all differ clearly from the actually measured S_{min}.

Table 4 Measured and calculated values of the minimum concentration for growth. S_{min} values were calculated with the kinetic parameters from tables 1, 2 and 3. All values are given in $\mu mol \cdot l^{-1}$.

Growth substrates	S_{min} measured	S_{min} calculated		
	recycling fermentor, phase IV	recycling fermentor, phases II-IV	chemostat	batch, resting cells
3CB	10 ± 1[A] 7.1 ± 0.4[A]	7.4 ± 2.0[A] 9.0 ± 3.2[A]	2.8 ± 1.0	35 ± 15[C]
3CB + acetate:				
3CB	4.4 ± 0.3	4.6 ± 1.7	1.6 ± 0.7	19 ± 9[C]
acetate	13 ± 3	---[B]	48 ± 37	---[D]
acetate	26 ± 12	---[B]	109 ± 78	---[D]

[A] Parameters for 3CB were determined in two independent recycling fermentor runs

[B] No kinetic parameters could be estimated in recycling fermentor

[C] Calculated with a maintenance coefficient of 0.12 $\mu mol \cdot mg\ dw^{-1} \cdot h^{-1}$ (Table 2)

[D] Not determined

Discussion

The observed growth patterns in the recycling fermentors correspond with literature descriptions (Fig. 1). After the transition from exponential growth to SPR limited growth, the cells were subject to a continuously dwindling rate of substrate supply per cell, causing a decreasing specific growth rate (Fig. 2). The nucleotides guanosine 3'-diphosphate 5'-diphosphate and guanosine 3'-diphosphate 5'-triphosphate (ppGpp and pppGpp) have been shown to increase during growth phase II (25, 75). The inflection point between phase II and III is known as the stringent response, at which high levels of these regulatory nucleotides cause an inhibition of the ribosomal protein synthesis and an increase in the fidelity of protein synthesis during the third growth phase (5, 117).

Different growth substrates have in general a limited influence on the biomass composition and energy-spilling reactions. Tijhuis et al. (124) found that the values of the Gibbs energy consumption for maintenance were comparable for a large number of different chemotrophic organisms, growing on different substrates at constant temperature. Based on this finding, no significant difference should be found between the maintenance energy requirements during growth on acetate, 3CB, or a mixture of both substances. The energy normalized maintenance coefficients from the recycling fermentor at zero growth show about the same value (between

0.3 and 0.4 J·mg $dw^{-1}·h^{-1}$) for all substrate combinations (Table 2). However, the maintenance energies during exponential growth in a chemostat are two times higher for acetate than for 3CB. Varying maintenance respiration rates with the carbon source were previously found for chemostat growth (40). The authors argued that the efficiency of energy conservation associated with the respiration may vary with the substrate. Since the maintenance energy for acetate is three times higher in chemostat than in the recycling fermentor, this indicates that the efficiency of energy conservation may also depend on the growth rate.

According to Pirt (86), the maintenance coefficient is: "the substrate of which the consumption does not produce growth". Thus, the maintenance coefficient clearly varies throughout the recycling fermentor run (Fig. 2). The actual maintenance coefficient may deviate from the calculated value when the true growth yield in the recycling fermentor is different from the Y_{max}, as determined in chemostat experiments (6). Still, the abrubt changes in both μ and m at the transition of the growth phases, and the slow decrease of both parameters during phase II and III will be the trend as long as Y_{max} is a constant. To our knowledge, no other methods than the use of chemostat data are available to estimate the Y_{max}. The upshift of the maintenance coefficient at the stringent response (phase II to III) has been explained as the energy demand of the stringent regulation itself: the manufacture of regulatory nucleotides and the editing of translation are typical maintenance processes (5, 119). Just before the transition to phase IV, the specific growth rates in the different recycling fermentors vary between 0.002 and 0.008 h^{-1}. In this range, only 1 cell out of 100 to 500 cells divides per hour. Using our approach, an upshift of the maintenance coefficient between phases III and IV (with $\mu = 0$; Fig. 2), could also be derived from the data of Müller and Babel (75). They measured no significant change in the ppGpp concentration at this transition. It would be interesting to obtain more details about the mechanism that makes a bacterial population stop growing, and instead use all substrate for non-growth purposes.

In this last stage, micromolar amounts of substrate were still measured in the culture liquid of the recycling fermentor. We considered the possibility that diffusion of the substrate through the boundary layer of the single cells (89) was limiting the substrate consumption (14, 103). We estimated that a concentration difference of about 1 nM 3CB is sufficient to satisfy the actual maintenance flux of 0.12 μmol 3CB·mg $dw^{-1}·h^{-1}$ under conditions of zero growth, using the approach of Pasciak (85) and a molecular diffusion coefficient of 8.10^{-10} $m^2·s^{-1}$ (145). This concentration gradient is insignificant compared to the actual residual concentration of 9 μM 3CB. Calculation of acetate diffusion yielded a similar result. It can therefore be concluded that the kinetics of substrate consumption and not diffusion were limiting in the recycling fermentors.

No residual substrate concentrations could be detected in batch experiments with resting cells of *Pseudomonas* sp. B13 (129). The reason for this difference is the non-steady-state nature of batch experiments. Substrate concentrations dropped below S_{min} because the substrate was not resupplied after utilization. In contrast, substrate is resupplied in continuous systems. In the stationary phase in the recycling fermentor this resupply exactly meets the maintenance consumption rate of the bacterial population. This period of zero growth can be regarded as a steady state. The cells are able to maintain themselves, but will still have a finite lifetime. The constant plateau of the growth curves could never be observed for longer than 2 days, probably because of cell death and cryptic growth.

The kinetic parameters for 3CB consumption (Table 3), and hence, the calculated S_{min} values (Table 4), were dependent on the experimental conditions. The maximum consumption rate was highest during exponential growth of the cells, irrespective of experimental system. At the transition from exponential to linear growth in a recycling fermentor (time in the recycling fermentor runs from right to left on the substrate axis of Fig. 3), the q_{max} dropped instantaneously to about 25% of the previous value. This shows that a substrate stringency almost immediately affects the uptake and transformation capacity of the cells. The q_{max} levels determined in batch systems with resting cells can be interpreted likewise: the measured q_{max} did not reflect the high activity of the exponentially growing cells, but the low activity of the cells immediately after the harvesting procedure of about 2 h. In this time span, substrate stringency had probably already affected the cells' uptake and transformation capacity, which was demonstrated by the decrease in the maximum consumption rate. This corresponds with observations of Höfle, who saw the specific affinity for glucose of batch-grown cells of *Cytophaga johnsonae* drop in the the first few hours of the stationary phase to a level of 10% of the preceding affinity (42).

The cells manifested a higher specific affinity during slow growth in the recycling fermentor than in resting state in batch (Table 3 and Fig. 3). The respiration activity on 0.33 mM 3CB in the recycling fermentor samples (Fig. 2) remained constant during phases II-IV and corresponded to the q_{max} in these growth phases (Fig. 3) calculated with a stoichiometry of 7 O_2: 1 3CB. This indicates that 3CB was entirely mineralized and that the q_{max} did not change significantly during phases II-IV. An immediate 3- to 20-fold increase of metabolic enzyme concentration (μmol·mg dw^{-1}) occurred in an *E. coli* strain after transition to phase II (25). An elevated level of the rate limiting metabolic enzyme would have increased the affinity by an increase of the q_{max} (not a decrease of K_s), and could therefore not explain the higher specific affinity in the recycling fermentor. The existence of multiple uptake or transformation systems has been reported in several studies (47, 67, 138). These systems were described by Michaelis-Menten kinetics

and were active in different concentration ranges. In our study, at least three uptake or transformation systems in the micromolar to millimolar range must be involved to give the observed 3CB transformation kinetics. The experimental growth conditions, and not substrate concentration would determine the followed kinetics. Thus far, the underlying mechanisms of the variability of 3CB consumption kinetics remain unknown.

The presented results lead to the conclusion that biotransformation studies should be done under conditions as close as possible to the ones in the biodegradation system under consideration. The extent to which the biomass is able to grow seems to be an important criterion for the kinetics of substrate transformation. S_{min} is the aqueous substrate concentration in a given system at which all transformed substrate will be used for maintenance purposes. An energy normalized maintenance coefficient m_e seems a useful parameter to describe the maintenance requirement of a mixed microbial population living on the substrate pool in bioreactors, aquifers or soil systems. Many different substrates will probably be used simultaneously when present at low concentrations (29, 78). Consequently, the maintenance contribution, and thus the S_{min}, of each individual substrate is expected to be lower than in single substrate use, which is supported by our observations (Table 4). Thus, in continuously operated bioremediation techniques the residual pollutant concentrations may be reduced by the provision of additional energy sources. The contaminant concentration can be brought below the S_{min} of the single pollutant when the maintenance requirement of the population determines the residual concentration, and bioavailability is not limiting.

Acknowledgments

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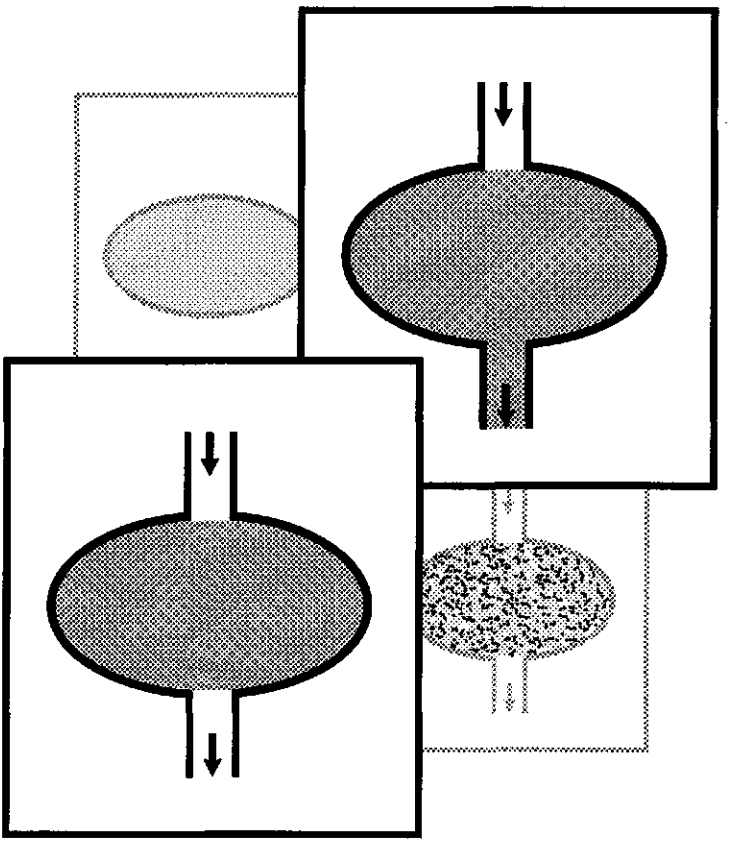
We thank Geert-Jan Ankoné, Ronald Eimers and Gerco Hoogeweg for providing part of the data, and Wim Roelofsen en Frits Lap for technical assistance.

Appendix Chapter 3

Effects of temperature on kinetic parameters and S_{min} of *Pseudomonas* sp. strain B13 during growth in chemostat and recycling fermentor



Setup

Fermentor experiments were also performed at 30°C to obtain information on the temperature dependence of the kinetic parameters and S_{min} of *Pseudomonas* sp. strain B13. The chemostats were run with 3-chlorobenzoate (3CB) and acetate, provided as single substrates. Recycling fermentors were fed with 3CB alone. The methods and equipment were the same as used at 20°C (Chapter 3) and the results required at both temperatures are compared and discussed.

Results

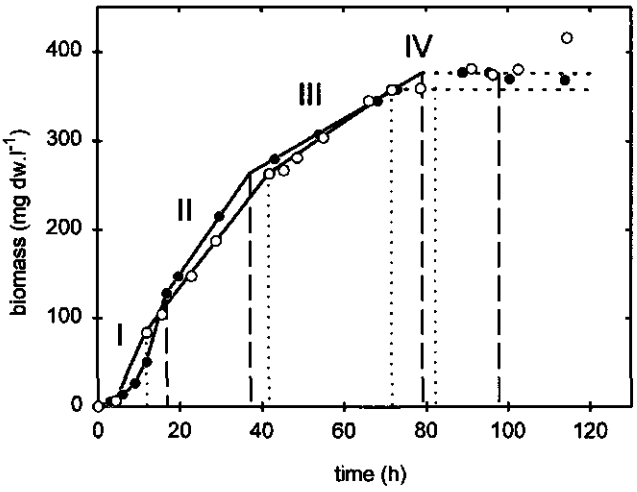
Table 5 lists the growth kinetic parameters of *Pseudomonas* sp. strain B13 at 30°C. The three different methods for μ_{max} determination produced comparable values. No constant wash-out rate could be obtained within 30 volume changes with acetate, resulting in a relatively high standard deviation of the μ_{max} value. The μ_{max} values from batch cultures were used for further calculations. Both, μ_{max} and maintenance coefficient increased with temperature, the increase in m was not significant for acetate (compare Tables 5 and 1). A higher K_s and a lower Y_{max} were observed for growth on 3CB at 30°C. No significant modulation of the K_s and Y_{max} with the temperature could be observed for growth on acetate.

Table 5 Kinetic parameters of *Pseudomonas* sp. B13 on 3CB and acetate at 30°C.

Growth substrate	μ_{max} (h^{-1})	K_s ($mmol \cdot l^{-1}$)	Y_{max} ($mg\ dw \cdot mmol^{-1}$)	m ($\mu mol \cdot mg\ dw^{-1} \cdot h^{-1}$)
3CB	0.296 ± 0.004^A	0.30 ± 0.08	50.9 ± 3.1	0.181 ± 0.027
	0.27 ± 0.01^B			
	0.26 ± 0.06^C			
acetate	0.626 ± 0.001^A	1.57 ± 0.37	17.6 ± 1.8	1.27 ± 0.17
	0.67 ± 0.06^B			
	0.59 ± 0.07^C			

[A] from batch experiments; [B] from chemostat wash-out experiments;
[C] estimated with NPE on chemostat data

Figure 4 Growth curves of *Pseudomonas* sp. strain B13 with 3CB at 30°C in two duplicate recycling fermentor runs. Roman numbers indicate the four growth phases.



Four distinct growth phases were observed in the recycling fermentor at 30°C (Fig. 4), similar to the data at 20°C (Fig. 1). The behavior of the substrate concentration, the specific growth rate, the substrate consumption rate and the maintenance coefficient followed also the same pattern during the various growth phases at 30°C (not shown) as was seen for growth at 20°C (Fig. 2). The maintenance coefficients in the fourth growth phase (zero growth) and the maintenance values calculated from chemostat data are given in Table 6. At both 20°C and 30°C in chemostats, the energy based maintenance coefficients for acetate

Table 6 Maintenance coefficients at 30°C, determined in chemostat and recycling fermentor (zero growth).

Growth substrate	Recycling fermentor		Chemostat	
	(μmol·mg dw^{-1}·h^{-1})	(J·mg dw^{-1}·h^{-1})	(μmol·mg dw^{-1}·h^{-1})	(J·mg dw^{-1}·h^{-1})
3CB	0.32 ± 0.04[A] 0.34 ± 0.04[A]	1.02 ± 0.12 1.09 ± 0.14	0.18 ± 0.03	0.58 ± 0.09
acetate	---[B]	---[B]	1.3 ± 0.2	1.1 ± 0.1

[A] Parameters for 3CB were determined in two independent recycling fermentor runs

[B] Not determined

Table 7 Kinetic parameters of 3CB consumption at 30°C in chemostat and recycling fermentor.

Experimental conditions	q_{max} (μmol·mg dw^{-1}·h^{-1})	K_s (μmol·l^{-1})	a^0 (10^{-3} l·mg dw^{-1}·h^{-1})
chemostat, exponential growth	6.0 ± 0.5	296 ± 77	20 ± 7
recycling fermentor, exponential growth	5.3 ± 2.1	---[A]	---[A]
recycling fermentor, phases II-IV	1.66 ± 0.16	55 ± 10	30 ± 8

[A] Not determined

exceed the ones for 3CB with a factor of about 2 (compare Tables 6 and 2). The increase of the maintenance coefficients at 30°C compared to 20°C is about a factor 3 for the recycling fermentor values, and a factor 1.5 for the chemostat values.

Table 7 shows the kinetic parameters of 3CB consumption at 30°C under different growth conditions in chemostat and recycling fermentor. Similar to the kinetics at 20°C (Table 3), the q_{max} during exponential growth in both fermentor types was about a factor 4 higher than the q_{max} in the linear growth phases of the recycling fermentor. The specific affinities for 3CB were about the same during both, chemostat growth and very slow growth in recycling fermentor. The S_{min} values that were calculated with the kinetic parameters from the two different fermentor systems did not differ significantly (Table 8). The S_{min} values measured at 30°C were a factor 2 higher than the values at 20°C (compare Tables 8 and 4).

Table 8 Measured and calculated values of the minimum concentration for growth at 30°C. S_{min} values were calculated with the kinetic parameters from tables 5, 6 and 7. All values are given in μmol.l^{-1}.

Growth substrate	S_{min} measured	S_{min} calculated	
	recycling fermentor, phase IV	recycling fermentor, phases II-IV	chemostat
3CB	19 ± 5[A] 19 ± 1[A]	13 ± 6[A] 14 ± 7[A]	9.2 ± 4.5
acetate	---[B]	---[B]	56 ± 27

[A] Parameters for 3CB were determined in two independent recycling fermentor runs

[B] Not determined

Discussion

The growth kinetic parameters μ_{max}, Y_{max}, K_s and m are often found to depend on temperature according to the Arrhenius equation (22, 60, 124, 125). In our case, both the maintenance coefficient m and μ_{max} increased with temperature (Tables 1, 2, 5 and 6). For acetate, the increase in the maintenance coefficient was not significant. If μ_{max} depends on temperature according to the Arrhenius equation, a minimum of two different growth temperatures suffices to give a rough estimation of the activation energy E_a of the rate limiting enzymatic step: $\ln(\mu_{max}) = E_a/RT + C$. For 3CB and acetate, the E_a values were calculated to be 61 and 56 kJ/mol, respectively. These values are in agreement with the activation energies found for a large number of other organisms (124). Calculation with the maintenance coefficients from the chemostat data yields 2 to 4 times lower E_a values. The maintenance coefficients at zero growth in the recycling fermentor with 3CB as substrate generate an E_a value of 75 kJ/mol, however.

The growth parameters K_s and Y_{max} did not show the same trend for both substrates (Tables 1 and 5). For 3CB a higher K_s and a lower Y_{max} were found at 30 °C, whereas for acetate the differences in these parameters were not significant. Growth of *E. coli* strains on glucose also displayed a more or less constant or slightly decreasing Y_{max} over a temperature range from 17 to 30°C (71, 79). Several studies with K_s values at different temperatures are available, the results varying from a positive (55, 146) via no significant (59, 143) to a negative (53, 125) temperature dependence.

The different kinetics of 3CB consumption at various growth conditions at 30°C (Table 7) are consistent with the results at 20°C (Table 3) and have been discussed in Chapter 3. For both exponential and very slow growth the q_{max} values at 20°C and 30°C differed a factor of about 3. In our case, the specific affinities do not vary substantially between the different growth conditions and temperature (Tables 3 and 7).

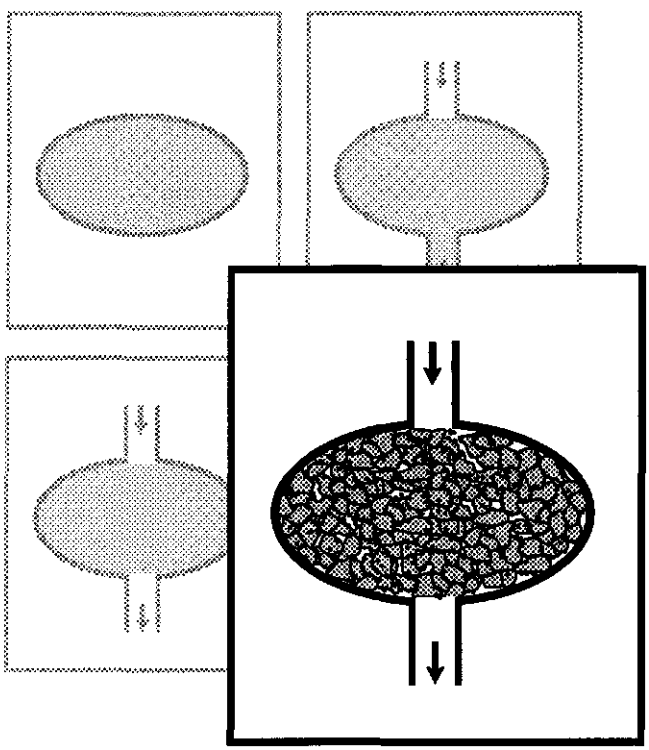
Since the bacterial metabolism represents a network of reactions, it is difficult to interpret temperature modulations of complex growth parameters like m, Y_{max} and K_s. The positive correlation of q_{max}, μ_{max} and m with temperature are, however, understandable when we consider the main metabolic processes during growth. The rates of metabolic reactions decrease with temperature, as long as its activation energy is rate limiting. At lower temperatures, the rate of substrate consumption and ATP generation will decrease, as well as the rate of biomass synthesis, coupled to the hydrolysis of ATP. As a result, the μ_{max} will decrease with temperature. Analogously, the rate of energy-spilling reactions, like the leakage of ions across the membrane and spontaneous denaturation and hydrolysis of proteins and other polymers, will decrease with temperature. As a result, a lower restoration

activity is needed to maintain the membrane gradients and the steady-state intracellular concentrations, which may result in lower maintenance coefficients at lower temperatures.

Temperatures in natural environments like aquifers and soil systems are often lower than 20°C. These lower temperatures will lead to lower maintenance requirements and probably to lower S_{min} values under field conditions than in laboratory experiments. Anderson and Domsch indeed found 10 times lower maintenance requirements at 15°C than at 28°C, determined *in situ* for the indigenous bacterial populations surviving in three types of soil ecosystems (2). In addition to mixed substrate utilization (Chapter 3), also lower temperatures may therefore reduce residual pollutant concentration in environments where the maintenance demand determines the aqueous concentration.

Chapter 4

Anomalies in the transformation of 3-chlorobenzoate in percolation columns with Pseudomonas sp. strain B13



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submitted to Environmental Science & Technology

Abstract

The biotransformation of 3-chlorobenzoate by attached cells of *Pseudomonas* sp. strain B13 was studied in percolation columns operated at various flow rates and biomass contents. Steady-state residual effluent concentrations were compared with predictions from spreadsheet models describing the combined action of microbial and mass transfer kinetics. Literature data on 3-chlorodibenzofuran degradation in percolation columns with *Sphingomonas* sp. strain HH19k [Harms & Zehnder, Appl. Environ. Microbiol. 60(1994)2736-2745] were also evaluated. In both studies, the effluent concentrations were successfully predicted above a critical ratio of flow rate and biomass. Below this critical point the steady-state residual effluent concentrations were higher than predicted and this deviation increased with decreasing flow rate:biomass ratios. The results are discussed in the light of convection-diffusion processes limiting the transport of both substrate and oxygen to the attached cells.

Introduction

The extensive production and use of chlorinated organic compounds in the last few decades has led to a widespread occurrence and persistence of these compounds in the environment. Numerous chlorinated organic contaminants are ubiquitously present at low concentrations in soil, groundwater, waste water and natural waters (nanomoles to micromoles per liter), though many are intrinsically biodegradable. Obviously, there are factors preventing a complete degradation of these contaminants. The aspect of low persistent concentrations of a pollutant is one of the factors limiting the application of bioremediation techniques for the clean-up of contaminated sites.

Residual concentrations of a chemical in the aqueous phase (i.e. concentrations that remain after initial biodegradation) have been observed in various laboratory experiments (16, 38, 95, 106, 127, 135). The complexity of the studied experimental systems varied from suspended cell batches (106) and fermentors (127) to percolation columns with a solid matrix (16, 38, 95, 135).

Growth kinetic parameters of microorganisms determine the steady-state residual concentration in chemostats at a fixed dilution rate. The lowest attainable substrate concentration in fermentors with 100% biomass retention was determined by the maintenance requirement of Pseudomonas sp. strain B13 (127). At this concentration, S_{min}, the substrate utilization equaled the total maintenance demand of the biomass and net growth was zero. Steady-state effluent concentrations were also detected in column studies with river sediment and dune sediment in which chlorinated benzenes were degraded (16, 135). Both studies showed that the residual effluent concentrations were independent of the influent concentration. Repeated

inoculation of active biomass into the columns did not lower the residual substrate in the effluent (135). Bosma et al. (16) speculated that sorption affected the availability of substrate. Harms and Zehnder studied 3-chlorodibenzofuran (3CDF) conversion by cells of *Sphingomonas* sp. strain HH19k attached to glass beads in percolation columns (38). Higher steady-state effluent concentrations were measured than were predicted with a spreadsheet program describing the microbial activity in the column. This deviation was larger with lower flow rates and with higher cell densities, which indicated a limitation by convective-diffusive substrate transport to the cells. However, theoretically predicted mass transfer rates could not explain the observed deviation.

The objective of the present study was to further examine the possible effects of mass transfer on the biodegradation of contaminants present in water flowing through sand columns, and to see whether the same anomaly - slower biotransformation than predicted using established expressions to predict the mass transfer rate - would occur. As model system, we used columns with *Pseudomonas* sp. strain B13 attached to iron coated sand (116) which were percolated with a medium containing 3CB. The intrinsic kinetics of 3CB transformation were established in previous studies with suspended cell systems (127, 129). The rate of mass transfer was varied by applying different flow velocities and biomass levels (38). The observed biotransformation rates and residual effluent concentrations during steady-state operation were compared to model predictions. The results on 3CDF transformation by *Sphingomonas* sp. strain HH19K (38) were included in the data analysis.

Materials and methods

Bacteria, media and culture conditions

Pseudomonas sp. B13 has been isolated from a sewage plant and is able to grow on 3-chlorobenzoate (3CB) as a sole carbon and energy source (28). Cells were pregrown on 3CB at 20°C and were washed after harvesting with the incubation medium of the subsequent experiment. Growth medium, preculture conditions and harvesting procedure have been described earlier (129). A MOPS-NaCl buffer was used in the column experiments, containing (per liter of demineralized water) 10.46 g MOPS, 0.72 g NaOH and 4.79 g NaCl (pH 6.95).

Transformation experiments in columns

Transformation of 3CB by attached resting cells of strain B13 was studied in column systems with ferrihydrite (FeOOH) coated sand. Previous experiments had shown that strain B13 possessed relatively poor adhesion properties (93). Adhesion of the negatively charged cells was therefore enhanced by the positively charged iron oxide (FeO^+) (116). Two types of glass columns were used: one with a fixed length (10 cm; inner diameter, 0.9 cm) and one with plunger end parts which allowed to vary the length (inner diameter, 1.0 cm or 2.5 cm). The column void volumes varied

between 2 and 6 ml. The columns were operated in a vertical position and the influent was pumped in a downward direction. Ferrihydrite coated sand, tubing and columns did not adsorb 3CB significantly.

Two methods were used to attach strain B13 to the column material. (i) Ferrihydrite coated sand (60 $g \cdot l^{-1}$) was mixed end-over-end (2 rpm) with a cell suspension in MOPS-NaCl buffer for 2 h. The cell concentrations ranged from about 10^7 to 10^9 colony forming units per ml (CFU $\cdot ml^{-1}$) in order to reach different amounts of attached cells. The sand was washed with clean buffer and was wet-packed in the columns. The biomass on the sand was calculated from the difference in the cell suspensions and in the washing buffer, between the biomass density before and after adhesion.
(ii) Columns were wet-packed with cell-free ferrihydrite coated sand and a cell suspension ($2 \cdot 10^8$ CFU $\cdot ml^{-1}$ in MOPS-NaCl) was pumped through the columns (1 h, 20 $ml \cdot h^{-1}$) and replaced with a cell-free solution afterwards. Effluent samples were taken every 10 min. The biomass on the sand was determined from the difference between the total biomass fed to the column and collected in the effluent. The biomass attachment method did not affect the biodegradation results.

Immediately after the attachment procedure, a 3CB solution (influent concentration, 5 or 20 μM) was fed to the columns to study 3CB transformation. The flow velocities in the different columns varied between 0.2 and 70 $cm \cdot h^{-1}$ (flow rates, 0.2 to 20 $ml \cdot h^{-1}$) and the biomass ranged from 35 to 2000 μg of protein per column (surface coverage, 0.4 to 22 %). Effluent samples were taken regularly during at least 7 porevolume changes and were analyzed for 3CB and biomass concentrations. Conversion in the 3CB samples was stopped by acidification with HCl (final pH, 1.5). A decrease in transformation activity was observed after about 35 to 40 h and subsequently the experiments were stopped. The total wash-out of biomass during the transformation experiment was less than 1% for all columns. Experiments with the lowest flow rates were also carried out using influent medium aerated with 98% O_2 (final concentration, 38 $mg \cdot l^{-1}$) in order to satisfy the total oxygen demand of the biomass. Results of these experiments did not deviate from the ones using air-saturated influent solutions.

Batch controls

The conditions in the column experiments differed on several points from our previous batch studies (129). Three control experiments were done to check for the influence of these factors on the transformation rate. (i) The transformation rates in growth medium and in MOPS-NaCl buffer were compared. (ii) The influence of cell adhesion to ferrihydrite coated sand on the transformation rate was investigated. (iii) The influence of varying amounts of oxygen in solution was studied. In all three experiments, initial concentrations were about 20 μM 3CB and incubations were done at 20°C.

In the first experiment, washed cells were resuspended in mineral growth medium or MOPS-NaCl buffer. Experimental handling and conditions were as described previously (129). In the second experiment, a series of 8 ml cell suspensions ($2 \cdot 10^8$ CFU $\cdot ml^{-1}$) in MOPS-NaCl were mixed end-over-end with 0, 0.2, 0.4, 0.6 and 0.8 g of ferrihydrite coated sand in 9 ml serum flasks. After 2.5 h, 3CB was added and both 3CB and biomass concentrations were followed for 3 h by sacrificing incubations at regular time intervals. The attached portions of biomass were 0%, 35%, 50%, 57% and 65% during incubation with 3CB, respectively (coverage, 5.7 to 12.3%). In the third experiment, cells were resuspended in 24 ml of MOPS-NaCl in a series of gas tight 100 ml serum bottles ($2 \cdot 10^8$ CFU $\cdot ml^{-1}$). After a starvation period of about 20 h, the suspensions were brought under 20%, 40%, 60%, 80% or 96% O_2 atmosphere and were mixed for 1 h on a rotary shaker to saturate the suspension with O_2. The 3CB was added and the transformation was followed during 6 h. Conversion of 3CB was stopped in all samples by acidification with HCl (final pH, 1.5).

Preparation of ferrihydrite coated sand

Ferrihydrite was synthesized by rapid hydrolysis of an acid solution of $Fe(NO_3)_3$ with 1 M of KOH (110). The ferrihydrite was centrifuged at pH 7 (6500 × *g*, 10 min) and washed 3 times with 0.1 M of $NaNO_3$. Preparation and washing were done under N_2 or N_2/H_2 (98:2 %) atmosphere using N_2 flushed solutions to prevent interference of CO_2 (116). Commercial acid purified lake sand (40 to 200 μm mesh; Fluka Chemie, Buchs, Switzerland) (250 $g \cdot l^{-1}$), was mixed end-over-end with a ferrihydrite suspension (*10* $g \cdot l^{-1}$ *in 0.1 M of* $NaNO_3$) for 24 h (101). The coated sand was washed with 0.1 M of $NaNO_3$, dried at 60°C, autoclaved before use and contained about 50 μmol of ferrihydrite per gram (116).

Analytical methods

Samples were centrifuged at 10,000 × *g* with a table centrifuge (Biofuge 13; Heraeus AG, Zürich, Switzerland). 3CB analysis was done by high performance liquid chromatography (HPLC) (PU-980 pump and UV-970 detector; Jasco, Tokyo, Japan). HPLC column, eluent and further operation were as described previously (129).

The oxygen concentration was determined with a iodometric titration according to Winkler (151).

Biomass was determined by protein measurements according to Lowry or Bradford (20, 70) with bovine serum albumin as standard (Sigma Chemical Company, St. Louis, Mo.). Low biomass concentrations were determined by optical density measurements at 280 nm (A_{280}) and sixfold plate counting. A_{280} measurements were corrected for absorption by 3CB and a slight decrease in absorption due to ageing of the cells. The methods were calibrated against each other. All samples were analyzed for biomass with at least two methods. Viability of the cells remained the same throughout the experiments.

Chemicals

Acetonitrile supra gradient HPLC grade (EGT Chemie AG, Tägerig, Switzerland) was used as mobile phase for HPLC. All other chemicals were of analytical grade and were used without further purification.

Model calculations

The biotransformation of a chemical results from the combined action of mass transfer, microbial uptake and transformation (17). The diffusive flux of substrate to a cell, Q_d ($M \cdot T^{-1}$) is determined by the mass transfer coefficient, k ($L^3 \cdot T^{-1}$), and the difference between the distant aqueous concentration (C_d) and the cell surface concentration (C_c; $M \cdot L^{-3}$):

$$Q_d = k(C_d - C_c) \quad (1)$$

In a packed bed with advective flow, k can be calculated as

$$k = \eta_T A_p U/n \quad (2)$$

which assumes a monolayer of cells on spherical collectors (17, 38). The influence of flow rate and cell density, as observed by Harms (38), is represented by U/n, the ratio of the liquid's linear velocity U ($L \cdot T^{-1}$) and the number of cells per collector, n. A_p is the cross sectional area of the collector (L^2) and η_T is the single collector efficiency, which is the fraction of molecules flowing to the collector surface which actually collides with it (91). The approach assumes laminar flow and mass transfer

from the bulk liquid to the collector due to just diffusion (91). A comparison with other empirical formulae to estimate mass transfer (150) revealed that eq. (2) predicts the slowest kinetics for our experimental conditions. The substrate flux due to uptake and transformation by the cells, Q_c ($M \cdot T^{-1}$) is a function of the cell surface concentration C_c. The 3CB transformation rate of one cell was calculated using the linear relationship that was observed for resting cells of strain B13 in the low micromolar range, > 1 μM:

$$Q_c = -a + bC_c \qquad (3)$$

where $a = 5.55 \cdot 10^{-14}$ $\mu mol \cdot s^{-1}$, and $b = 1.12 \cdot 10^{-16}$ $l \cdot s^{-1}$ (129). Microbial 3CDF transformation by strain HH19k was calculated with Michaelis-Menten kinetics as described previously (38).

The steady-state 3CB transformation resulting from combined mass transfer and microbial activity ($Q_d = Q_c = Q$) was obtained by combining eqs. (1) and (3):

$$Q = (bC_d - a)k/(b+k) \qquad (4)$$

The mass transfer limited transformation of 3CDF was calculated by combination of eq. (1) and the Michaelis-Menten equation. This gives an expression which was previously described as the 'Best' equation (17, 56).

Calculations were carried out with standard spreadsheet programs, adapted from (38). In the calculations, the column was divided in 1000 sections and biotransformation was calculated in each section. The effluent concentration of each section was used as the influent concentration for the next. Effluent concentrations were predicted using two versions of the model: one incorporating microbial kinetics only, and a second combining both microbial and mass transfer kinetics.

Results

Batch controls

The specific transformation rates of resting cells were equal in growth medium and MOPS-NaCl buffer. Cell adhesion to ferrihydrite coated sand did not influence the specific removal rates, nor did the presence of higher oxygen concentrations.

Development of steady-state in the column experiments

Figure 1A to 1D show the final part of the 3CB breakthrough curves of four columns at different flow rates. After breakthrough (not sampled), the residual effluent concentrations decreased with time until steady state was observed. Steady state was observed after 3 to 80 pore volume changes, depending on the experiments. The time needed to reach steady state (t_{ss}, expressed in units of time) appeared to correlate well with the volumetric flow rate F (Fig. 2A). The correlations with the linear flow rate (U) or the flow rate:biomass ratio (U/n) were

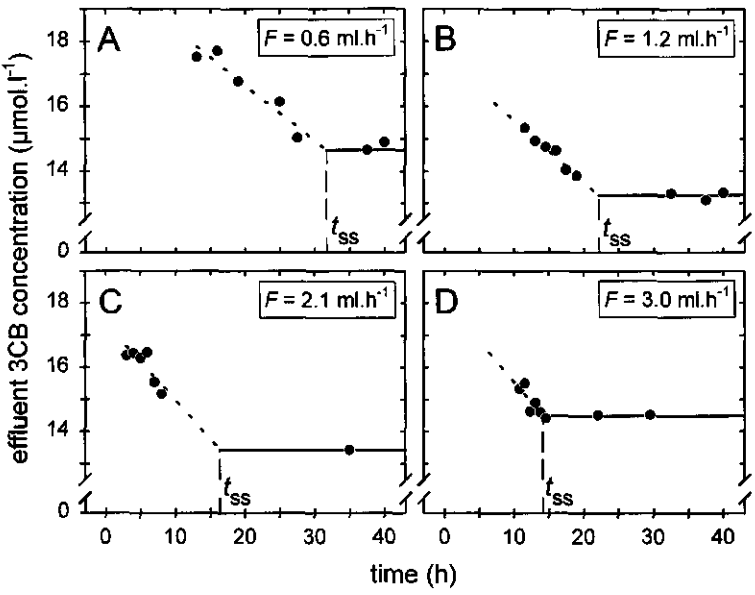
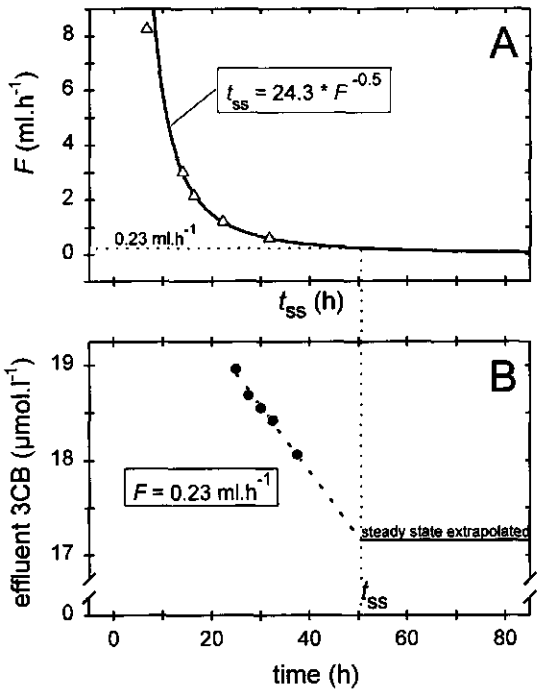


Figure 1 Time dependent 3CB effluent concentrations in column experiments with *Pseudomonas* sp. strain B13 at the flow rates 0.6 (A), 1.2 (B), 2.1 (C) and 3.0 ml·h^{-1} (D). Regression line (short dash) and steady-state effluent level (solid line) are given for each column experiment. The moment of steady state achievement, t_{ss} is indicated in each graph (long dash).

Figure 2
Exponential relationship between t_{ss} and the flow rates $F \leq 3$ ml·h^{-1} in column experiments with *Pseudomonas* sp. strain B13 (A). For columns in which steady state was not measured, steady-state levels were extrapolated (B, solid line) by extending the regression line through the measured effluent concentrations (B, dashed line) to the estimated t_{ss}.

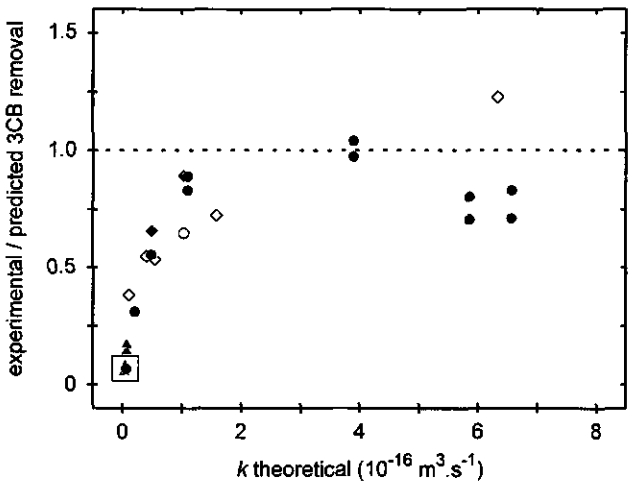


less good. No correlations were found with the dilution rate (porevolume changes per time) or the substrate loading (neither as mass per time nor mass per time and unit of biomass; not shown). An exponential relationship between t_{ss} and F could be fitted ($t_{ss} = 24.3 \cdot F^{-0.5}$) from the columns operated at $F \leq 3$ ml·h^{-1} that reached steady state. The steady-state concentration levels for the columns that did not reach steady state were predicted with the help of this relationship by a linear extrapolation of the measured effluent concentrations until t_{ss} (Fig. 2B). The extrapolated effluent concentrations at t_{ss} (experimental results) were taken for comparison with the model results.

Comparison of experimental and modeled steady-state concentrations

The steady-state removal by strain B13 in a column was calculated as the difference between influent and steady-state effluent concentration. Figure 3 shows the ratio between the experimental and the predicted 3CB removal in the column experiments with strain B13, without considering mass transfer kinetics. The experimental data corresponded with the predictions in experiments with a theoretical k above a critical value of about $1 \cdot 10^{-16}$ m^3·s^{-1} (see also Fig. 4A). Below this critical point, the removal is overestimated and experimental effluent concentrations were much higher than calculated (Figs. 3 and 4A). In several columns,

Figure 3 Ratio between experimental and predicted 3CB removal in steady-state columns with *Pseudomonas* sp. strain B13. Presented data were measured (circles), or estimated after intrapolation (diamonds) or extrapolation of t_{ss} (triangles). Open symbols represent results from column experiments with equal flow rates and varying biomass densities. Symbols within the box are the results from column experiments aerated with 98% O_2 (final concentration, 38 mg·l^{-1}).



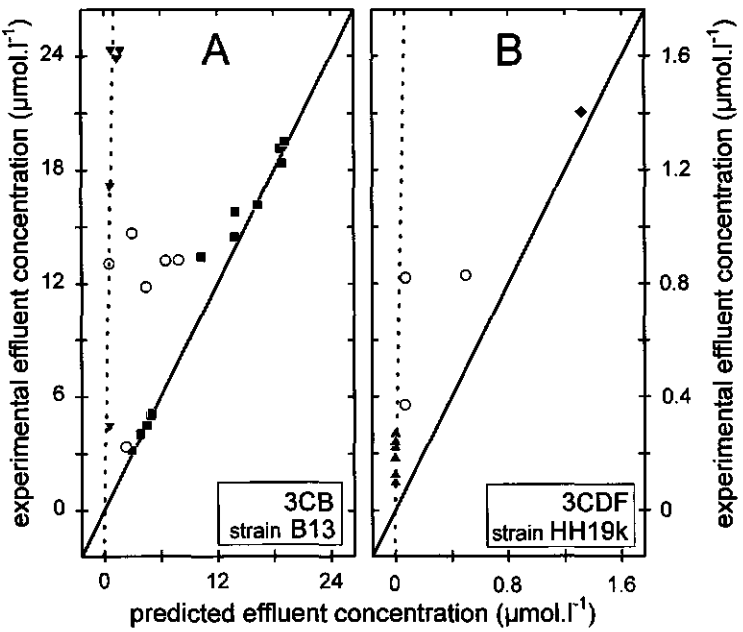


Figure 4 Effluent concentrations in all column experiments performed with *Pseudomonas* sp. B13 transforming 3CB (A) and with *Sphingomonas* sp. strain HH19k transforming 3CDF (B, adapted from (38)). Experimental effluent concentrations are plotted against the predicted values. The lines with an experimental:predicted effluent concentration ratio of 1 (solid line) and 25 (dotted line) are given in both graphs. Column data with strain B13 (A) are categorized in $U/n < 1 \cdot 10^{-10}$ (▼), $1 \cdot 10^{-10} < U/n < 1 \cdot 10^{-9}$ (○) and $U/n > 1 \cdot 10^{-9}$ m·s^{-1} (i.e. theoretical $k > 1 \cdot 10^{-16}$, ■). Categories of column data with strain HH19k (B) are $U/n < 7 \cdot 10^{-8}$ (▲), $7 \cdot 10^{-8} < U/n < 2 \cdot 10^{-7}$ (○) and $U/n > 2 \cdot 10^{-7}$ m·s^{-1} (◆).

experimental effluent concentrations exceeded the predicted concentrations with a factor of more than 25 (Fig. 4A). The results of the column experiments aerated with 98% O_2 (final concentration, 38 mg·l^{-1}) did not deviate from the ones using air-saturated influent solutions (Fig. 3). Similarly, higher 3CDF effluent concentrations were measured than were predicted in the column experiments with strain HH19k (Fig. 4B). The inclusion of mass transfer in the model affected neither the prediction of 3CB transformation by *Pseudomonas* sp. strain B13 (Fig 5A) nor the prediction of 3CDF transformation by *Sphingomonas* sp. strain HH19k (38) Fig 5B). Thus, the established expression for the mass transfer coefficient, eq. (2), did not describe the experimental data. Nevertheless, the observed deviations between experiments and model predictions appeared to correlate with part of this expression: the ratio of the linear flow rate and the number of cells per collector (U/n). 3CB transformation was overestimated at $U/n < 1 \cdot 10^{-9}$, 3CDF transformation at $U/n < 2 \cdot 10^{-7}$ m·s^{-1} (Fig. 4).

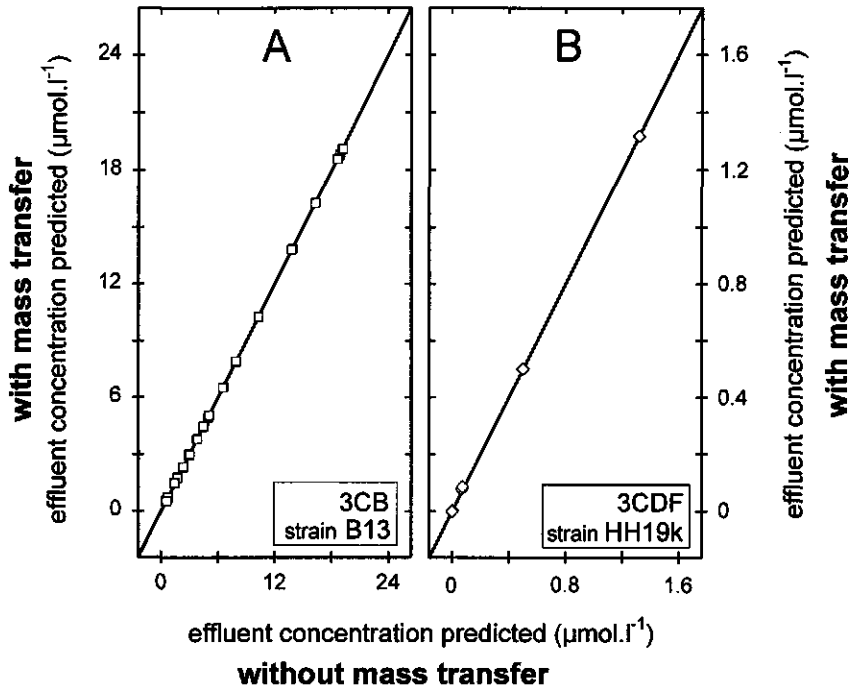


Figure 5 Predicted effluent concentrations in all column experiments performed with *Pseudomonas* sp. strain B13 transforming 3CB (A) and with *Sphingomonas* sp. strain HH19k transforming 3CDF (B, adapted from (38)). Effluent concentrations predicted with the model combining microbial and mass transfer kinetics are plotted against the predictions using microbial activity alone (symbols). The solid line is the 1:1 prediction line.

Discussion

The 3CB transformation in percolated soil columns with resting cells of strain B13 was modeled using previously determined kinetics in resting cell suspensions (129). This model predicted steady-state effluent concentrations correctly in column experiments with $U/n > 1 \cdot 10^{-9}$ m·s^{-1} (Figs. 3 and 4A). The scattering of these data around the line of prediction are within the experimental error. These results indicate that the intrinsic kinetics of resting cells are the same in suspension and attached to soil in a percolation column.

However, our experimental results are also characterized by two anomalies. (i) The time needed to reach steady state depended on the flow rate (Figs. 1 and 2). The number of pore volumes that was changed until steady state varied from less than 3 to about 80. Five pore volume changes should be sufficient from a hydrodynamic point of view. The value of t_{ss} correlated well with the volumetric

flow rate F. Worse correlations existed with the linear flow rate (U) or the flow rate:biomass ratio (U/n). No correlations were found with the dilution rate or the (specific) substrate loading. (ii) In columns with $U/n < 1 \cdot 10^{-9}$ m·s^{-1}, the experimental steady-state effluent concentrations were higher than predicted on the basis of independently established microbial and mass transfer kinetics (Figs. 3 and 4A). U/n (or F/n) appeared to give the best correlation with the observed deviation. Hence, the flow in the column seems to affect both the time needed to establish steady state and the level of the steady-state concentration in the effluent. The biomass density seems to affect the attainable steady-state concentration only. The controls exclude the possibilities that attachment or medium composition caused the anomalies.

Resting cells of strain B13 always showed an immediate 3CB transformation after a starvation period of $\leq$ 24 hours in batch system (129). Therefore, a lag phase or adaptation period causing the late steady state adjustment in the columns is not very likely. At lower flow rates (i.e. lower pressure on the column system) the resistance of the narrowest flow paths may not be overcome and flow may become more preferential. As a result, regions with immobile water develop, leading to an enhanced diffusion limitation. Hence, a longer time would be needed to involve all biomass in the transformation. This would result in a later steady state adjustment and a reduction of the steady-state removal activities. Empirical k values were estimated by fitting the η_T for 3CB on the data of the individual column experiments. The empirical k was $< 4 \cdot 10^{-20}$ m^3·s^{-1} for experiments with an experimental:predicted removal below 0.5 (U/n below about $5 \cdot 10^{-10}$ m·s^{-1}). This value is 3 orders of magnitude lower than the theoretically predicted value. Thus, if substrate diffusion actually limits the steady-state removal activities, then the mass transfer in the columns is clearly not described by the established expression for k.

Different mass transfer characteristics will also affect the mass transfer of oxygen. Corresponding oxygen-k values of $< 8 \cdot 10^{-20}$ m^3·s^{-1} were estimated for these experiments, based on the difference in molecular diffusion coefficients of 3CB and oxygen. The oxygen need for endogeneous respiration is about 7 nmol O_2·mg protein^{-1}·min^{-1} for resting cells of strain B13. Transformation of 20 μM 3CB at a rate according to eq. (3) would require an additional oxygen flux of about 12 nmol O_2·mg protein^{-1}·min^{-1}. We calculated that at this oxygen-k value of $8 \cdot 10^{-20}$ m^3·s^{-1} the oxygen supply would be just enough to satisfy both endogeneous and substrate respiration. In the experiments aerated with 98% O_2, the calculated oxygen fluxes were still below the required flux for endogeneous respiration. We therefore suggest that the actual mass transfer coefficients in these experiments indeed lie in the range of 10^{-20} m^3·s^{-1} and that the lower observed transformation activities are due to mass transfer effects concerning both 3CB and oxygen.

The role of mass transfer processes is also supported by the influence of the

biomass density. Experiments carried out at equal flow rates showed decreasing experimental:predicted steady-state removal ratios with increasing biomass content (Fig 3). Thus, the variation in the experimental:predicted steady-state removal was not directly related with the flow rate, but a dominant association was observed with the flow:biomass ratio. Since this ratio is an important parameter determining mass transfer (eq. 2), this correlation stresses the role of mass transfer limitation in our experiments. From our observations we are not able to determine whether oxygen fluxes only were insufficient for adequate 3CB transformation, or if the cells also lost activity due to oxygen deficiency. A loss of active biomass or transformation activity can only be verified with the direct determination of specific transformation rate of the biomass from the soil columns in steady state.

Harms and Zehnder expressed mass transfer limitation as an apparent upshift in the half-saturation constant for uptake, K_t (38). They calculated theoretical K_t upshifts for 3CDF transformation in column experiments with *Sphingomonas* sp. strain HH19k, although a theoretical mass transfer limitation is hardly visible in the predicted effluent concentrations (Fig. 5B). This is caused by the relatively small influence of mass transfer on the higher predicted effluent concentrations (at high flow:biomass ratios), and the relatively large influence on predicted effluent concentrations of about zero (at low flow:biomass ratios). Similar to the observations with strain B13, the experimental effluent concentrations and thus the experimental K_t upshifts were higher than was described by the available expressions for η_T and k (38) (Fig. 4B). This deviation from predicted values increased with lower flow:biomass ratios (Fig. 4B). The hypotheses described above for the column experiments with 3CB at low flow:biomass ratios are not plausible for the experiments with 3CDF. The lowest flow rates used in the 3CDF experiments were in the highest range of the experiments with 3CB. Also fitted values of η_T and k showed that oxygen fluxes could not be limiting in the 3CDF transformation. Since strain HH19k possesses a much higher specific substrate affinity than strain B13, strain HH19k is expected to be much more susceptible to mass transfer limitation (17, 38). Deviation of the experimental from predicted data was indeed observed in a much higher range of flow:biomass ratio in experiments with strain HH19k than with strain B13, which supports the role of diffusion limitation.

This study contains several indications for mass transfer limitation of biotransformation in a column system, although no indisputable evidence could be established from our and published results. Apparently, convective-diffusive nutrient transport to the bacteria limits the transformation in a percolated packed bed system below a certain flow rate:biomass ratio. Moreover, this effect may be enhanced if the nutrient flux to the cells is too low to meet the maintenance requirement and leads to loss of active biomass. In groundwater systems with low flow rates, mass transfer may therefore be the critical process determining residual contaminant concentrations.

Acknowledgments

The authors thank Hauke Harms for providing the original spreadsheet and raw data of reference (38).

Chapter 5

General Discussion

Introduction

The world-wide production and use of synthetic organic compounds has led to a wide distribution of these compounds in our environment. This has raised serious concern about the hazard of organic pollution to existing life forms. Detoxification of these chemicals may occur by transformation into harmless products by both biotic and abiotic processes (4). However, transformation may also lead to products which can even be more hazardous to the environment than the original contaminant (141). Microbial degradation is often required for a complete mineralization and eventual elimination of the compounds. Many potentially biodegradable contaminants are present in soil, groundwater and natural waters at typical aqueous concentrations in the nanomolar to micromolar range. Since easily degradable organic compounds are also often present at low concentrations, aquatic and terrestrial environments are potentially oligotrophic (74). Predicting the fate of micropollutants therefore requires knowledge about biodegradation at low substrate concentration levels and under conditions without, or with very slow bacterial growth. The next paragraphs shortly discuss the present knowledge in this field. It is divided into three parts: (i) the kinetics of uptake and biotransformation, (ii) the occurrence and possible causes of residual concentrations, and (iii) the simultaneous utilization of multiple substrates.

Uptake and conversion kinetics

Diversity of kinetics in different concentration ranges

The kinetics of biotransformation in environments with low substrate concentrations can differ substantially from the transformation kinetics under conditions of ample substrate availability. When a biotransformation process cannot be described by one set of kinetic parameters covering all different concentration ranges, the kinetics of the conversion are called multiphasic. Two phenomena may account for multiphasic kinetics: (i) the kinetic diversity of a mixed population of coexisting microorganisms and (ii) the multiphasic kinetic behavior of single bacterial strains.

The transformation kinetics of a mixed microbial population is a resultant of the kinetic behavior of the coexisting strains. In different concentration ranges, different subpopulations of bacteria may be active and govern the kinetics, depending on their specific affinity for the substrate. The specific affinity a of an organism for a substrate was defined as the ratio between the specific uptake or transformation rate V and the extracellular substrate concentration S (62). At subsaturated concentrations, the specific affinity approaches a^0, which equals the

slope of the first-order part of the *V*-versus-*S* plot (21). Organisms from oligotrophic environments are generally characterized by lower values for the half-saturation constant K_s or K_m and higher values for a^0 as compared to eutrophic organisms (112). Bacterial isolates from low nutrient environments like ocean and drinking water showed K_s values in the nanomolar range (23, 131, 134). Oligotrophic and eutrophic organisms represent the extremities of a broad range of organisms with diverse kinetics, but can still be isolated from the same environment (130). Microbial assemblages in natural water samples demonstrated almost continuously increasing a^0 values for several substrates at decreasing substrate concentrations (8, 46, 68). Multiphasic kinetics have also been observed for mixed bacterial populations in soil systems and biofilm reactors (7, 30, 84, 105, 111). Schmidt and Gier showed that two soil bacteria were apparently responsible for the observed multiphasic mineralization kinetics of 2,4-dinitrophenol in this soil type (104, 105).

Multiphasic kinetics have also been observed in single bacterial strains. They may for instance result from varying concentrations of a given transport system or the use of transport enzymes with multiphasic behavior (35, 81). Another mechanism is the use of multiple uptake systems in different concentration ranges, which has been shown for amino acid transport in both oligotrophic and eutrophic bacterial strains (33, 47, 54, 90, 148). The individual systems were in most studies described by separate sets of Michaelis-Menten parameters and the systems operating in the lower concentration ranges were characterized by higher values for the specific affinity (a^0) and lower values for the uptake capacity (V_{max}). Ishida et al. reported multiphasic growth kinetics of two facultatively oligotrophic and two eutrophic organisms (47). The distinct K_s values in the different concentration ranges were remarkably lower for the facultative oligotrophs than for the eutrophs. Few studies are available on multiphasic transformation of xenobiotic compounds by pure bacterial cultures. At least two uptake or transformation systems were involved in the conversion of methyl parathion by a *Flavobacterium* species (67). Indications for the existence of two systems for 3-chlorobenzoate (3CB) uptake or transformation were found for *Pseudomonas* sp. strain B13 (Chapter 2). The transformation system operating above 1 μM 3CB possessed an apparent threshold concentration for transformation of 0.50 $\pm$ 0.11 μM 3CB. The transformation system that was active below 1 μM 3CB could not be characterized as a high-affinity, low-capacity system. This system showed only first-order and no saturation kinetics, which was different from the literature data we had available.

Kinetics in different states of growth

The growth state of microorganisms can have a noticeable influence on bioconversion kinetics. Powell has shown that apparent kinetic parameters may depend on the growth rate history of the organism (88). In the following paragrahps,

we distinguish the influence of long-term and of short-term growth rate history on substrate transformation kinetics.

Since carbon substrates are severely limited in many natural environments, it must be expected that microbial biomass has experienced a long-term selection under slow or no-growth conditions. The selective potential of long-term chemostat growth at a fixed dilution rate is generally accepted and is based on the competition for the limiting substrate between organisms which differ in specific affinity and/or their efficiency of substrate utilization. Jannasch used this method to select many single bacterial strains from the indigenous bacterial population in seawater samples (49). However, also chemostat cultures starting from one single bacterial strain have shown a selection of descendants with a higher substrate affinity (43, 57, 58, 82, 114). In most of these studies 50 volume changes or more were needed to observe the change in kinetic behavior. Höfle found a first sudden change in glucose uptake kinetics after about 30 volume changes in continuous culture of *Cytophaga johnsonae* (D = 75% of μ_{max}). Prolonged culturing finally revealed five levels of 'steady-state' residual glucose concentrations, and each stepwise decrease of this concentration was accompanied by a stepwise increase of the specific affinity a^0. These observations were interpreted as shifts to higher-affinity glucose uptake systems or their regulation by mutations (43, 87).

The short-term growth history of microorganisms may primarily be of importance for the biodegradation kinetics in engineered environments. The *Cytophaga johnsonae* strain described above also possessed multiple glucose uptake kinetics dependent on the short-term growth history of the cells (42). A low-affinity uptake system was working in cells from the exponential phase of a batch culture and from glucose-limited chemostats at high dilution rates (after 5 volume changes; D = 75% of μ_{max}). The active uptake system of cells grown in chemostats at low dilution rates (D = 15% of μ_{max}) had an about 60 times higher specific affinity a^0 for glucose and a 10 times higher V_{max} (42). An opposite effect of growth rate history was observed for *Torula utilis* cells, which showed lower maximum respiration rates on a variety of substrates when they were grown at lower rates in a chemostat. This effect was explained as an economy in enzyme synthesis (122). Similarly, cells of *Pseudomonas* sp. strain B13 showed much lower maximum 3CB conversion rates in resting state and during very slow growth in a recycling fermentor than during exponential growth. A difference in enzyme concentration alone would not explain the differences in K_s values in the three experimental systems, however (Chapter 3). A sudden 75% decrease in the maximum conversion rate, q_{max}, occurred within a few hours after the onset of 3CB stringency in recycling fermentor and batch system (Chapter 3). This corresponds with the observations of Höfle, who saw the specific affinity a^0 for glucose of batch grown cells drop in the first few hours of the stationary phase to a level of 10% of the preceding affinity

(42). This effect coincided with the exhaustion of the glucose and a 50% drop in ATP content of the cells (44).

Residual concentrations: role of thresholds and process kinetics

Introduction

In the preceding chapters, the term residual substrate concentration has been used as the substrate concentration that remains in the aqueous phase of a biodegradation system. Residual substrate concentrations have been observed in a variety of experimental systems (13, 16, 41, 52, 61, 80, 95, 135) (Chapter 3, Chapter 4). Most of these authors refer to the observed residual concentrations as 'thresholds'. Several types of thresholds have been accredited in literature and are reviewed in short in the following paragraphs. Not all thresholds do necessarily mean that a compound cannot be utilized below this value: a change in experimental conditions may enable degradation below a certain threshold value. On the other hand, not all residual concentrations are related to a threshold concentration. According to our definition, a residual concentration may result from any mechanism preventing a (further) decrease of the concentration. Examples of such mechanisms are thresholds for degradation or growth, inhibiting factors or substrates, and the kinetics of the processes involved in transport and degradation of the compounds.

Threshold for growth or S_{min}

The presence of relatively constant concentration levels of dissolved organic carbon in ocean waters gave rise to the first postulation of the possible existence of a threshold. The carbon concentrations were supposed to being too low to support microbial proliferation and hence a detectable carbon mineralization (50). It was questioned whether such a threshold for microbial growth is actually the cause of steady-state natural carbon levels in oceans (1, 29). Nevertheless, a threshold concentration for growth is considered to play an important role in the persistent low levels of synthetic carbon compounds in the environment (1, 13, 103). The substrate threshold for growth is also called S_{min}, and has been defined as the substrate concentration at which the transformation rate equals the maintenance energy demand of the bacterial population. Therefore, in batch systems and natural environments without a regular input of the considered substrate, S_{min} can only explain a persisting residual concentration level when the population size is so small that the transformation for maintenance purposes does not significantly affect the bulk concentration. In batch experiments, threshold concentrations for growth have

been determined as the highest substrate concentrations at which the bacterial growth curves did not differ significantly from the growth curves in batches without added substrate (83, 102, 132-134). This method yielded S_{min} values of four bacterial strains for *p*-nitrophenol ranging from 2 to 100 $\mu g \cdot l^{-1}$ (83). Growth of a *Flavobacterium* species, isolated from drinking water, was still enhanced by additional glucose and starch at concentrations below 1 $\mu g \cdot l^{-1}$ of carbon (132).

Thresholds for utilization

Several types of thresholds have been described below which no uptake or degradation was observed. In methanogenic systems, observed threshold concentrations for degradation are often explained by reaction thermodynamics. A lower concentration limit exists below which the overall change in free energy of the degradation reactions is too low for converting it to biologically useful energy (45, 52, 147). Aerobic degradation reactions are usually sufficiently exergonic (Chapter 2).

An adaptation threshold was found for freshwater and sediment samples that started degrading *p*-nitrophenol only when they had been preexposed to a concentration higher than about 10 $\mu g \cdot l^{-1}$ (118). Roughly the same concentration was found as a supposed threshold for induction of enzymes necessary for the uptake or degradation of *p*-nitrophenol by growing cells of a *Pseudomonas* sp. (106). Studies on the enzyme level also revealed induction thresholds for some metabolic enzymes in the degradation of xenobiotic compounds (48, 63).

A threshold for 3CB uptake or transformation (0.50 ± 0.11 μM 3CB) seems to exist for one of the 3CB uptake or transformation systems of resting cells of *Pseudomonas* sp. strain B13 (Chapter 2). These cells were well adapted and fully induced for 3CB transformation, and limitation by thermodynamics could not play a role. Moreover, 3CB was still transformed at concentrations below this apparent threshold (down to the detection limit of 1.0 nM 3CB), probably by another transformation system (Chapter 2). The author is not aware of literature reporting a similar threshold of an uptake or transformation system.

It must be said, however, that most studies cannot distinguish the different thresholds described above. For instance, a threshold of an uptake or transformation system, as observed in Chapter 2, may very well be part of the supposed induction threshold reported by Schmidt (106) or play a role in an observed adaptation threshold (118). Furthermore, thresholds for growth and induction can be part of an adaptation threshold, since induction is without doubt part of the adaptation process, and growth of a small subpopulation may be necessary to adapt an indigenous population to xenobiotic degradation.

Role of process kinetics on residuals

Residual concentrations can be observed without the role of any of the aforementioned thresholds in systems with a continuous substrate replenishment. It is generally accepted that the growth kinetic parameters of microorganisms determine the steady-state residual substrate concentrations in carbon-limited chemostats at a fixed dilution rate (i.e. growth rate) (109, 121; Chapter 3). For transient growth in a chemostat (slowly increasing growth rate), the residual substrate concentration could be predicted with about the same kinetic parameters as determined in steady state (76). During growth of *Pseudomonas* sp. strain B13 in recycling fermentors (decreasing growth rate), residual 3CB concentrations were also observed and their relation with the 3CB utilization rate was clearly described by a Michaelis-Menten-type kinetics (Chapter 3).

Roughly, two main processes establish the overall substrate utilization by bacteria in aqueous environments: (i) mass transfer of substrate to the cells surface and (ii) the combined process of uptake and transformation by the cells. Even in turbulent cell suspensions the substrate still has to diffuse through an unstirred layer around the cells (14, 38, 56, 85). Under certain circumstances, diffusion can become the rate limiting process and diffusion kinetics, instead of microbial kinetics, will determine the eventual utilization rate. In cell suspensions, diffusion may limit the substrate utilization rate especially at low substrate concentrations and at very high specific affinities (37, 38, 103). Limitation of substrate utilization by diffusion could not be calculated for any of the suspended cell experiments with *Pseudomonas* sp. strain B13, and residual concentrations observed in the fermentor experiments were therefore determined by microbial kinetics (Chapter3).

Mass transfer has been recognized as a critical factor for the biodegradation in various engineered and natural environments, however. Several studies have reported the limitation of the degradation activity by advection (69), sorption/desorption (27, 92), diffusion (27, 38, 78, 85, 96, 149) and dissolution of the substrate (31, 123, 142). Depending on the biodegradation system under consideration, the combination of one or more mass transfer mechanisms will determine the overall mass transfer kinetics (17). Column experiments with attached cells of *Pseudomonas* sp. strain B13 suggested limitation of 3CB transformation by convective-diffusive transport and showed a noticeable similarity with the study of Harms and Zehnder (38) (Chapter 4). Below a critical ratio of flow rate and biomass, the residual effluent concentrations were higher than predicted with microbial kinetics. This deviation increased with decreasing flow rate:biomass ratios, suggesting mass transfer limitation. Interestingly, our results indicated that 3CB degradation was limited by the combined mass transfer of 3CB and oxygen to the cells.

A special case of steady state is the situation in which the substrate flux into the cells is so low, that the transformation just satisfies the maintenance requirement and net growth is zero. The aqueous substrate concentration at which this occurs is S_{min}, the treshold or minimum concentration for growth. S_{min} has been correctly predicted in continuous biofilm reactors as the aqueous substrate concentration below which no steady-state biofilm activity could occur anymore (95, 96). The most frequently used method of S_{min} calculation, however, is extrapolation of steady-state chemostat growth data to zero growth rate (22, 59, 62). In our study, S_{min} was measured directly at zero net growth in a recycling fermentor. *Pseudomonas* sp. strain B13 was cultured under substrate stringency in a fermentor with 100% biomass retention. The residual substrate concentrations and the specific rates of transformation and growth decreased progressively until the substrate transformation rate equaled the biomass' maintenance demand. At this point, growth became zero and the residual substrate concentration equaled S_{min} (Chapter 3). In fact, S_{min} can be considered as a lower limit to the residual concentration of a compound in a relatively stable environmental or experimental system.

A prominent example of a non-stable system is of course a batch incubation. In batches with *Pseudomonas* sp. strain B13 no residual concentration of 3CB and acetate could be detected (Chapter 2). Additionally, biofilms that had developed with a high substrate concentration could in non-steady-state degrade this substrate below S_{min}, after a sudden decrease of the influent concentration (72, 97).

From the previous paragraphs it should be clear, however, that also in a stable or steady-state situation S_{min} has not a fixed value for one particular substrate/organism combination, but depends on the conditions in the biodegradation system. The combined action of microbial and mass transfer kinetics will determine the bulk aqueous concentration S_{min}, that is required for establishing the flux necessary to maintain a microbial population. Additionally, the required maintenance substrate flux in stable natural environments may deviate from the values determined in pure culture studies. Anderson an Domsch found that *in situ* maintenance coefficients for the indigenous bacterial populations surviving in three types of soil ecosystems at environmental temperatures (15°C) were some 3 orders of magnitude lower than the values determined in pure culture suspensions and actively metabolizing soil microorganisms (2, 3; Chapter 3). Therefore, the S_{min} values may also be much lower for indigenous populations in natural environments than for pure culture studies.

Mixed substrate utilization

Mixed substrates at high concentrations

A xenobiotic compound is generally one of many carbon compounds in natural or engineered environments. Numerous microorganisms are able to utilize mixtures of carbon substrates (9, 34). At high concentrations, a diauxic growth pattern is often observed due to the inhibitory effect of one carbon source on the other (36, 144). Inhibitory effects of additional substrate on xenobiotic substrate degradation rates were reported by several authors (10, 99, 100, 106, 120, 126). A possible mechanism is the inhibition of enzyme synthesis for xenobiotics conversion by the presence of readily metabolized additional substrate (catabolite repression). This mechanism may have played a role in slurries of aquifer solids, in which the mineralization of toluene, *p*-nitrophenol or ethylene bromide at 0.1 $mg \cdot kg^{-1}$ were inhibited when glucose or amino acids (at 0.1 and 2 $mg \cdot kg^{-1}$, respectively) were added (120). Another mechanism for inhibition is for instance the interference with the uptake or mineralization by a substrate analog (competitive inhibition) (24, 100, 106). A concentration of 100 mg of phenol per liter caused a sixfold lower specific affinity a^0 of a *Pseudomonas* sp. for *p*-nitrophenol (initial concentration, 4 $mg \cdot l^{-1}$) (106).

An effective enhancement of pollutant degradation by relatively high concentrations of additional substrate may result from extra growth of the microbial population which is able to degrade the contaminant (41, 106). Other stimulatory effects of supplementary substrate on contaminant degradation were for example ascribed to an elevated intracellular level of reduced cofactors (11, 32, 94).

Mixed substrates at low concentrations

Readily utilizable carbon/energy substrate is often severely limited in aquatic and terrestrial environments, which are therefore potentially oligotrophic (29, 74). In addition to an increase of specific affinity for the substrate, as described in the first section of this chapter, many organisms living in these environments use the strategy of a broader substrate spectrum. When a wide range of catabolic enzymes is expressed at a low level, a multiplicity of substrates can be utilized simultaneously (36, 87). This was illustrated by the respiration of 29 out of 39 tested substrates by a freshwater bacterium pregrown in a chemostat at $D = 0.001\ h^{-1}$. When pregrown at $D = 0.34\ h^{-1}$, only 9 of these substrates could be respired (115). Another study showed that oligotrophic isolates from Antarctic freshwater were nutritionally much more versatile than copiotrophic isolates from the same water sample (130). Thus, the simultaneous utilization of mixtures of carbon substrates at low concentration is rather the rule than the exception in natural environments (29).

Considering all environments with low pollutant concentration, it is interesting to know the influence of mixed substrate utilization on the pollutant degradation rate. A small increase of the methylene chloride degradation rate was observed during simultaneous acetate mineralization in batch experiments with a *Pseudomonas* sp. (61). In resting cell batches with *Pseudomonas* sp. strain B13, transformation rates of 3CB at nanomolar and low micromolar concentrations were not affected by the addition of 50 to 5,000 μM acetate (Chapter 2). A similar lack of effect was previously described for the simultaneous utilization of 560 μM glucose and 30 μM *p*-nitrophenol by resting cells of a *Pseudomonas* sp. (106).

Perhaps even more important is the question whether mixed substrate degradation affects residual pollutant concentrations. Until now, a considerable number of studies demonstrates that residual substrate concentrations are lower in simultaneous utilization than in single substrate use. Batches with low cell densities were only able to degrade their substrate below the thresholds for growth, when the simultaneous utilization of one or more additional substrates allowed the cells to grow (83, 102, 134). Similarly in continuous flow systems, very low concentrations of xenobiotics can only be degraded when steady-state biofilms are supported by a primary substrate (18, 19). In biofilm reactors degrading two substrates in the same concentration range, residual concentrations were found to be lower than in reactor runs with a single substrate (77). For *E. coli* growing in chemostat on a mixture of three sugars, Lendenmann demonstrated clearly that the steady-state residual concentrations of the individual sugars added up to the residual substrate concentration, which was obtained with only one of the sugars as the single substrate. (65). In Chapter 3, we presented a similar result for strain B13 in a recycling fermentor fed with a mixture of 3CB and acetate. The S_{min} values reflected the relative energy contribution of the two substrates in the influent and were about 50% of the residual concentrations in the runs with the single substrates (Chapter 3). Thus, the probable contribution of many substrates to the maintenance energy of a microbial population living in a natural environment, allows the S_{min} of individual xenobiotic substrates to be much lower than in single substrate use.

Concluding remarks

Biotransformation kinetics and residual pollutant concentrations may depend on concentration level, bacterial growth state, maintenance energy demand, thresholds, other degradable carbon substrates and mass transfer kinetics. In carbon-limited aquatic and terrestrial environments, mixed substrate utilization, the use of high-affinity transformation systems and much lower *in situ* maintenance coefficients together determine the transformation kinetics and the residual concentrations.

Therefore, if microbial kinetics limit the biodegradation, much lower S_{min} values for the individual substrates are expected in natural environments than in single substrate use by pure cultures. Moreover, microbially determined S_{min} values may be far below a detection limit, when we consider the 1000 times lower maintenance coefficients under field conditions compared to the values determined in pure culture suspensions (2; Chapter 3). Since intrinsically degradable pollutants are still detected in the environment, we suggest that in most cases mass transfer kinetics, and not microbial kinetics establish the S_{min} in the bulk liquid. This is important for the application and controlling of (*in situ*) bioremediation techniques. Residual pollutant concentrations are then best reduced by improving the compounds' bioavailability, provided that the microbial population is adapted to the degradation of this compound.

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Summary

and Concluding Remarks

The pollution of our environment with a large number of synthetic organic chemicals has raised serious concern about their toxicity to existing life forms. Microorganisms play an essential role in the breakdown of xenobiotic compounds by using them as a carbon and energy source. However, pollutants that are intrinsically biodegradable are still widespread in soil, groundwater and natural waters at typical concentrations at the nanomolar to micromolar level. This has raised questions about microbial degradation kinetics at low concentration levels and about factors which prevent complete biotransformation.

The project of this thesis was initiated to study the biodegradation of xenobiotic compounds in the low concentration range. The work presented here focussed on three main topics: (i) biotransformation kinetics at low concentration levels, (ii) the lowest attainable or residual concentration, and (iii) the effects of experimental conditions on the kinetics and residual concentrations. We examined the influence of the growth state of the organisms, the presence of additional readily degradable substrate, the temperature, the liquid flow regime and the process of mass transfer. The studies were done using a pure culture of *Pseudomonas* sp. strain B13 in a defined medium. 3-Chlorobenzoate (3CB) was used as a model pollutant and acetate as a model compound for easily degradable substrate. We used experimental systems with different levels of complexity, this to be able to distinguish the influence of intrinsic microbial properties and system-linked factors on the biotransformation kinetics and residual concentrations.

Batch experiments with resting cell suspensions of strain B13 are presented in Chapter 2. Transformation of 3CB is described by Michaelis-Menten kinetics in the wide concentration range of 1.5 μM to 16 mM (K_m, 0.13 mM; V_{max}, 24 nmol·mg protein^{-1}·min^{-1}). Experiments at the nanomolar and low micromolar concentration level indicated that probably a second system for uptake or transformation at low concentrations is present in strain B13. The system operating above 1 μM 3CB possessed an apparent threshold concentration of 0.50 $\pm$ 0.11 μM. The system that was active below 1 μM showed first order kinetics and no saturation, as is typical for Michaelis-Menten kinetics. This system had a rate constant of 0.076 liter·g protein^{-1}·min^{-1} and no detectable threshold concentration. No residual concentrations could be detected for 3CB and acetate when they were spiked as single substrates (detection limits, 1.0 and 0.5 nM, respectively). The addition of various concentrations of acetate as a second, readily degradable substrate neither affected the 3CB transformation kinetics, nor did it generate a detectable residual concentration of either substrate.

Residual concentrations of 3CB and acetate were measured in well-mixed suspended cell fermentors with continuous substrate supply (Chapter 3). Growth kinetic parameters of strain B13 determined the steady-state residual substrate concentrations in chemostats. In a fermentor with 100% biomass retention (recycling fermentor), the lowest residual concentration measured was S_{min}, the minimum

concentration required for growth. S_{min} was measured in a stationary phase of net zero growth, and was determined by the maintenance requirement and the transformation kinetic parameters of strain B13 in the recycling fermentor. Both maintenance coefficient and S_{min} were lower in recycling fermentors at 20°C than at 30°C. When provided as a mixture, the individual S_{min} values of 3CB and acetate were lower than the measured S_{min} values during single substrate use. The S_{min} values with the mixture reflected the relative energy contribution of the two substrates in the fermentor feed. The kinetic parameters of 3CB transformation, and thus predicted S_{min} values, varied remarkably with the different growth states of strain B13 in batch (resting state), recycling fermentor (very slow growth) and chemostat (exponential growth).

The kinetics of substrate transfer to the microorganisms, and not the intrinsic microbial kinetics, may under certain conditions determine the biodegradation rate. The possible effect of mass transfer on residual concentrations of 3CB was studied in percolated soil columns with attached resting cells of strain B13 (Chapter 4). The rate of mass transfer was varied by applying different flow rates and biomass levels. The observed residual concentrations in steady state were compared with predictions from spreadsheet models describing the combined action of microbial and mass transfer kinetics. The effluent concentrations were successfully predicted above a critical ratio of flow rate and biomass. Below this critical point, the experimental concentrations were higher than predicted and this deviation increased with decreasing flow rate:biomass ratios. These results corresponded remarkably with literature data on 3-chlorodibenzofuran degradation in percolation columns with *Sphingomonas* sp. strain HH19k. It was calculated that 3CB transformation was probably limited by convective-diffusive transport of both 3CB and oxygen to the cells.

The relevance of the results presented in this thesis is discussed in Chapter 5. The results are included in a short review of literature data reporting on the kinetics of uptake and transformation, on residual concentrations and on the factors which are of influence, such as concentration level, bacterial growth state, thresholds, maintenance energy requirement, other carbon substrates and mass transfer kinetics.

The gathered knowledge on these topics supports the following concluding remarks for pollutant degradation in natural and engineered environments. S_{min} can be considered as the lower concentration limit of a compound in relatively stable or steady-state environments. In non-stable systems like batch incubations, even lower residual concentrations are expected, providing that bioavailability of the pollutant or thresholds for utilization are not limiting the degradation. The significance for bioremediation techniques may lie in the application of batch-like or pulsing systems (Chapter 2) and/or the provision of low concentrations of additional substrates (Chapter 3) to reduce residual pollutant concentrations.

Microbially determined S_{min} values are probably much lower in natural carbon-limited environments than in studies with one organism/substrate combination. Such S_{min} values may even be far below a detection limit, if we consider the 1000 times lower *in situ* maintenance coefficients compared to pure culture suspensions (Chapter 3). Intrinsically biodegradable pollutants are still detected in many environments, however. It is therefore suggested that mass transfer kinetics (Chapter 4), and not microbial kinetics determine the S_{min} in these environments. Residual pollutant concentrations can then only be reduced by improving the bioavailability of the compounds.

Samenvatting

Bacteriële afbraak van microverontreinigingen - welke factoren beïnvloeden resteoncentraties ?

Milieuverontreiniging en bacteriën

In de laatste helft van deze eeuw heeft de chemische industrie een hoge vlucht genomen met de wereldwijde produktie van organische chemicaliën. Deze produkten vonden hun toepassing in onze moderne samenleving, maar hebben tevens geleid tot een wijdverbreide verontreiniging van ons leefmilieu. Sinds enkele tientallen jaren is gelukkig ook de bezorgdheid over de voortschrijdende vervuiling sterk toegenomen. Uit bepaalde onderzoeken, maar helaas ook door 'schade en schande' is namelijk duidelijk geworden dat organische verontreinigingen giftig kunnen zijn voor levende organismen, inclusief de mens. Een belangrijke vraag waar we voor zijn komen te staan is hoe we weer van deze schadelijke verbindingen af kunnen komen.

Ontgifting van organische verbindingen kan plaatsvinden door deze om te zetten in andere, minder schadelijke produkten. Een omzetting van een verbinding kan echter ook een produkt geven dat even schadelijk, of zelfs nog schadelijker is dan de uitgangsstof. De veiligste omzetting is een volledige 'verbranding' van een organische verontreiniging tot de onschadelijke eindprodukten water, koolzuurgas (CO_2), en eventueel wat mineralen. Deze laatste reactie is in de praktijk vaak van biologische aard, en wordt vooral uitgevoerd door bacteriën.

Bacteriën zijn kleine eencellige organismen met afmetingen van rond eenduizendste millimeter. Ze leven overal: in en op andere organismen, in de lucht, in oppervlaktewater en in de ondergrond. Sommige bacteriesoorten zijn schadelijk voor mens en dier (ziekteverwekkers), maar de meeste zijn juist heel nuttig. Zij breken allerlei organische verbindingen (hun substraat ofwel voedsel) af en winnen daaruit energie en bouwmateriaal voor de instandhouding van hun cel (onderhoud) en voor de vorming van nieuwe cellen (groei). Uit laboratoriumtests is gebleken dat de meeste organische stoffen, waaronder vele giftige chemicaliën die door de mens zijn geproduceerd, kunnen worden afgebroken door bacteriën. De bacteriën vormen daardoor een belangrijke groep afvalverwerkers in de natuur, maar ook in reinigingsinstallaties voor bijvoorbeeld vervuild grondwater, of huishoudelijk of industrieel afvalwater.

Omzetting van microverontreinigingen

We hebben net genoemd dat vele schadelijke verontreinigingen kunnen worden afgebroken en ontgift door bacteriën, en dat bacteriën bovendien 'overal' zijn. Desondanks worden vele van deze verbindingen nog steeds in bodem, grondwater en oppervlaktewater aangetroffen. Hoe vallen deze twee waarnemingen te rijmen? Om deze vraag te beantwoorden zullen we de levensomstandigheden van de bacteriën in laboratoriumtests moeten vergelijken met die in werkelijkheid. Eén van de belangrijke verschillen tussen veel laboratoriumtests en de praktijk is de concentratie waarin de organische verontreiniging voorkomt. Veel milieuveront-

reiniging is namelijk van een zogenaamd gespreide of diffuse vorm en de heersende concentraties zijn vaak aanzienlijk lager dan in veel laboratoriumexperimenten. Karakteristieke concentraties in grondwater en oppervlaktewater liggen in de grootteorde van microgrammen per liter of zelfs lager (een microgram is een miljoenste gram). Dit is ook te zien in Tabel 1. Verontreinigingen bij zulke lage concentraties worden daarom vaak ook microverontreinigingen genoemd.

Verwijdering van microverontreinigingen uit het milieu is meestal gewenst omdat veel verbindingen ook bij 'lage' concentraties schadelijk kunnen zijn voor levende organismen. Tabel 1 laat slechts een fractie zien van alle organische verontreinigingen die zijn aangetroffen in Nederlands oppervlaktewater. Toch wordt dit water gebruikt voor de bereiding van drinkwater en zal het moeten worden gezuiverd om te voldoen aan de drinkwaternormen. Voor een belangrijk deel van de drinkwatervoorziening van Amsterdam wordt bijvoorbeeld gebruik gemaakt van Rijnwater. Dit water wordt onder andere geïnfiltreerd in een speciaal duingebied, waarbij vele organische verontreinigingen worden afgebroken door de populatie bacteriën in het duinzand. Na deze biologische reinigingsstap worden er echter nog steeds (te hoge) restconcentraties van een aantal van deze verbindingen in het water gevonden. In het algemeen kan gesteld worden dat bij het toepassen van een biologische reinigingstechniek niet alleen het percentage verwijdering van een verontreiniging belangrijk is, maar vooral hoeveel van de verontreiniging er na afloop van de behandeling nog resteert. Er is echter nog weinig bekend over de factoren die een restconcentratie bepalen.

Opzet van het onderzoek

In dit onderzoek is de omzetting van verontreinigingen bij lage concentraties als onderwerp gekozen. De studie heeft zich daarbij gericht op drie aspecten: (i) de kinetiek van omzetting en groei (hierbij is de snelheid van omzetting, respectievelijk van groei, bij verschillende concentraties verontreiniging bestudeerd), (ii) de laagst mogelijke restconcentratie verontreiniging, en (iii) de invloed van experimentele omstandigheden op kinetiek en restconcentratie. De factoren die hierbij in de verschillende proeven zijn gevarieerd, zijn gebaseerd op de omstandigheden die in het natuurlijk milieu of tijdens een biologische reinigingsprocedure kunnen heersen. Deze factoren zijn: de gelijktijdige aanwezigheid van een makkelijk afbreekbare voedingsstof, de temperatuur, de mate waarin de bacteriën kunnen groeien, een voortdurende vloeistofstroom, en het mechanisme en de snelheid waarmee de verontreiniging de bacteriecellen bereikt.

Om al deze factoren te kunnen onderzoeken is gebruik gemaakt van verschillende experimentele opstellingen, die varieerden van heel eenvoudig tot behoorlijk complex. In de volgende paragrafen worden deze systemen nader toegelicht. We hebben met één bacteriesoort gewerkt, *Pseudomonas* B13, om de

Tabel 1 Maximale concentraties van (gechloreerde) aromatische verontreinigingen, aangetroffen in Nederlandse oppervlaktewateren (de Rijn, de Maas en het IJsselmeer) en in het daaruit bereide drinkwater. De verontreinigingen zijn aangetroffen in de periode van 1987 tot 1991. (Bron: Rapport SWO 93.261, 1993, KIWA N.V., Nieuwegein).

Verontreiniging	Oppervlaktewater[4]	Drinkwater[4]
anilines		
aniline	xx	x
2-chlooraniline	xx	x
3-chlooraniline	x	n.b.
4-chlooraniline	x	<
2,3-dichlooraniline	xx	<
2,4-dichlooraniline	x	<
3,4-dichlooraniline	xx	<
3,5-dichlooraniline	xx	<
2,4,5-trichlooraniline	xx	x
benzenen		
benzeen	xxx	<
chloorbenzeen	x	<
1,2-dichloorbenzeen	xx	xx
1,3-dichloorbenzeen	x	n.b.
1,4-dichloorbenzeen	xx	n.b.
trichloorbenzeen	x	<
tetrachloorbenzeen	x	<
benzoaten		
benzoaat	xx	xxx
3-chloorbenzoaat	xx	n.b.
2,4-dichloorbenzoaat	xx	n.b.
tetrachloorbenzoaat	x	x
fenolen		
fenol	x	n.b.
chloorfenol	x	n.b.
2,4- of 2,5-dichloorfenol	xx	<
3,4-dichloorfenol	x	<
3,5-dichloorfenol	xx	<
2,4,5-trichloorfenol	x	<
2,4,6-trichloorfenol	x	<
3,4,5-trichloorfenol	x	<
2,3,4,5-tetrachloorfenol	x	<
2,3,4,6-tetrachloorfenol	x	<
2,3,5,6-tetrachloorfenol	x	<
pentachloorfenol	x	<

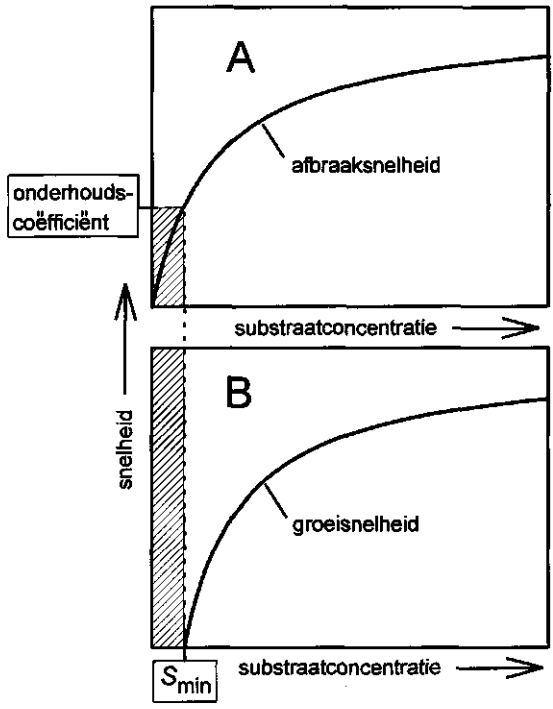
[4] x : lager dan 0,1 microgram per liter; xx : tussen 0,1 en 1 microgram per liter;
xxx : tussen 1 en 10 microgram per liter; < : beneden aantoonbaarheidsgrens;
n.b.: niet bepaald.

resultaten in de verschillende experimenten goed te kunnen vergelijken. Deze *Pseudomonas* is oorspronkelijk uit een afvalwaterzuiveringsinstallatie geïsoleerd en kan onder andere 3-chloorbenzoaat (afgekort als 3CB) als substraat (voedsel) gebruiken. Wij hebben 3CB, een gechloreerde aromatische verbinding, als een voorbeeldverontreiniging gebruikt, en azijnzuur als een model voor een makkelijk afbreekbaar substraat.

Kinetiek van voedselafbraak en bacteriegroei

De snelheid waarmee bacteriën een verbinding kunnen afbreken en gebruiken is onder andere afhankelijk van de concentratie van deze verbinding. Hoe die afbraaksnelheid precies afhangt van de concentratie noemen we in het kort de kinetiek van afbraak. Figuur 1A laat de afbraaksnelheid zien als functie van de substraatconcentratie. Deze vorm van kinetiek is karakteristiek voor de meeste verbindingen en voor de meeste bacteriën. De steilheid van het stijgende deel en de

Figuur 1 Kinetiek van substraatafbraak en bacteriegroei. De afbraaksnelheid (A) en groeisnelheid (B) zijn weergegeven als functie van de substraatconcentratie. Bij de concentratie S_{min} is de afbraaksnelheid van het substraat net voldoende om de bacteriën te onderhouden (onderhoudscoëfficiënt). De groeisnelheid is dan nul.



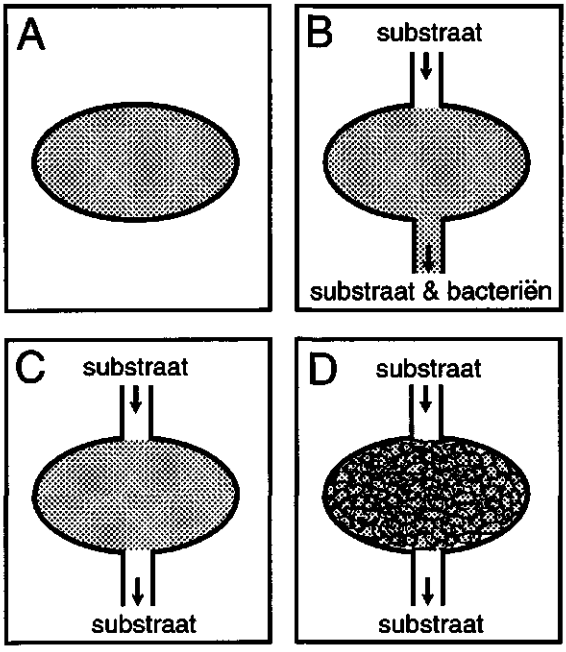
hoogte waarop de curve afvlakt kunnen wèl erg sterk variëren tussen verschillende bacteriën en substraten. De precieze vorm wordt gekarakteriseerd door de zogenaamde kinetische parameters.

Hetzelfde geldt voor de curve van de groeisnelheid als functie van de substraatconcentratie (Fig. 1B). De vorm van deze groeicurve is grofweg gelijk aan die van de afbraakcurve in Fig. 1A. Dit is misschien niet erg verrassend, aangezien er immers pas groei kan optreden als er substraat is afgebroken voor de nodige energie en bouwstenen. Als we echter goed kijken naar de lage substraatconcentraties (hierin zijn we per slot geïnteresseerd) zien we toch een afwijking. In de meeste experimenteel bepaalde curven zal deze afwijking niet duidelijk te zien zijn, maar in de figuur is deze overdreven weergegeven. Bij een zekere substraatconcentratie S_{min} zien we dat er nog wel substraat wordt afgebroken (snelheid groter dan nul), maar dat er geen groei meer optreedt. Dit verschijnsel kunnen we begrijpen door te bedenken dat de bacteriecellen ook een zekere hoeveelheid energie en bouwstenen nodig hebben om zichzelf in stand te houden. Ze moeten onderhoud plegen aan celdelen: eiwitten vervangen en repareren en bepaalde belangrijke mineralen, die langzaam uit hun cel weglekken, weer naar binnen halen. Bij de concentratie S_{min} is de afbraaksnelheid zo laag dat precies in deze onderhoudsbehoefte wordt voorzien. De afbraaksnelheid bij S_{min} heet dan ook de onderhoudscoëfficiënt en S_{min} zelf noemen we de drempelconcentratie voor groei. Er heerst bij S_{min} dus nog steeds bacteriële bedrijvigheid. Er zal af en toe eens een bacteriecel sterven, en er zal af en toe eens één bijkomen, maar de netto groei van de bacteriepopulatie is nul.

Het effect van kinetiek op restconcentraties

Wat heeft nu de hierboven beschreven kinetiek van bacteriële omzetting of groei te maken met de restconcentraties waarin we zo geïnteresseerd zijn? Zoals in de onderzoeksopzet al is aangestipt, hebben we gewerkt met een aantal verschillende experimentele systemen. Deze zijn schematisch weergegeven in Figuur 2. De eerste twee systemen (Fig. 2A en B) zullen we gebruiken om te illustreren wat voor effect kinetiek op restconcentraties kan hebben. In beide systemen zweefden de bacteriecellen gelijkmatig verdeeld ('in suspensie') in een goed gemengde voedingsoplossing. Voor de rest waren de twee systemen echter heel verschillend.

Het batch-systeem (Fig. 2A) wordt gekarakteriseerd door het feit dat het vaatje met de bacteriesuspensie gesloten is voor zowel bacteriën als substraat. Dit wil zeggen, nadat de *Pseudomonas*-cellen en een éénmalige hoeveelheid 3CB in het batch-vaatje waren gebracht, was er geen uitwisseling meer van bacteriën of 3CB. Tijdens het mengen van dit bacterie-3CB-mengsel werd het 3CB afgebroken en aan het einde van de proef kon er geen restconcentratie 3CB worden aangetoond (Hoofdstuk 2).



Figuur 2 Schematische weergave van de gebruikte proefopstellingen. Het batch-systeem (A) bevatte een goed gemengde bacteriesuspensie en een starthoeveelheid substraat. Gedurende het experiment bleef het systeem gesloten. Door de chemostaat (B) werd een constante vloeistofstroom met substraat gepompt. De suspensie met bacteriecellen en resterend substraat werd met dezelfde snelheid afgevoerd. De bacteriecellen zijn 'opgesloten' in de recycling fermentor (C). Er vinden een voortdurende toevoer van vers substraat en afvoer van resterend substraat plaats. In zandkolommen (D) zijn de bacteriecellen gehecht aan de zandkorrels, en blijven zo in de kolom. Vers substraat werd voortdurend toegevoegd en resterend substraat werd afgevoerd. Per kolom werd de vloeistofstroom constant gehouden.

Het chemostaatsysteem (Fig. 2B) is juist open voor zowel bacteriën als substraat. Er vindt een voortdurende toevoer van voedingsoplossing met substraat plaats, en een voortdurende afvoer van voedingsoplossing met bacteriecellen en resterend substraat. Het principe van zo'n chemostaat is dat de groeisnelheid van de bacteriën ermee ingesteld kan worden. Er ontstaat een evenwichtssituatie, waarbij er even snel cellen bijgroeien als er cellen uitspoelen. Deze groeisnelheid hangt samen met de heersende substraatconcentratie in de chemostaat via een groeicurve als is geschetst in Figuur 1B. De restconcentratie substraat in de chemostaat wordt dus bepaald door de ingestelde groeisnelheid en de kinetische parameters van groei. We hebben zo restconcentraties 3CB gemeten voor een aantal verschillende groeisnelheden van *Pseudomonas* B13 (Hoofdstuk 3).

Hiermee is geïllustreerd dat bacteriën in een systeem met voortdurende substraattoevoer restconcentraties kunnen 'overlaten', terwijl dezelfde bacteriën dit substraat helemaal tot beneden de aantoonbaarheidsgrens afbreken in een gesloten batch-systeem. In het batch-vaatje daalde de 3CB-concentratie tijdens de omzetting steeds verder. Daling beneden de S_{min} (Fig. 1) was mogelijk doordat er bij deze concentraties nog steeds afbraak plaatsvond (Fig. 1A), waardoor de concentratie weer daalde, enzovoort. Dat deze afbraaksnelheid te laag was om volledig het onderhoud van de bacteriën te verzorgen was binnen het tijdsbestek van deze proef niet te merken. Daarvoor waren er veel teveel bacteriën aanwezig en was de afsterfsnelheid veel te laag. De restconcentraties in de chemostaten waren te danken aan de voortdurende aanvulling met vers substraat. De hoogte van deze restconcentraties werd bepaald door de verversingssnelheid en door de kinetische parameters van groei.

Het effect van bijkomende factoren op restconcentraties

De hierboven besproken batch- en chemostaatsystemen hebben geen grote gelijkenis met natuurlijke of gereguleerde biologische reinigingssystemen. In de praktijk zullen we niet te maken hebben met een éénmalige substraatpuls zoals in batch. We zullen echter evenmin te maken hebben met een evenwichtssituatie met voortdurende groei en uitspoeling van bacteriën, zoals in chemostaat. In natuurlijke systemen is meestal niet alleen de verontreiniging diffuus en in lage concentraties aanwezig, maar ook andere, makkelijk afbreekbare voedingsstoffen zijn schaars. De toevoer van substraat is vaak langzaam, zoals bijvoorbeeld in de hiervoor aangehaalde duininfiltratie of in grondwater dat de organische stoffen met het infiltrerende regenwater krijgt aangevoerd. Bacteriegroei is in veel situaties dus niet, of maar in heel beperkte mate mogelijk. De derde proefopstelling die we hebben gebruikt, de recycling fermentor (Fig. 2C), staat al een stapje dichter bij deze situatie.

(i) De onderhoudsbehoefte. De recycling fermentor (Fig. 2C) is open voor substraat (zoals de chemostaat), maar gesloten voor bacteriën (zoals de batch). Er wordt voortdurend voedingsoplossing met substraat toegevoerd, en met dezelfde snelheid 'gebruikte' voedingsoplossing met resterend substraat afgevoerd. De bacteriemassa die hierop groeit, blijft achter in het fermentorvat en neemt zodoende steeds meer toe. De constante substraattoevoer wordt dus verdeeld over een steeds toenemend aantal bacteriën. Hierdoor neemt de gemiddelde substraataanvoer per bacterie, en dus de groeisnelheid, steeds meer af, totdat er een stabiele fase aanbreekt waarin de gemiddelde groeisnelheid nul is. De substraataanvoer is dan zo laag geworden, dat het juist genoeg is om het onderhoud van de bacteriemassa te verzorgen en er niets meer overblijft voor groei. De substraatconcentratie die dan in het fermentorvat heerst is de S_{min} (Fig. 1B). S_{min} is de laagst mogelijke

restconcentratie in de recycling fermentor, en deze kan, net als in de chemostaat, gemeten worden dankzij de voortdurende aanvoer van vers substraat. De hoogte van deze restconcentratie S_{min} wordt bepaald door de onderhoudscoëfficiënt en de kinetische parameters van afbraak die gelden voor de betreffende bacterie en het substraat (Fig. 1A). We hebben zo de onderhoudscoëfficiënten en de S_{min} van *Pseudomonas* B13 voor de afzonderlijke substraten 3CB en azijnzuur gemeten (Hoofdstuk 3).

(ii) Gelijktijdig gebruik van meerdere substraten. De S_{min}-waarden en onderhoudscoëfficiënten van 3CB en azijnzuur zijn eveneens gemeten in recycling fermentors, waarin beide substraten gelijktijdig geconsumeerd werden (Hoofdstuk 3). De S_{min}-waarden voor de substraten in de fermentor met het mengsel waren beide de helft van de S_{min}-waarden die voor de substraten afzonderlijk waren gemeten. Hetzelfde werd waargenomen voor de onderhoudscoëfficiënten voor beide substraten. Dit illustreert dat de bijdrage van een substraat aan het onderhoud van de bacteriën kleiner is als er meerdere substraten gelijktijdig worden gebruikt. De som van de verschillende bijdragen dekt dan de totale onderhoudsbehoefte van de bacteriemassa.

(iii) Kinetiek van substraattransport. Het belang van de kinetiek van substraatafbraak op restconcentraties is in de vorige paragrafen steeds weer gebleken. Tot nu toe hebben we het alleen gehad over de kinetische eigenschappen van de bacteriën zelf. Deze konden we namelijk goed bestuderen in de proefopstellingen met goed gemengde bacteriesuspensies. In de praktijk hebben we echter meestal te maken met bacteriën die zich op een vaste fase van bijvoorbeeld zandkorrels bevinden (vergelijk Fig. 2D). Daartussen beweegt dan de vloeibare fase met de microverontreinigingen. De ruimtelijke scheiding tussen bacteriën en substraat is hierdoor veel groter dan in gemengde bacteriesuspensies. Substraatafbraak kan pas plaatsvinden nadat deze afstand tussen waterstroom en de bacteriën overbrugd is, en de bacteriën zullen het substraat niet sneller kunnen afbreken dan het wordt aangevoerd. De uiteindelijke afbraakkinetiek kan daardoor worden bepaald door de kinetiek van substraattransport naar de bacteriën toe, en niet door de kinetische parameters van de bacteriën zelf.

Met onze vierde proefopstelling, een reeks zandkolommen, hebben we deze situatie geprobeerd na te bootsen (Fig. 2D). Cellen van *Pseudomonas* B13 waren gehecht aan de zandkorrels, en er werden met verschillende stroomsnelheden 3CB-oplossingen door de kolommen gevoerd. Gemeten restconcentraties in de uitstromende vloeistof waren in veel gevallen hoger dan we konden voorspellen op grond van de afbraakkinetiek van de bacteriën zelf. We berekenden verder dat in deze gevallen zeer waarschijnlijk sprake was van een beperking van de afbraaksnelheid door het langzame transport van 3CB èn zuurstof (nodig voor de afbraak van 3CB) naar de bacteriecellen (Hoofdstuk 4).

Conclusies

De resultaten uit deze studie zijn relevant in verband met het optreden van restconcentraties verontreiniging in natuurlijke en toegepaste biologische reinigingssystemen. S_{min} kan worden beschouwd als de laagst bereikbare concentratie in relatief stabiele milieus of in evenwichtssystemen. De hoogte van S_{min} is afhankelijk van de omstandigheden in het systeem waar de omzetting plaatsvindt. In instabiele systemen, zoals in batch, zijn zelfs concentraties beneden de S_{min} te verwachten, zolang de verontreiniging beschikbaar is voor omzetting door de bacteriën. Een mogelijke toepassing van dit verschijnsel is het gebruik van biologische reinigingssystemen met een schoksgewijze (pulserende) vloeistofstroom. Deze fungeren dan als een serie van achtereenvolgende batches, en er zullen lagere restconcentraties kunnen worden bereikt dan met een gelijkmatige (continue) vloeistofstroom (Hoofdstuk 2). Een andere mogelijke toepassing is de toevoeging van kleine hoeveelheden van andere, onschadelijke substraten bij de reiniging van verontreinigd water. De restconcentraties verontreiniging zouden zo omlaag kunnen worden gebracht (Hoofdstuk 3).

De meeste natuurlijke milieus worden gekenmerkt door de aanwezigheid van lage concentraties van vele verschillende substraten, waaronder verontreinigingen. De gelijktijdige omzetting van meerdere substraten zal daarom eerder regel zijn dan uitzondering. Onderzoek heeft bovendien laten zien dat de onderhoudscoëfficiënten van bodembacteriën onder veldomstandigheden duizend keer lager waren dan die van actieve bodembacteriën in het laboratorium. Deze beide aspecten wijzen erop dat de afzonderlijke S_{min}-waarden voor verontreinigingen veel lager zullen zijn in natuurlijke milieus, dan in systemen met één bacteriesoort en één substraat. Sterker nog, met een duizend keer lagere onderhoudscoëfficiënt zouden de 'veldwaarden' van S_{min} waarschijnlijk ruim beneden de aantoonbaarheidsgrens liggen. Dat wil zeggen, dit is de verwachting zolang de kinetische parameters van de bacteriën de uiteindelijke omzettingssnelheid in het veld bepalen. We hadden voor het begin van onze studie echter al geconstateerd dat vele, biologisch afbreekbare verontreinigingen juist wèl in ons milieu kunnen worden aangetoond. Het is daarom waarschijnlijk dat de S_{min} in de meeste milieus wordt bepaald (en 'omhooggeschroefd') door de kinetiek van substraattransport naar de bacteriën toe, en niet door de omzettingskinetiek van de bacteriën zelf (Hoofdstuk 4). Deze conclusie is van belang voor de uitvoering van een eventuele biologische reiniging van deze milieus. De restconcentraties van de verontreinigingen kunnen dan alleen worden verlaagd door het transport van de verontreiniging naar de bacteriën te versnellen, en zo de biologische beschikbaarheid te verbeteren.

Curriculum vitae

In de ontluikende lente van 1963, na één van de koudste winters van de 20e eeuw, werd ik op 28 maart te Veendam geboren als Maria Elizabeth (Marijke) Tros. Achttien lentes later, in 1981, behaalde ik het VWO-diploma aan de Rijksscholengemeenschap Winkler Prins te Veendam. In datzelfde jaar begon ik met de studie Milieuhygiëne aan de Landbouwuniversiteit Wageningen, waar ik vier jaren later het kandidaatsdiploma behaalde. In de doctoraalfase koos ik de hoofdvakken Bodemverontreiniging (Prof. Dr. F.A.M. de Haan) en Microbiologie (Prof. Dr. A.J.B. Zehnder). Mijn stages verrichtte ik bij de productgroep Bodem van Raadgevend Ingenieursbureau DHV, en aan het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM). Na het behalen van het ingenieursdiploma in 1988, werd ik aangesteld als assistent onderzoeker aan de vakgroep Microbiologie van de Landbouwuniversiteit te Wageningen. Ik was aan deze vakgroep verbonden van juli 1988 tot november 1992, en van maart 1993 tot januari 1994. Van augustus 1994 tot juli 1996 was ik werkzaam aan de 'Eidgenössische Anstalt für Wasserversorgung, Abwasserreinigung und Gewässerschutz' (EAWAG), locatie Kastanienbaum, in hartje Zwitserland. De vruchten van mijn onderzoekswerk presenteer ik in dit proefschrift.

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