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Workshop

Microbiology of Polluted Aquatic Ecosystems

Petra Maria Becker (Editor)

Proceedings
of the Workshop
held on the 4th and 5th December 1997
at the
UFZ Centre for Environmental Research
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Foreword

The host of the workshop *Microbiology of Polluted Aquatic Ecosystems*, the Centre for Environmental Research UFZ, was founded primarily to assess and improve the current state of the environment in the Leipzig-Halle-Bitterfeld region. For over 50 years, vast areas of Germany and Eastern Europe were economically exploited while neglecting the environmental consequences. The majority of the current damage to the environment was caused by extensive mining activities in the quest for lignite, potash, uranium and copper slate, which were coupled with ecologically irresponsible industrialization and waste management. Some current treatment strategies as well as preliminary investigations concerning the different kinds of regional pollutions are presented among the UFZ's contributions to the workshop.

Although each of the eleven departments of the UFZ deal with a range of individual research tasks, all the UFZ's research and development projects contribute to three main areas:

- stock-taking
- remediation research
- development strategies

Stock-taking involves the description of the structure, function and dynamics of heavily contaminated systems. Concepts for the remediation of heavily polluted landscapes focus on the regenerative behavior and capacity of polluted and denaturized cultivated landscapes. The third field of research consists of strategies for the conservation and sustained development of ecosystems.

Because of the complexity of the problems with which we are dealing, interdisciplinary approaches are the key to environmental research. Hence, the departments of Hydrogeology, Inland Water Research, Remediation Research and Soil Sciences each employ microbiologists along with chemists, geologists, limnologists, physicists, etc., to improve the cooperation and exchange among specialist fields. The Department of Environmental Microbiology consists almost entirely of microbiologists and thus forms the microbiological backbone of the UFZ. Nevertheless, its staff work on projects relating to the contaminated areas of the region and cooperate with researchers from other institutions, as do the smaller microbiological workgroups in the other departments.

We decided to hold a workshop on the *Microbiology of Polluted Aquatic Ecosystems* because

- ... *Aquatic Ecosystems* are of concern to the Water/Wastewater Section of the VAAM (Vereinigung für Allgemeine und Angewandte Mikrobiologie - German Association of General and Applied Microbiology) which promoted the workshop
- ... *Polluted Aquatic Ecosystems* are of interest to the Departments of Environmental Microbiology, Hydrogeology, Inland Water Research and Remediation Research at the UFZ
- *Microbiology of Polluted Aquatic Ecosystems* gave the microbiologists of the UFZ from the aforementioned departments (which are located at some distance from each other; namely in Bad Lauchstädt, Leipzig and Magdeburg) a chance to get together in order to discuss our current research interests and future prospects

The workshop was conceived as a forum for both exchange and discussion of materials, methods, results, ideas and prospects in current microbiological research on contaminated aquatic environments.

Contributions to the following issues were requested:

- Microbial communities dependent on the type and degree of pollution
  - taxonomic diversity - structure of communities
  - physiological diversity - functional interrelations
- Potentials and activities of species and communities to attenuate or remove a contaminant
  - *in vitro*
  - *in situ*
- Strategies for increasing degradation abilities
  - phenotypically / physiologically
  - genotypically
- Fate of released microorganisms

As can be seen from the following pages, due to the broad range of relevant workshop issues, the topics of the contributions were quite heterogeneous. The backgrounds of the participants were just as varied, ranging from pure research to applied microbiology.

Pure, sound research forms the basis for an extension of our current knowledge about the building blocks of the environment. The knowledge gained should then be used to develop new evaluation and monitoring methods for field application; since an investigator of a polluted site (usually short of time and working capacity) is mainly interested in the questions 'What must I know about the microbiology of the polluted site?' and 'Are there appropriate methods to get an insight into what I must know?'. He or she will soon realize that the current methods for studying natural microbial communities are insufficient in certain respects. Excellent techniques have been developed for studying the taxonomic structure of communities (FISH-technique and DGGE, for example). However, the spectrum of methods which can be applied to study community functioning or *in situ* activities is rather small and unsatisfactory.

When going into the field in order to treat a polluted site, the first step should be to try to understand what is hindering the natural self-cleansing capacity. When considering a possible remediation strategy, the fact that a symptom may have more than one possible cause should never be overlooked. The symptom 'pollutant isn't degraded', for example, may be caused by its unavailability, its toxicity, the lack of electron acceptors, the lack of degraders or other reasons. Last but not least (and self-evidently for biologists), an ecologically and economically friendly measure should be preferred.

I am very much indebted to the willing reader because of the excessive length of this foreword. It just happened to grow uncontrollably when writing.

P.M. Becker
Leipzig,
Feb. 1998

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Assessment of the biological self purification of the River Rhine

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Abstract

The importance of enzymatic processes in aquatic habitats has received increasing recognition. The capability to measure enzymatic activities in vivo in natural water samples are based on degradation processes of contaminants or nutrients by microorganisms. The methods proposed are based on the enzymatic cleavage of synthetic chromogenic or fluorogenic substrates. The methods are easy to perform and well established. A test protocol to detect inhibition effects of the enzymatic activities was developed too. Classes of inhibition as criteria for the intensity and indices of inhibition as criteria for the frequency of hazard effects were also introduced. These test modifications allow, therefore, the assessment of natural purification processes and of the biological self purification in surface water. In this paper, the influence of pollutants and nutrients in the river Rhine on the enzymatic activities will be described. The first survey was performed to detect the influence of specific industrial emission on the tested enzymes. In the second survey the alteration of the water quality in a detailed longitudinal profile of the river Rhine will be described. Finally, results of investigations on the whole reach of the river Rhine will be presented.

1. Introduction

In Germany and other well mechanized countries household and industrial sewage were cleaned with a high technical standard in modern waste water plants. Thus, deoxygenating and biological degradable nutrients and toxicants in acute toxicological concentrations in flowing waters such as the river Rhine are up to now no or only a little problem. But toxicants and nutrients in low doses, which have the possibility to confirm chronical damages or which are able to create inhibition effects on biological processes are more and more important in the

whole water system. Natural and anthropogenic organic substances in surface water will normally be reduced by physical, chemical and biological mechanisms. But these substances will be eliminated completely only by the biological way. Microorganisms especially bacteria are mainly involved in the degradation of particular and dissolved organic material. They catabolize nutrients and pollutants extracellularly by exo-enzymes or they transport them into the lumen of the cell for their intracellular metabolism and for the generation of biological energy. Sometimes, however, pollutants prevent their own degradation by inhibition of the enzymatic activities or by direct impairment of the microorganisms. Thus, the bacterial activity has a strong influence on the concentration and speciation of organic molecules in water. The determination of enzymatic activities in vivo is therefore an indicator for microbial degradation of water contaminants and allows the control of natural biological purification processes in water samples [1].

Recently, the importance of enzymatic processes in aquatic habitats has received increasing recognition and the methods proposed are simple and well established [2 - 4]. The enzymatic activities in vivo of the aquatic microorganisms were routinely determined as substrate turnover per time of synthetic chromogenic or fluorogenic substrates. In ecotoxicological studies the harmful influence of pollutants will normally be measured by dose-response curves. A similar test protocol using linear dilution of the water under test and measuring the enzyme activities in each dilution step was developed. Moreover, classes of inhibition as criteria for the intensity and indices of inhibition as criteria for the frequency of the hazard effects are introduced and enables a graduated assessment of the measured results. These methods, therefore, allow the monitoring and the assessment of degradation processes and the biological self purification in natural water samples. Besides that, it is possible to discriminate between real inhibition effects and nutritional requirements (lack of nutrients) in the tested samples. In the following chapters, surveys in water samples of the river Rhine (Germany) over a period of four years will be described. Different examples for the influence of pollutants in the river Rhine and the corresponding alterations in the biological self purification and the water quality will be presented.

2. Materials and Methods

2.1. Description of the sampling sites

A first survey was performed in 1993 and 1994 on water samples of different sampling stations from Rhine river (Germany). The sampling sites are specific for industrial emission. The sampling took place regularly upstream and downstream from points of discharge into the receiving water of chemical pulp factories e.g. Stracel near Strasbourg (France) and Holtzmann near Karlsruhe (Germany) or upstream and downstream of the discharge from the industrial waste water treatment plant of the BASF AG near Ludwigshafen [5].

In order to get information about the alteration of the water quality in a detailed longitudinal profile of the river Rhine, a navigation with the laboratory ship "Max Honsell" from the "Landesanstalt für Umweltschutz Baden-Württemberg" took place from the

06.March 1995 to the 16. March 1995. The trip began upstream of the navigation lock of Rheinfelden (Rhine River, km 155) and finished at the Rhine near the city of Mainz (Rhine River, km 502). Every day we pass 30 - 50 kilometers. Sampling of 68 transverse sections at about four samples, respectively, took place - one sample of the right side, one sample of the left side, one sample of the middle of the river and one sample as a mixture of these three samples. Besides that, eight single samples of discharges from different provenances were analyzed: waste water treatment plant of Pratteln (km 156.7); factory Sandoz (km 168.3); household treatment plant of Mulhouse (km 194.5); factory Keyserberg (km 228.2); factory Stracel (km 292.1); household treatment plant of Strasbourg (km 300.5); factory OMW (km 367.5) and industrial waste water treatment plant of the BASF AG (km 433.2) [5, 6].

In a third program the whole reach of the river Rhine was examined. Over a period of a half year from November 1995 to March 1996 samples from the river Rhine at Öhningen, Karlsruhe, Mainz and Düsseldorf (Germany) as well as samples from Lobith and Hagestein (Netherlands) were collected and analyzed monthly [6].

2.2. Measurement of enzyme activities

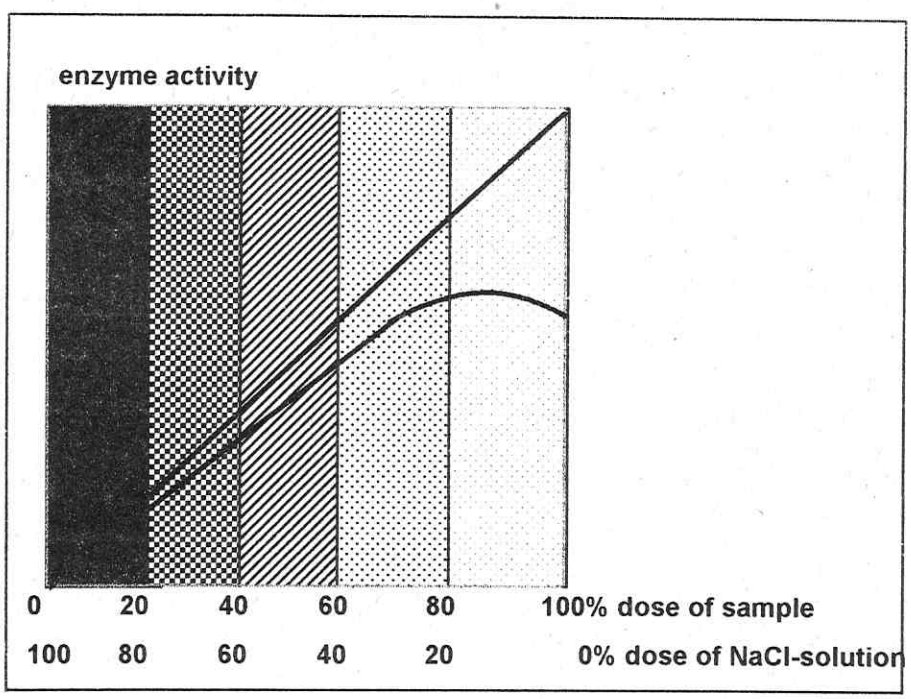
The applied methods for measuring enzyme activities in vivo are based on adding chromogenic substrates to the native water samples. After incubation under standardized test conditions, the color of the enzymatically cleaved dyes were measured spectrometrically and the results were calculated by the aid of calibration curves. In order to confirm results and to carry out numerous tests with dilutions of the samples, the tests were modified to an application on microtiter plates [4, 7]. In each assay esterases, alanine-aminopeptidases and β-glucosidases activities were measured in triplicate. The detailed description of the test procedures are presented in Table 1.

Inhibition of enzymatic activities was detected using linear dilution of the water under test and the correlation of the enzyme activities in each dilution step to the concentration of the particular samples. Therefore, the native water sample was diluted linear in 12 steps with physiological sodium chloride solution. The enzyme activities were measured in each dilution step. The activity is plotted versus the dilution. A linear correlation shows an uninhibited enzyme activity. A decreased slope in higher concentration of the sample indicates inhibition of enzyme activity [1, 4, 8].

2.3. Assessment of inhibited enzymatic activities

Assessment of the enzymatic inhibition effect is based on the dose response curve. Five classes of inhibition are defined: class 1: no inhibition (straight line of the dilution slope or deviation from linearity between sample portion 81 to 100 %); class 2: slight inhibition (deviation from linearity between sample portion 61 to 80 %); class 3: moderate inhibition (deviation from linearity between sample portion 41 to 60 %); class 4: severe inhibition deviation from linearity between sample portion 21 to 40 %); class 5: more severe inhibition

(deviation from linearity between sample portion 0 to 20 %) (Figure 1). The deviation from the linear relationship of a dose response curve (graphical analysis) and the corresponding sample concentration determines the inhibition class into which the specific water sample will be grouped. The frequency a inhibition class will occur at one location (measured several times) was given in percent.



	dose of sample in %	assessment	factor
		no activity detected	
	81 - 100	no inhibition	0
	61 - 80	slight inhibition	1
	41 - 60	moderate inhibition	2
	21 - 40	severe inhibition	3
	0 - 20	more severe inhibition	4

Fig. 1. Assessment of enzymatic inhibition effects [1].

Table 1. Testprotocol for measuring the esterases, alanin aminopeptidases and ß-glucosidases activities.

	Esterases	Alanin Aminopeptidases	β-Glucosidases
Volume of the samples per assay	200 µL	200 µL	200 µL
Substrate	Fluorescein -diacetate	L-Alanine-4-nitroanilide hydrochloride	4-Nitrophenyl-ß-D-glucopyranoside
Concentration of the substrate per assay	mol/assay $2,4 \times 10^{-8}$	mol/assay $1,2 \times 10^{-7}$	mol/assay $3,32 \times 10^{-8}$
Incubation time	24 h	24 h	24 h
Incubation temperature	room temperature	30°C	30°C
Stop-reagent		40 µL Trichloracetic Acid (10%)	40 µL Sodium Carbonate Solution
Wavelength	490 nm	405 nm	405 nm

In order to assess not only the frequency, but also the intensity of the detected inhibition, the defined inhibition classes are graduated additionally with a factor from zero to four. The factors will be multiplied with the frequency of the inhibition classes. The sum of all values is defined as an index of inhibition. The indices of inhibition range from 0 to 400. If all samples (100 %) of one sample station measured several times never show enzymatic inhibition effects, the index of inhibition will be zero, if all samples (100%) show the severest inhibition effects, the index of inhibition will be 400. The greater the amount of the index of inhibition is, the worse is the water quality on the specific sampling site. [1, 8].

3. Results

As a result of our work, three examples for the application of the enzyme activity tests in vivo are given as follows. All examples describe the influence of pollutants on the tested enzyme activities in the river Rhine and the occurring alterations in the biological self purification and the water quality.

In Figure 2 the frequencies of the inhibition classes occurring at specific locations measured several times upstream and downstream of industrial affluents at the river Rhine are shown. It was obvious, that downstream of the different affluents there are much more inhibition effects than in the samples collected upstream. But, it was not only possible to observe an increase in the frequency of inhibition, the intensity of the inhibition effects was much more pronounced too. For example: In samples downstream of the chemical pulp factory

Stracel or Holtzmann all defined inhibition classes (class zero to four; Figure 1) could be observed.

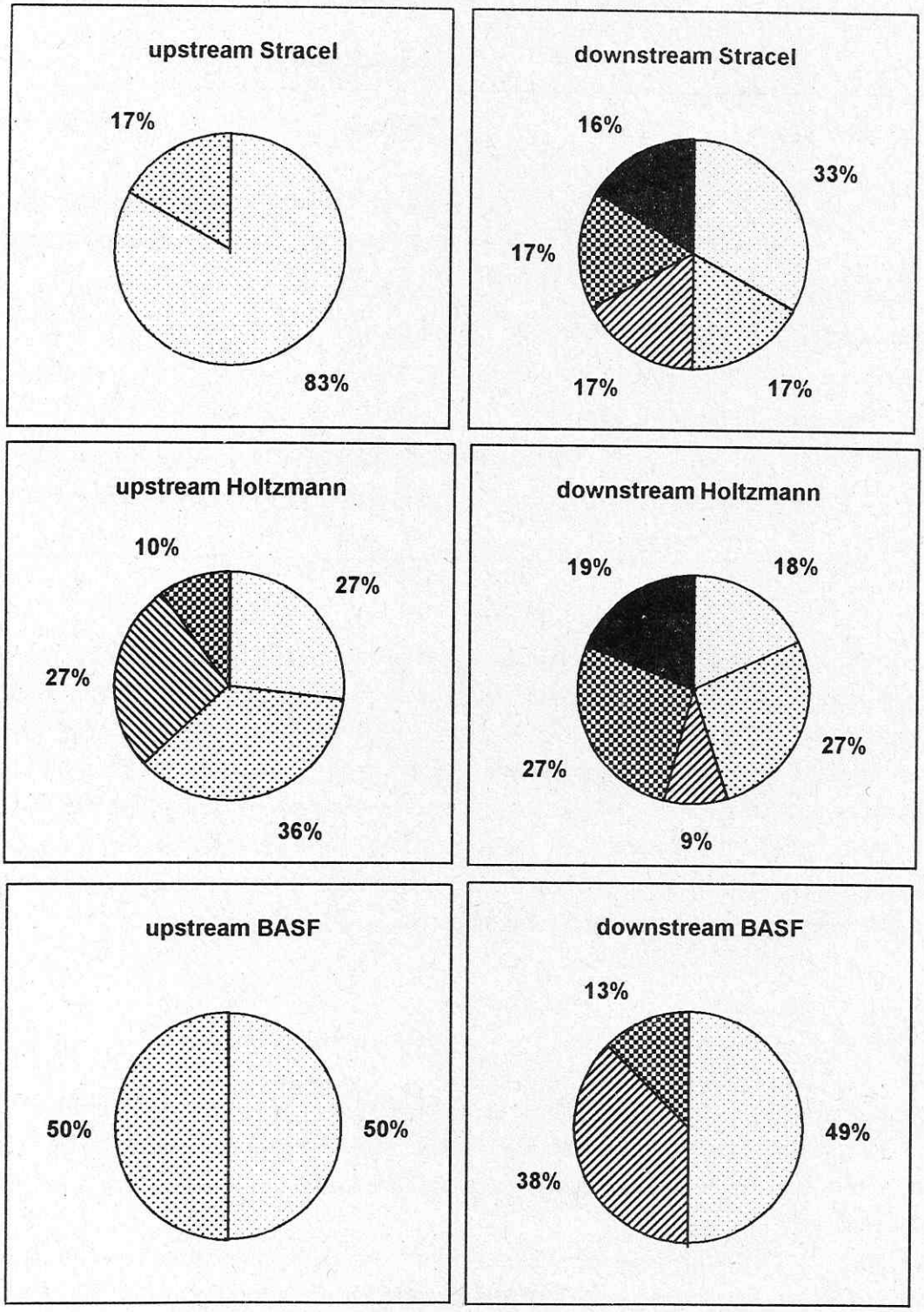


Fig. 2. Frequencies (graduated in percent) of the inhibition classes of the alanin aminopeptidases activities measured 8 times a year from the sampling sites upstream and downstream of the factory Stracel, Holtzmann AG and of the wastewater treatment plant from the BASF AG at the river Rhine [5, 6].

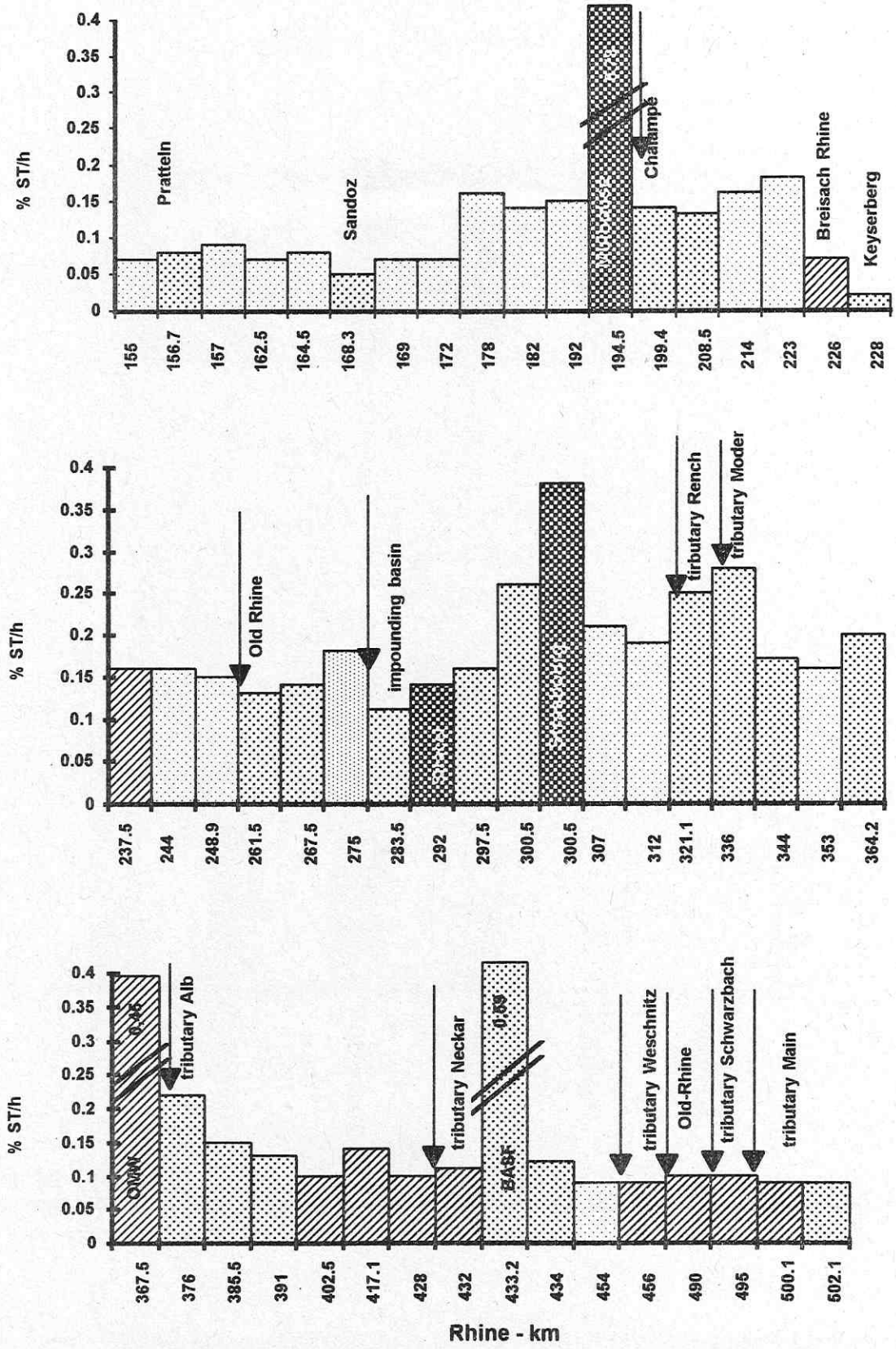


Fig. 3. Changes of the alanin aminopeptidases activities determined as substrate turnover in percent (%ST/h) from samples of the detailed longitudinal profile of the river Rhine in March 1995. Inhibition of the enzymatic activities presented as the shape of the bars [5, 6].

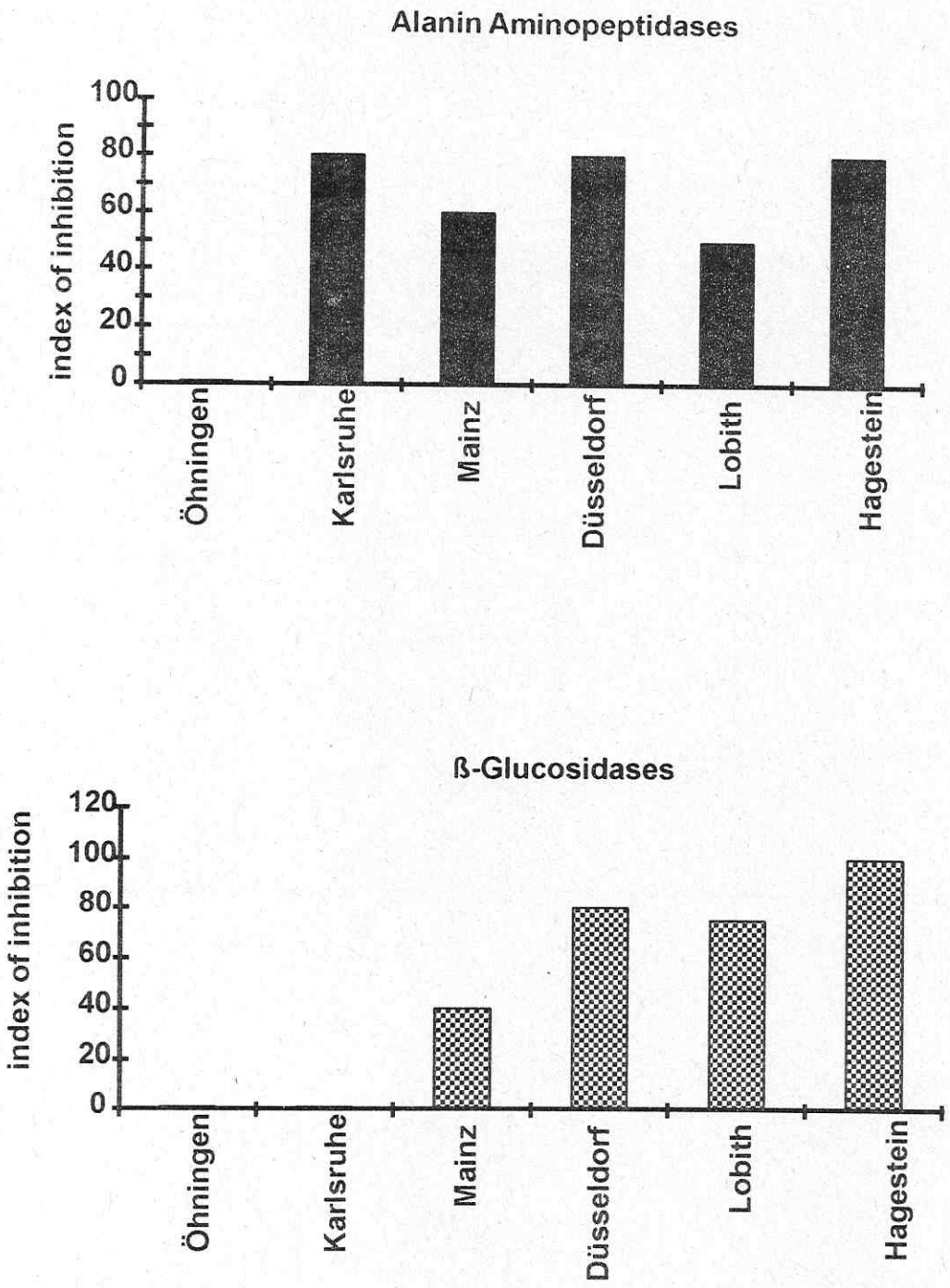


Fig. 4. Indices of inhibition of the alanin aminopeptidases and the ß-glucosidases activities of the six

In Figure 3 the alanine aminopeptidases activities of the mixture samples from the longitudinal profile and the single samples of the different discharges of the river Rhine are presented. In Figure 3 the activities, described as substrate turnover in percent, of the undiluted water samples as well as the detected inhibition class (presented as the hatching of the bars) determined by using the correlation of the linear relationship of the dose response curves were

shown. It was obvious, that the water quality of the river Rhine was dependent on the tributary rivers and the affluent of specific discharges from different provenances. Especially downstream of the discharges from the household treatment plants of Mulhouse and Strasbourg or downstream of the paper pulp factory Stracel much more pronounced inhibition effects (inhibition class 3) could be observed. The affluent of different tributaries such the River Rench, Moder or Weschnitz etc. influences the activity of this enzyme in the River Rhine considerably too. But sometimes however, recreating effects could be observed in the tested longitudinal profile. After a more or less quiet reach the intensity of enzymatic inhibition effects decrease. For example, downstream the affluent of the household treatment plant at Mulhouse (km 199.4 to km 223) only the inhibition classes one to zero could be determined.

In Figure 4 the inhibition indices of the alanine aminopeptidases and ß-glucosidases activities of the samples collected over the period of six month from Öhningen (Germany) to Hagestein (Netherlands) are presented. It was obvious, that in the tested samples from Öhningen in comparison to samples from the other sites no inhibition could be observed. As a result, the sampling site Öhningen must be classified as a relative unloaded point. The inhibition effects of the ß-glucosidases activities increase continuously from Öhningen to Hagestein influenced by the increasing load of the river water with industrial and household wastes. The gaging stations Hagestein must therefore be classified as the sampling site with the worst water quality regarding the measured enzymes.

4. Discussion

Criteria for evaluation, characterizing and assessing the quality of surface water are unavoidably necessary. Up to now, biological quality mapping of surface water has been based on the saprobic system [9, 10]. Analysis of single contaminants and summarizing parameters such as DOC and AOX is the method from chemists to measure the water quality. A comparison of results gathered in 1970 and the following years using these methods shows, that the water quality of the Rhine River is much better now, than in previous years [11, 12]. However, in the last few years the hard detectable trace contaminants and the micro-organics are the main source of pollution [12]. These pollutants in most cases are responsible for an inhibition of the biological self purification. The here suggested methods and criteria for evaluation and assessment supply an additional aspect of the biological water quality as well as the responsibility to control natural treatment processes in surface water.

Assessing the gaging stations at the Rhine River with the new criteria it is obvious, that downstream of industrial emission or downstream from household or industrial waste water treatment plants a deterioration of the water quality and of the biological self purification is detectable. These results could be confirmed downstream the direct discharges of the companies from e.g. Sandoz, Stracel, Keyserberg or OMW as well as the discharges of the household or industrial waste water treatment plants such as Mulhouse, Strasbourg or the BASF AG. But not only the discharges from different provenances into a river influence the water quality, the affluent of tributaries influences the activity of the tested enzymes too. The frequency and the intensity of the observed inhibition effects increase downstream of these

discharges and affluents. Sometimes however, after a more or less quiet reach the frequency and intensity of enzymatic inhibition effects decrease. These results indicate, that not only the impairment but also the recreation of the microbial biocenosis could be determined. Nevertheless of this temporary recreating effects, a remarkable deterioration of the water quality along the complete longitudinal profile of the river Rhine could be observed (Figure 3 and 4). These results correspond good with results gathered from the traditionally water quality mapping or with results from chemical analysis of summarizing parameters or single contaminants [11].

The results of this study show clearly, that the suggested criteria are useful for the assessment of the water quality and the biological self purification as well as for the control of discharges into the receiving water. They deliver an urgent aspect of biological effects of pollutants to the microbial biocenosis.

Acknowledgment

This study was financially supported by the Arbeitsgemeinschaft Rhein-Wasserwerke e.V. and the Project: Water - Sewage - Soil (PWAB) in Baden-Württemberg, Germany. We thank for the assistance at sampling the Landesanstalt für Umweltschutz in Baden-Württemberg, Germany, as well as the colleagues from the Stadtwerke Düsseldorf (Germany) and from WRK Nieuwegein (Netherlands).

References

[1] Obst, U., Weßler, A. and Wiegand-Rosinus, M. (1998, in press) Enzyme inhibition of toxic effects in aquatic systems. In: Microscale Aquatic Toxicology - Advances, Techniques and Practice. (Well, P.G., Lee, K. and Blaise, C. Eds)

[2] Burton, G.A. (1984) Sediment microbial activity tests for the detection of toxicant impacts. American society for testing and materials. Special technical testing publication, 864, 214-228

[3] Meyer-Reil, L.A. (1986) Measurements of hydrolytic activity and incorporation of dissolved organic substrates by microorganisms in marine sediments. Marine Ecology Progress Series, 31, 143-149

[4] Chrost, R.J. Eds. (1991) Microbial enzymes in aquatic environment. Springer Verlag, Berlin, Heidelberg

[5] Weßler, A., Dämgen, K., Hryk, R. and Obst, U. (1997) Gewässergütebewertung und Bewertung von Abwasserfahnen in Rhein und Main auf der Basis mikrobieller Enzymaktivitäten. Acta hydrochim. hydrobiol. 25, 17-26

[6] Weßler, A. and Obst, U. (1997) Gewässergütebewertung auf der Basis mikrobieller Enzymaktivitäten und Bewertung von Abwasserfhanen in Rhein und Main mit biochemische Methoden. FZKA-PWAB 21, Karlsruhe

[7] Wiegand-Rosinus, M., Obst, U. and Herzberger, E. (1992) Miniaturized and half automatic methods to determine enzymatic activities in vivo for water research. Pro. DECHEMA Biotechnol. Conf. 5, 903-911

[8] Weßler, A., Dämgen, K. und Obst, U. (1994) Untersuchungen mikrobieller Enzymaktivitäten in Fließgewässern unterschiedlicher Güte als Ergänzung der biologischen Gewässergütekartierung. Vom Wasser 83, 69-79

[9] Pantle, K. and Buch H. (1955) Die biologische Überwachung der Gewässer und die Darstellung der Ergebnisse. Gas- und Wasserfach. Wasser/Abwasser *96*, 609-620

[10] Liebmann, H. (1962) Handbuch der Frischwasser- und Abwasserbiologie. Bd.1, 2. Aufl. Gustav Fischer Verlag, Jena

[11] Länderarbeitsgemeinschaft Wasser/Abwasser (LAWA) (1996) Gewässergüteatlas der Bundesrepublik Deutschland

[12] Schalekamp, M. (1993) Vater Rhein: Die Sonne geht auf! Gas/Wasser/Abwasser. Sonderdruck Nr. 1292 des Schweizerischen Vereins des Gas- und Wasserfaches (SVGW), Zürich

Responses of the bacterioplankton of river Helbe to desalinization in comparison to its tributary Urbach (Thuringia, Germany)

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Abstract

In December 1995/January 1996, termination of the introduction of salt-loaded waters led to a considerable decrease in salinity and to changes in the ion ratios of river Helbe. Its tributary Urbach (Thuringia, Germany) was also concerned by desalinization. In 1996, water samples were taken during an annual cycle and analyzed for chemical and bacteriological parameters. Results were opposed to data available from 1995. Whereas the results of the years 1995 and 1996 did not show distinct changes in the bacteriological parameters for river Helbe, those of the tributary Urbach were remarkably different. This holds especially good for the parameters saprophyte numbers, bacterial biomass, mean cell volume and, as a trend, activity of alanine-aminopeptidase. These differences can be attributed to desalinization.

1. Introduction

Potassium mining was a main branch of industry in Thuringia in the past. An important area was called "mining area Südharz" and was situated about 20 kilometers distant from the south side of the Harz mountains. Here potassium salts were mined in the mine of Volkenroda near the city of Ebeleben since 1906, and manufacturing started in 1923. After deposition of the production residues by means of deep well disposal had been terminated in the 1960's, solid deposition was practiced, leading to a considerable enlargement of the salt heaps. Since their components were soluble to a high degree, they were washed out by rain, stocked up in a storage basin and discharged into the creek Urbach and, by this, into river Helbe. A smaller part of the heap effluents reached the running waters directly by diffusion. Both sources led to a considerable salinization of the Urbach and, to a lesser extent below its mouth, of river Helbe. Mining was terminated in 1990.

Changes in the salt-load management rendered a termination of the introduction of salt brines possible . This caused a distinct decrease in salinity in December 1995/January 1996. Salinization may affect aquatic communities strongly due to a combined effect of concentration and ion ratios [1], and numerous investigations concerning algae and macrozoobenthon have been performed with this regard (e.g., see [2]). However, only little is known about the effects of salinization to bacteria [3-5]. A previous investigation had shown that the desalinization had distinct consequences for the bacterial populations of the tributary Urbach [6]. The aim of this

study was to find out how far this holds also good for the river Helbe. For this purpose, water samples were taken in 1996 and analyzed for chemical and bacteriological parameters. Results were compared to data available from summer 1995 when salinity was still high, and the effects observed were compared to those observed at the Urbach.

2. Materials and methods

2.1 Sampling and sampling site

The source of river Helbe is situated in a mountain chain called "Hainleite" in Northern Thuringia, Germany. Total length of the river is 74.4 kilometers, and catchment area is 496.4 km^2. The subsoil along the upper course is marked by karst. Due to this reason the water seeps away some kilometers below the source, and over a distance of 40 kilometers the bed of river Helbe is dry over a period of several months each year. From the city of Ebeleben on where the Urbach joins river Helbe discharge is permanent again. At times all the discharge may be contributed by the Urbach.

The sampling site was located about 12 kilometers downstream Ebeleben near the village of Wasserthaleben. It was chosen because chemical and especially bacteriological parameters had been determined at this location for four times in 1995, thus a few comparative data being present. MQ is 1.29 m^3 s^{-1} here (Staatliches Umweltamt Sondershausen, unpublished data). Between Ebeleben and Wasserthaleben the salt-containing water of the Helbe is diluted by several creeks joining the Helbe as well as by springs. Untreated domestic wastewater and agriculture causes an input of organic substances and inorganic nutrients. Trees are missing at the sampling location where a ford made of concrete slabs crosses the river. Besides the passage the sediment is sandy, partly muddy. Many of the pebbles lying on the ground are covered with brown diatom films. *Cladophora spp.* and *Enteromorpha spp.* formed dense mats. In September 1996 a mass development of *Lemna gibba* was noticed.

The Urbach originates at the mine of Volkenroda near the village of Menteroda by the confluence of three ditches draining the mining area. It forms a tributary of river Helbe which joins river Unstrut. For further details, see [6].

Water samples were taken once a month from January to December 1996. Glass bottles were used for sampling, and those designed for the determination of bacteriological parameters were sterilized by autoclaving before use (20 min at 121°C, 1.1 atm.). Samples were cooled during transport to the laboratory and processed immediately here. Transport time was about an hour.

2.2 Hydrographical and chemical parameters

Water temperature and oxygen concentration were measured at the sampling location by means of a suitable probe (Oxi 92 WTW company, Weilheim, Germany). Conductivity and pH were determined in the laboratory using the probes Lf 90 and pH 96 (WTW company), respectively. - Salts and nutrients were determined according to DIN [7]: DOC (DIN 38 409

part 3); K^+ and Na^+ (DIN 38 406 part 27/ISO 9964-3); Ca^{2+} (DIN 38 406 part 3-2); Mg^{2+} (DIN 38 406 part 3-3); Cl^- (DIN 38 405 part 1-2); HCO_3^- (DEV D 8); SO_4^{2-} (DIN 38 405 part 5-2); NH_4^+ (DIN 38 406 part 5); NO_2^- (EN 26 777); NO_3^- (DIN 38 409 part 9); o-PO_4^{3-} (DIN 38 405 part 11-1).

2.3 Bacteriological parameters

Numbers of saprophytic bacteria were determined by means of the plate method by Koch. A peptone-yeast extract-agar was used (medium 2216 E [8]), modified, consisting of 1.0 g yeast extract (Difco), 5.0 g bacto peptone (Difco), bacto agar 15.0 g (Difco), aged seawater 250 ml, deionized water 750 ml, pH 7.2 (ZB, S ≈ 8 °/oo). A second medium was prepared by omitting aged seawater and taking 1,000 ml of tap water instead of 750 ml deionized water (ZL). - Total bacterial numbers were determined using epifluorescence microscopy [9]. DAPI was used for staining of the cells, and evaluations were made by use of a a Nikon microscope. 50 of the 400 cells counted were measured by comparing both length and width of the cells to the globes of a calibrated eyepiece graticule (Patterson Globe and Circle, GI, Eyepiece Graticules Ltd., Great Britain). From these counts total bacterial numbers, bacterial biomass and mean cell volume were calculated. The following equation was used for estimating the cell volumes: biovolume $\mu m^3 = (\pi/4)W^2(L-W/3)$, where L = cell length, W = cell width and for cocci L = W. A nonlinear equation derived from Simon and Azam ([10]) was used for estimating bacterial biomass: cell carbon = cell volume$^{0.59}$ x 88.6 x 1.04878. - Activities of the following three bacterial enzymes were determined photometrically [11]: alanine-aminopeptidase, phosphatase and β-glucosidase. Measurements were made in triplicate. Results were divided by the corresponding BBM value and are declared as % substrate turnover x µg bacterial biomass^{-1}. - Respiratory activities were determined using CTC (5-cyano-2,3-ditolyl tetrazolium chloride, purchased from Polysciences Europe, Eppelheim, Germany) as a substrate [12]. Samples were supplemented with 1 % (w/v) glucose and incubated for four hours.

3. Results

3.1. Hydrographical and chemical parameters

Water temperatures varied from 9.0 to 14.9°C in summer 1995 and from 11.4 to 15.1°C in summer 1996. In the latter year, minimum and maximum temperatures were 3.5 (February) and 15.3°C (August), respectively. Data are presented graphically in Figure 1.

The pH was balanced all the time. In summer 1995, it was between 7.45 and 7.95, and in summer 1996 values ranged from 7.45 to 7.59. With regard to the total year 1996, values ranged from 7.21 (February) to 8.10 (December).

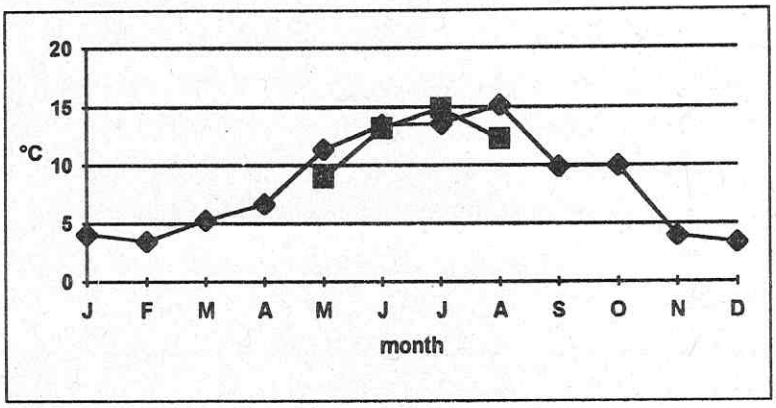

Fig. 1. Water temperature. Rhombi: 1996, squares: 1995

Oxygen concentrations ranged from 6.4 to 11.0 mg l^{-1} in summer 1995 and from 7.1 to 9.7 mg l^{-1} in summer 1996. Referring to the total year, minimum was 5.5 (October) and maximum was 15.0 mg l^{-1} (April).

Conductivity displayed much more differences. Values ranged from 3.26 to 5.64 mS cm^{-1} in summer 1995, remaining within this order of magnitude till the end of the year. In 1996, between 1.3 and 1.8 mS cm^{-1} were measured. A distinct decrease is to be seen from the data (Figure 4). Those measured from September to December 1995 by the chemical laboratory of the "Staatliches Umweltamt Sondershausen" are also shown to demonstrate the decrease that took place.

In summer 1995 and 1996, organic load (DOC) did not differ very much. Values ranged from 2.2 to 2.4 (average value: 2.3) mg l^{-1} in summer 1995, whereas in summer 1996 it were between 2.1 and 3.5 (average value: 2.8) mg l^{-1}. Annual mean of the total year 1996 was 3.0 (1.4-5.3) mg l^{-1}.

Concentrations of the nutrients NH_4^+, NO_2^-, NO_3^- and o-PO_4^{3-} are compiled in Table 1.

Table 1. Nutrient concentrations

	1995	1996
	summer (n=4) average value (min-max)	summer (n=4)/total year (n=12) average value (min-max)
NH_4^+ (mg l^{-1})	0.075 (0.02-0.13)	0.032 (0.01-0.09)/ 0.038 (0.01-1.33)
NO_2^- (mg l^{-1})	0.1 (0.1-0.3)	0.075 (0.01-0.1) / 0.23 (0.01-0.8)
NO_3^- (mg l^{-1})	23.5 (29-41.5)	25.2 (23.0-26.9) / 25.9 (23.0-36.7)
o-PO_4^{3-} (mg l^{-1})	0.62 (0.11-1.79)	0.35 (0.23-0.52) / 0.31 (0.20-0.52)

Salt concentrations developed differently in 1995/1996. Decrease of sodium and chloride was most pronounced: concentrations in 1995 were about tenfold higher than in 1996. Concentrations of other ions decreased less (e. g., potassium), remained unchanged such as bicarbonate or showed even somewhat increase (e. g. calcium). The changes found caused a shift from a NaCl- to a $Ca(HCO_3)_2$- water. Data are given in Table 2.

Table 2. Salt concentrations

	1995 summer (n=4) average value (min-max)	1996 summer (n=4)/total year (n=12) average value (min-max)
Na^+ (mg l^{-1})	600 (380-879)	60 (49-71) / 63 (49-96)
K^+ (mg l^{-1})	147 (103-220)	20 (17-24) / 21(17-31)
Ca^{2+} (mg l^{-1})	143 (111-172)	189 (180-204) / 184 (142-219)
Mg^{2+} (mg l^{-1})	109 (52-173)	67 (63-69) / 65 (52-78)
Cl^- (mg l^{-1})	1,130 (688-2,030)	134 (112-152) / 127 (102-194)
SO_4^{2-} (mg l^{-1})	578 (481-763)	400 (374-424) / 377 (215-440)
HCO_3^- (mg l^{-1})	365 (349-370)	370 (349-392) / 374 (327-392)

3.2. Bacteriological parameters

3.2.1. Numbers of saprophytic bacteria

In general, saprophyte numbers on tap water agar tended to be lower than those obtained from brackish water agar. Unusual high numbers were found once on both media in November 1996.

Saprophyte numbers on tap water agar (ZL) were balanced in summer 1995, and 30 (18-44) x 10^3 cfu ml^{-1} were counted on an average. Numbers showed greater fluctuations in summer 1996 when the average value was 110 (27-290) x 10^3 cfu ml^{-1} . With regard to the total year 1996, 94.5 (3.5-344) x 10^3 cfu ml^{-1} were counted (annual mean).

On the brackish water medium (ZB), average numbers were 40.5 (30-52) x 10^3 cfu ml^{-1} in summer 1995 and 206 (67-350) x 10^3 cfu ml^{-1} in summer 1996. With regard to the total year 1996, average number was 240 (22-1,160) x 10^3 cfu ml^{-1} , a single peak being found in November 1996 (1,160 x 10^3 cfu ml^{-1}).

A distinct uniform trend was missing. Partly, order of magnitude found in 1995 was comparable to that found in 1996 (ZL: May, June, August; ZB: July and August), however, in part, numbers were lower (ZL: July; ZB: May and June). Data are presented in Figure 2.

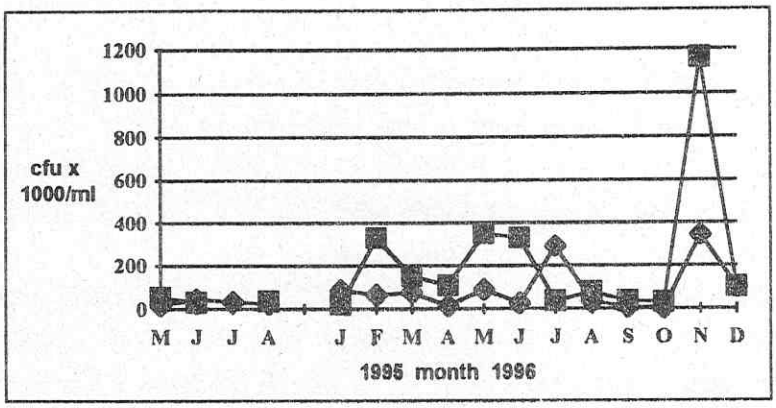

Fig. 2. Saprophyte numbers. Rhombi: ZB, squares: ZL

3.2.2. Total bacterial numbers, bacterial biomass, mean cell volume and frequency of dividing cells

Total bacterial numbers (TBN) were comparable all the time. This holds also good for the baterial biomass (BBM), whereas mean cell volume (MCV) tended to be somewhat higher in 1995 in comparison to 1996. Here a distinct peak was visible in November 1996 coinciding with a high DOC, high saprophyte numbers and high respiratory activities. The frequency of dividing cells (FDC) was comparable in May and June 1995 vs. 1996, whereas it was somewhat higher in July and August 1996 in comparison to 1995. Data are given in Table 3.

Table 3. Bacteriological parameters

	1995 summer (n=4) average value (min-max)	1996 summer (n=4)/total year (n=12) average value (min-max)
TBN (x 10^6 ml^{-1})	1.034 (0.529-1.303)	1.432 (1.084-2.032) / 1.138 (0.430-2.236)
BBM (µg C l^{-1})	42 (23-53)	50 (33-75) / 48 (30-75)
MCV (µm^3)	0.256 (0.208-0.287)	0.189 (0.152-0.213) / 0.354 (0.130-1.30)
FDC (% of TBN)	1.13 (0.94-1.23)	2.88 (1.43-4.30) / 4.53 (1.28-13.4)

The annual means of MCV and FDC were strongly influenced by the peak in November 1996 and would be distinctly lower otherwise (0.230 µm^3 and 3.73%, respectively).

3.2. 3. Enzymatic and respiratory activities

Activities of the three bacterial exoenzymes alanine-aminopeptidase, β-glucosidase and phosphatase were within the same order of magnitude during both summers 1995 and 1996, the level of activity found in summer 1996 being the same one as given during the other seasons of this year. Great differences with regard to the summers 1995 and 1996 were missing. Results are graphically presented in Figure 3.

Percentage of actively respiring cells as measured by the CTC assay was 2.57 % (1.0-5.3 %) in summer 1995 and 4.03 % (0.25-6.8%) in summer 1996. For the total year 1996, 7.45% (0.25-30.2%) were determined. Usually ≤ 10 % of the bacteria were found to be actively respiring. A remarkably high peak was found only once in November 1996 (30.2 %).

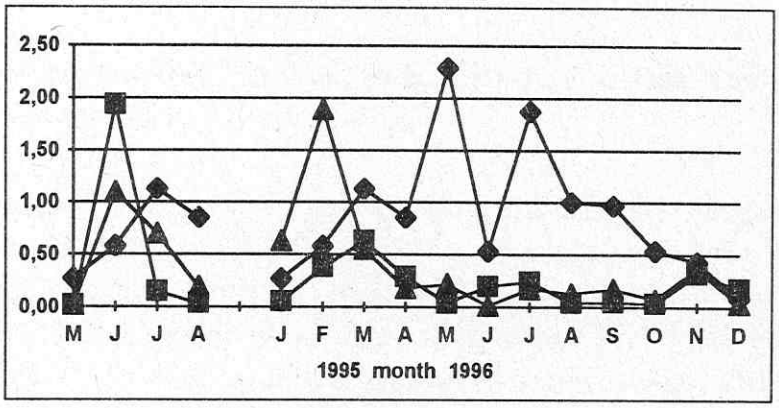

Fig. 3. Enzymatic activities. Squares: alanine-aminopeptidase, rhombi: β-glucosidase, triangles: phosphatase. Y-axis: % substrate turnover µg biomass^{-1}

4. Discussion

In the turn of the year 1995/1996, desalinization led to distinct changes in the water chemistry of river Helbe: the nutrient level remaining unchanged, (i) concentrations of sodium and chloride were reduced to about 10% of the former values; (ii) typical limnetic waters being characterized by a Na/Ca^{2+}-ratio < 0.4 [1], the ratio found in 1996 was 0.3; thus, the NaCl-water became a $Ca(HCO_3)_2$-water; (iii) however, the bacterioplankton seems to be unaffected by these events. All what was most obvious was a single peak in DOC in November 1996, coinciding with remarkably high saprophyte numbers, a high MCV and FDC and respiratory activity what may be attributed to the introduction of wastewater. However, hints for changes in structure and/or function of the bacterial populations due to desalinization were rare. Comparing the data from summer 1995 to summer 1996, saprophyte numbers were of the same order of magnitude as were TBN, BBM and exoenzymatic activities. FDC was partly lower, partly higher, whereas only FDC and respiratory activity showed an increase.

In contrast to this, remarkable changes and a much more uniform tendency were observed in the tributary Urbach [6]. In this case comparative data from 8 samples were

available (May-December 1995). Levels of the organic load and nutrients also remaining unchanged, (i) the reduction of conductivity and salt concentrations was much more pronounced (Figure 4, Table 4); (ii) however, in contrast to river Helbe, a shift to a $Ca(HCO_3)_2$- water was missing; (iii) the desalinization had marked consequences for the bacterioplankton: in 1996, especially saprophyte numbers increased two-fold to two hundred-fold, MCV and consequently BBM were distinctly higher than in 1995. As a tendency, this holds also good for the activities of alanine aminopetidase (see [6]).

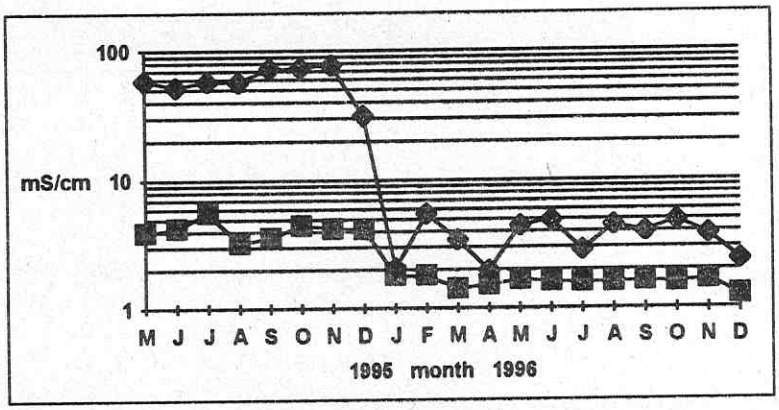

Fig. 4. Decrease in conductivity of river Helbe (squares) in comparison to the tributary Urbach (rhombi)

Table 4. Salt concentrations of the Urbach (after [6], modified)

	1995 summer (n=4) average value (min-max)	1996 summer (n=4)/total year (n=12) average value (min-max)
Na^+ (mg l^{-1})	10,100(4,100-14,600)	426 (106-682)
K^+ (mg l^{-1})	2,460 (2,000-3,280)	132 (41-192)
Ca^{2+} (mg l^{-1})	160 (111-191)	133(102-178)
Mg^{2+} (mg l^{-1})	1,450 (690-2,210)	99 (61-149)
Cl^- (mg l^{-1})	8,200 (7,540-25,400)	800 (222-1,210)
SO_4^{2-} (mg l^{-1})	6,220 (2,290-8,910)	420 (236-663)
HCO_3^- (mg l^{-1})	400 (349-436)	353 (261-632)

Figure 5 illustrates the increase in saprophyte numbers enumerated on ZB. The same trend was obtained from ZL and W 50 plates, in the latter case results from 8 samplings being available. Results of the bacteriological parameters from the Urbach are given in Table 5.

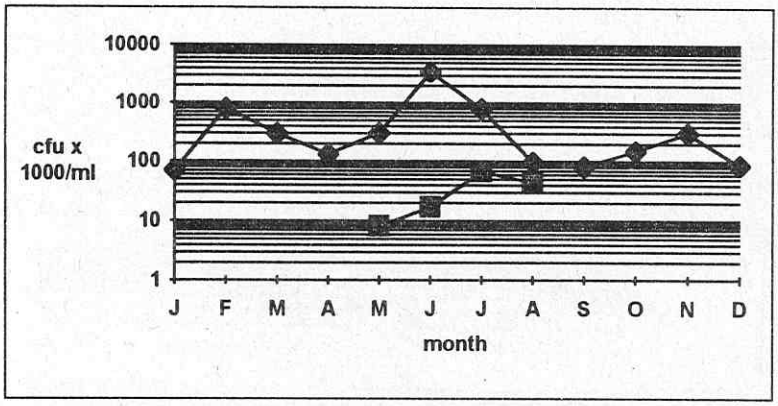

Fig. 5. Saprophyte numbers of the Urbach on ZB in 1995 (squares) and 1996 (rhombi).

Table 5. Bacteriological parameters of the Urbach 1995/1996 (after [6], modified)

	1995 summer-autumn (n=8) average value (min-max)	1996 summer-autumn (n=8)/total year (n=12) average value (min-max)
TBN (x 10^6 ml^{-1})	2.79 (1.46-7.58)	3.78 (1.07-9.11) / 2.91 (0.91-9.11)
BBM (µg C l^{-1})	76 (37-189)	143 (41-492) /110 (19-492)
MCV (µm^3)	0.139 (0.107-0.268)	0.213 (0.106-0.400) / 0.228 (0.106-0.426)
FDC (% of TBN)	1.16 (0.14-3.45)	2.23 (1.00-4.28) / 2.71 (1.00-6.05)
CTC (% of TBN)	1.25 (0.10-3.49)*	0.29 (0.1-0.75)* / 1.38 (0.1-5.08)

* summer only (n=4)

The reason for this different pattern might be the different levels of salinity given in both cases. Rheinheimer names a threshold value of 10°/oo for many freshwater bacteria [3]. Ziemann concluded from laboratory experiments with diluted potassium brines salinity to produce inhibitory effects to freshwater bacteria above a threshold value of 10,000 to 20,000 mg Cl^- l^{-1}, depending of the degradability of the substrate used [13]. No inhibitory effects were detected at all up to a concentration of 5,000 mg Cl^- l^{-1}. In 1995, annual mean concentrations of Cl^- were 1,130 (Helbe) and 18,200 mg l^{-1} (Urbach), respectively, the former value falling distinctly below and the latter one exceeding the levels of 5,000 and 10,000 mg l^{-1}. Thus it seems likely that the bacterial populations of river Helbe were unaffected by salinity already in

1995 and therefore the decrease in salinity could not cause great changes any more. In contrast to this it is to be assumed that the high salt concentrations of the tributary Urbach in 1995 affected bacteria significantly and that therefore the changes observed in the bacterial parameters are due to desalinization. This is confirmed by a previous study performed along river Wipper which is situated in the same area of investigation [14]. Samples were taken at 6 locations, average concentrations of Cl^- ranging from 13 to 1,810 mg l^{-1}. No inhibitory effects that might be due to salinity were detectable also in this case. It may be assumed that many of the bacteria of the Urbach and the Helbe derive from untreated wastewater and are able to tolerate osmolarities of a wide range as part of their survival strategy [15,16].

The results show that the desalinization which changed the water chemistry of both river Helbe and its tributary Urbach favored conditions for bacterioplankton of the Urbach, whereas for the bacteria of river Helbe no distinct effects became visible.

Acknowledgments

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References

[1] Ziemann, H. (1997): The influence of different ion ratios on the biological effect of salinity in running waters of Thuringia (Germany). Limnologica 27(1), 19-28

[2] Schulz, Claus-Jürgen (ed.) (1997): Salinization of running waters and its consequences for aquatic communities. Limnologica 27(1). 144 pp.

[3] Rheinheimer, G. (1991): Mikrobiologie der Gewässer. 5.ed. G. Fischer Verlag Jena Stuttgart, 294 pp.

[4] Prieur, D., Troussellier, M., Romana, A., Chamroux, S., Mevel, G. and Baleux, B. (1987): Evolution of bacterial communities in the Gironde Estuary (France) according to a salinity gradient. Estuarine Coastal Shelf Sci. 24, 95 - 108

[5] Wilde, V. and Rheinheimer, G. (1981): Mikrobiologische Untersuchungen in Flüssen. IV. Der Einfluß von Salzgehaltsänderungen im Mündungsgebiet auf Zusammensetzung und Aktivität der Mikroflora. Arch. Hydrobiol. 93, 21-31

[6] Schulz, Claus-Jürgen: Desalinization of running waters. I. Effects of desalinization on the bacterio- and viroplankton on the running water of a creek in Northern Thuringia (Germany). Limnologica (in press)

[7] DIN (Deutsches Institut für Normung e.V.): Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung. State 1994. Verlag Chemie Weinheim

[8] Oppenheimer, C. H. and ZoBell, C. E. (1952): The growth and viability of sixty-three species of marine bacteria as influenced by hydrostatic pressure. J. Mar. Res. 11, 10-18

[9] Zimmermann, R. and Meyer-Reil, L. A. (1974): A new method for fluorescence staining of bacterial populations on membrane filters. Kieler Meeresforsch. 30, 24-27

[10] Simon, M.. and Azam, F. (1989): Protein content and protein synthesis rate of planktonic marine bacteria. Mar. Ecol. Prog. Ser.51, 201-213

[11] Obst, U. and Holzapfel-Pschorn, A. (1988): Enzymatische Tests für die Wasseranalytik. Oldenbourg Verlag München. 86 pp.

[12] Schaule, G., Flemming, H.-C. and Ridgway, H. F. (1993): Use of 5-cyano-2,3-ditolyl tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. Appl. Env. Microbiol. 59(11), 3850-3857

[13] Ziemann, H. (1973): Untersuchungen über den Einfluß verdünnter Kaliendlaugen auf den Abbau organischer Substanzen im Wasser. Acta hydrochim. hydrobiol. 1, 257-265

[14] Schulz, C. J., Baumgart, J., Karrsch, B. and Baborowski, M. (1997): Effects of salinization on the structure and function of bacterio- and phytoplankton of the salt-loaded river Wipper (Thuringia, Germany). Limnologica 27(1), 43-53

[15] Foster, J. W. and Spector, M. P. (1995): How *Salmonella* survive against the odds. Ann. Rev. Microbiol. 49, 145-174

[16] Galinski, E. (1994): Halophile und halotolerante Eubakterien. *In*: Hausmann, K. and Kremer, B. (eds.): Extremophile: Mikroorganismen in ausgefallenen Lebensräumen pp. 89-112. Verlag Chemie, Weinheim

Bacterial activity and morphological diversity in the water and in the stream-bed sediments along differently polluted sections of a small mountain stream (Horloff/Vogelsberg)

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Abstract

Model sediments (sand bags) were exposed in the stream-bed of the Horloff at several sites downstream the outfall of a wastewater treatment pond. The parameters examined included the total number of bacteria, the bacterial morphology and the activities of esterase, β-glucosidase, L-alanin-aminopeptidase and phosphatase. Water samples were taken simultaneously and examined in the same way. The bacterial morphological diversity in the water samples was higher than in the sediments. Based on the enzyme activities in the model sediments the self-purification processes in the different sections could be well distinguished. The relative enzyme activity was used to describe the relationship between the enzyme activities in the water samples compared to the sediments. Some enzymes seemed to be closer related to the sediment compartment than others. Moreover, the relative enzyme activity was influenced by the water level rather than the section.

1. Introduction

Sediments are the essential compartments for the mineralization of organic compounds in small streams. The self-purification process is caused by micro-organisms, which form biofilms on the particles leading to microbial food chains in the sediment of the stream-bed. When investigating microbial activity and density in different sediments of small streams, however, heterogeneous size, structure and distribution of the particles in the stream-bed are limiting factors. Microbial processes depend on the sediment type and structure, which make it difficult to compare different sections within one stream.

2. Materials and methods

Microbial studies have been carried out in the Horloff, a little mountain stream in the Vogelsberg (Hesse) from July 1996 to July 1997. The investigations focused on downstream sections, which were polluted differently by the effluents of a municipal wastewater treatment pond. The facility has a capacity of 1200 EGW and a discharge of 4 $L \cdot s^{-1}$. Five sampling sites

(site 2 - 6) downstream of the effluents and one reference site (1) upstream of the outfall were compared. Table 1 shows the main chemical characteristics of the study site. Especially the high concentrations of ammonia caused by the wastewater treatment pond affected the ecosystem seriously with regard to toxicity and the oxygen demand of nitrification.

Table 1. Mean and extreme values of characteristic chemical parameters in the flowing water of the Horloff from July 1996 to July 1997 (n = 15)

site	O_2 [%]		COD ($KMnO_4$) [mg $O_2 \cdot L^{-1}$]		BOD_5 [mg $O_2 \cdot L^{-1}$]		NH_4^+ [mg $N \cdot L^{-1}$]		o-PO_4^{3-} [mg $P \cdot L^{-1}$]	
1	**89**	(41 - 103)	**14**	(8,5 - 25)	**2,1**	(0,75 - 5,5)	**0,04**	(0,01 - 0,14)	**0,1**	(0,01 - 0,5)
2	**86**	(64 - 103)	**15**	(9,1 - 26)	**2,3**	(1,2 - 4,9)	**1,5**	(0, 9 - 4,8)	**0,3**	(0,02 - 0,8)
3	**76**	(47 - 101)	**15**	(8,9 - 24)	**3,5**	(1,2 - 5,8)	**1,3**	(0,08 - 4,7)	**0,3**	(0,02 - 0,8)
4	**79**	(50 - 111)	**14**	(8,6 - 22)	**3,3**	(1,1 - 6,0)	**0,8**	(0,05 - 3,9)	**0,2**	(0,02 - 0,6)
5	**71**	(45 - 100)	**13**	(9,1 - 20)	**2,6**	(0,95 - 4,6)	**0,3**	(0,03 - 2,5)	**0,2**	(0,01 - 0,6)
6	**81**	(66 - 99)	**14**	(8,9 - 20)	**2,2**	(0,70 - 4,6)	**0,2**	(0,02 - 1,4)	**0,2**	(0,02 - 0,7)

Water samples were taken monthly at each sampling site. At each site nylon bags (ca. 10 · 10 cm) filled with carefully washed and sieved (0,2 - 5 mm) sand (200 g) were exposed for 8 weeks on the surface of the stream-bed to allow a comparison between the different. Three bags were spread out over the cross section of the stream and fixed with 75 cm long steel needles into the sediment.

The total number of bacteria (AODC) was determined microscopically by fluorescence staining with acridin-orange [1]. The bacteria were divided into 6 morphological classes (cocci, rods < 1 µm, rods < 2 µm, rods > 2 µm, curved rods < 2 µm, and curved rods > 2 µm), which were counted separately. Esterase and phosphatase activities were measured as indicators for the general heterotrophic activity, while the activity of β-glucosidase was selected as a measure of the carbohydrate turn-over. Correspondingly, the L-alanin-aminopeptidase activity reflected the protein metabolism. For details of the method the reader is referred to Obst & Holzapfel-Pschorn [2]. Sediments were incubated for 5 h and water samples for 20 h.

In order to compare enzyme activities from different compartments in stream ecosystems, relative enzyme activities were used to characterize the interactions between the sediments and the flowing water. The relative enzyme activity describes the volume of water that has the same activity as one square meter of sediment surface area [3] and is calculated as follows:

$$REA\left[L \cdot m^{-2}\right] = \frac{EA - sediment\left[\mu mol \cdot m^{-2} \cdot h^{-1}\right]}{EA - water\left[\mu mol \cdot L^{-1} \cdot h^{-1}\right]}$$

REA = *relative enzyme activity*
EA = *enzyme activity measured in the water or sand, respectively*

The higher the REA, the less active is the water body in comparison to the sediment. The area of the sand surface was estimated according to Marxsen & Witzel [4].

3. Results and discussion

3.1. Total number of bacteria (AODC) and morphology

No relationship between the total number of bacteria and the concentration of organic compounds along the self-purification section could be detected either in the water or in the model sediment. The observed numbers of bacteria varied from $3,3 \cdot 10^6$ to $1,7 \cdot 10^7$ bacteria $\cdot$ mL^{-1} in the water samples and from $2,1 \cdot 10^8$ to $1,9 \cdot 10^9$ bacteria $\cdot$ g dry $matter^{-1}$ in the sand samples.

Comparing the different compartments, the bacterial population in the water column showed the greatest morphological diversity though small forms of bacteria (cocci and rods < 1 µm) dominated in the water samples as well as in the model sediment. Nevertheless, the average cell volume of the pelagic bacteria (0,173 μm^3) was significantly higher than the volume of the benthic ones (0,078 μm^3).

The portion of cocci decreased in the water column from sampling site 1 to 6 from 54 % to 41 %. Simultaneously, the portion of curved rods > 2 µm doubled. Unlike the bacterial population in the water, the portions of the different morphological groups remained nearly constant in the sediment. On the other hand, seasonal variations did strongly influence the biodiversity in the water. The groups of medium-sized (< 2 µm) and large (> 2 µm) bacteria reached over 40 % of the AODC in July and August 1996. Thereof, the portion of curved rods was with 20 % about three times higher than during the rest of the investigation period. During autumn and winter the cocci group ("starving forms") increased again.

The bacterial morphology changed dramatically downstream the effluents (site 2 - 6) due to freezing of the wastewater treatment pond in January 1997. Large and medium-sized bacteria reached a portion of about 60 % in the water body, while the cocci group decreased from 45 % to 17 %. Apart from bacterial forms observed commonly, chain and thread like bacteria occurred in masses. No changes in the bacterial population were detected at the reference site (1).

An increasing portion of cocci and a decrease of small (< 1 µm) and medium-sized (< 2 µm) rods were characterizing the winter situation in the model sediment. Apparently, the wastewater effluents did not affect the biodiversity in the sand samples.

3.2. Extracellular enzyme activities

About 10 to 100 times higher enzyme activities were obtained in the model sediment than in the water samples (Table 2). The different enzymes in the water column showed divergent reactions to the pollution. The wastewater effluents did not influence the activity of the esterases significantly. On the other hand, the β-glucosidase activity doubled downstream the outfall of the wastewater treatment pond (site 2) and decreased slightly afterwards. The L-alanin-aminopeptidase activity increased from site 1 to 6.

However, a closer relationship between enzyme activities and self-purification processes could be detected in the sand samples. The development of the enzyme activity in the investigated stream section is shown exemplary for the L-alanin-aminopeptidase activity. After increasing from 206 µmol · kg dm^{-1} · h^{-1} (site 1) to 286 µmol · kg dm^{-1} · h^{-1} downstream of the effluents (site 3), the extracellular peptidase activity decreased again to 151 µmol · kg dm^{-1} · h^{-1} at site 6. This effect was found for all enzymes in the model sediment.

Table 2. Means and extremes of the enzyme activities of the esterases, β-glucosidases, and phosphatases in the water (n = 15) and the model sediment (n = 13) from July 1996 to July 1997

			water [µmol L^{-1} h^{-1}]			
site	esterase		β-glucosidase		phosphatase	
1	**0,027**	(0,006-0,058)	**4,66**	(0,76-15,5)	**0,58**	(0,07-1,36)
2	**0,032**	(0,011-0,071)	**10,20**	(1,47-37,1)	**0,70**	(0,11-1,75)
3	**0,031**	(0,005-0,066)	**9,24**	(2,07-37,7)	**0,70**	(0,14-1,93)
4	**0,029**	(0,006-0,069)	**8,86**	(1,92-33,1)	**0,64**	(0,09-1,54)
5	**0,029**	(0,006-0,057)	**7,03**	(1,80-79,4)	**0,77**	(0,16-1,34)
6	**0,033**	(0,011-0,065)	**8,02**	(0,16-32,8)	**0,57**	(0,16-1,07)

			sand [µmol kg dm^{-1} h^{-1}]			
site	esterase		β-glucosidase		phosphatase	
1	**0,97**	(0,45-1,71)	**23,0**	(8,36-48,5)	**109,0**	(39,2-227,5)
2	**0,84**	(0,42-1,35)	**27,4**	(3,21-65,8)	**86,4**	(22,7-175,0)
3	**1,01**	(0,46-1,39)	**22,4**	(1,86-43,3)	**100,1**	(36,7-234,7)
4	**0,92**	(0,49-1,38)	**19,1**	(2,06-34,5)	**75,2**	(29,3-177,5)
5	**0,76**	(0,37-1,11)	**17,7**	(1,01-40,1)	**61,9**	(17,9-115,2)
6	**0,76**	(0,18-1,22)	**20,1**	(4,74-53,7)	**77,4**	(31,1-225,4)

3.3. Relative enzyme activity (REA)

In general, the REAs showed a similar development for all enzymes tested. In most cases the highest REAs were found at sampling point 1. After decreasing at site 2 the REAs stayed nearly constant within the following section of the Horloff. For the phosphatase the highest REA (60 - 1102 L · m^{-2}) was measured followed by the peptidase (17 - 450 L · m^{-2}), esterase (11 - 233 L · m^{-2}) and β-glucosidase (0,6 - 30 L · m^{-2}). These results indicate that the phosphatase activity might be closer related to the sediment than the esterase or glucosidase activity, for example. However, this sand to water activity ratio was closely connected to the water level. High REAs were detected at high water levels in winter 1996 and spring 1997 (Figure 1). Dilution effects probably caused a decrease of enzyme activity in water in comparison to the sediment activity. Two explanations for this effect might be possible. On the one hand the high water could have diluted the enzymes, which were set free by benthic microflora. On the other hand the decrease of enzymatic activity may be ascribed to a decrease in the concentration of nutrients. In fact, both explanations may be valid.

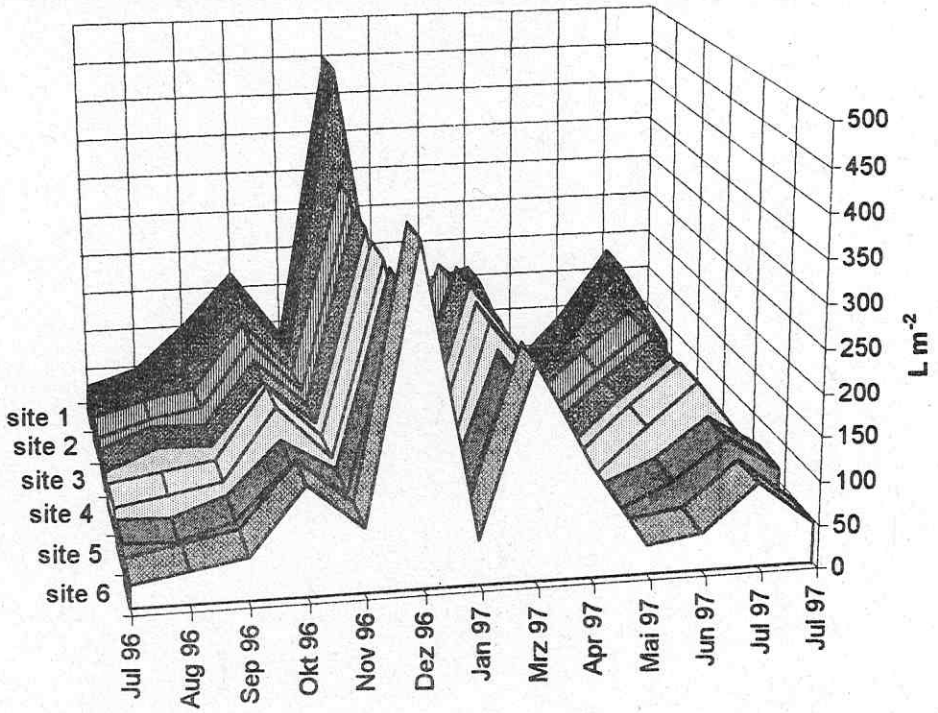

Fig. 1. Relative enzyme activity of the L-alanin-aminopeptidase activity at site 1 to 6 of the river Horloff during the course of the year

4. Summary

Concerning the morphology, the bacterial population seemed to be more constant in the model sediment, while variation in the pelagic population could be caused by seasonal influences and

heavy pollution. The comparison of the enzymatic assays in the different sites the model sediments were well reflecting the self-purification processes. The relative enzyme activity indicated relations of different significance between each enzyme and the model sediment. Simultaneously a close relationship between the REA and the water level was observed.

References

[1] Zimmermann, R. & Meyer-Reil, L.-A. (1974) A new method for fluorescence staining of bacteria populations on membrane filters. Kieler Meeresforsch. 30, 24-27

[2] Obst, U. and Holzapfel-Pschorn, A. (1988) Enzymatische Tests für die Wasseranalytik, R. Oldenburg Verlag, München

[3] Chappell, K.R. and Goulder, R. (1994) Seasonal variation of epilithic extracellular enzyme activity in three diverse headstreams. Arch. Hydrobiol. 130, 195-214

[4] Marxsen, J. and Witzel, K.-P. (1991) Significance of extracellular enzymes for organic matter degradation an nutrient regeneration in small streams. *In*: Microbial enzymes in aquatic environments (Chrost, R. J., Ed.), pp. 270-285. Springer-Verlag, New York

Simulation of substrate utilization in the benthic biofilm of small running waters with RIOVAL

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Abstract

The computer model RIOVAL has been developed to simulate oxygen, ammonia and the biochemical oxygen demand in small running waters. Substrate utilization in the small brooks take place in the benthic biofilm. It could be shown, that the turnover of substrate in the water could be neglected. The brook was treated as a plug flow reactor in the model. Several reference brooks were used to verify the model. The simulated concentration gradients in the brooks were in good agreement with the measured results. The results show, that biofilms have a great turnover of substrate in the aquatic environment.

The model could be used to predict the influence of wastewater discharge out of treatment plants on the water quality in the country side, where usually small brooks were polluted by discharge effluent.

1. Introduction

In the most states of Germany the discharge of wastewater is accompanied by a high priority for the water quality in the rivers. Until now the water quality in the running waters is only checked by the ratio of waste water and river water in the planning stage of a treatment plant. The self purification processes in the running waters were mostly neglected or only considered qualitatively.

In particular in small running waters with a flow up to 0.5 m^3/s the discharge of treated waste water has a remarkable effect on the water quality. In this rivers or brooks the main processes of self purification take place in the benthic biofilm [1, 2]. The biomass in the water body could be neglected when modelling the substrate degradation in the river. Such a river biofilm system could be described with the following compartments:

- air
- water
- boundary layer
- benthic biofilm
- substratum (sediment, macrophytes , stones)

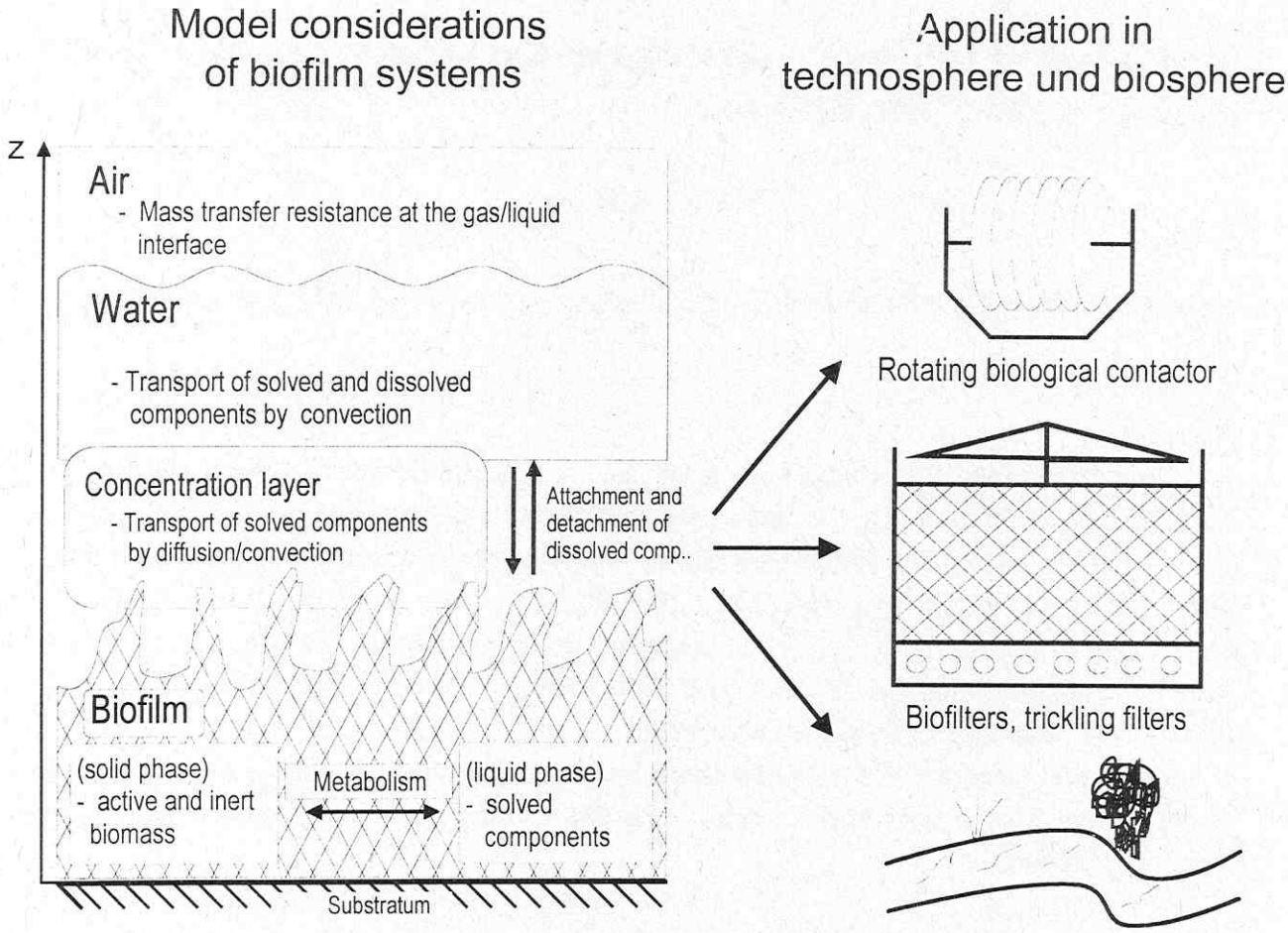

Fig. 1. Model consideration and possible application in technosphere and biosphere

Figure 1 gives an impression about the processes in a biofilm system. In the last decade it has become possible to model the complex processes of substrate utilization and mass transport in biofilm systems [3, 4]. Appropriate models allowed the solution of the main problems in biofilm systems:

- transport of substrate in the water
- mass transfer at the interface air/water
- mass transfer at the interface water/biofilm
- substrate utilization in the biofilm
- growth and decay of the microorganisms
- transport and displacement of the microorganisms

Furthermore, the use of new methods like micro electrodes and confocal laser scanning microscopy (CSLM) allows a better understanding of the intrinsic processes of biofilms [5, 6]. On the one hand the measurement with micro electrodes show substrate concentration profiles inside the biofilm system, on the other hand the confocal laser scanning microscopy gives an idea about the structure of biofilms.

In the last years both methods yielded results which play an important role for the simulation of substrate utilization and mass transport in biofilm systems. The concentration profiles were used to fit the diffusion coefficients of substrate and oxygen inside the biofilm.

Furthermore, the growth rates and the decay of the micro organisms inside the biofilms have been determined [4]. Finally, the mass transfer at the interface water/biofilm has been investigated intensively by micro electrodes [5].

The structure of biofilms especially the localization of the volume fractions of the different types of micro organisms and inert material was investigated by the CSLM [6]. There is no doubt that the structure of biofilms influenced the performance of substrate utilization and mass transport. It has to be investigated whether simplifications of the biofilm structure in the model are allowed and to which extend these simplifications influence the simulation results.

The purpose of this paper is to show that research results out of the laboratory could be used for the modeling of real processes in the biosphere. The computer model RIOVAL has been developed to simulate self purification in small running waters.

2. Model

The presented model considers main parameters for the ecological water quality classification:

- Oxygen
- Biochemical Oxygen Demand
- Ammonia
- pH
- Temperature

The degradation of the considered parameters in the benthic biofilm is described with a simple Monod kinetic (see Table 1). Complex processes like maintenance, endogenous respiration and inactivation of the micro-organisms were neglected.

Table 1. Process matrix

Process	Solved components			Solid components		Process rate r	
	c_{NH_4}	c_{BOB_5}	c_{O_2}	X_A	X_H		
Growth (autotrophic)	$-\frac{1}{Y_A}$	-	$-\frac{\alpha - Y_A}{Y_A}$	+1	-	$\mu_A \frac{c_{NH_4}}{K_{NH_4} + c_{NH_4}} \frac{c_{O_2}}{K_{O_2} + c_{O_2}}$	X_A
Growth (heterotrophic)	-	$-\frac{1}{Y_H}$	$-\frac{1 - Y_H}{Y_H}$	-	+1	$\mu_H \frac{c_{BOD_5}}{K_S + c_{BOD_5}} \frac{c_{O_2}}{K_{O_2} + c_{O_2}}$	X_H

The biomass distribution of the autotrophic and heterotrophic micro-organisms in the biofilm and the biofilm thickness is fixed in the model. Figure 2 shows the model biofilm. The chosen distribution fits best with the results out of several reference rivers in the state of Hessen and Saxony Anhalt. It could be shown that a dynamic simulation of the biomass distribution leads to the same results than the simulation with the steady state biofilm in RIOVAL [7]. The advantage of this simplification is a reduction of the calculation time which is of great interest when several variants for the planed waste water treatment have to be simulated.

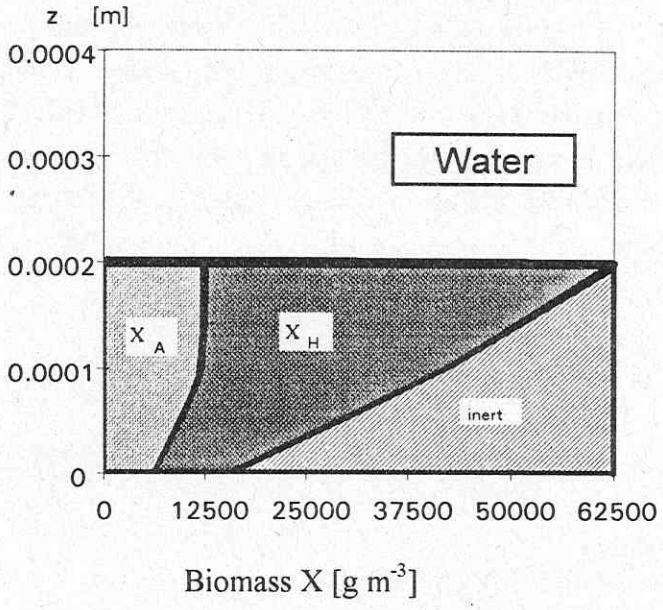

Fig. 2. Model biofilm with steady state distribution of auto- and heterotrophic biomass

The temperature dependence of the process growth of the autotrophic and the heterotrophic micro organisms is considered with the following formula:

$$\mu = \mu\,(20°C)\ e^{(\theta*(T-20)/T*20)} \qquad (1)$$

Inside the biofilm the solved components are transported by diffusion, the mass balance of the substrate i is described by:

$$\frac{\partial c_i}{\partial t} = -\frac{\partial j_i}{\partial z} + r_i \qquad (2)$$

j is the mass flux of the solved component i in the biofilm, r is the process rate (see Table 1). This leads to a system of partial differential equations with diffusion, reaction and time dependable boundary conditions.

A Galerkin-h-p-method was developed for the solution of this problem. The method has already been used successfully for similar problems in polymer reaction simulation [8]. This algorithm is a fully adaptive method and because of that short calculating times of round about 2 to 5 minutes on a Pentium-Processor (133 MHz) could be realized.

A Neumann - boundary condition is chosen to describe the mass transfer at the water/biofilm interface:

$$D_i \left.\frac{d c_i}{d z}\right|_{L_F} = \beta_i \ (c_{iB} - c_{iF}) \tag{3}$$

The mass transfer coefficient β is calculated by a flow velocity dependent formula:

$$\beta = 2281 * v^{1/2} * Sc^{1/2} * D * (1+ 23.33\ v) \tag{4}$$

Equation (4) has been developed empirical on the base of several papers which dealt with mass transfer to river beds [2, 9, 10, 11].

The transport of the solved components in the water is considered by convection. Dispersion can be neglected if the width of the running water ranges between 0.5 and 3 m. A plug flow model is applied. RIOVAL starts with the simulation of the substrate and oxygen profiles along the river axis after the boundary condition (see equation 3) together with the start concentrations after mixing of waste water discharge and river water achieves constant substrate profiles in the local biofilm. The time axis is then changed with the local axis in the river. The degradation of the solved substrate in the river is given by:

$$c_i(t) = k_{River} \ (c_i(t) - c_{iF}) \tag{5}$$

k_{River} is a river specific constant:

$$k_{River} = \frac{\beta * b * v}{Q} \tag{6}$$

This constant determines the individual self purification of the river. The used parameters are the mean width (b), the flow velocity (v) and the volumetric flow rate (Q) of the river.
The aeration of the river water is formulated with the saturation function and the aeration coefficient k_{Aer} :

$$c_{O_2}(t) = k_{Aer} \ (c_{O_2}(t) - c_{O_2sat}) \tag{7}$$

k_{Aer} is calculated in dependence on the flow velocity of the river, it has been shown that the depth of the water can be neglected in small running waters [12]:

$$k_{Aer} = 3100 * v^2 \tag{9}$$

The kinetic parameters which are used in RIOVAL are shown in Table 2. Most of the parameters are taken from laboratory scale studies of an auto-/heterotrophic biofilm [4].

Table 3 shows the parameters which are necessary to perform a simulation with RIOVAL.

Table 2. Kinetic parameters

Parameter		Value	
Growth rate:			
heterotrophic	μ_H	3,0	d^{-1}
autotrophic	μ_A	0,15	d^{-1}
Yield coefficient:			
heterotrophic	Y_H	0,2	gX g^{-1}BOD5
autotrophic	Y_A	0,06	gX g^{-1}NH4-N
Monod constant:			
BOB_5	K_S	10	g m^{-3}
Ammonia	K_{NH4-N}	0,5	g m^{-3}
Oxygen	K_{O2}	0,5	g m^{-3}
Diffusion coefficient:			
Oxygen	D_{O2}	2,1	cm^2 d^{-1}
Ammonia	D_{NH4}	1,8	cm^2 d^{-1}
BOB_5	D_S	0,58	cm^2 d^{-1}
Stochiometr.			
Factor O_2 / NH_4-N	α	4,4	-
Temperature coefficient	θ	0,0105	

3. Results

3.1. Reference rivers

Three reference rivers in the State of Hessen have been investigated and the experimental results have been used to fit the model parameters [12]. The volumetric flow rate ranged between 1 and 60 l s^{-1} including the discharge. All three rivers were strong polluted by the discharge of waste water treatment plants from small towns in the country side. The ratio of river water and waste water has values between 30 and 0.

The width of the rivers ranged between 0.6 and 2 m, the substratum was stony respectively clay like. The flow velocities ranged between 0.1 and 0.3 m s^{-1}.

The rivers were investigated two times in the year (spring/summer and autumn). Batch tests with river water in the laboratory showed that the substrate degradation in the water body could be neglected (< 1%). The results of one investigation is shown in Figure 3. The simulated results fitted very fine with the experimental results.

All three rivers could be modeled with one set of kinetic parameters which were shown in Table 2.

3.2. Example for the planning practice

In the catchment area of two small running waters the waste water treatment was planned. It has to be decided whether a central treatment plant or two decentralized waste water treatment plants should be build up.

Variant 1: The waste water of the small villages should be treated in two decentralized plants (Plant I and II) near the villages, the treated waste water was discharged in place. On the one side river I and river II were polluted. On the other side the pollution was divided and the self purification of the rivers were used in an optimal way.

Variant 2: The waste water was collected together and then treated in one treatment plant (central plant) with the discharge of waste water in only one river. This option was optimized with respect to the investment and operating costs.

The simulation with RIOVAL should be used to take the self purification of both rivers into account. It could be shown that in river I the ammonia concentration is already on a higher level due to discharge of waste water upstream and the discharge of the plant I increases the pollution only very little.

Table 3. Necessary parameters for a simulation of self purification in small running waters with RIOVAL

Parameter		Waste water treatment plant	Running water
Volumetric flow Q	[$m^3 s^{-1}$]	+	+
Substrate concentration c_{NH_4}, c_{BOD_5}, c_{O_2}	[$g m^{-3}$]	+	+
pH, alkalinity	[mmol L^{-1}]	+	+
Temperature air/water T	[°C]	+	+
Flow velocity v	[$m s^{-1}$]	-	+
Mean river width b	[m]	-	+
Structure of the river substratum		-	+

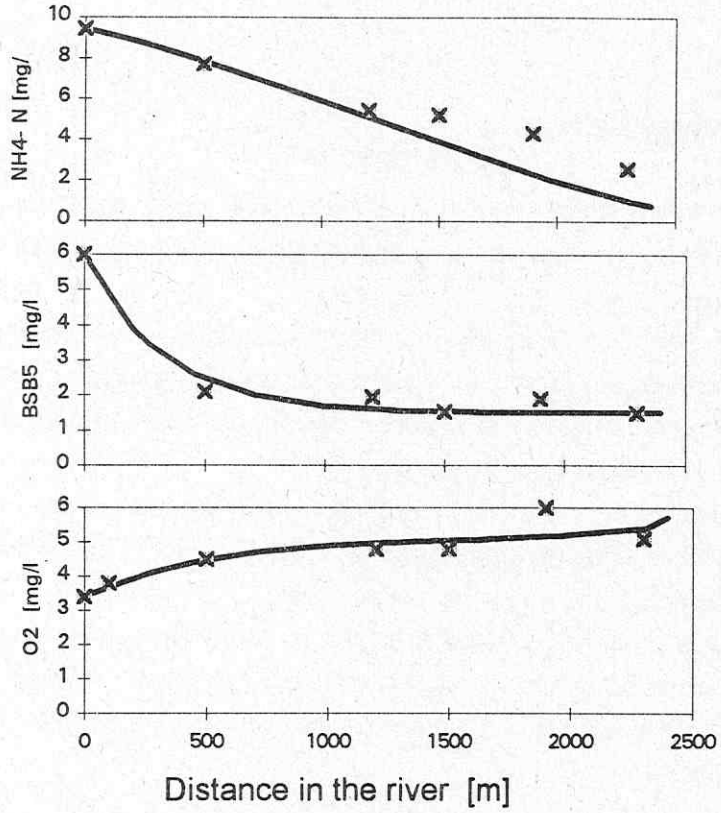

Fig. 3. Experimental results and simulation of a small running water

In river II the central plant leads to a higher pollution than the decentralized treatment plant II. The simulation of self purification gives an idea of what could happen in the rivers. When the final decision is made on the one hand the costs for each variant have to be taken into account. On the other hand the simulation showed very fine the possible pollution of the rivers, which must be an integral part of the waste water treatment in the country side.

4. Conclusion

The simulation of substrate degradation and mass transport in small running waters is very easy when a suitable computer software is provided. RIOVAL represents on the one hand the state of art in biofilm modeling, on the other hand it is an optimized software for the application in engineering and for environmental administrative tasks.

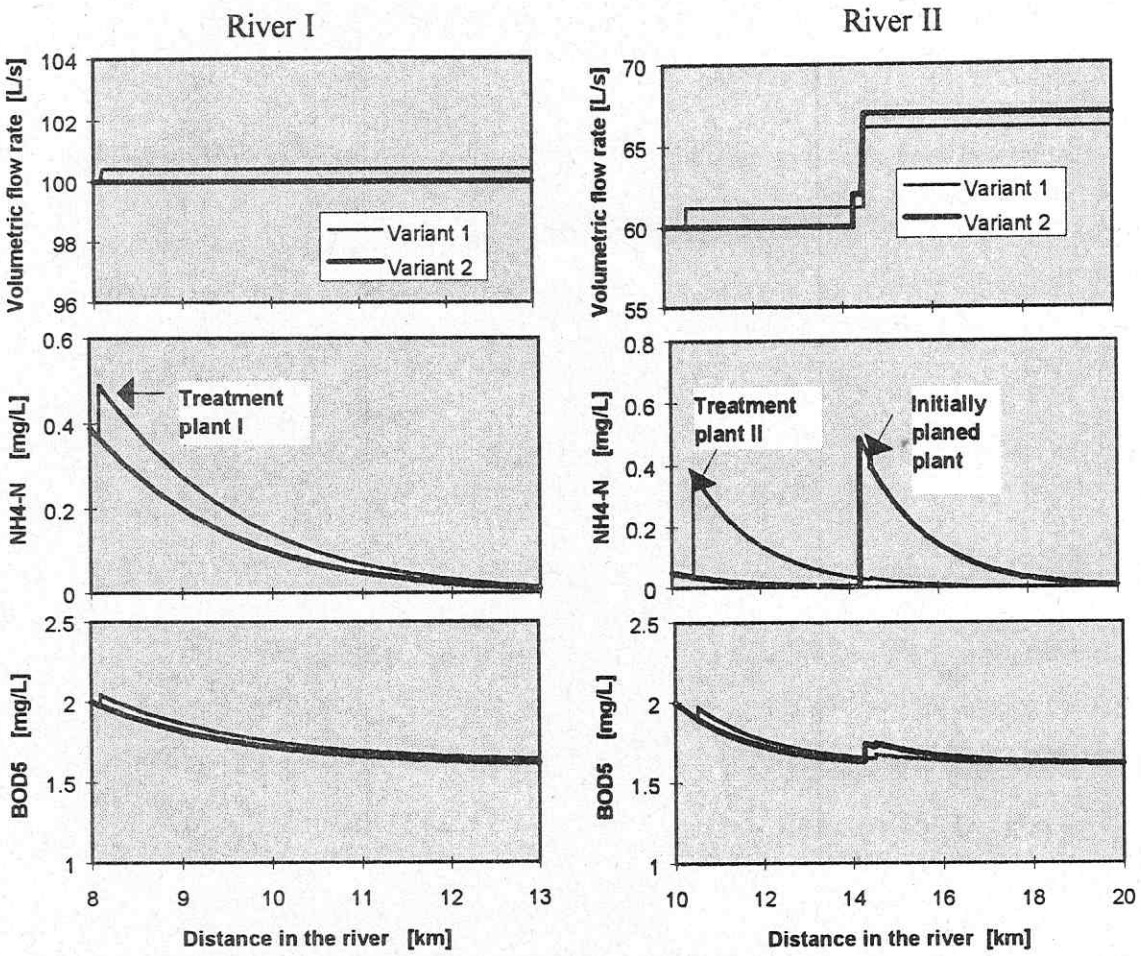

Fig. 4. Simulated variants of the waste water treatment in the catchment area of two small running waters

Symbols

c = Concentration of the solved components (g m^{-3})
b = River width (m)
D = Diffusions coefficient (m^2 d^{-1})
j = Flux (g m^{-2} d^{-1})
K = Monod constant, (g m^{-3})
k_{Aer} = Aeration constant in the river (d^{-1})
k_{River} = River constant (d^{-1})
L_F = Biofilm thickness (m)
Q = Volumetric flow rate (m^3 s^{-1})
r = Process rate (g m^{-3} d^{-1})
Sc = Schmidt number = υ/ D
T = Temperature (°C)
t = Time (d)
v = Flow velocity in the river (m s^{-1})

X = Concentration of particulate components in the biofilm (g m^{-3})
Y = Yield coefficient (gX $g^{-1}S$)

α = Stochiometric coefficient (gO_2 $g^{-1}NH_4$-N)
β = Mass transfer coefficient (m s^{-1})
μ = Growth rate (d^{-1})
θ = Temperature coefficient
υ = Kinematic viscosity (m^2 s^{-1})

Subscripts :

A = Autotroph
H = Heterotroph
NH_4 = Ammonia
BOD_5 = Biochemical Oxygen Demand
O_2 = Oxygen
O_{2sat} = Oxygen saturation concentration

References

[1] Gujer, W. (1978) : Nitrit in Fließgewässern - ein erweitertes Nitrifikationsmodel, Schweiz. Z. Hydrol. 40/2, 211 - 230

[2] Lau, Y.L. (1990): Modelling the Consumption of Dissolved Contaminants by Biofilm Periphyton in Open-Channel Flow, Wat. Res., Vol.24, No.10, 1269 - 1274

[3] Gujer, W. and Wanner O. (1990) Modeling mixed population biofilms. Eds. Characklis W. G. and Marshall K. C., Biofilms. John Wiley & Sons, 397-445

[4] Horn, H. und Hempel, D.C. (1997): Growth and decay in an auto-/heterotrophic biofilm, Water Research, 31 (9), 2243 - 2252

[5] Horn, H. (1997) Microelectrodes and Tube Reactors in Biofilm Research, *In*: Flemming, H.-C., The Investigation of Biofilms, Springer, Berlin, in press

[6] Neu, T. and Lawrence, J.R. (1997), Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy, FEMS Microbiology Ecology 24, 11 - 25

[7] Horn, H.; Telgmann, U. und Wulkow, M. (1996): Simulationsverfahren für Biofilmsysteme in Festbettreaktoren und kleinen Fließgewässern, *In*: GVC VDI-Gesellschaft Verfahrenstechnik und Chemieingenieurwesen: Verfahrenstechnik der Abwasser- und Schlammbehandlung. Bd. 1. Düsseldorf: VDI, 1996, S. 325-342

[8] Wulkow, M. (1996), The simulation of molecular weight distributions in polyreaction kinetics by discrete Galerkin methods, Macromol. Theory Simul. 5, 393-416

[9] Hickey, C.W. (1988): River Oxygen Uptake and Respiratory Decay of Sewage Fungus Biofilms, Wat. Res., Vol. 22, No. 11, 1375 - 1380

[10] Schöl, A. (1991): Experimentelle Untersuchungen zur Abhängigkeit der Selbstreinigung von der Naturnähe in Fließgewässern - Teil II: Laborversuche, F+E Vorhaben im Auftrag des DVWK

[11] Perchtold, K.; Kirchesch, V. and Müller, D. (1988): Modellversuche zur Nitrifikation am Gewässerbett, gwf-Wasser/Abwasser 129 (1), 7 - 12

[12] Horn, H. and Wulkow, M. (1996): Simulation von Einleitungen in kleine Fließgewässer, gwf - Wasser/Abwasser , 137 (10), 557 - 564

- Polluted groundwater and sediments -

Bioremediation of groundwater polluted by a herbicide production plant

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Abstract

The feasibility of remediating the groundwater below a former herbicide production plant contaminated with various chlorinated and/or methylated phenoxyalkanoic acids and phenols was investigated. The pH value in the vicinity of the production site was alkaline and the water had a deep brown color. Microcosm experiments showed that indigenous microorganisms did not show metabolic activities for the degradation of these compounds. The inoculation of the groundwater with biodegradative strains, e.g. *Ralstonia eutropha* JMP 134, or alkaliphilic/alkalitolerant biodegradative bacteria including strains of *Comamonas acidovorans, Rhodoferax fermentans, Aureobacterium testaceum* and *Rhodococcus erythropolis* led to the almost complete degradation of the phenolic contaminants and herbicides present. The color of the water was shown by synchronous fluorescence spectroscopy to be caused by dissolved organic matter (humic substances). Both acidification and the application of trivalent cations were suitable for removing these compounds by flocculation. Thus, remediation of this site should be feasible by applying specialist inocula in an appropriate bioreactor technology, combined with flocculation treatment.

1. Introduction

Production sites used by the chemical industry are often highly polluted especially the surrounding soil, underlying groundwater and the buildings. In a feasibility study we focused on the groundwater polluted by a former phenoxy herbicide production plant which was heavily contaminated with the spectrum of products including the source compounds, intermediates and by-products. A second

source of pollutants resulted from a period of diesel fuel production at this site more than five decades ago. The groundwater below the herbicide production site itself was alkaline and exhibited a deep brown color, whereas in the more peripheral areas of the pollution plume it was slightly acidic and clear.

These pollutants must be removed in order to prevent their spreading and toxification of large areas of the environment. The aim of the investigations summarized subsequently therefore was the development of an *in situ* or a *on site* bioremediation technology for groundwater heavily polluted with herbicides and intermediates of their industrial production. The experiments were thus focused on eliminating the toxic chlorinated aromatic compounds. Microbial activities degrading various chlorinated phenols and phenoxyalkanoic acids are well known under neutral pH conditions [1-3]. As a result of our investigations of the decontamination of herbicide-polluted building materials [4, 5], we have isolated bacterial strains which also degrade these compounds under (very) alkaline pH conditions [6-8]; these were hence used. An additional problem arose from the deep brown color of the groundwater observed in certain areas. Data from synchronous fluorescence spectroscopy, which has been proven a useful tool for monitoring the distribution and changes of dissolved organic matter (humic substances) in groundwater [9, 10], have indicated that this is most probably derived from humic matter. Measures were subsequently applied for their removal (cf. [11]).

2. Results and discussion

The pollution problem as observed by annual routine measurements is summarized up in Table 1. High contamination was found immediately below the former herbicide plant (Well 45). Evidently, the main problem was caused by the chlorinated phenoxyalkanoates and phenols with AOX values as high as 6 mg/l. Pollution by hydrocarbons (mineral oils) amounted to about 5 mg/l. The identification of 2,4-dichlorophenol (DCP) and 4-chloro-2-methylphenol (MCP) was not always possible in routine HPLC analysis, as both showed similar retention times under the conditions applied. However, the concentration of MCP was higher by an order of magnitude in comparison to DCP. The analysis of the dominant pollutants within the groundwater was optimized for *on site* field monitoring purposes on the basis of solid phase extraction and subsequent GC-MS measurement. With this method, a recovery of about 100 % was obtained for DCP and MCP. In particular, MCP reached concentrations of up to 14.6 mg/l (Well 45, 10 m depth; Sept. 1996). A similar spectrum of pollution but at a lower level was found at a distance of about 1-2 km from this position (Well 40). We tested if the degradation of MCP at the polluted site was limited by the catabolic potential of the indigenous microbial community or by the availability of oxygen. To this end, we performed experiments with microcosms made up from groundwater samples of the polluted site. All microcosms were oxygenated by gentle shaking and than inoculated with *Ralstonia eutropha* JMP134 (formerly *Alcaligenes eutrophus* JMP 134), a strain able to mineralize a wide spectrum of substituted aromatics. Controls remained untreated. The survival of *R. eutropha* JMP 134 in the microcosm was followed by plate counts on selective minimal media, and the degradation of the target compounds analyzed by GC measurement as described above. In microcosms inoculated with strain JMP134 (initial density of approximately 10^7 cfu/ml), MCP and DCP were degraded within 24 hours. Final concentrations after 216 h were 0.29 mg/l (DCP) and

0.077 mg/l (MCP), respectively (Figure 1). No degradation occurred in uninoculated control microcosms. Thus, biodegradation of MCP and DCP is limited by the catabolic potential of the indigenous community of the polluted aquifer. *R. eutropha* JMP134 might contribute in an inoculum to complete degradation of the herbicides present [12].

Table 1. Characteristics of the groundwater from a herbicide site

	Well 45	Well 40
Sum characteristics		
Color	dark brown	colorless
pH	8.5	5.4
TOC (mg/l)	33	4.7
AOX (mg/l)	6.1	0.25
IR Hydrocarbons (mg/l)	4.8	n.d.
Chlorinated compounds (mg/l)		
2,4-D	< 0.1	< 0.05
2,4-DP	1800	220
2,4-DB	1000	< 0.05
MCPA	290	8
MCPP	3200	120
MCPB	970	1
2,4,5-TP	60	n.d.
2-Chlorophenol	93	8.5
2,4-Dichlorophenol	52	< 0.3
2,4,6-Trichlorphenol	35	< 0.3

n.d., no data
Data provided by BASF Schwarzheide.

Besides *R. eutropha* JMP134, which is active under neutral pH conditions strains were applied which are characterized by alkaliphilic/alkalitolerant properties. These should be especially suited for treating the highly polluted alkaline groundwater with pH values up to 10 in the deepest layers of the aquifer. The degradative properties of these strains are shown in Table 2. Consequently, metabolic activity for the degradation of the main pollutants are available. Several strains and combinations of strains were applied for bioremediation purposes. The prime concern at this phase of the investigation was to establish the feasibility of bioremediation; detailed kinetic investigations are aimed at in further experiments. The application of the alkaliphilic strain *Comamonas acidovorans* P4a, for instance, resulted in the degradation of the phenoxyacetate herbicides (2,4-D and MCPA). In addition, chlorinated/methylated phenols, which are present in this groundwater and

are also intermediates in the microbial degradation of the phenoxy herbicides (e.g. DCP/MCP) were also utilized (Figure 2). *Rhodoferax fermentans* strain P230 proved to be a versatile bacterium which degraded a broad spectrum of the chloro-organic pollutants (Figure 3). Its application in the groundwater resulted in the removal of almost all of these pollutants, only leaving the phenoxybutyrates unutilized. This problem, however, might be solved by the metabolic activity of strains such as *Aureobacterium testaceum* K2-17 or *Rhodococcus erythropolis* K2-12, which were shown to attack substituted phenoxybutyrates by liberating the respective substituted phenols. These products/intermediates in turn are degradable by the activity of one of the above-mentioned bacteria. Thus, at the present state of investigation the degradation of a large spectrum of chlorophenols and phenoxyalkanoates would require the application of a consortium of two species, namely *R. fermentans* P230 and either *A. testaceum* K2-17 or *R. erythropolis* K2-12. The application of a mixture of these strains, including *C. acidovorans* P4a, indeed resulted in an almost complete elimination of the phenolic pollutants (Figure 4). The hydrocarbon contaminants arising from the previous diesel fuel production were not considered at this state of investigation. Initial inspection of the IR adsorption signal before and after the treatment of groundwater with the herbicide-degrading trinary consortium did not indicate an essential decrease of the hydrocarbon content during this treatment. Control experiments on groundwater supplied with ammonium and phosphate did not show a significant degradation of phenoxyalkanoates within this period (Figure 5).

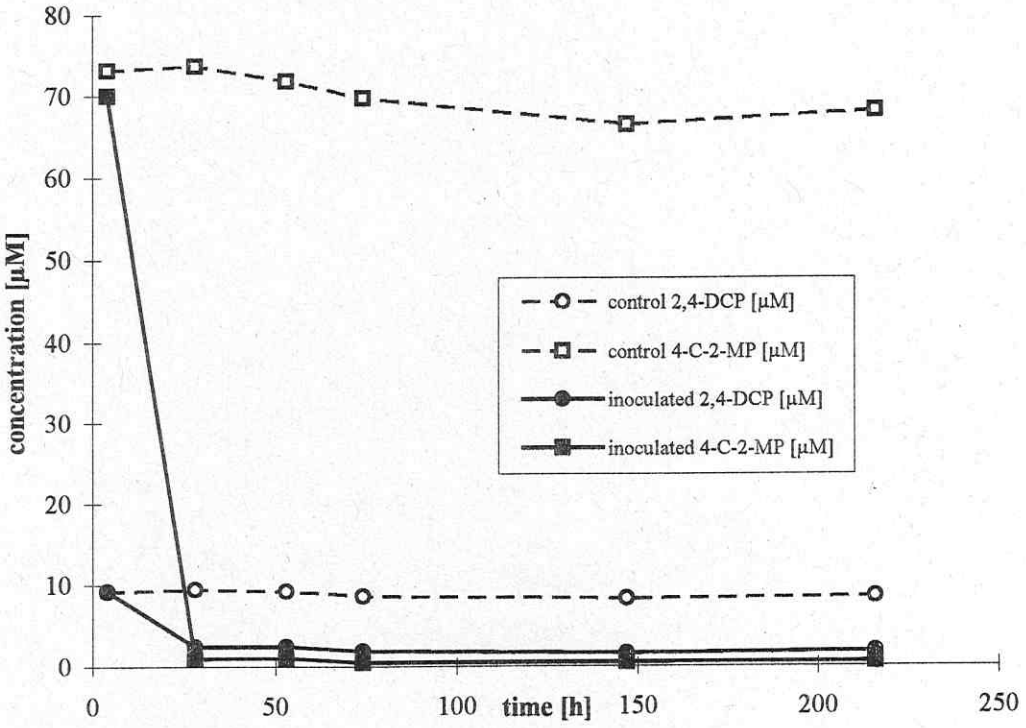

Fig. 1. Degradation of 2,4-dichlorophenol and 4-chloro-2-methylphenol in groundwater microcosms inoculated with *Ralstonia eutropha* JMP 134
Circle, DCP; Square MCP; Open symbols, control; Filled symbols, inoculated

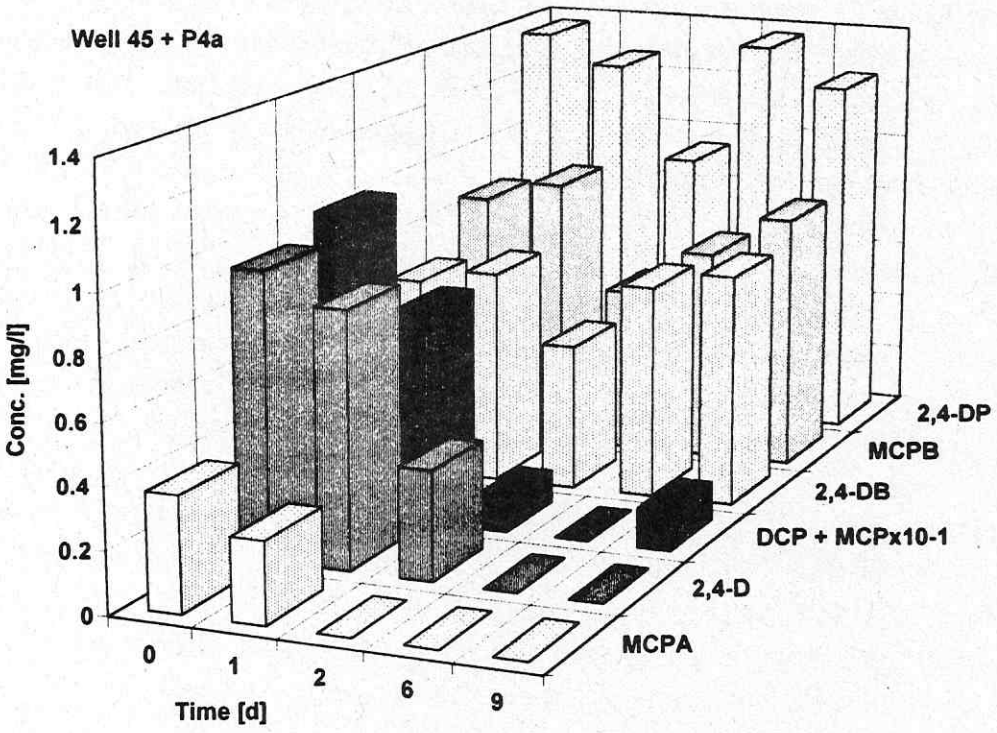

Fig. 2. Degradation patterns of the phenolic contaminants after application of *Comamonas acidovorans* P4a

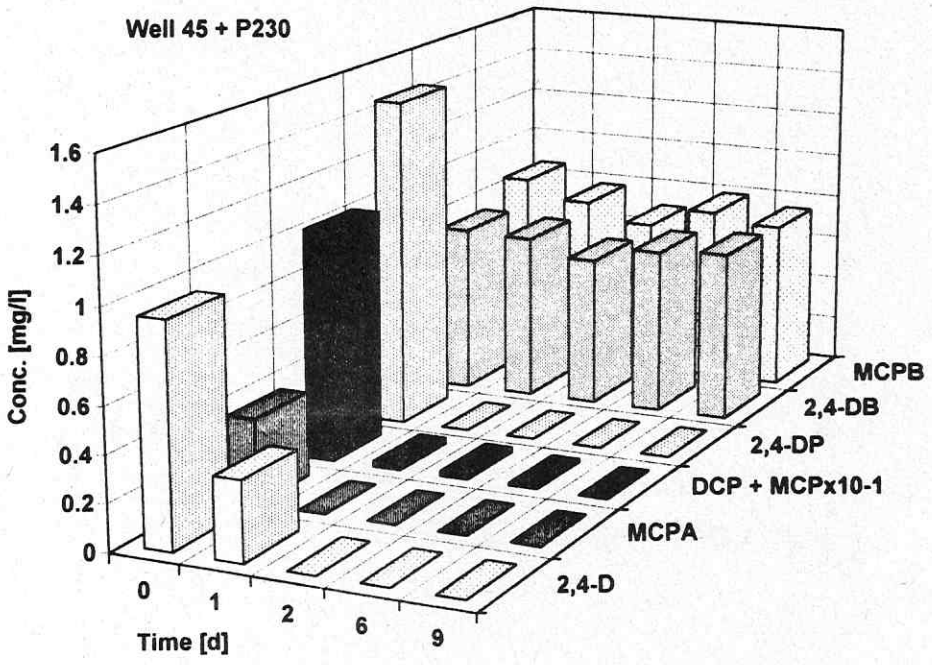

Fig. 3. Degradation patterns of the phenolic contaminants after application of *Rhodoferax fermentans* P230

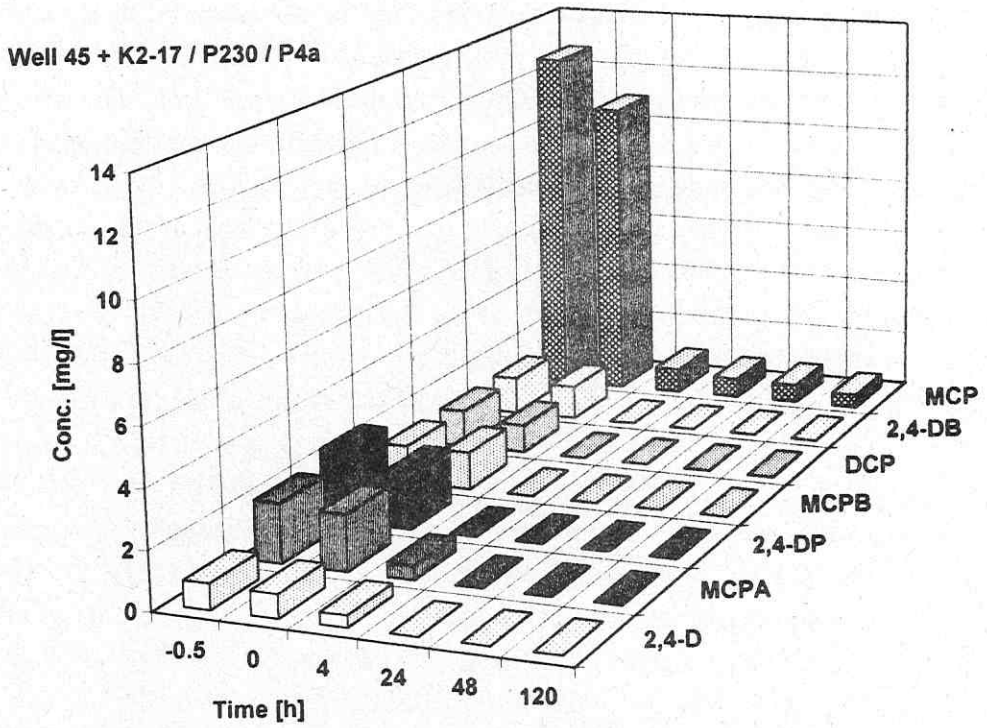

Fig. 4. Degradation patterns of the phenolic contaminants after application of a bacterial mixed culture consisting of *Comamonas acidovorans* P4a, *Rhodoferax fermentans* P230 and *Aureobecterium testaceum* K2-17

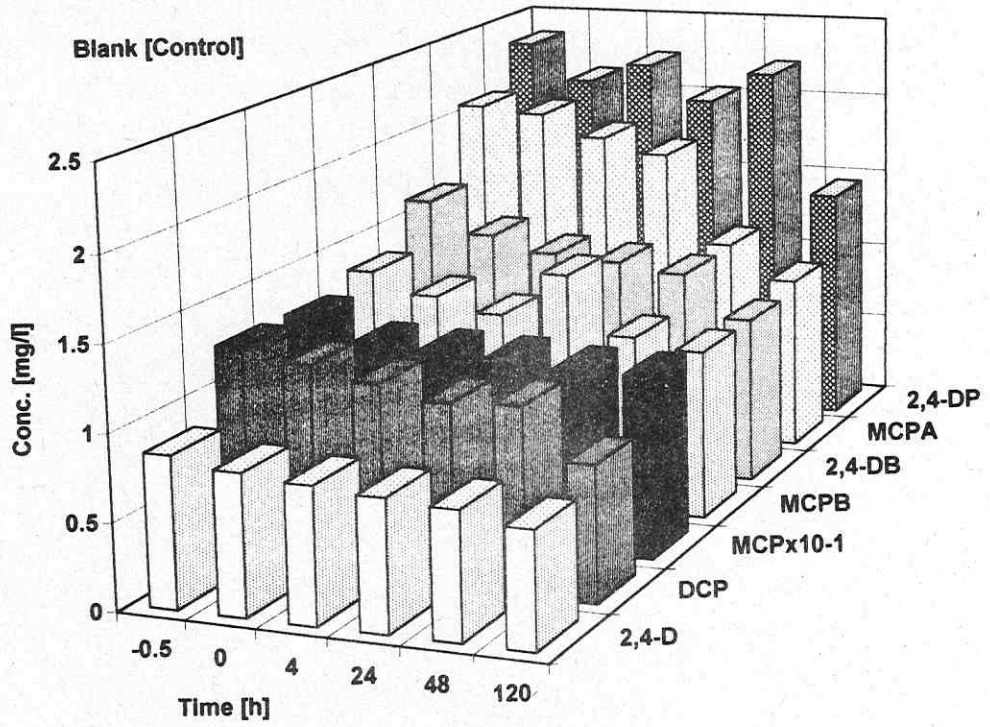

Fig. 5. Concentration profile of the phenolic compounds during aerobic treatment of groundwater supplemented with ammonium and phosphate without inoculation

An additional problem concerns the deep brown color of the highly polluted groundwater which remains unchanged after microbiological treatment. Synchronous fluorescence spectra reveal mayor peaks at 350, 395 and 470 nm indicating the presence of dissolved organic material (humic matter) [9, 10]. Although these data are not suitable for chemical identification, they are useful for pattern recognition. This is shown for the groundwater from the herbicide site in comparison to the solution of aquatic humic acid standards (Swannee River humic acid, IHSS International Humic Substances Society). Apparently, the two almost coincide (Fig. 6). Comparing these data to a soil humic acid standard from the IHSS which is characterized by a higher content of high-molecular weight structures, reflected in the high intensity of the peak at 470 nm [15] indicates that the content of low and high molecular weight humic matter is more balanced in the 1996 water samples. Surprisingly, samples taken in October 1997 exhibited a very different picture. A peak at 275 nm became dominant in the fluorescence spectra corresponding to a high content of low moleculare weight structures (Fig. 7) [15]. The pH of the groundwater had dropped to below 7 and the content of humic matter was very low. The data of this water sample resemble the figures found in a lake of wastewater from low temperature carbonization (Schwelvollert) after treatment (precipitation of humic matter). The reasons for the change in the hydrochemical characteristics are actually unknown but may result from a stratification of the aquifer. The highly polluted water (1996 sample) was treated by chemical measures in order to remove these humic compounds. Acidification by HCl, for instance, was successful. At a pH of about 3.5 almost all of these compounds were removed by flocculation. This rather strong acidification is unsuitable for technical application. Flocculation was also caused by the addition of trivalent cations [11]. With Al^{3+}, for instance, an equivalent of these ions as few as 2-2.5 mM led to the almost complete elimination of the precipitable material in the aqueous phase while only decreasing the pH to about 6.7 (Table 3). This decrease in the load of humic matter is reflected by the fluorescence spectrum (Fig. 7).

To summarize, preliminary microbial investigations have shown that strains are available and applicable which are active in eliminating the main and most toxic compounds in this groundwater under the resident conditions. The problem arising from a high content of humic matter might subsequently be solved by the application of conventional technical measures.

Table 3. Treatment of the groundwater by $Al_2(SO_4)_3$

Concentration (g/l)	pH	Extinction at 352 nm
0	8.7	1.95
0.05	8.24	1.93
0.1	7.7	1.83
0.2	7.28	1.54
0.4	6.85	0.152
0.5	6.58	0.111
0.6	6.37	0.085
0.8	5.98	0.066

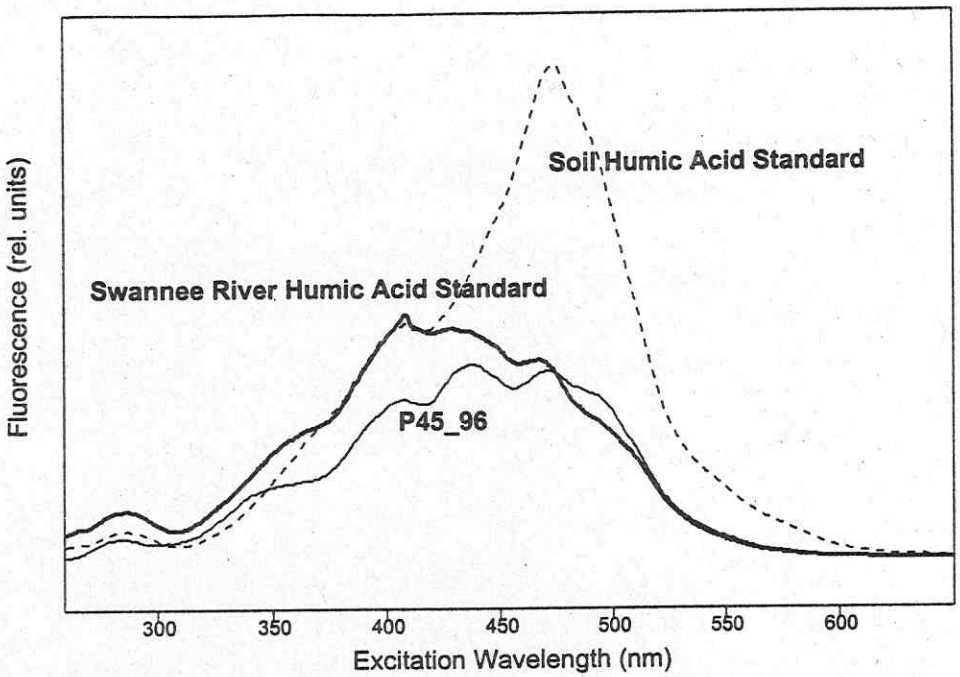

Fig. 6. Synchronous fluorescence spectra of groundwater from a herbicide site (September 1996) in comparison to solutions of humic acid standards from the IHSS (pathlength of the cuvette 5 mm, $\Delta\lambda = 18$ nm)

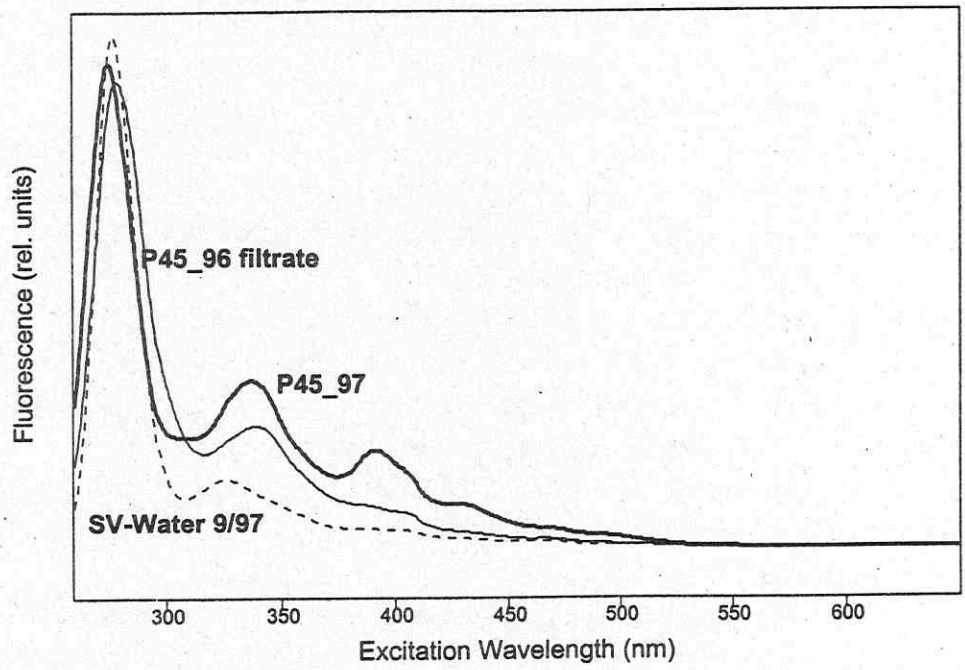

Fig. 7. Synchronous fluorescence spectra of groundwater after treatment by Al^{3+} and from the herbicide site in October 1997. The data were compared to the spectrum of a carbonization waste water (Schwelvollert) after flocculation (conditions see Fig. 6)

Abbreviations

DCP - 2,4-Dichlorophenol
2,4-D - 2,4-Dichlorophenoxyacetate
2,4-DB - 2,4-Dichlorophenoxybutyrate
2,4-DP - 2,4-Dichlorophenoxypropionate
MCP - 4-Chloro-2-methylphenol
MCPA - 4-Chloro-2-methylphenoxyacetate
MCPB - 4-Chloro-2-methylphenoxybytyrate
MCPP - 4-Chloro-2-methylphenoxypropionate

References

[1] Sinton, G.L., Fan, L.T., Erickson, L.E. and Lee, S.M. (1986) Biodegradation of 2,4-D and related compounds. Enzyme Microb. Technol. 8, 395-403

[2] Reineke, W., Knackmuss, H.J. (1988) Microbial degradation of haloaromatics. Annu. Rev. Microbiol. 42, 263-287

[3] Häggblom, M.M. (1992) Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. 103, 29-72

[4] Müller, R.H. and Babel, W. (1994) Biodegradative properties of alkaliphilic microorganisms from building materials of herbicide production plants. *In*: IChem^{E}, 2nd UK Congress of Biotechnology, Brighton July 4-6 1994. Chameleon Press London, pp. 73-75

[5] Müller, R.H., Müller, R.A., Zeika, O., Gleitsmann, R. and Babel, W. (1997) Mikrobielle Dekontamination von herbizidbelastetem Bauschutt, Erfahrungen aus dem Labor- und Technikumsmaßstab. TerraTech 4/1997

[6] Hoffmann, D., Müller, R.H., Kiesel, B. and Babel, W. (1996) Isolation and characterization of an alkaliphilic bacterium capable of growing on 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid. Acta Biotechnol. 16, 121-131

[7] Ehrig, A., Müller, R.H. and Babel, W. (1979) Isolation of phenoxy herbicide-degrading *Rhodoferax* species from contaminated building material. Acta Biotechnol. 17, 351-356.

[8] Müller, R.H., Mertingk, H. and Babel, W. Etherolytic cleavage of 4-(2,4-dichlorophenoxy)butyric acid and 4-(4-chloro-2-methylphenoxy)butyric acid by grampositive bacteria isolated from an alkaline environment. (submitted for publication)

[9] Geyer, W., Brüggemann, L. and Schmitz, U. (1996) An approach for classification of groundwaters by synchronous fluorescence spectroscopy and discrimination analysis. Acta hydrochim. hydrobiol. 24, 206-211

[10] Kalbitz, K., Popp, P., Geyer, W. and Hanschmann, G. (1997) ß-HCH mobilization in polluted wetland soils as influenced by dissolved organic matter. Sci. Total Environ. 204, 37-48

[11] Walther, H.J. and Winkler, F. (1981) Wasserbehandlung durch Flockungsprozesse. Akademie Verlag Berlin

[12] Wagner-Döbler, I., Erb, R., Rapp, P., Brümmer, I., Hauser, B., Gleitsmann, R. and Müller, R.H. (1997) On site assessment of microbiological diversity and activity. Field Screening Europe, First Internat. Conference, Karlsruhe 20.09. - 01.10.

[13] Müller, R,H., Jorks, S., Kleinsteuber S. and Babel, W. Degradation of chlorophenols under alkaline conditions by gram-negative bacteria related to *Ochrobactrum anthropi*. (submitted for publication)

[14] Pieper, D.H., Reineke, W., Engesser, K.H. and Knackmuss, H.J. (1988) Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP 134. Arch. Microbiol. 150, 95-102

[15] Ferrari, G.M. and Mingazzini, M. (1995) Synchronous fluorescence spectra of dissolved organic matter (DOM) of algal origin in marine waters. Mar. Ecol. Prog. Ser. 125, 305-315

Degradation of chlorobenzenes by autochthonous bacteria from a polluted aquifer

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Abstract

Anaerobic groundwaters and sediments of a site contaminated with chloroorganics in Bitterfeld (Germany) were colonized with bacteria down to the maximum drilling depth of 50 meters. In the autochthonous biocenoses denitrifying and iron-reducing bacteria predominated, but sulfate-reducing bacteria were also present. A surprisingly high abundance of aerobic bacteria was found throughout. The main contaminants in the quaternary aquifer were monochloro-, 1,2- and 1,4- dichlorobenzene. These substances were degraded simultaneously by the autochthonous biocenoses after addition of different terminal electron acceptors. Under aerobic conditions monochlorobenzene and 1,4-dichlorobenzene had disappeared completely and 1,2-dichlorobenzene to about 88 % after 20 days. Using nitrate-, sulfate-, iron-, and manganese-reducing conditions all three chlorobenzenes were degraded by about 80 % within 40 days.

Trichloroethane, predominantly occurring in the groundwater of a lower, tertiary aquifer, was not modified by the autochthonous biocenosis of this aquifer under any of the conditions tested.

1. Introduction

The contamination and potential danger to the groundwater in the area of Bitterfeld (Germany) results from leakages during chemical production and dumping of huge amounts of waste products from the chemical industry (mainly chloroorganic compounds) in disused open cast coal mines. With the closure of the open cast mines the groundwater is no longer lowered. A decontamination process in that particular region has been proposed based on the "funnel and gate" principle for the off-flowing anaerobic groundwater which combines physical-chemical techniques for the dehalogenation and microbiological bioremediation (Project SAFIRA).

Because of its importance in bioremediation and environmental protection the microbiological degradation of chloroaromatic compounds has been subject of a number of investigations in the past few years [for reviews see 1 - 3]. Anaerobic degradation is not fully understood yet. Under anaerobic conditions the reductive dehalogenation is the only well known degradation mechanism for higher chlorinated aromatics [4, 5]. Anaerobic

mineralization of halogenated phenols and benzoates using the terminal electron acceptors NO_3^-, SO_4^{2-} or Fe^{3+} has been demonstrated [6,7]. In contrast to benzene chlorobenzenes have not been reported to be degraded through the use of electron acceptors other than oxygen [8]. The aim of this study was to characterize the groundwater biocenoses of two contaminated aquifers and to evaluate the degradation potential of autochthonous bacteria for the primary contaminants monochloro- (MCB), 1,2- dichloro-(1,2-DCB), 1,4- dichlorobenzene (1,4-DCB) and trichloroethane (TCE) under aerobic and anaerobic conditions.

2. Materials and methods

2.1. Enumeration of microorganisms

Colony forming units (CFU) of aerobic bacteria were determined using R2A agar (Difco, Germany) and for CFUs of anaerobes the bacteria were grown on triple sugar iron (TSI) agar (Merck, Germany). Fungi were counted on MYP-agar pH 5.0 (7.0 g malt extract, 0.5 g yeast extract, 1.0 g peptone from soya, and 15 g agar per liter).

Most probable numbers (MPN) were estimated in four replicates using enrichment media for denitrifying bacteria [9], iron-reducing bacteria [10] and sulfate-reducing bacteria [11]. Incubation temperature was 16°C.

2.2. Experimental set-up

Degradation experiments were carried out in 5 ml suspension cultures. Anaerobic cultures were incubated in 11 ml headspace vials with 6 ml N_2/H_2 (95/5, v/v), aerobic cultures in 20 ml vials with 15 ml air. Each of the vials was closed with crimp seals (PTFE-coated butyl rubber).

Chlorobenzenes were added simultaneously to final concentrations of 30 mg MCB, 10 mg 1,2-DCB and 10 mg 1,4-DCB per liter; TCE (trichloroethene) was added to separate cultures to a final concentration of 30 mg per liter.

Cultures contained per liter 250 ml inoculum, 250 ml xenobiotics stock solution in distilled water and a total of 500 ml substrates-, electron acceptors-, and reductants- solutions prepared with sterilized groundwater of the corresponding aquifer.

The influence of auxiliary substrates and macronutrients was investigated by adding per liter medium: 50 mg sodium acetate · 3 H_2O, 50 mg sodium lactate, 5 mg yeast extract, 25 mg NH_4Cl, and 10 mg $NaH_2PO_4 \cdot H_2O$. Each liter denitrifying medium contained 0.5 g KNO_3, sulfidogenic medium contained 0.5 g $FeSO_4 \cdot 7\ H_2O$, 100 mg thioglycollate, 100 mg ascorbic acid, and 20 mg sodium dithionite. The medium for iron-reducing bacteria contained approximately 5 g amorphous FeOOH [preparation according to 12] and 200 mg thioglycollate per liter.

Inoculation was performed with fresh groundwater, which had been used to extract bacteria from 100 g wet mixed aquifer sediments per liter for 20 hours in an overhead shaker (1 min^{-1}) under N_2/H_2 (95/5, v/v)-atmosphere and room temperature.

2.3. Chemical analysis

Chloroaromatic compounds were extracted with n-hexane (Merck, Germany) directly from culture vials and detected using a GC/MS system (EM 640, Bruker Franzen-Analytik, Germany).

TCE was measured by headspace-GC after enrichment by solid phase micro extraction (85 µm polyacrylate, adsorption 20 min at room temperature, desorption 3 min at injection temperature 250°C). The separation was performed with an DB-5 apolar capillary column (30 m × 0.25 mm) at 40°C, carrier gas hydrogen, FID detection.

3. Results and discussion

3.1. Microbiological characterization of the site

Bacteria were found in the groundwater and sediments throughout the profile down to the maximum drilling depth of 50.5 m below the surface (Figure 1a).

A more comprehensive picture of the physiological diversity of the autochthonous bacterial biocenoses was obtained using the MPN method combined with selective growth conditions for the different physiological groups (Figure 1b). In the autochthonous biocenoses denitrifying and iron-reducing bacteria predominated, but sulfate-reducing bacteria were also present and have been enriched from every sediment sample. Surprisingly a large number of (facultative) aerobes were found. Colony forming units of fungi were found in few samples and only in very low concentrations ($<10^2$ ml^{-1}, data not presented).

3.2. Degradation of chloroorganics

Evaluation of the degradation potential of the different groups of autochthonous bacteria was carried out by selective stimulation through the addition of various electron acceptors and in some cases reductants to the media.

Experimental controls contained autoclaved groundwater/sediment extract instead of inoculum. They showed a decrease in the concentrations of about 20-30 % for MCB and 1,2-DCB and of about 40-50 % for 1,4-DCB during a period of 80 days. This could be due to diffusion through the lids of the vials and/or irreversible adsorption to surfaces.

Under aerobic conditions MCB and 1,4-DCB disappeared completely during the first 10 days of incubation (Figure 2a), while 1,2-dichlorobenzene was degraded at a lower rate than the other chlorobenzenes, but in contrast to investigations of Feidieker et al. [13] it did not persist. The addition of nutrients did not accelerate the degradation of any of the chlorobenzenes, not even under anaerobic conditions. It seemed to have a weak inhibitory effect at least for the first steps of bioconversion.

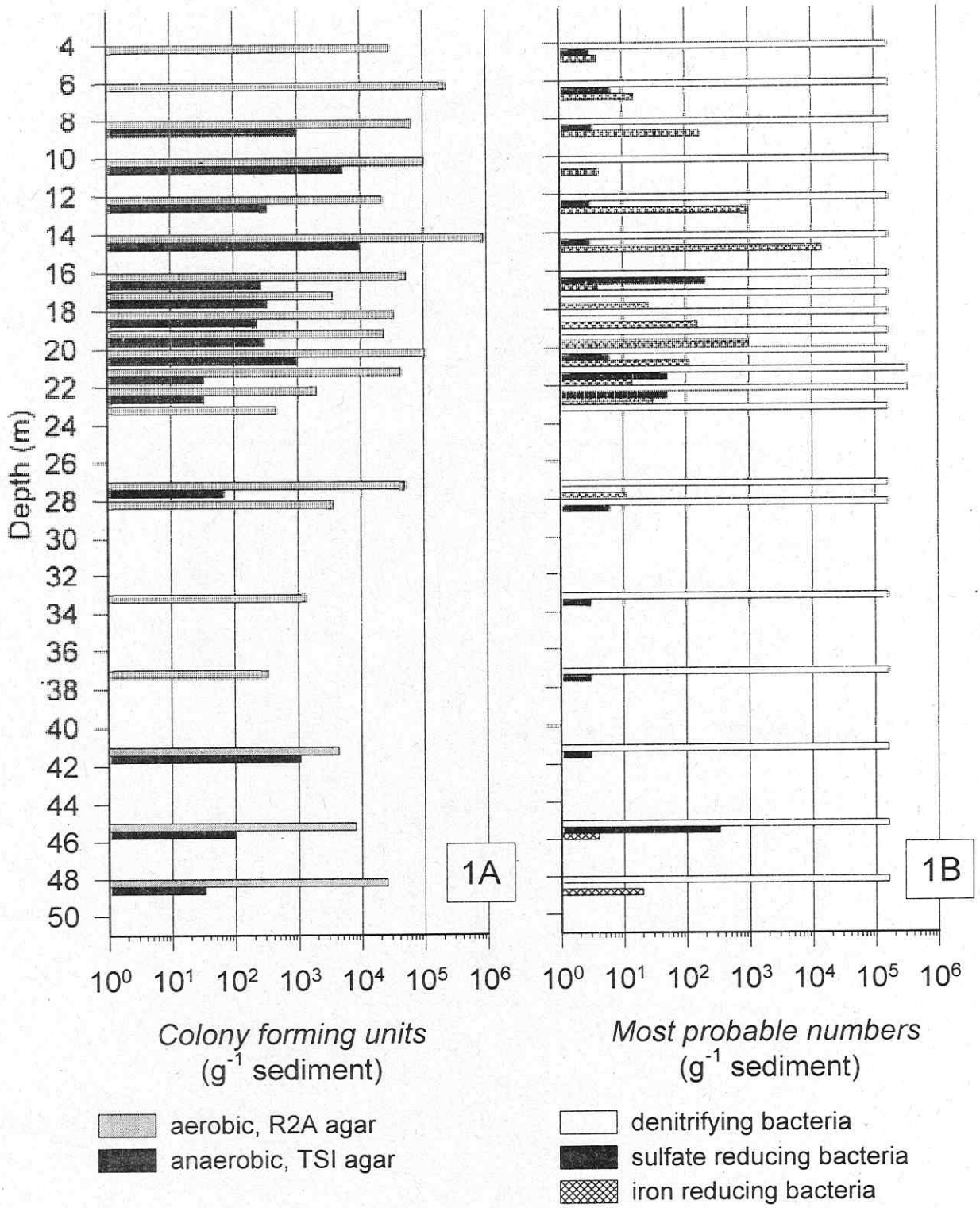

Fig. 1. Abundance of selected groups of bacteria in quaternary and tertiary sediments of a contaminated site (Bitterfeld, Germany)

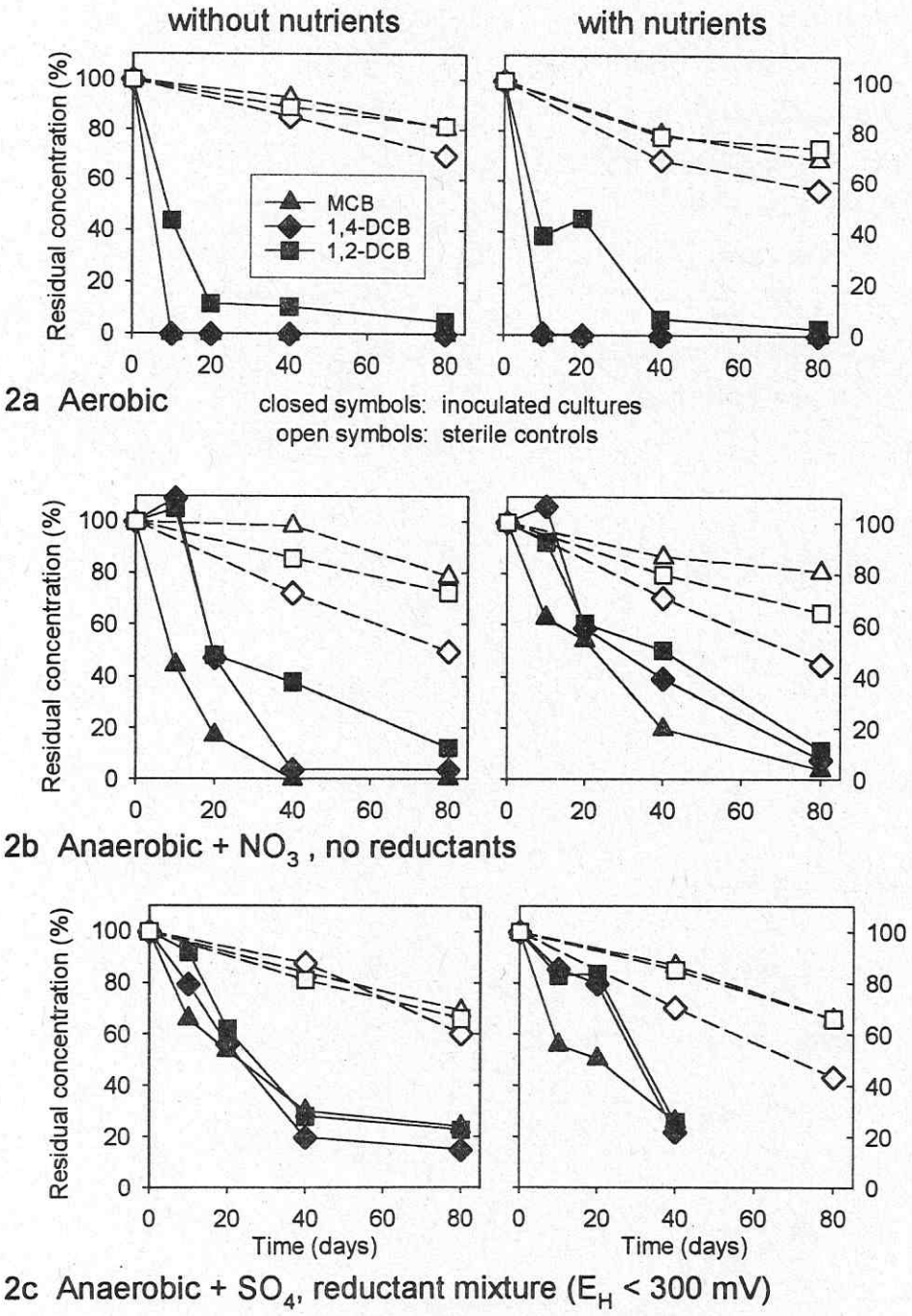

Fig. 2a-c. Simultaneous degradation of mono- and dichlorobenzenes under different culture conditions in groundwater medium at 16°C. All points represent the average of 2 - 3 replicate cultures (5 ml). For legends see figure 2a

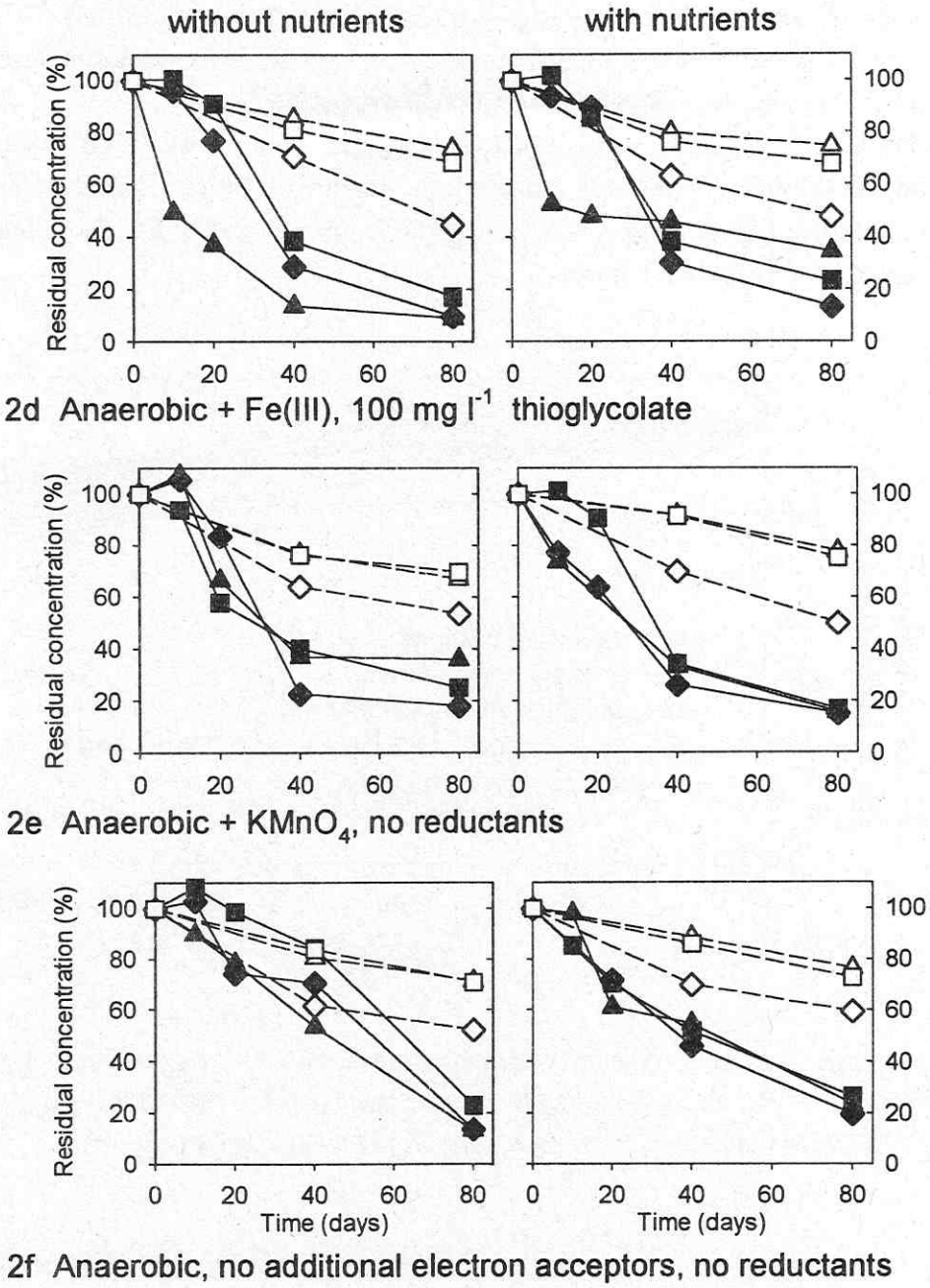

Fig. 2d-f. Simultaneous degradation of mono- and dichlorobenzenes under different culture conditions in groundwater medium at 16°C. All points represent the average of 2 - 3 replicate cultures (5 ml). For legends see figure 2a

Under nitrate-, sulfate-, manganese- and iron- reducing conditions (Figures 2b - e) the 3 chlorobenzenes under investigation disappeared to at least 60 % after 40 days of incubation,

when no other nutrients were added. MCB and 1,4-DCB were degraded in most cases (with the exception of sulfate reducing conditions) at a higher rate than 1,2-DCB. There was no evidence for a long-term persistence of 1,4-DCB which was found by Barber [14]. If no electron acceptors and reductant were added to anaerobic cultures (Figure 2f) the degradation was slowest of all, possibly indicating unsuitable redox conditions for sulfate reduction (sulfate concentrations in the aquifer range from 600 - 1000 mg per liter).

Degradation of TCE by autochthonous bacteria was investigated under identical experimental conditions. It was not modified or degraded neither under aerobic nor any anaerobic conditions. Figure 3 shows data for aerobic, nitrate- and sulfate reducing conditions which are representative for all cultures.

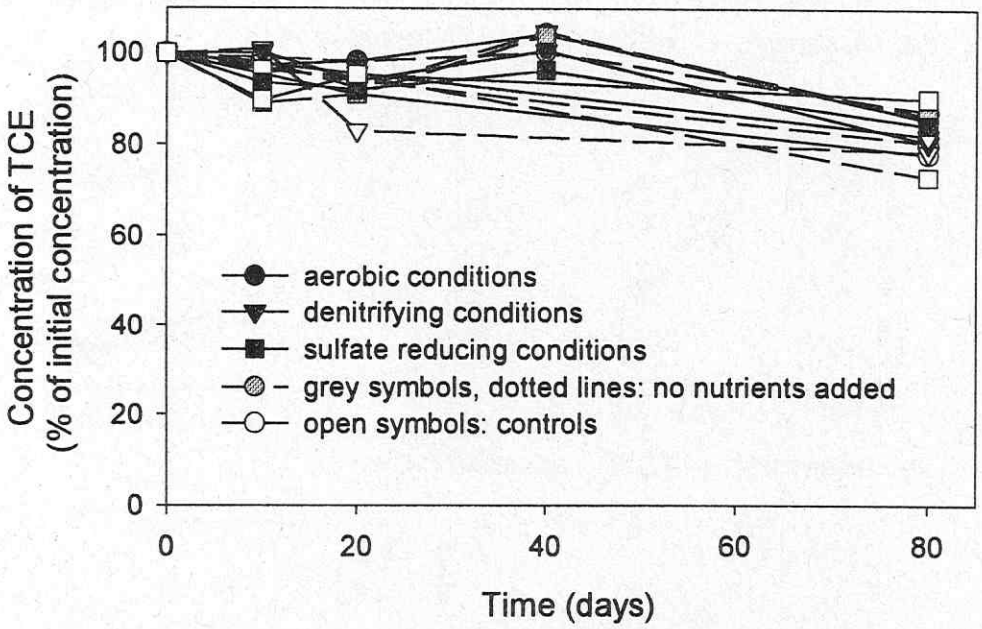

Fig. 3. Persistence of trichloroethene (TCE) under different culture conditions in groundwater medium at 16°C. Each point represents the mean of 2-3 replicate cultures (headspace GC vials with 5 ml medium); sterile controls are single determinations.

The results of this study show that the autochthonous groups of anaerobic/facultatively anaerobic bacteria of this contaminated anoxic aquifer have the potential to degrade the main chloroaromatic contaminants under anaerobic conditions. Identification of metabolites and final products is in progress. In contrast to chlorobenzenes TCE persists under all conditions tested which might indicate *in situ* persistence. This problem needs further attention including the evaluation of methanogenic conditions.

Acknowledgments

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References

[1] Reineke, W., Knackmuss, H.-J. (1988) Microbial degradation of haloaromatics. Ann. Rev. Microbiol. 42, 263-287

[2] Chaudry, G.R., Chapalamadugu, S. (1991) Biodegradation of halogenated organic compounds. Microbiol. Rev. 55, 59-79

[3] Hardmann, D.J. (1991) Biotransformation of halogenated compounds. Crit. rev. Biotechnol. 11, 1-40

[4] Fathepure, B.Z., Tiedje, J.M., Boyd, S.A. (1988) Reductive dechlorination of hexachlorobenzene to tri- and dichlorobenzenes in aerobic sewage sludge. Appl. Environ. Microbiol. 54, 327-330

[5] Masunaga, S., Susarla, S., Yonezawa, Y. (1996) Dechlorination of chlorobenzenes in anaerobic estuarine sediment. Water Sci. Technol. 33, 173-180

[6] Häggblom, M.M., Rivera, M.D., Young, L.Y. (1993) Influence of alternative electron acceptors on the anaerobic biodegradability of chlorinated phenols and benzoic acids. Appl. Environ. Microbiol. 59 1162-1167

[7] Kazumi, J., Häggblom, M.M., Young, L.Y. (1995) Degradation of monochlorinated and nonchlorinated aromatic compounds under iron-reducing conditions. Appl. Environ. Microbiol. 61, 4069-4073

[8] Williams, R.A., Shuttle, K.A., Kunkler, J.L., Madson, E.L., Hooper, S.W. (1997) Intrinsic bioremediation in a solvent-contaminated alluvial groundwater. J. Ind. Microbiol. 18, 177-188

[9] Alef, K. (1991) Methodenhandbuch Bodenmikrobiologie. Ecomed Landsberg/Lech, Germany

[10] Ottow, J.C.G., Glathe, H. (1970) Isolation and identification of iron-reducing bacteria from gley soils. Soil Biol. Biochem. 3, 43-55

[11] Postgate, J.R. (1963) Versatile medium for enumeration of sulfate-reducing bacteria. Appl. Environ. Microbiol. 11, 265-267

[12] Lovley, D.R., Phillips, E.J.P. (1986) Organic matter mineralisation with reduction of ferric iron in anaerobic sediments. Appl. Environ. Microbiol. 51, 683-689

[13] Feidieker, D., Kämpfer, P., Dott, W. (1994) Microbiological and chemical evaluation of a site contaminated with chlorinated aromatic compounds and hexachlorocyclohexanes. FEMS Microbiol. Ecol. 15, 265-278

[14] Barber, L.B. (1988) Dichlorobenzene in groundwater: Evidence for long-term persistence. Groundwater 26, 696-702

Anaerobic bacteria from sediments dechlorinate dioxin congeners

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Abstract

An enrichment culture from Saale river sediment dechlorinated 1,2,3,4-tetrachloro-dibenzo-p-dioxin via 1,2,4-trichloro- (1,2,4-TrCDD) to 1,3-dichlorodibenzo-p-dioxin (1,3-DCDD) and traces of 2-chlorodibenzo-p-dioxin (2-MCDD). This enrichment culture was further studied using 1,2,4-TrCDD as model compound. The dechlorination activity was completely inhibited by pasteurization. Suppression of methanogenesis with 5 mM 2-bromoethanesulfonic acid and addition of 1 mM molybdate exhibited no influence on the dechlorination rate. Dechlorination was positively influenced by fumarate or yeast extract (0.1% w/v). A vibroid, motile, gram-negative bacterium able to dechlorinate 1,2,4-TrCDD was isolated from agar dilution series. Hydrogen and formate (in the presence of acetate as carbon source) supported growth with thiosulfate. The isolate grew fermentatively with fumarate and malate.

1. Introduction

Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) became one of the most known environmental contaminants since the accident in a herbicide producing plant in Seveso in 1976. They are ubiquitous environmental contaminants emitted into the environment as unwanted by-products of anthropogenic processes like the production of herbicides and pentachlorophenol (PCP), bleach processes in the pulp and paper industry, metal smelting and the incineration of municipal and industrial waste [1]. In vitro experiments with chlorophenols and hydrogen peroxide in the presence of peroxidases gave first evidence that PCDD/Fs were biochemically formed from chlorophenol precursors [2]. The production of PCDD/Fs during composting and wastewater treatment has also been reported [3, 4]. The de novo synthesis of chloroaromatics as putative precursors for the biogenic formation of dioxins was reported for common wood- and forest litter-degrading fungi [5]. They produced chlorinated anisyl metabolites up to 61 mg kg (dry weight) of woody material-1. Several ligninolytic basidomycetes were capable to form tetrachloro-1,4-dimethoxybenzene and tetrachloro-4-methoxyphenol [6]. The natural occurrence of dioxins makes it likely, that biological processes of their destruction or transformation have been developed during evolution.

Microbial reductive dechlorination of high-chlorinated organics under anaerobic conditions provides low-chlorinated compounds more susceptible to mineralization by

anaerobic or aerobic bacteria and, in most cases, possessing a lower toxicity than the parent compounds. Recently, laboratory studies have demonstrated, that PCDDs were reductively dechlorinated by sediment and aquifer microorganisms under anaerobic conditions [7-11]. Reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-p-dioxin (1,2,3,4-TCDD) to 1,3-dichlorodibenzo-p-dioxin (1,3-DCDD) as main product and to minor amounts of the 1,2,4- and 1,2,3-trichlorodibenzo-p-dioxins (TrCDD) was observed in enrichment cultures inoculated with sediment of the river Saale as depicted in Figure 1. 1,2,4-TrCDD was identified as main intermediate of the dechlorination route according to thermodynamic predictions of Huang et al. [12]. The dechlorination of 1,2,4-TrCDD to 1,3-DCDD and traces of 2-MCDD was an order of magnitude faster compared with 1,2,3,4-TCDD. Therefore, 1,2,4-TrCDD was a suitable model compound for the investigation of the dehalogenating bacteria.

The objective of this study was the further enrichment of the 1,2,3,4-TCDD-dechlorinating bacteria. The physiological characterization of the mixed culture and preliminary results obtained with a dioxin reducing pure culture are presented.

2. Methods

2.1. Growth conditions

Bacteria were usually grown at 20°C (except for the temperature optimum experiment) in a low-sulfate minimal medium prepared and handled as described previously [11] and essentially consisted of mineral salts, trace elements, 0.005% (w/v) yeast extract (YE), vitamins, resazurin, bicarbonate-phosphate buffer (pH 7.0) and was pre-reduced with 2 mM Na2S. For single colony isolation the medium was solidified with 0.8 % (w/v) agar. 1,2,4-TrCDD was added to autoclaved empty vessels in different aliquots from a 17.2 µg ml-1 filter-sterilized stock solution in acetone to obtain the desired final concentrations. The acetone was vaporized under a gentle stream of sterile N2 / CO2-(80 / 20) gas mixture as described previously [11]. To test the utilization of electron donors in batch-experiments with 1,2,4-TrCDD as electron acceptor, the medium was supplemented from sterile stock solutions of lactate, acetate, pyruvate, formate, benzoate or fumarate (to give 20 mM), respectively, or with a mixture of ß-hydroxybutyrate, acetate, butyrate, propionate, ethanol, iso-valerate, valerate and iso-butyrate (3 mM each). Additionally, one vessel with basal medium was flushed with hydrogen, one with H2/CO2 (80 / 20), and one was supplemented with 1% (w/v) YE. The control did not receive any supplements. For experiments with alternative electron acceptors in addition to 1,2,4-TrCDD, an electron donor mixture of acetate, benzoate (5 mM each) and lactate 10 mM was used. Sulfate, nitrate, sulfite or thiosulfate were added at 5 mM concentrations. To examine the influence of different microbiological groups on the dechlorination, the inhibitors of methanogenesis and sulfate reduction 2-bromoethanesulfonic acid (BrES) and molybdate were supplied in a concentration of 5 or 1 mM, respectively, together with an electron donor mixture of pyruvate, acetate, fumarate and benzoate (5 mM each). Inhibition and most probable number experiments were carried out by using replicate cultures (volume of 3-10 ml in Hungate tubes), which were sacrificed in triplicate at the times

indicated and stored at -20°C. Samples (1 ml) were taken from the batch cultures with sterile syringes in duplicate and stored at -20°C until analysis. Rich media like BBL nutrient broth (Becton Dickinson, Heidelberg), M17 broth (Difco Lab., USA) and M.R.S. (Oxoid, Basigstoke, UK) were used for some growth experiments.

2.2. Analytical procedures

The clean-up procedure for the analyses of chlorinated dioxins and the measurements by capillary GC-ECD were done as described previously [11]. Organic acids were analyzed by the method according to [13] on a FFAP-capillary column (25 m x 0.25 mm i. d., film thickness 0.25 µm), methane and hydrogen concentration in headspace samples were determined by gas chromatography [11]. Formate and sulfide concentrations were determined colorimetrically using an assay according to [14] and [15], respectively. Cell protein was measured [16] with bovine serum albumin as the standard.

3. Results and Discussion

3.1. Composition of the enrichment as determined by the most probable number (MPN) method

Cells from the second transfer of the dioxin dechlorinating consortium served as inoculum for a dilution series up to 10-6 in five replicates to enumerate the electron donor-consuming bacteria and dioxin reducers, respectively. A mixture of lactate, acetate, benzoate and fumarate served as potential electron donor. After statistical evaluation the portion of 1,3-DCDD producing and benzoate utilizing bacteria were 5,000 and 1,300 cells ml-1, respectively. High numbers of fumarate and lactate consuming bacteria (>9 105 cells ml-1) were found. Succinate was formed in almost the same tubes, where fumarate was consumed (according to 1.3 105 cells ml-1). Methane production occurred until the highest dilution according to the low amounts of free hydrogen measured. Low numbers of acetate consumers (1,3 102 cells ml-1) and only traces of propionate and ethanol as fermentation products were indicated.

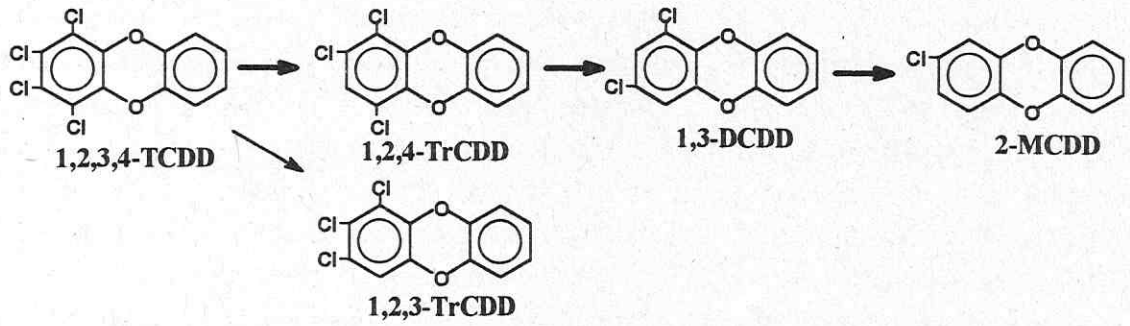

Fig. 1. Proposed pathway of the dechlorination of 1,2,3,4-TCDD by the anaerobic consortium from Saale river sediment.

3.2. Temperature dependence and influence of 1,2,4-TrCDD concentration

Dechlorination activity was demonstrated at temperatures between 10°C and 30°C with an optimum at 20°C by comparing the amount of 1,3-DCDD formed from 10 µM 1,2,4-TrCDD using parallel subcultures grown at different incubation temperatures. 20 °C was the incubation temperature of the primary enrichment. No dechlorination activity occurred at 37°C. Another property of the mixed culture was the increasing dechlorination rate with higher initial concentrations of 1,2,4-TrCDD. The dechlorination rate was the highest (5.4 µM d^{-1}) with 250 µM as the highest initial concentration tested. One reason might be the larger total surface of the 1,2,4-TrCDD crystals supplied, which probably enhances the bioavailability, as was also demonstrated for the degradation of phenanthrene [e.g. 17].

3.3. Participation of methanogens, sulfate reducers and sporeforming bacteria in the dechlorination process

To study the influence of methanogens on the dechlorination process, bromoethanesulfonic acid (BrES) was added (Figure 2A). BrES completely inhibited methanogenesis, whereas dechlorination was not affected. This was in accordance with several studies on the dechlorination of chlorobenzenes or polychlorinated biphenyls [18, 19], where obviously methanogens were not involved. Further enrichments were therefore done in the presence of BrES. Involvement of spore-forming bacteria in dechlorination was examined after heat treatment of the inocula at different temperatures (75°C; 80°C; 85°C) for 15 minutes. Dechlorination was not maintained (Figure 2B). Thus, non-sporulating bacteria seem to be essential for dechlorination. Molybdate is known to inhibit sulfate reducing bacteria. In the enrichment culture, molybdate did not significantly influence dechlorination (Figure 2B).

3.4. Effect of different electron donors and alternative acceptors on the dechlorination of 1,2,4-TrCDD

Comparing the dechlorination of 1,2,4-TrCDD in the presence of different electron donors, fumarate and 0.1% (w/v) yeast extract were found to be most suitable for dechlorination. Acetate and lactate seemed to inhibit the dechlorination. The presence of alternative acceptors such as sulfate, thiosulfate, sulfite, nitrate or < 5% oxygen resulted in inhibition of dechlorination and utilization of the alternative acceptors. One could speculate, that the non-dechlorinating members of the consortium consumed the electron donors added (acetate, lactate, benzoate) by reduction of the different acceptors. The relatively slow process of dechlorination might be inhibited by the lack of electron donors. In contrast, addition of fumarate positively influenced the dechlorination.

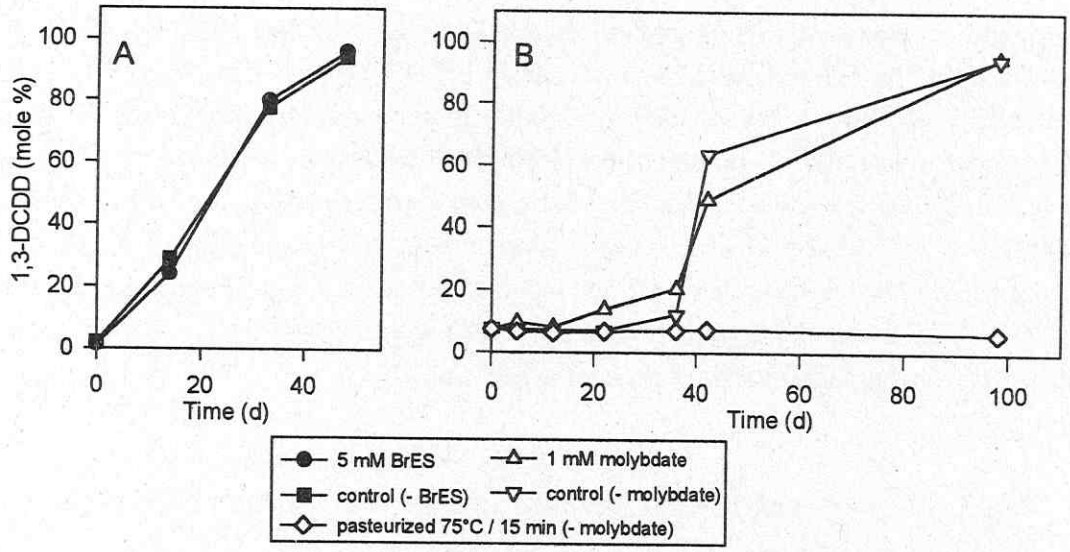

Fig. 2. Effects of 5 mM BrES (A), heat treatment (B) and 1 mM molybdate (B) on the reductive dechlorination of 50 µM 1,2,4-TrCDD by a mixed culture enriched from Saale river sediment.

3.5. Isolation and characterization of a pure culture

Dilution series solidified with 0.8% (w/v) agar and supplemented with sterile 1,2,4-TrCDD crystals (50 µM), 0.1% YE, 5 mM BrES, fumarate and pyruvate (10 mM each) were inoculated with an actively 1,2,4-TrCDD-dechlorinating enrichment. After two weeks of incubation, 21 different colonies were picked from the 10^{-4} to 10^{-6} dilutions and incubated in 3 ml batch cultures ("mini-cultures") containing 10 µM 1,2,4-TrCDD. After 8 weeks 500 µL were transferred as inoculum into fresh media. The rest was sacrificed for analyzes as described before [11]. Five of the 21 colonies tested produced more than 10 mol percent 1,3-DCDD from 1,2,4-TrCDD. Screening of the positive mini-cultures by means of phase contrast microscopy and fluorescence microscopy (after acridine orange and DAPI staining, respectively) revealed, that culture EK7 was phenotypically uniform. Only this strain was studied in detail.

Cells of the isolate were vibroid, highly motile, approximately 0.2 - 0.4 wide and 1-2 µm long. The cells stained gramnegative. The isolate was checked for purity on media containing 0.1% (w/v) YE. Slow growth occurred (OD_{660} 0.3 after 7 days). EK7 did not grow on rich media like BBL nutrient broth, M17 broth and M.R.S. Pale white colonies developed on the surface of mineral agar supplied with fumarate and pyruvate (10 mM each). The colonies appeared light yellow below the agar surface.

A number of electron acceptors (10 mM each) was tested in batch cultures in the presence of the electron donors formate plus acetate, butyrate, fumarate and lactate, respectively. Growth, utilization of the donor and reduction of NO_3^- to NO_2^-, SO_4^{2-}, SO_3^{2-}, or $S_2O_3^{2-}$ to H_2S and fumarate to succinate, respectively, were monitored. Growth and reduction of the

acceptor were obsered with formate plus acetate combined with O_2, NO_3^- and thiosulfate. Cells grew also with fumarate, thereby reducing NO_3^- and O_2. Therefore, fumarate seemed to serve as electron donor.

A broader range of different electron donors such as sugars, amino acids, alcohols and organic acids was studied in combination with thiosulfate. Only formate or hydrogen (each with acetate as carbon source) supported the reduction of thiosulfate to sulfide. Fermentative growth occurred in the presence of fumarate (20mM), which was converted to succinate (13.57 mM) and acetate (5.62 mM) and on malate (20mM), which was metabolized via fumarate to succinate and acetate in the same ratio. The properties observed were similar to those reported for some members of the ε-subclass of the *Proteobacteria* [20].

Acknowledgments

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References

[1] Hagenmaier, H., Lindig, C. and She, J. (1994) Correlation of environmental occurrence of polychlorinated dibenzo-*p*-dioxins and dibenzofurans with possible sources. Chemosphere 29, 2163-2174

[2] Öberg, L.G. and Rappe, C. (1992) Biochemical formation of PCDD/Fs from chlorophenols. Chemosphere 25, 49-52

[3] Krauß, T., Krauß, P. and Hagenmaier, H. (1994) Formation of PCDD/PCDF during composting? Chemosphere 28, 155-158

[4] Schramm, K.W., Klimm, C., Henkelmann, K. and Kettrup, A. (1996) Formation of octa- and heptachlorodibenzo-*p*-dioxins during semi anaerobic digestion of sewage sludge. Organohalogen Compounds 27, 84-87

[5] De Jong, E., Field, J.A., Spinnler, H.E., Wijnberg, J.B.P.A. and De Bont, J.A.M. (1994) Significant biogenesis of chlorinated aromatics by fungi in natural environments. Appl. Environ. Microbiol. 60, 264-270

[6] Teunissen, P.J.M., Swarts, H.J. and Field, J.A. (1997) The de novo production of drosophilin A (tetrachloro-4-methoxyphenol) and drosophilin A methyl ether (tetrachloro-1,4-dimethoxybenzene) by ligninolytic basidiomycetes. Appl. Microbiol. Biotechnol. 47, 695-700

[7] Beurskens, J.E.M., Toussaint, M., De Wolf, J., van der Steen, J.M.D.V., Slot, P.C., Commandeur, L.C.M. and Parsons, J.R. (1995) Dehalogenation of chlorinated dioxins by an anaerobic microbial consortium from sediment. Environ. Toxicol. Chem. 14, 939-943

[8] Adriaens, P. and Grbic-Galic, D. (1994) Reductive dechlorination of PCDD/F by anaerobic cultures and sediments. Chemosphere 29, 2253-2259

[9] Adriaens, P., Fu, Q. and Grbic-Galic, D. (1995) Bioavailability and transformation of highly chlorinated dibenzo-*p*-dioxins and dibenzofurans in anaerobic soils and sediments. Environ. Sci. Technol. 29, 2252-2260

[10] Barkovskii, A.L. and Adriaens, P. (1996) Microbial dechlorination of historically present and freshly spiked chlorinated dioxins and diversity of dioxin-dechlorinating populations. Appl. Environ. Microbiol. 62, 4556-4562

[11] Ballerstedt, H., Kraus, A. and Lechner, U. (1997) Reductive dechlorination of 1,2,3,4-tetrachlorodibenzo-*p*-dioxin and its products by anaerobic mixed cultures from Saale river sediment. Environ. Sci. Technol. 31, 1749-1753

[12] Huang, C.-L., Harrison, B.K., Madura, J. and Dolfing, J. (1996) Gibbs free energies of formation of PCDDs: Evaluation of estimation methods and application for predicting dehalogenation pathways. Environ. Toxicol. Chem. 15, 824-836

[13] Nanninga, H.J. and Gottschal, J.C. (1985) Amino acid fermentation and hydrogen transfer in mixed cultures. FEMS Microbiol. Ecol. 31, 261-269

[14] Lang, E. and Lang, H. (1972) Spezifische Farbreaktion zum direkten Nachweis der Ameisensäure. Z. Anal. Chem. 260, 8-10

[15] Trüper, H.G. and Schlegel, H.G. (1964) Sulfur metabolism in *Thiorhodaceae*. 1. Quantitative measurements on growing cells of *Chromatium okenii*. Antonie van Leeuwenhoek 30, 225-238

[16] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurements with Folin phenol reagent. J. Biol. Chem. 193, 265-275

[17] Köhler, A., Schüttoff, M., Bryniok, D. and Knackmuß, H-J. (1994) Enhanced biodegradation of phenanthrene in a biphasic culture system. Biodegradation 5, 93-103

[18] Holliger, C., Schraa, G., Stams, A.J.M. and Zehnder, A.J.B. (1992) Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. 58, 1636-1644

[19] Rhee, G.Y., Bush, B., Bethoney, C.M., Denucci, A., Oh, H.M. and Sokol, R.C. (1993) Anaerobic dechlorination of Aroclor 1242 as affected by some environmental conditions. Environ. Toxicol. Chem. 12, 1033-1039

[20] Schumacher, W., Kroneck, P.M.H. and Pfennig, N. (1992) Comparative systematic study on "Spirillum" 5175, *Campylobacter* and *Wolinella* species. Arch. Microbiol. 158, 287-293

- Mining wastewaters -

Removal of aluminium from mining water using sulfate-reducing bacteria

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Abstract

The mechanism of biosorption of aluminium by 5 strains of sulfate-reducing bacteria isolated from uranium mine tailings was examined. One strain, designated UFZ B 406 not of mining site origin was also used. The mechanism of aluminium biosorption was found to be a passive one. Freezing and thawing of the cells resulted in higher biosorption of aluminium, whereas heat treatment or the uncoupler carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) showed no effect. The pH-value had significant influence on aluminium ion adsorption, the highest was found at pH 3 and 5, the lowest at pH 7. Aluminium biosorption decreased after the addition of magnesium or the presence of iron sulfide precipitates. The suitability of the isolates for the removal of aluminium from waste water is discussed.

1. Introduction

Acid mine drainage from abandoned uranium mines and dumps poses a significant threat to the environment. As result of bacterial leaching these drainage waters are very acidic with pH values between 1 and 2 and contain high concentrations of metals, particularly iron, uranium, aluminium and magnesium [1]. A microbial decontamination process to increase the pH and to immobilize metals as insoluble sulfides utilizes the ability of sulfate-reducing bacteria to reduce sulfate ions to sulfide ions [2, 3].

Aluminium, known to have toxic effects [4] cannot be removed from the water by precipitation since the sulfides are soluble in water; they have to be immobilized by other methods. As the pH increases through the reduction of sulfate by sulfate-reducing bacteria a large amount of aluminium precipitates as the hydroxide. Depending on the concentration of inorganic (e.g. F^-) or organic (humic acids) complexing agents present high concentrations of aluminium ions (as Al^{3+}) remain in solution. Reports on aluminium accumulation have so far been restricted to *Bacillus* species [5, 6].

In this work we investigated the ability of sulfate-reducing bacteria to remove aluminium from waste water and the mechanism behind it.

2. Materials and methods

2.1. Organism isolation and cultivation

Water samples were taken from sampling wells on a waste dump of a uranium processing plant in Dresden-Gittersee (Germany). For the isolation of sulfate-reducing bacteria a modification of Postgate's medium was used [3]. For biosorption experiments on plates 10 mM aluminium sulfate was added to the medium. Iron sulfate was omitted to exclude any interaction or competition for binding sites between aluminium and iron. The plates were inoculated with 1 ml of cell suspension and incubated for 7 to 10 days at 30°C in anaerobic jars. UFZ B 406 was isolated from the mud of a waste water pond of a disused sugar factory as described previously [2].

2.2. Protein measurements

Protein was determined according to the modified method of Bradford [3].

2.3. Qualitative aluminium biosorption experiments

For a fast qualitative assessment of the ability of the isolates to accumulate aluminium, a modification of the method of Pümpel et al. [7] was used. After the colonies had grown to a size of approximately 1 to 2 mm in diameter the plates were overlaid with a layer of molten agar. After 12 to 24 hours the plates were covered with 3 ml of a 0.1 % (w/v) alizarin red S solution (sodium alizarin sulfonate; Sigma, Germany). 1 % acetic acid (v/v) was added until the original orange red color had disappeared. After a few minutes a bright red aluminium-alizarin complex formed and isolates which have the ability to adsorb aluminium were at this stage seen as purple colonies. After 24 hours light circles were recognizable around the colonies. More alzarin s solution was added to check for possible degradation of the dye by bacteria. As the light circles around the colonies remained unchanged the aluminium had to have been accumulated by the bacterial cells.

2.4. Quantitative aluminium biosorption assay

The aluminium sulfate solution used in all experiments had a concentration of 10 mM and a pH value of 7 unless stated otherwise.

For the direct and indirect assays 1 ml cell suspension samples were incubated for 2.5 hours with 30 ml or 10 ml of 10 mM $Al_2(SO_4)_3$. After incubation cells were harvested by centrifugation at 10 000 *g* for 10 minutes and washed with distilled H_2O. The supernatant and the control were used for indirect biosorption measurements by inductively coupled plasma-atomic emission spectroscopy (ICP-AES).

For direct biosorption assays the pellet was resuspended in 10 M nitric acid (1 ml) and the volume made up to 10 ml. The samples were centrifuged at 10 000 *g* for 10 minutes. Aluminium concentrations in the supernatants were measured on the same day using ICP-AES. All determinations were carried out in triplicate.

2.5. Staining procedures and microscopy

The Gram-stain was carried out using a commercial Gram-staining kit by Merck (Germany). For the volutin staining procedures Neisser-dyes were applied: Solution A: 0.1 g methylene blue, 5 ml glacial acetic acid (100 %), 5 ml ethanol (96 %, v/v), 100 ml dH_2O. Solution B: 10 ml crystal violet (3.3 %, v/v in 96 % ethanol, v/v). Solution C: 0.33 g chrysoidine Y, 100 ml distilled H_2O. The cells were dried on a fatfree microscope slide, heat fixed and covered with a freshly prepared mixture of solution A and B (2:1). After 15 minutes the dye was removed by tilting the slide for the excess dye to run off. Solution C was applied for counterstaining for 45 seconds and then washed off with water. The preparation was air dried and examined under a light microscope (Axioskop, Zeiss, Germany).

3. Results and discussion

3.1. Isolation of strains for biosorption of aluminium

Agar plates were inoculated with sample material and after 10 days of incubation a number of colonies had formed on all plates. Some of the colonies were black indicating the reduction of sulfate to sulfide. From these plates single black colonies were taken, transferred to new plates and incubated with 10 mM aluminium sulfate.

Five isolates were selected for further investigation: Iso 2 was chosen because of its fast growth compared to the other isolates and its ability to eliminate aluminium from the medium. Al-Tol 4 was isolated directly from plates with aluminium and tested positive, however it was found to grow comparatively slowly. Iso 16 as well as Iso 21 were tolerant to aluminium, yet both isolates were unable to accumulate aluminium. Iso 21 formed colonies which were visible after only 3 days. Even though Iso D was not tolerant to aluminium, it showed the highest level of aluminium sorption. It grew very slowly on the original isolation medium described above.

3.2. Measurement of aluminium biosorption

The amount of aluminium which is taken out of the medium by bacterial cells can be determined using two different approaches. The direct method whereby the aluminium which has been fixed by the cells is brought back into liquid phase using a suitable eluent. This however will in most cases destroy the cells. The indirect method measures the concentration of aluminium before and after incubation of the cells and determines fixation through the difference. This method is the one that has been most commonly used in the past for experiments carried out with other metals [8]. There have been no reports so far on a suitable eluent for detaching aluminium from bacterial cell walls or washing it out of cells. EDTA cannot be used as an eluent as it binds aluminium which could influence the determination of aluminium with ICP-AES.

In Figure 1 fixation of aluminium ions by the different isolates is shown. Nitric acid at a concentration of 10 M was found to be useful for the purpose of getting the aluminium off the cells and data of the aluminium measurements were compared to data from the indirect method. The values obtained using the indirect method are slightly higher. This could be due to aluminium adsorption to the walls of the glass, dilution or other experimental errors. For these reasons all further measurements in this study were carried out using the direct method.

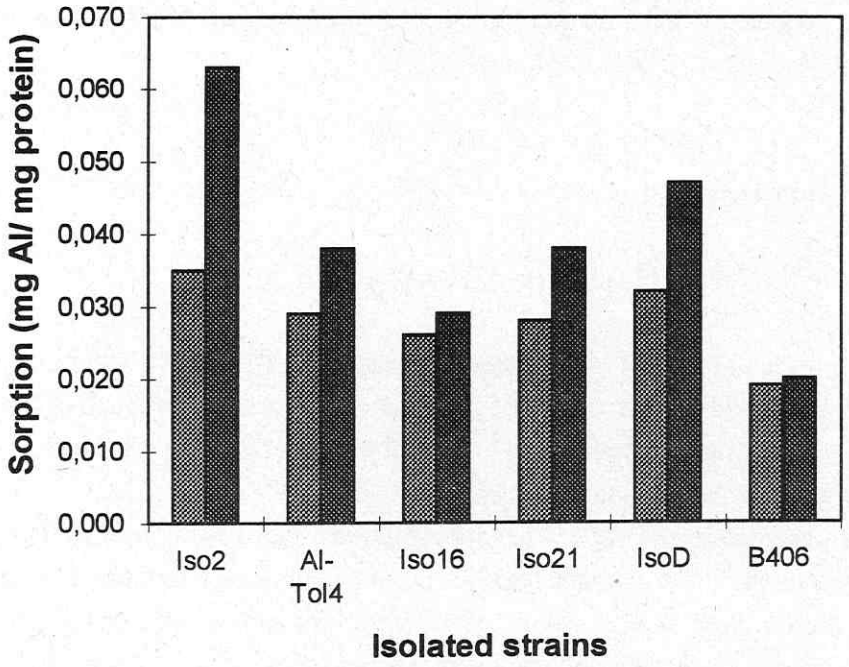

Fig. 1. Comparison of the direct and indirect method for quantifying aluminium biosorption. Data represents mean of 5 batch cultures of each isolate in this and all subsequent experiments unless stated otherwise. ▒ direct method, ▓ indirect method

3.3. Influence of iron sulfide on biosorption of aluminium

The enrichment medium for the selection of sulfate-reducing bacteria contained iron sulfate. During growth of the bacteria it was reduced to iron sulfide which precipitated as black flakes. Figure 2 shows a comparison of elimination of aluminium from the medium by cells grown in the presence and absence of iron sulfide. While there is little difference in the fixation of aluminium by strain Iso 2, more aluminium is fixed by cells of strain UFZ 406 in the absence of iron sulfide. Therefore all subsequent experiments were carried out with cells grown without iron.

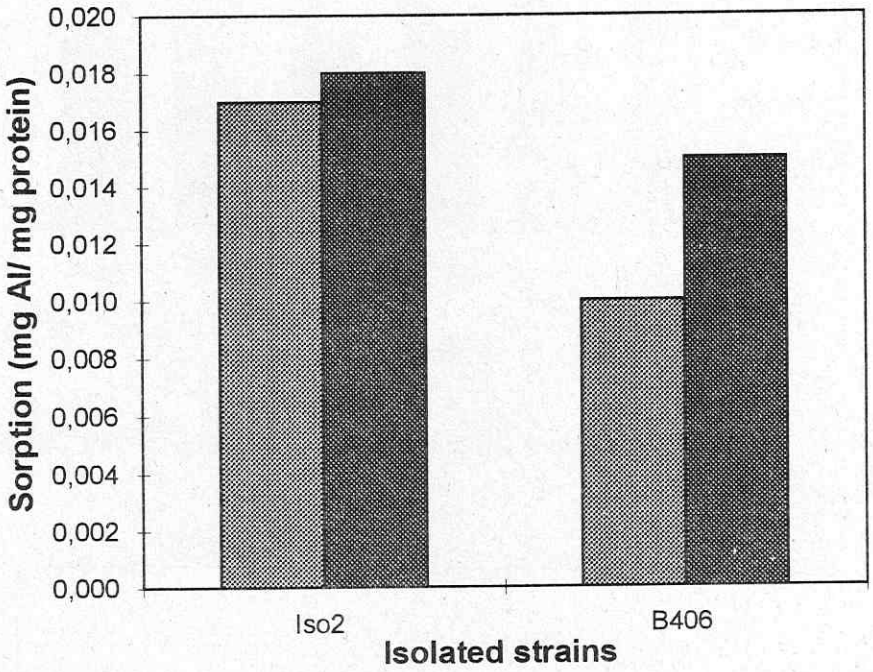

Fig. 2. Influence of iron sulfide on the accumulation of aluminium by strains Iso 2 and UFZ B 406. ▒ medium with iron sulfide, ▓ medium without iron sulfide

3.4. Mechanism of elimination of aluminium

In order to investigate whether the process of elimination is uptake and subsequent accumulation inside the cell or adsorption and accumulation outside the cell, 20 mM lactate as energy source was added to the samples (Figure 3). The values for samples with added lactate were slightly higher than controls. Only Iso 16 showed a greater difference, 0.007 mg aluminium/mg protein.

Incubation of the cells of the strains Iso 2 and UFZ B 406 with the uncoupler carbonyl-cyanide-m-chlorophenylhydrazone, CCCP (50 µM) or heat treatment (98°C, 30 minutes) showed little or no effect compared to the controls (Figure 4). There was no difference in aluminium biosorption between the treated and untreated samples of Iso 2 and little difference (0.002 mg aluminium/mg protein) between control samples of UFZ B 406 and treated ones.

The relationship between biomass concentration and total aluminium fixed was linear, higher protein concentrations resulted in higher biosorption values (data not shown).

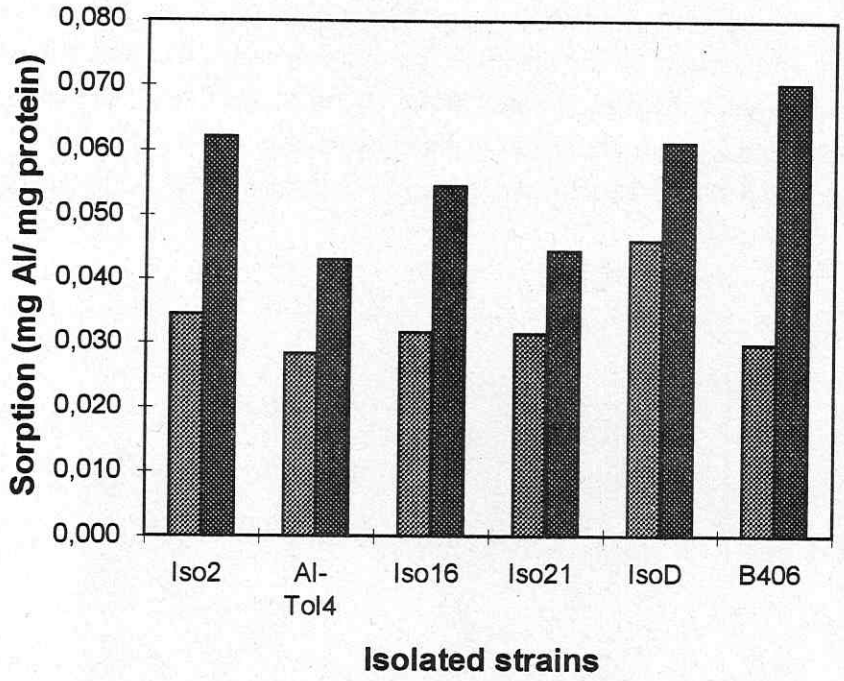

Fig. 3. Aluminium ion fixation with and without additional lactate. ▒ with 20 mM lactate, ■ control

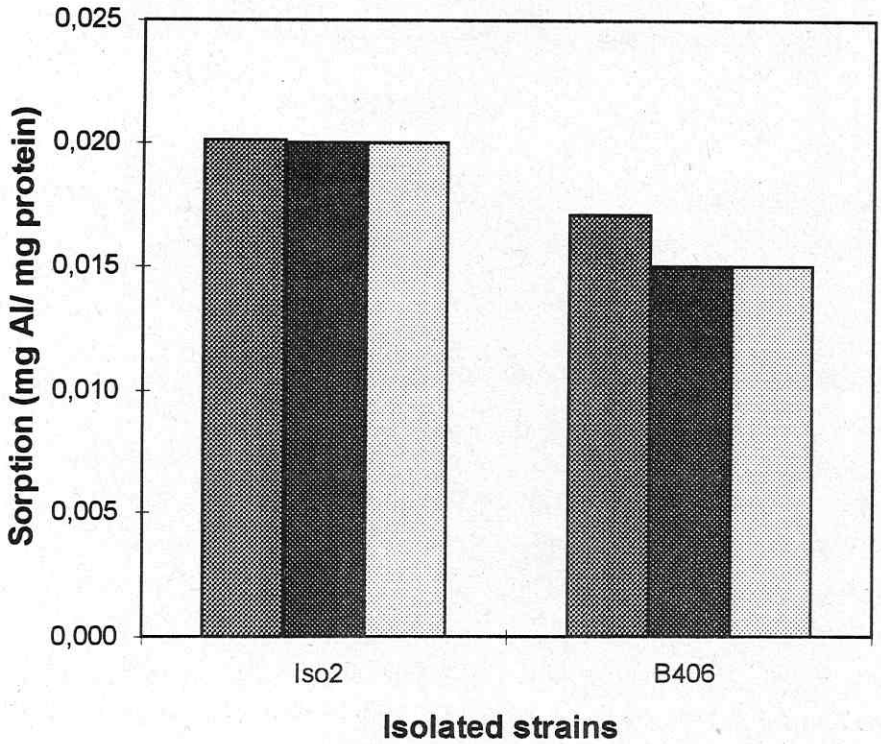

Fig. 4. Influences of CCCP or heat treatment on aluminium ion sorption by strains Iso 2 and UFZ B 406. ▒ 50 mM CCCP, ▒ heat treatment, ■ control

3.5. Influence of freezing and thawing of cells on the removal of aluminium from the medium

Cells of 5 isolates and strain UFZ B 406 were frozen and the effect of freezing and thawing on fixation of aluminium was investigated (Figure 5). More aluminium was bound to treated cells than to untreated cells. There was no correlation between the accumulation of aluminium to living cells or to frozen cells. Strain UFZ B 406 which removes the least in the control experiments showed the highest values with frozen and thawed cells.

Frozen and thawed cells of both strains used accumulated more aluminium than untreated ones. This is possibly due to a breaking of the cells and subsequent availability of additional binding sites. It indicates that process of fixation of aluminium by sulfate-reducing bacteria is a passive one.

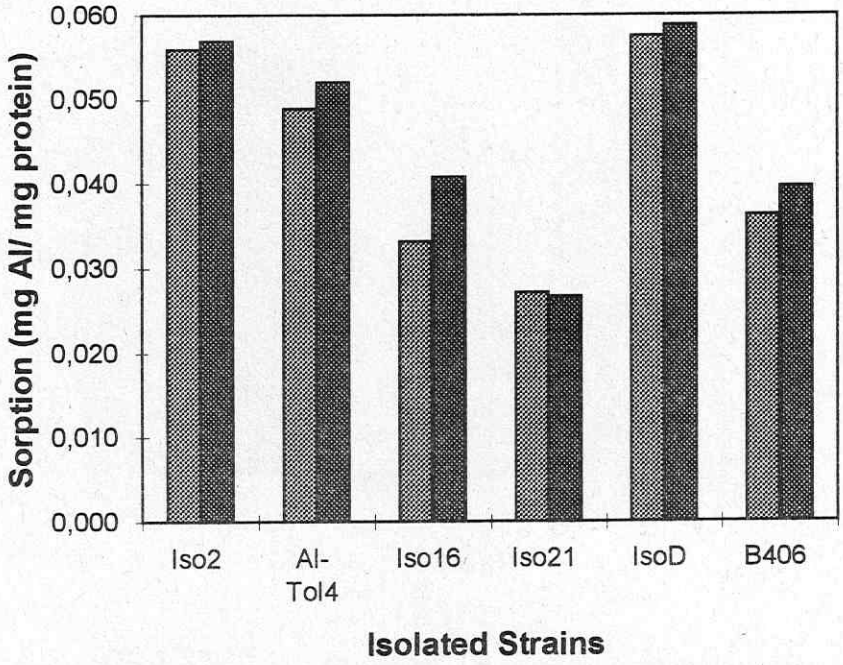

Fig. 5. Effect of freezing and thawing on aluminium accumulation by different isolates ▒ cells frozen and thawn, ▓ control

3.6. Influence of pH on biosorption

The influence of the pH on biosorption of aluminium is shown in Figure 6. Cells of isolates Iso 2 and UFZ B 406 were incubated in 10 mM aluminium sulfate solution at pH 3, pH 5, pH 7 and pH 9. The highest accumulation of aluminium by the cells were found at pH values of 3 and 5, the least at pH 7.

This quite significant influence of the pH on the metal-cell interaction is in agreement with studies by Hughes and Poole [9]. At first sight it seems contradictory that biosorption is lowest

at neutral pH where the cellular complex formers as well as the metal ions are dissociated and highest at lower pH values where the complex forming (acid) groups are increasingly protonated. Lower biosorption at pH 7 could be due to the presence of tartaric acid as soluble complexing agent in addition to the cellular ligands. At neutral pH much of the aluminium is in a stable aluminium-tartrate complex and not free for adsorption to cellular structures. Lowering the pH results in more free Al^{3+}-ions which are now available for binding, and tartaric acid in protonated form does no longer act as complex former. At alkaline pH aluminium forms very stable $Al(OH)_4^-$ complexes which, being anions do not react with anionic cellular ligands.

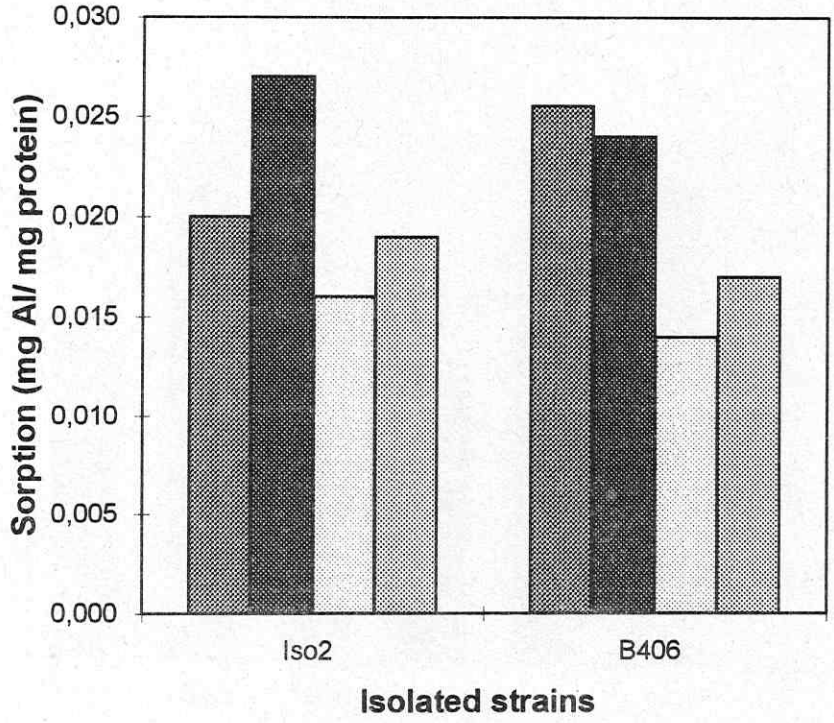

Fig. 6. Effect of pH on aluminium fixation by strains Iso 2 and UFZ B 406.
pH 3, pH 5, □ pH 7, pH 9

3.7. Influence of Mg^{2+}-ions on biosorption

Most waste waters are contaminated with more than one metal species. Competition for binding sites between metals is therefore an important issue in biosorption processes. Acid mine water from uranium mines in eastern Germany contains high concentrations of aluminium (around 90 mM) as well as high concentrations of magnesium (around 130 mM). The influence of different concentrations of magnesium ions on the biosorption of aluminium by strain Iso 2 was investigated. Figure 7 shows the highest accumulation at 10 mM and 50 mM magnesium ions, higher magnesium concentrations in the medium result in lower biosorption of aluminium. The presence of magnesium ions had negative influence on the accumulation of aluminium

even though magnesium and aluminium ions differ in charge and size. However, because both have similar chemical properties, they are hard ions and not readily polarized, there seems to be competition for binding sites at the cell surface. With the aim of removing aluminium from waster water it means that the presence of high concentrations of magnesium has strong influence on the magnitude of biosorption and has to be taken into consideration.

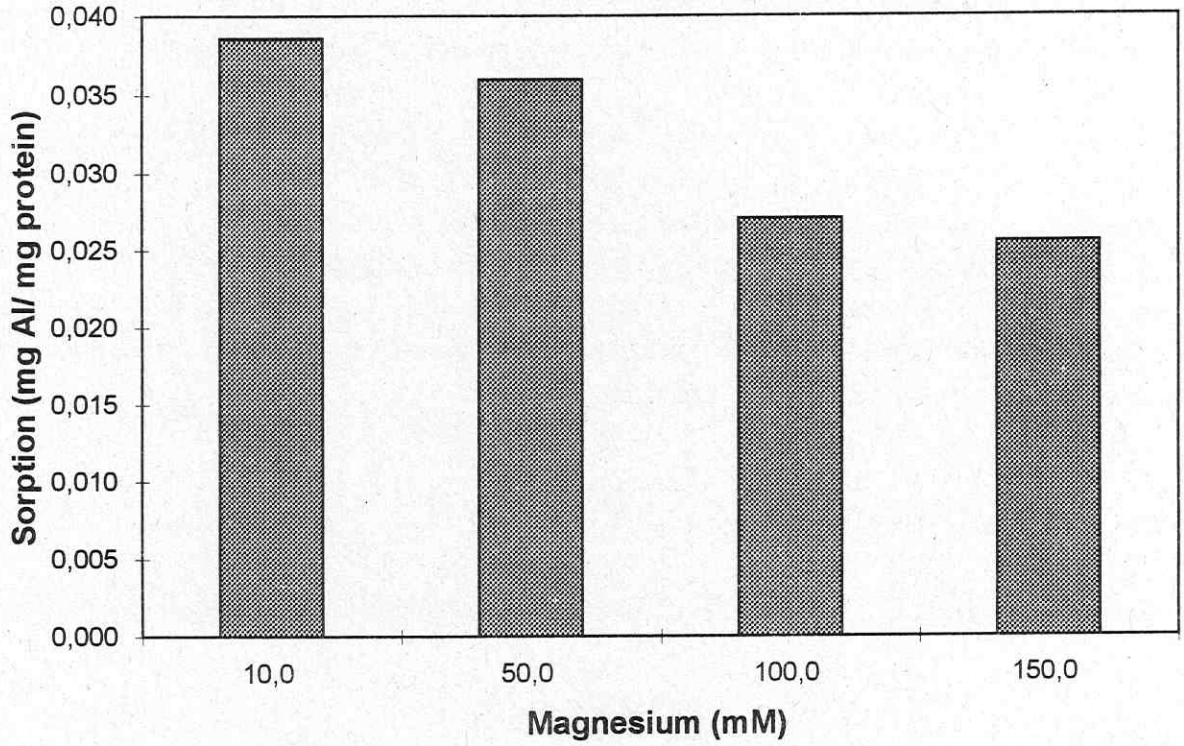

Fig. 7. Influence of different magnesium concentrations in the medium on aluminium ion biosorption by strain Iso 2.

3.8. Staining

Isolate Iso 2 stained Gram-positive. A number of cells with spores in the center were observed. UFZ B 406 stained Gram-negative and spores were not found.

Peptidoglycan is the major site of metal complexation, in particular the carboxylgroups of the glutamate rests [10]. As Iso 2 is a Gram-positive bacterium it is highly probable that the peptidoglycan layer is the main site of biosorption. Peptidoglycan is structurally quite stable and not susceptible to moderate heat treatment. Because of the murein layer which makes up to 50 % of the cell wall dry mass Gram-positive bacteria should passively bind more metal ions than Gram-negative bacteria. This confirms the findings in this study where Iso 2, a Gram-positive bacterium adsorbs more aluminium ions than UFZ B 406 which is Gram-negative.

Volutin could have been another possible site for aluminium accumulation. However, the results of the volutin staining procedures were negative, no polyphosphate granules were detected.

4. Conclusions

Bioremediation of acidic waste water with high concentrations of metals using sulfate-reducing bacteria is a viable option [2, 3, 11]. In the past biosorption of aluminium has not yet been examined.

The results of this study suggest that the process of aluminium biosorption by sulfate-reducing bacteria is a passive one and occurs on the surface of the cells. It is independent of the physiological status of the cells, they can be alive or dead, intact or broken.

Whether elimination of aluminium from waste water by biosorption is a realistic approach remains to be seen. However, using sulfate-reducing bacteria would solve three problems simultaneously: the acidic waters are neutralized, metals are precipitated as sulfides or hydroxides and aluminium is fixed and collected.

Acknowledgments

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References

[1] Leduc LG, Ferroni GD (1994) The chemolithotrophic bacterium *Thiobacillus ferro-oxidans*. FEMS Microbiol Rev 14,103-120

[2] Hard B.C., Friedrich S., Babel W. (1996) Bioremediation of acid mine water using facultatively methylotrophic metal-tolerant sulfate-reducing bacteria. Microbiol. Res. 152, 65-73

[3] Hard B.C., Babel W. (1995) Characterization of a methanol-utilizing sulfate-reducing bacterium isolated from a wastewater pond. J. Basic Microbiol. 35, 385-392

[4] Savory J., Wills M.R. (1991) Aluminium. *In*: Metals and their compounds in the environment (Merian, E., Ed.), pp. 715-741, VCH, Weinheim.

[5] Hu X., Boyer G.L. (1996) Siderophore mediated aluminium uptake by *Bacillus megaterium* ATCC 19213. Appl. Environ. Microbiol. 92, 4044-4048

[6] McLean R.J.C., Beauchemin D., Clapham L., Beveridge T.J. (1990) Metal-binding characteristics of the gamma-glutamyl capsular polymer of *Bacillus licheniformis* ATCC 9945. Appl. Environ. Microbiol .56, 3671-3677

[7] Pümpel T., Pernfuß B., Pigher B., Diels L., Schirner F. (1995) A rapid screening method for the isolation of metal accumulating microorganisms. J. Ind. Microbiol. 14, 213-217

[8] Mullen M.D., Wolf D.C., Ferris F.G., Beveridge T.J., Flemming C.A., Bayley G.W. (1989) Bacterial sorption of heavy metals. Appl. Environ. Microbiol. 55, 3143-3149

[9] Hughes M.N., Poole R.K. (1991) Metal speciation and microbial growth-the hard and soft facts. J. Gen. Microbiol. 137, 725-734

[10] Shumate II S.E., Strandberg G.W. (1985) Accumulation of metals by microbial cells. In: Comprehensive Biotechnology, vol. 4 (Robinson, C.W., Howell, J.A., Eds.), pp. 235-247, Pergamon Press, Oxford.

[11] Wakao N., Takahashi T., Sakurai Y., Shiota H. (1979) A treatment of acid mine water using sulfate-reducing bacteria. J. Ferment. Technol. 57, 445-452

Aquatic hyphomycetes at extremely polluted sites in the Mansfelder Land area

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Abstract

Aquatic hyphomycetes are essential contributors to the decomposition of organic matter in lakes and streams. They are believed to be highly sensitive to pollution. Little is known about the distribution of the fungi in polluted aquatic systems. In the copper shale mining district of Mansfeld (Central Germany) heaps and dumps from mining and smelting are subject to weathering resulting in surface and groundwater with extremely high metal pollution (Cd, Cu, Mn, Pb, Zn). In a survey of four sites in this area, a total of 17 species were found to colonize and sporulate on alder leaves. Even at the most highly polluted site, which also contains high sulfate concentrations, we observed at least five species. *Heliscus lugdunensis, Tetracladium marchalianum* and *Tricladium angulatum* occurred at all locations. The studies are prerequisites for characterizing the fungal potential for the elimination and degradation of xenobiotics in aquatic systems.

1. Introduction

The oldest traces of copper shale mining in the district of Mansfeld in Central Germany date back to the stone age, about 5,000 years ago [1]. Systematic mining and smelting was initiated in the early Middle Ages and continued until 1990 when mining was abandoned for economic reasons. In total, more than 2.2 million tons of copper and 11,000 tons of silver were recovered in the districts of Mansfeld and Sangerhausen. The large-scale smelting of copper shale began developing in the late 19th century at Eisleben (1870) and Helbra (1880). During the final years of production up to 1.4 million tons of ore were processed annually. One ton of polymetallic copper shale yielded 35-38 kg of metals, 720-740 kg of slag, 20 kg of flue dust and 1,300-1,500 m³ of furnace top gas [2]. Most of the precious metals from the copper shale were concentrated in the sulfidic melt (copper stone) and subsequently recovered by

electrolysis. Metals such As, Pb, Se, Sn and Zn volatilized during the smelting process and were emitted as a constituent of the furnace top gas into the atmosphere. These losses were not only economic significant but also gave rise to the extensive pollution of environment [3]. Beginning 20th century, metals such as As, Cd, Pb, Zn, etc. were scrubbed from the furnace top gas, resulting in the formation of a glutinous sludge. After the closure of a major metallurgical plant in 1978, more than 300,000 t of this metalliferous sludge was deposited in basins and ponds on slag heaps and mining waste dumps.

During some early studies hydrological and hydrochemical data of some sites in this area were measured. In addition, the heterotrophic bacterial communities of standing and running waters were characterized [4]. The sites revealed rich bacterial communities, whose ecophysiological properties partly reflected levels of contamination [4, 5].

Aquatic hyphomycetes are an important link between autumn shed leaves and detrivores in many streams. This group, also named Ingoldian Fungi to honor its discoverer Professor C.T. Ingold, today includes some 300 species [6]. Their spores settle on the leaves' surfaces, germinate, and the hyphae invade the tissue. Fungi have been found to account for approximately 97% of microbial biomass living on poplar leaves [7]. Microbial colonization of leaves in streams initiates a series of chemical changes. In the early stages, structural polysaccharides are decomposed by fungi. The resulting subunits (oligosaccharides, disaccharides, monosaccharides) and the microbial biomass 'condition' the leaves, which improves their digestibility to invertebrates [8].

Aquatic hyphomycetes have adapted morphologically and physiologically to aquatic habitats. Their conidia (asexual spores) are characteristically tetraradiate or sigmoid, and generally allow species identification. The term hyphomycetes indicates that they are asexually reproducing fungi (Deuteromycotina, or Fungi imperfecti) whose conidiophores occur singly or aggregated in various ways, but are never enclosed within a covered conidioma [6]. These fungi are most common in clean, well-aerated waters. They are believed to be highly sensitive to pollution, such as acidic coal-mine effluents or organic enrichment [6, 8, 9]. The effect of heavy metals on fungal communities in streams is unknown; in the laboratory, low concentrations of Cd, Cu, and Zn inhibit their growth and reproduction [10, 11, 12], and fungi respond by synthesizing specific stress peptides [12]. In the current study, we surveyed four highly polluted sites in the district of Mansfeld (Germany) (Figure 1). Bags of alder leaves were exposed to allow colonization by fungi. The leaves were subsequently aerated in distilled water, and released conidia were counted and identified. We also isolated several strains of *Heliscus lugdunensis* and tested their sensitivity against heavy metals.

2. Materials and methods

Standard methods were used for water chemistry analysis: Ion chromatography for the determination of nitrate, sulfate, and photometric methods for ammonium and phosphate.

TOC was determined according the German standard for the examination of water, waste water and sludge (DIN 38409 H3-1). X-ray fluorescence (WDXRF) was employed for the determination of the total concentration of heavy metals in solid samples whereas the

maximum acid leachable amount (aqua regia) (German standard methods, DIN 38414 -S7) of the elements were obtained by analysis of the resulting solutions. Applying pneumatic nebulization and multi-element standard solutions inductively coupled plasma atomic emission spectrometry (ICP-AES) was used for the determination of As , Cu, Fe, Mn, Ni, Pb, Sn, Zn in both aqueous and acidic solutions by standard addition technique. Atomic absorption techniques had been applied for the determination of mercury (cold vapor technique), arsenic (hydride generation coupled with graphite furnace atomization), cadmium and lead (both by Zeeman corrected graphite furnace AAS).

To determine fungal colonization, airdried leaves of *Alnus glutinosa* were placed in nylon mesh bags (10 x 10 cm, 5 mm mesh), and exposed at the various sites for 4 weeks. Sporulation from these leaves was characterized as described by Bärlocher [13]. To isolate pure cultures, individual spores were picked up with capillary pipets, and transferred aseptically to 1 % Malt Extract Agar. One strain of *Heliscus lugdunensis* was cultivated and its sensitivity against heavy metals (Cd, Cu, Pb, Zn as chloride salts) was tested according to [10, 12].

3. Results and discussion

Extensive leaching from metalliferous sediments on slag heaps resulted in extraordinarily high levels of heavy metals. Leachates contain up to 2,630 mg L^{-1} of Zn in the water column of site H4 corresponding to 16,660 mg kg^{-1} of Zn in the sediment of this site. Average amounts (from three samples) of Cd, Cu and Pb were given in Table 1.

Table 1. Heavy metals of four sites in the copper shale mining district of Mansfeld (Central Germany); (water-mg L^{-1}, sediment-mg kg^{-1})

	H 3	**H 3**	**H 4**	**H 4**	**H 6**	**H 6**	**H 8**	**H 8**
Heavy metals	water	sediment	water	sediment	water	sediment	water	sediment
As	0.007	**5,000**	0.003	**6,800**	0.001	64.00	0.006	290.00
Cd	0.13	63.00	2.80	29.00	< 0.05	6.00	< 0.05	9.0
Cu	0.27	**10,875**	13.25	**50,224**	< 0.02	737.00	< 0.02	460.00
Fe	0.25	7.77	0.05	2.09	0.17	2.59	0.80	3.60
Mn	2.25	**538.00**	19.08	178.00	0.12	**771.00**	0.37	420.00
Ni	0.09	76.00	2.23	**397.00**	< 0.08	26.00	< 0.08	**720.00**
Pb	0.74	**49,000**	1.90	**24,500**	< 0.1	372.00	< 0.1	720.00
Sn	0	4,500	0	528.00	0	14.00	0	0
Zn	**56.10**	**25,960**	**2,630**	**16,660**	0.427	**2,060**	0.98	**3,100**

At sites H3 and H4 1.2 µg L^{-1} and 2.9 µg L^{-1} PAH, respectivily, were found and showed characteristic profiles comparable with profiles in Theisen sludge (not shown).

Nitrate (63-81 mg L^{-1}) and ammonium (0.03-1.4 mg L^{-1}) levels substantially exceeded levels of unpolluted waters [14]. Very high sulfate were measured at site H3 (1,360 mg L^{-1}) and site H4 (6,750 mg L^{-1}). The phosphate levels of the four sites ranged between 0 and 0.45 mg L^{-1}, and oxygen levels ranged between 70% and 100% saturation; DOC values varied between 9 and 14.5 mg L^{-1}.

At the four selected sites (Figure 1) alder leaves had been exposed for 4 weeks, collected and examined for the presence of fungal reproductive structures. Table 2 lists the taxa observed in each sample, and Figure 2 give the number of conidia produced per unit leaf mass. Overall, 17 species of aquatic hyphomycetes were identified; within a given site, the numbers varied between 5 (site H4) and 14 (site H6) (Table 2). In addition, Oomycota and conidia of *Fusarium* were common (Figure 2).

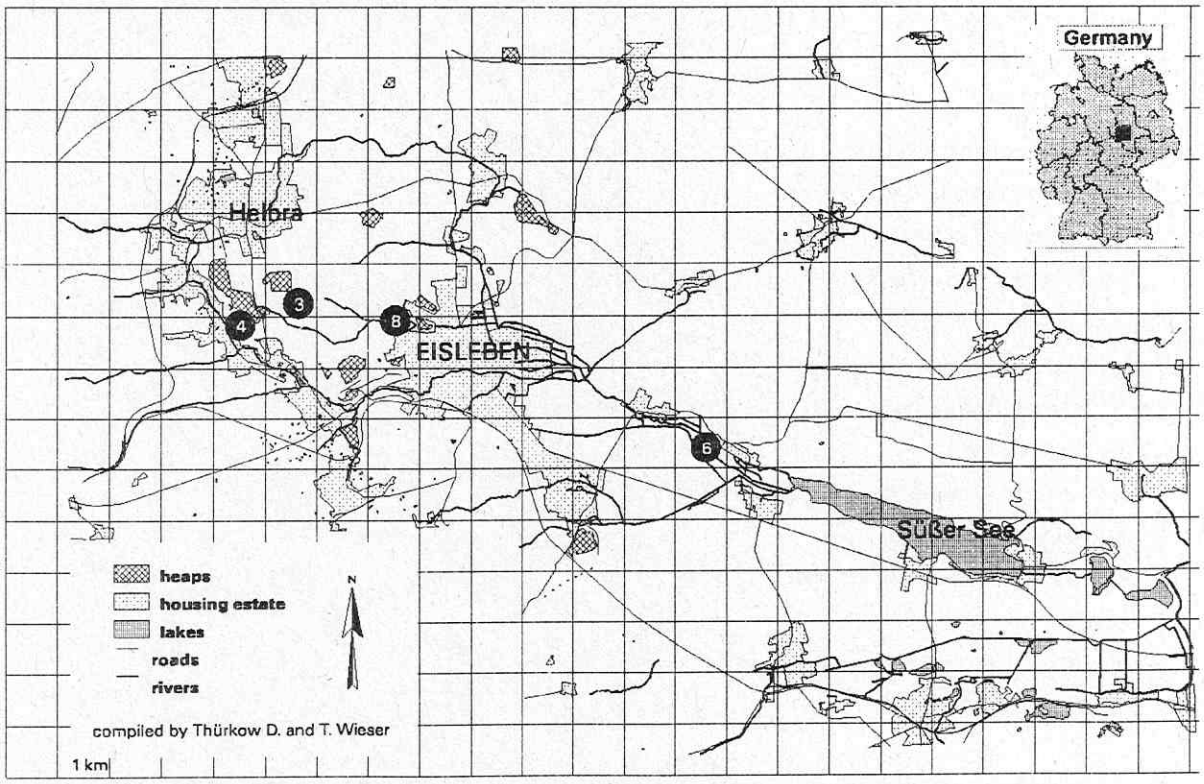


Fig. 1. Map of the sampling sites in the Mansfeld copper shale mining district (Site H3: leachate collecting pond close to former flue gas treatment plant; bottom covered by 20 cm of metalliferous sludge; Site H4: spring in a small valley next to the main slag heap in Helbra; Site H6: brook, main feeder stream of Sweet Lake (Süßer See); Site H8: brook running through base of slag heap)

Heliscus lugdunensis, Tetracladium marchalianum and *Tricladium angulatum* occurred in all locations (Table 2). Strains of *Heliscus* and *Tetracladium* species were shown earlier to be comparatively resistant to Cd [10].

Typically, alder leaves are colonized by 20 - 30 species during their decomposition in streams [8, 15], and there is little doubt that the habitats investigated here have impoverished fungal communities. But considering earlier reports, the damage in the presence of very severe pollution, which results in visible mineral precipitations (Glaukokerinit) with high Zn and Al content [16, 17] on leaves at site 4, seems to have been surprisingly limited.

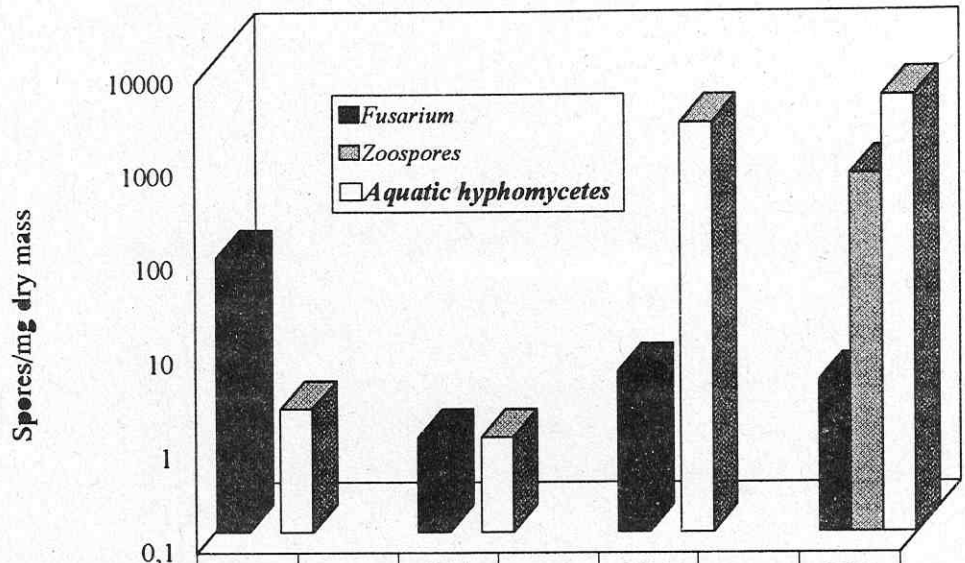
At the most polluted sites H3 and H4 spore production of aquatic hyphomycetes was much reduced (Figure 2). A very high number of zoospores was found at site H8 (Figure 2).

Table 2. Occurrence of aquatic hyphomycete species at four sites in the copper shale mining district of Mansfeld (recovery after 4 weeks field exposure)

	H 3	H 4	H 6	H 8
Anguillospora sp.	*	*	*	
A. filiformis	*			
A. longissima	*		*	*
Articulospora tetracladia			*	
Clavariopsis aquatica			*	
Clavatospora longibrachiata			*	
Culicidospora aquatica			*	
Flagellospora curvula			*	*
Heliscina stellata				*
Heliscus lugdunensis	*	*	*	*
Lambdasporium sp.	*		*	*
Lemnonniera aquatica			*	
Tetrachaetum elegans			*	
Tetracladium marchalianum	*	*	*	*
T. setigerum	*		*	*
Tricladium angulatum	*	*	*	*
Tumularia aquatica		*		
Total Number of Species	**8**	**5**	**14**	**8**

Several *Fusarium sp.* appear to be ubiquitous during leaf decomposition in clean streams; apparently, they also occur in severely polluted waters.

Pure cultures of strains of various fungal species were isolated by us from different polluted sites of the Mansfelder Land area.



	H 3		H 4		H 6		H 8
Fusarium	81		1		5		4
Zoospores	0		0		0		640
Aquatic hyphomycetes	**2**		**1**		**2260**		**4260**

Fig. 2. Spores production from alder leaves after field exposure (4 weeks) at four sites in the highly polluted area of Mansfelder Land (Average of 4 replicates)

A pure culture of a strain of *Heliscus lugdunensis* was isolated from site H 4. At concentration between 50 and 300 µM, the fungi were most sensitive against Cd and least sensitive against Pb (Cd > Zn > Cu > Pb). As recently shown, concentrations above 50 - 100 mg L^{-1} of Cd completely inhibited sporulation in pure culture, and reduced growth by 90 % [10, 12]; effects of similar magnitude were observed in the presence of copper (Figure 3).

Our study has demonstrated, for the first time, that substantial numbers of aquatic hyphomycetes species occur at some extremely polluted sites. Since these fungi are adapted to aquatic habitats, their apparent resistance to extremely high levels of metals and other pollutants makes them promising candidates for bioremediation. We are particularly interested in detailed studies of fungal biosorption of heavy metals and degradation of organic pollutants. In addition, these sites provide interesting communities to study connections between biodiversity and ecological functions in impoverished communities.

Acknowledgments

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References

[1] Eisenhut, K.H. and Kautsch, E. (1954) Handbuch für den Kupferschieferbergbau. Fachbuchverlag Leipzig

[2] Schreck, P. (1997) Environmentally sustainable disposal of radioactive sludge rich in heavy metals from copper shale smelting in Central Germany. *In*: Engineering geology and the environment (Marinos, P. G., Koukis, G. C., Tsiambaos, G. C., Stournaras, G. C. Eds) 2, pp. 2145-2149, Balkema, Rotterdam, Brookfield

[3] Fuchsloch, N. (1996) Rauchschäden im Mansfelder Land. In: Bergbau- und Umweltgeschichte in Mitteldeutschland (Meinicke, K. P. and Ebersbach, W. Eds.) Proceedings of a colloquium, Universitätsverlag Halle

[4] Krauss, G., Baerlocher, F., Schreck, P., Dermietzel, J., Krauss, G.J., Glaesser, W. (1997) Assessing ecotoxic levels of pollutants on microbial communities in leachates from metalliferous deposits. p. 163. Proceedings Seventh Annual Meeting of SETAC- Europe, Amsterdam

[5] Krauss, G., Baerlocher, F., Schreck, P., Dermietzel, J., Wennrich R., Glässer, W., Krauss, G.J. (1998) Aquatic hyphomycetes occur in hyperpolluted waters in Central Germany. FEMS Microb. Ecol. (submitted for publication))

[6] Dix, N.J. and Webster, J. (1995) Aquatic fungi. In: Fungal ecology. pp. 225-265, Chapman & Hall, London

[7] Weyers, H., S. and Suberkropp, K. (1996) Fungal and bacterial production during the breakdown of yellow poplar leaves in 2 streams. J. N. Benthol. Soc. 4, 408-420

[8] Bärlocher, F. (Ed.). (1992) The ecology of aquatic hyphomycetes. 225 pp, Springer-Verlag, Berlin, New York

[9] Maltby, L. and Booth, R. (1991) The effect of coal-mine effluent on fungal assemblages and leaf breakdown. Wat. Res. 3, 247-250

[10] Abel, T. and Bärlocher, F. (1984) Effects of Cd on aquatic hyphomycetes. Appl. Environ. Microbiol. 48, 245-251

[11] Abel, T. and Bärlocher, F. (1988) Accumulation of cadmium through water and food in *Gammarus fossarum* (Amphipoda). J. Appl. Ecol. 25, 223-231

[12] Miersch, J., Bärlocher, F., Bruns, I. and Krauss, G.-J. (1997) Effect of cadmium, copper, and zinc on growth and thiol content of aquatic hyphomycetes. Hydrobiologia. 346, 77-84

[13] Bärlocher, F. (1982) Conidium production from leaves and needles in four streams. Canad. J. Bot. 60, 1487-1494

[14] Wetzel, R.G. (1975) Limnology. 743, W.B. Saunders, London

[15] Bärlocher, F., Canhoto, C. and Graça, M.A.S. (1995) Fungal colonization of alder and eucalypt leaves in two streams in Central Portugal. Arch. Hydrobiol. 133, 457-470

[16] Witzke, Th. and Pöllmann, H. (1996) Neoformation of minerals in slags of Kupferschiefer smelting in the Mansfeld area, Sachsen-Anhalt. *In*: Hallesches Jahrbuch für Geowissenschaften- Reihe B: Geologie, Paläontologie, Mineralogie. (Bachmann, G. H., Borg, G., Haubold, H., Heinisch, H., Lempp, C., Pöllmann, H. and Wycisk, Eds.), 18, 109-118. Universitätsverlag Halle

[17] Witzke, Th. (1997) Sekundärmineralbildungen in Kupferschlacke als Indikator für Schwermetallmobilisierung und -fixierung. *In*: Reststoffe der Kupferschieferverhüttung, Teil 1: Mansfelder Kupferschlacken. (Schreck, P. and Gläßer, W. Hrsg.), UFZ Berichte, 23, pp. 25-29, UFZ- Umweltforschungszentrum Leipzig/Halle

- Transformation of aromatic pollutants -

Population dynamics of *Ralstonia eutropha* JMP 134 on different substrates

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Abstract

Ralstonia eutropha JMP 134, which is able to grow on phenol is an ubiquitous occurring gram-negative strain of the ß-group of bacteria. It is used as a model organism for the investigation of the growth characteristics of single bacterial strains in mixed populations. With respect to this aim it is exposed to conditions which are similar to natural settings.

Xenobiotica utilizing organisms from natural settings are part of mixed populations whose individuals must be distinguished specifically according to their class, subclass and species in order to get information about their metabolic activity. Group specific and species specific probes for *Ralstonia eutropha* JMP 134 are used.

The individuals were characterized regarding the proliferation activity (measurement of changes in the DNA and rRNA content) and the capacity of assimilation of metabolites via measurement of the membrane potential. The interpretation of the vitality of a bacterial cell by using this method performs data about the actual energetic state of a cell, and contains insofar more information than simple live/dead estimations.

Investigations to the metabolic and generative characteristics of the strain by using different carbon sources (batch cultivation on pyruvate, phenol and acetate) are shown first. Furthermore transient state cultivations in continuous processes were done to investigate the proliferative activity under increasing concentrations of phenol.

Flow cytometry is recommended as an extended monitoring method for optimizing biotechnical decontamination processes of polluted environment using constructed mixed populations.

1. Experiments with mixed populations

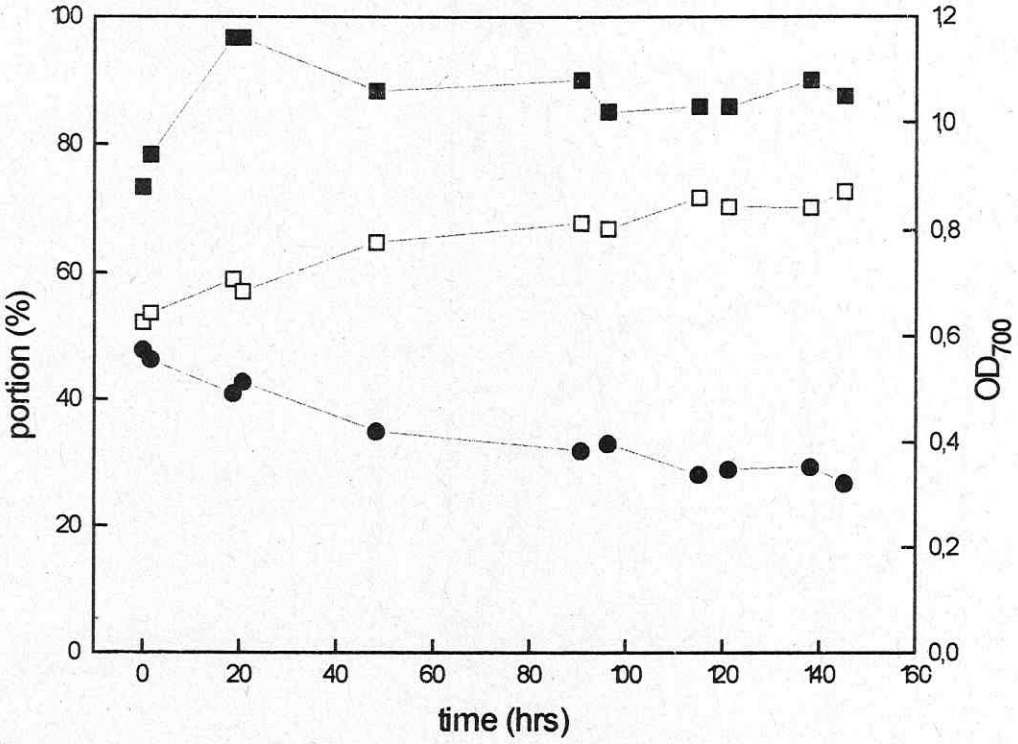


Fig. 1. Continuous cultivation of a mixed population and its optical density (■) consisting of *Ralstonia eutropha* JMP134 (●) and *Acinetobacter calcoaceticus* 69-V (□), which was added at t_0 to the chemostate (D = 0,1 h^{-1}). The population of *Acinetobacter calcoaceticus* 69-V remains in the system while the other population is washed out

The quantity of the membrane potential related fluorescence intensity (mprf) correlates obviously with the synthesis of rRNA and DNA as well as with the growth rates (Figure 2). Five hours later the fluorescence intensity of the membrane potential decreases despite of the increasing OD. The increase of the fluorescence intensity after seven hours is caused by non-specific cellular staining.

The quantity of the membrane potential related fluorescence intensity correlates with the synthesis of rRNA and DNA as well as with the growth rates similar to the growth on acetate (Figure 3). After depletion of phenol cell size, rRNA- and DNA content as well as the membrane potential related fluorescence intensity decrease.

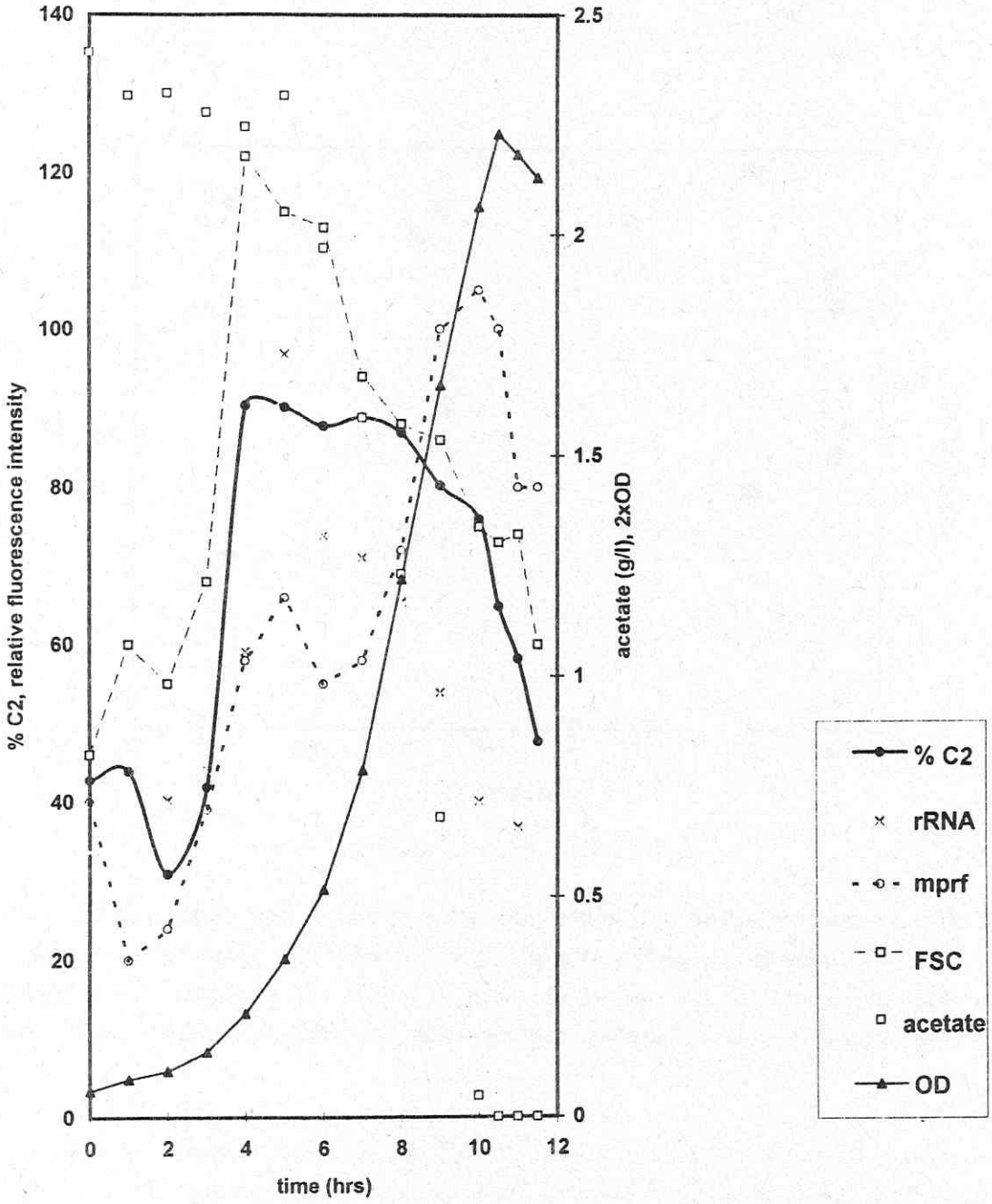
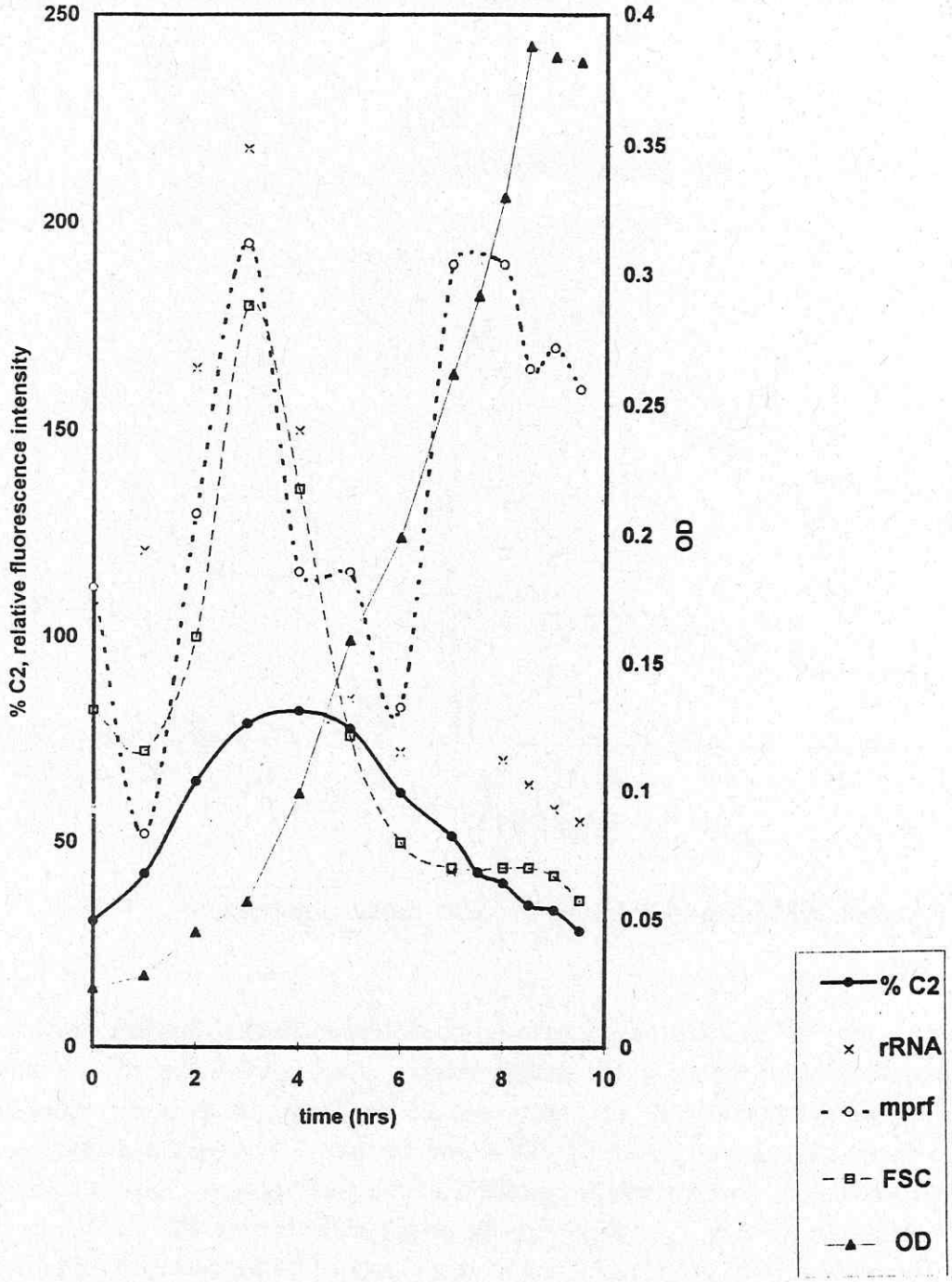


Fig. 2. Growth on acetate (batch-cultivation)

To reach steady state conditions chemostate cultivation must be performed (Figure 4). In the experiment shown the method of transient state cultivation is used. Via a successive increase in the nutrient flow stream in small periods quasi-stationary states are achieved. The conditions of constant growth allows firstly, a more distinct estimation of the generation time and secondly, an investigation of the changing growth characteristics of *Ralstonia eutropha* JMP 134 under known and controlled conditions. The effects of increasing concentrations of phenol on the proliferative activity of the bacterial cells were investigated.



measurement of the DNA: via counting the amount of cells with two chromosomes

measurement of the rRNA: via binding of oligonucleotide probes directed to a group (ß) and strain specific level

measurement of the membrane potential: see techniques

measurement of the FSC: via small angle scatter (3-9°)

measurement of acetate and phenol: via HPLC

Fig. 3. Growth on phenol (batch cultivation)

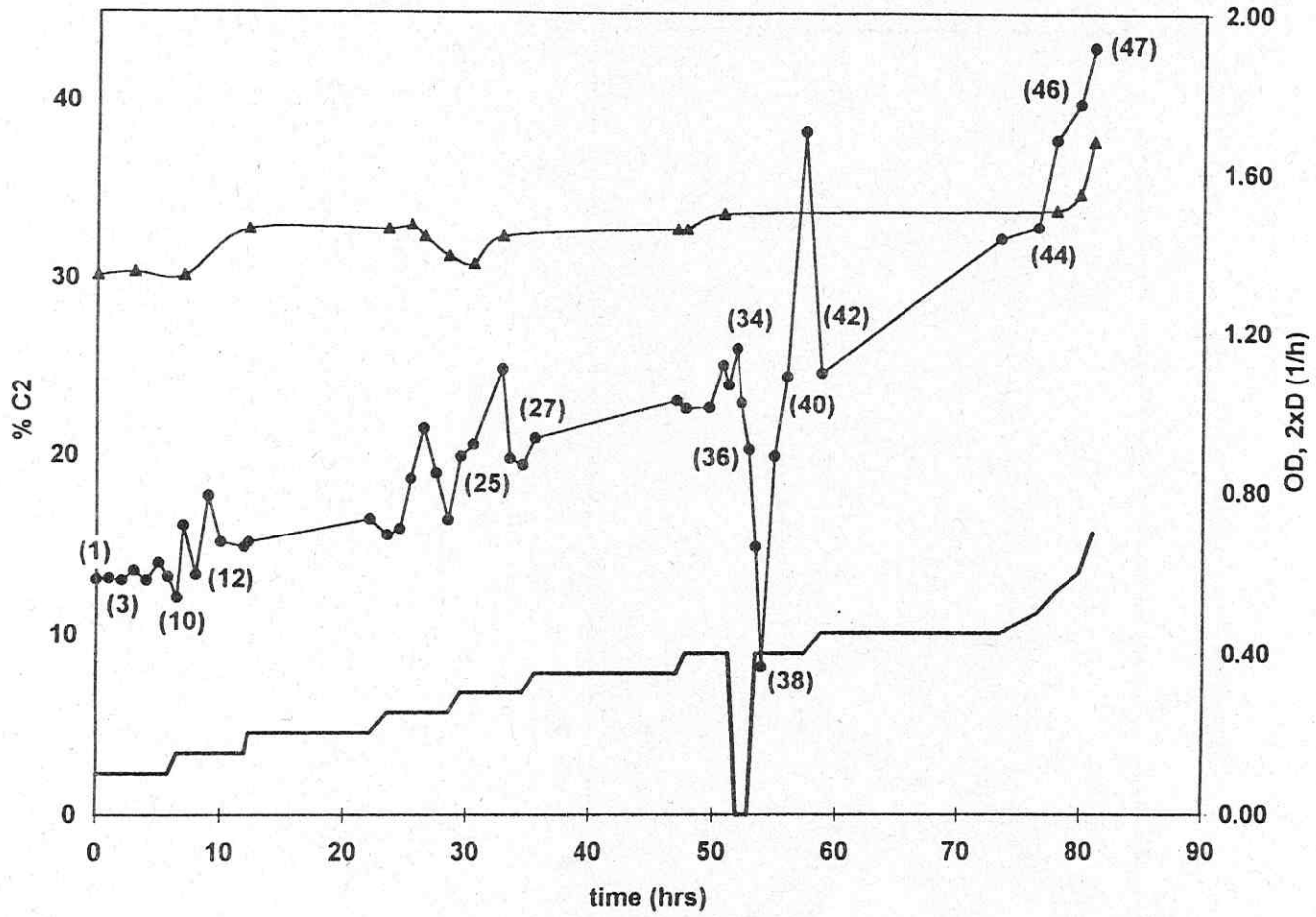


Fig. 4. Transient state cultivation of *Ralstonia eutropha* JMP134 on phenol

The typical course of bacterial cells grown on phenol by increasing nutrient flow rates. The given dilution rates correspond to the growth rates μ, whereby the actual concentrations of phenol are known to be very small. The increase in the proliferation activity correlates with an increase in the dilution rate very well. By this reason we used it as a reliable parameter for 'vitality' of the population, demonstrated as % cells with two chromosomes. The estimation of both the size of the cells and the optical density is not able to give the same value of information. The measurement of the membrane potential related fluorescence intensity is not workable for the control of such industrial decontamination processes because of logistical reasons. The degree of sensitivity of the method is proved by incorporating a carbon exhaustion phase. The state of vitality could be estimated via measurement of cell division and following replication by application of new substrate.

2. Techniques

Single bacterial cells are characterized by using the dye 3,3'-dihexyloxacarbocyanine, which is known to show the quantity of the actual membrane potential. $DiOC_6(3)$ is bound to the inner

lipid bilayer because of its lipophilic character and cationic charge. By this reason the dye functions for detection of living cells in contrast to dead cell dyes like from the oxonol class or dyes used for estimating enzyme activities.

The following parameters should be settled to establish the method for characterization of the physiological state of *Ralstonia eutropha* JMP 134:

1. Staining method for estimation of the membrane potential:
   The optical density of the bacterial culture is estimated by 700 nm. Without delay cells were centrifuged (3 min, 6000g) and adjusted to 3 x 10^8 cells/ml using imidazole-HCl-buffer (20 mM, pH 7.0). Following 5µl $DiOC_6(3)$ (0.12 µM) were applied and measured flow-cytometrically.

2. The disadvantage of the non-specific staining of the intracellularly lipophilic compound within the bacterial cell is observed in the case of to much concentrations of the dye as well as in the case of staining highly stationary phase cells. By these reasons hyperpolarization and depolarization of the membrane potential is provoked (via gramicidin and valinomycin), to prove the specificity of the dye binding.

3. The alteration in substrate uptake capability via measurement of the membrane potential is determined in dependence of both the kind of carbon source and the growth rate.

3. Conclusions

Using flow cytometry *Ralstonia eutropha* JMP 134 was characterized concerning proliferation activity (by measuring of the cellular DNA content), activity of the protein synthesis (by measuring the rRNA content) and capability of substance uptake (by measuring of the membrane potential). Information about changing cell sizes were obtained by measuring of the light scatter.

The analysis of these characteristics qualified for estimation of the temporary physiological state of a single cell within a bacterial culture. The physiological states could be observed in mixed cultures operating in-situ hybridization techniques.

In the future the application of the method should enable for process optimization during industrially performed decontamination of polluted soils running artificially mixed populations.

References

[1] Herrmann, C., Lösche, A., Müller, S., Bley, T., Babel, W. (1997) Flow cytometric differentiation of *Acinetobacter calcoaceticus* 69-V and *Alcaligenes eutrophus* JMP134

by fluorescently labeled rRNA-targeted oligonucleotide probes and DNA staining. Acta Biotechnol. 17, 19-38

[2] Müller, R.H., Babel, W. (1996) Growth rate-dependent expression of phenol-assimilation pathways in *Alcaligenes eutrophus JMP 134* - the influence of formate as an auxiliary energy source on phenol conversion characteristics. Appl. Microbiol. Biotechnol. 46, 156-162

[3] Müller, R.H., Bley, Th., Babel, W. (1995) Transient state cultivation as a means for determining maximum growth rates of microorganism in inhibition kinetics. J. Microbiol. Methods 22, 209-219

[4] Müller, S., Loffhagen, N., Bley, Th., Babel, W. (1996) Membrane-potential-related fluorescence intensity indicates membrane injury. Microbiol. Res. 151, 127-131

Comparison of the biodegradability of chloro- and methylphenol mixtures in model wastewater by mixed and pure cultures

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Abstract

A mixed bacterial culture which was enriched on different chloro- and methylphenols is composed of seven *Comamonas*, *Alcaligenes* and *Pseudomonas* species, named strain JH1 - JH7. The artificial consortium of these strains showed considerably the same degradation ability for substrate mixtures consisting of chloro- and methylphenols as the original mixed culture. Comparing the mixed culture and the strains *Alcaligenes xylosoxidans subspecies denitrificans* JH1 and *Comamonas testosteroni* JH5 the mixed culture degraded the chloro- and methylphenols mixtures more completely by combination of the different abilities of the strains and interactions between the strains. Strain *Alcaligenes xylosoxidans subspecies denitrificans* JH1 used only the *ortho* cleavage pathway for the degradation leading to the accumulation of methyllactones as dead end-metabolites. Strain *Comamonas testosteroni* JH5 used the protocatechuate pathway for the degradation of 4-methylphenol (4-MP) and the *meta* cleavage pathway for the degradation of the other methylphenols and 4-chlorophenol (4-CP). Mineralization of 4-MP and 4-CP by the mixed culture and strain JH5 occurred successively accompanied by diauxic growth. The activities of the key enzymes during the degradation indicate a regulation on the level of transcription. During the degradation of 4-methylphenol by the protocatechuate pathway the formation of the phenolhydroxylase is repressed.

1. Introduction

Biological treatment of wastewater involves degradation of substrate mixtures by mixed cultures rather than degradation of single compounds by pure cultures. However, our present understanding of the processes which accompany the mineralization of complex mixtures is limited [12, 17, 19, 21] and the interactions of several strains in a mixed culture during the degradation is investigated to a minor extent [4, 8, 10, 11]. Degradation of methyl- and chloroaromatic substrates in mixture is known to be incomplete. In general, methyl-substituted aromatic compounds are degraded via the *meta* cleavage pathway [6] whereas chloroaromatic compounds are mineralized via the *ortho* cleavage pathway [16] (Figure 1). The simultaneous transformation leads to the accumulation of dead-end metabolites like chlorocatechols, chlorinated hydroxymuconic semialdehydes or 4-carboxymethyl-methylbut-2-en-1,4-olides

(methyllactones) [20 - 22]. 3-chlorocatechol as possible metabolite of chloroaromatic breakdown reversibly inhibits the *meta* cleaving catechol 2,3-dioxygenase or totally blocks the *meta* cleavage pathway by irreversible suicide inactivation of this dioxygenase [5, 12, 15]. This study intended to analyze the degradation of mixtures of monomethyl- and monochlorophenols in model wastewater by a mixed culture enriched from activated sludge. The degradation ability of the mixed culture was compared with that of the different strains of the mixed culture and discussed in regard to the different pathways existing in these strains.

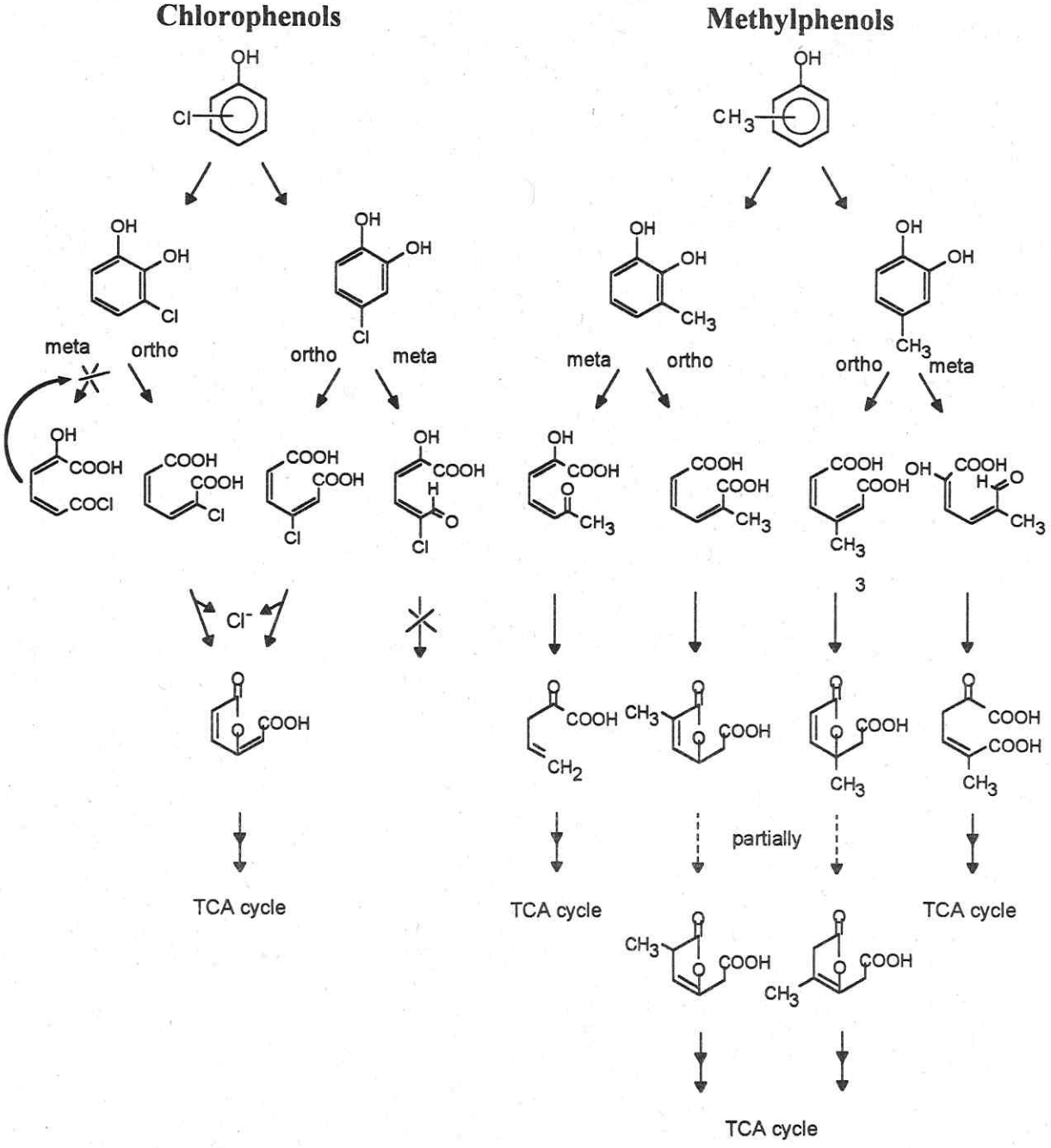

Fig. 1. *Ortho* and *meta* cleavage pathways for methyl- and chlorophenols

2. Materials and methods

Strain JH1 - JH7 (Table 1) were isolated from the enriched culture on R2A or phenol agar. The identification of the strains was done by phenotypic characterization and determination of the cellular fatty acids and ubiquinones. Batch culture studies were performed with phenols or mixtures of methyl- and chlorophenols (0.25 -1.0 mM) as sole sources of energy and carbon, and an inoculum of 2.5 - 10 %. The assays with substrate mixtures were incubated for 10 - 14 days in the dark at 25°C on a rotary shaker. Phenolic substrates and metabolites were analyzed by HPLC and chloride release was measured by potentiometric detection with an ion selective Ag/AgCl-electrode (details cf. Hollender et al. [12, 13]). Enzyme assays were conducted with the pure cultures grown on the appropriate substrates (0.5 -1 mM) to the late exponential phase. The cells were harvested, broken by ultrasonic treatment and the activities of the enzymes determined as described previously [12, 13].

Table 1. Characterization of the mixed culture

Strain No.	Identification	aerobic degradation of	
		4-Chlorophenol	4-Methylphenol
JH1	*Alcaligenes xylosoxidans subspecies denitrificans*	+	+
JH2	*Pseudomonas fluorescens*	-	-
JH3	*Pseudomonas stutzeri*	-	+
JH4	*Pseudomonas vesicularis* like*	-	-
JH5	*Comamonas testosteroni*	+	+
JH6	*Alcaligenes* like*	-	+
JH7	*Comamonas* like*	-	-

+, complete degradation; -, no degradation; *, clear identification needs further investigations

3. Results and Discussion

3.1. Characterization of the mixed culture

The mixed culture was enriched from activated sludge on a mixture of different methyl- and chlorophenols at first in an airlift reactor and later on in batch culture. Seven gram negative bacterial strains were isolated on solid media from the culture and identified as *Comamonas*, *Alcaligenes* and *Pseudomonas* species (Table 1). Four strains were able to grow on 4-MP as

sole source of energy and carbon and two of them, strain JH1 and JH5, utilized also 4-CP as growth substrate.

3.2. Degradation pathways for methyl- and chlorophenols by strain JH1, JH3, and JH5

The strains JH1, JH3, and JH5 were characterized in more detail. The degradation of all isomeric methyl- and chlorophenols by the strains were studied and the activities of the most important enzymes were measured. The results lead to the possible pathways for the different phenols by the strains shown in Figures 2 and 3. Phenolhydroxylases catalyzing the turnover of the phenols to catechols were induced in cells of strain JH1 grown on chlorophenols and methylphenols whereas a hydroxylase was only detected in 2-MP, 3-MP, and 4-CP grown cells of strain JH5. Instead of a phenolhydroxylase in cell extracts of strain JH5 and strain JH3 grown on 4-MP a methylhydroxylase and protocatechuate dioxygenases were detected. Protocatechuate are *meta* cleaved by a 4,5-protocatechuate dioxygenase in strain JH5 and *ortho* cleaved in strain JH3 by a 3,4-protocatechuate dioxygenase. Additionally the temporary accumulation of the metabolites 4-hydroxybenzyl alcohol, 4-hydroxybenzaldehyde and 4-hydroxybenzoic acid in degradation assays shows that the degradation of 4-methylphenol by both strains occurs via protocatechuate as central metabolite. In strain JH3 3-methylphenol (3-MP) was obviously also first attacked at the methyl substituent as shown by the induction of a gentisate dioxygenase. Aromatic ring-cleaving catechol dioxygenases were detected in strain JH1 and JH5. The catechol 2,3-dioxygenases were expressed constitutively in both strains whereas type I and II catechol 1,2-dioxygenases were only induced in phenol or chlorophenol grown cells of strain JH1. Since the *meta* cleaving dioxygenase of strain JH1 are inactivated by 3- and 4-chlorocatechol all isomeric chlorophenols are metabolized by the *ortho* catechol pathway as shown in Figure 3. Strain JH5 uses the *meta* cleavage pathway for the complete mineralization of 4-CP as recently shown for this strain [12, 13] and a few other strains [1 - 3, 14, 18].

3.3. Degradation of methyl- and chlorophenols in mixtures

The biodegradability of 10 different mixtures of monomethyl- and monochlorophenols as well as the biodegradability of the single compounds was studied in batch culture (Figure 4). The assays were characterized by the growth of the culture (not shown), the degradation of the substrates, the release of chloride, and the formation of HPLC-UV-detectable metabolites. The degradation abilities of the enriched culture and the artificial consortium of the strains JH1 - JH7 showed good correspondence. This indicates that all strains of the enriched culture which are significant for the phenol degradation could be isolated. However, in some assays of the artificial consortium the metabolites 3- and 4-methyllactone accumulated. The culture could not degrade these metabolites of the *ortho* pathway of methylphenols. In contrast, the enriched culture could degrade 3-methyllactone as single substrate so that this metabolite did not accumulated in the assays.

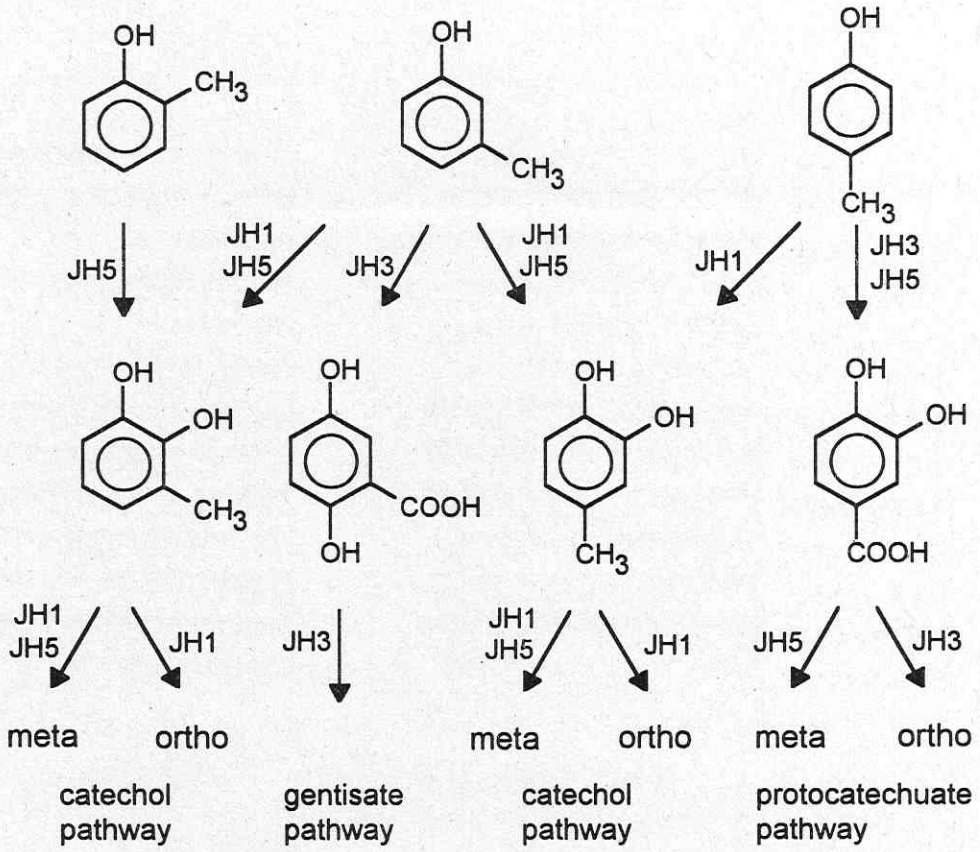

Fig. 2. Possible pathways for the isomeric methylphenols by strain JH1, JH3, and JH5

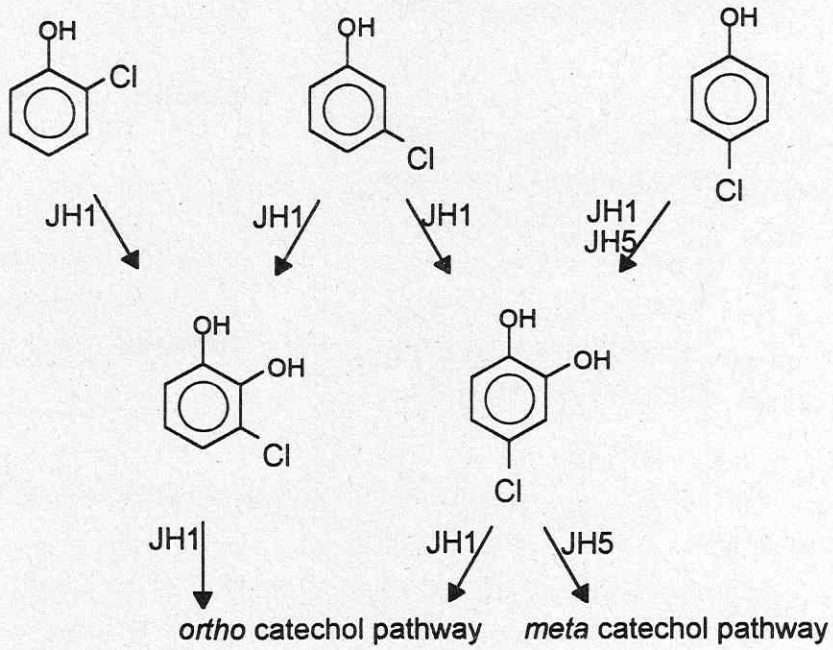

Fig. 3. Possible pathways for the isomeric chloropenols by strain JH1 and JH5

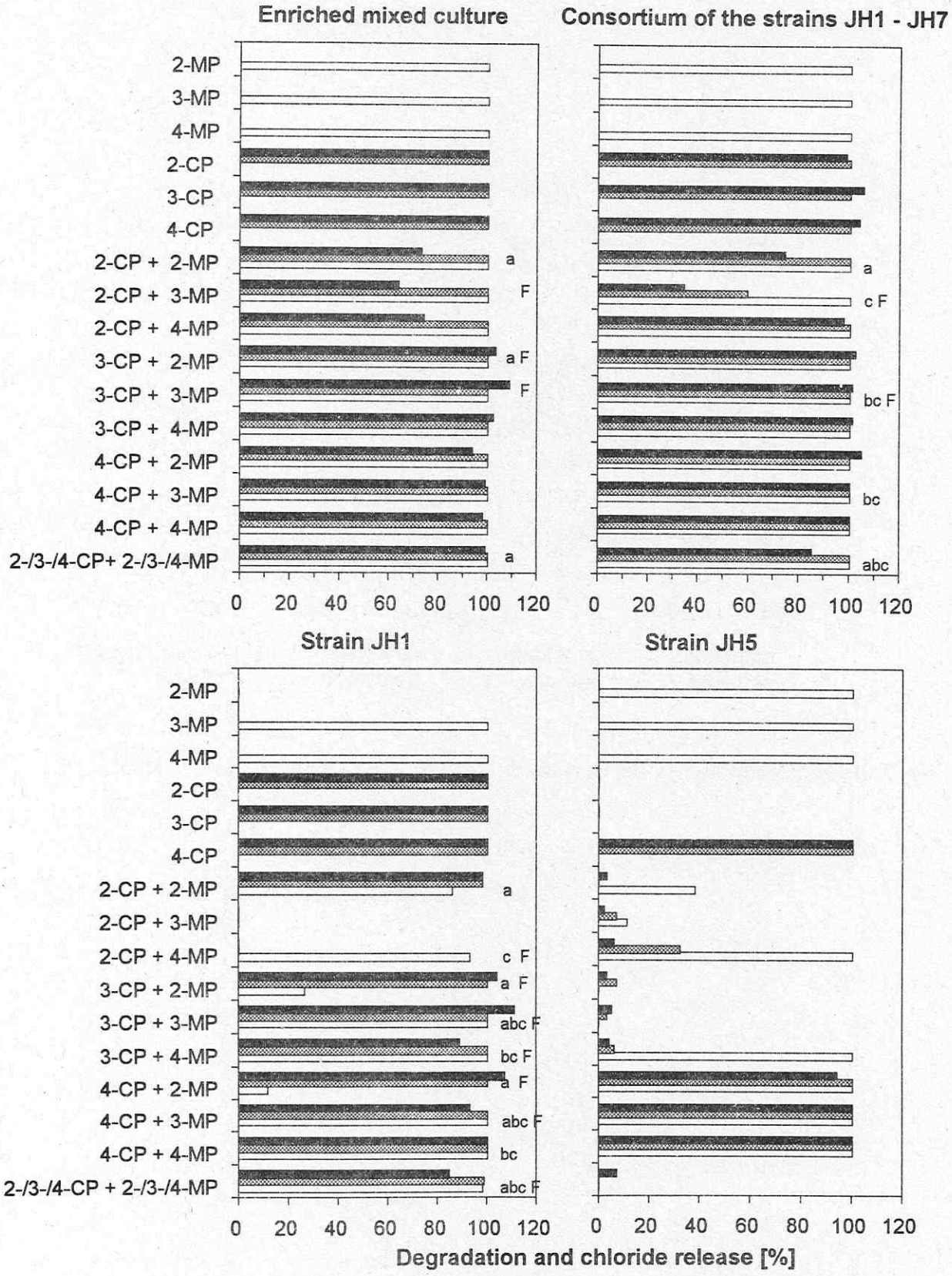

Fig. 4. Biodegradability of methyl- (MP) and chlorophenols (CP) as single compounds and mixtures by the enriched mixed culture, the consortium of the strains JH1 - JH7, strain JH1, and strain JH5. ☐, methylphenol; ▩, chlorophenol; ■, chloride; a,b,c, formation of 2-, 3-, 4-methyllactone, respectively; F, coloring of the assay.

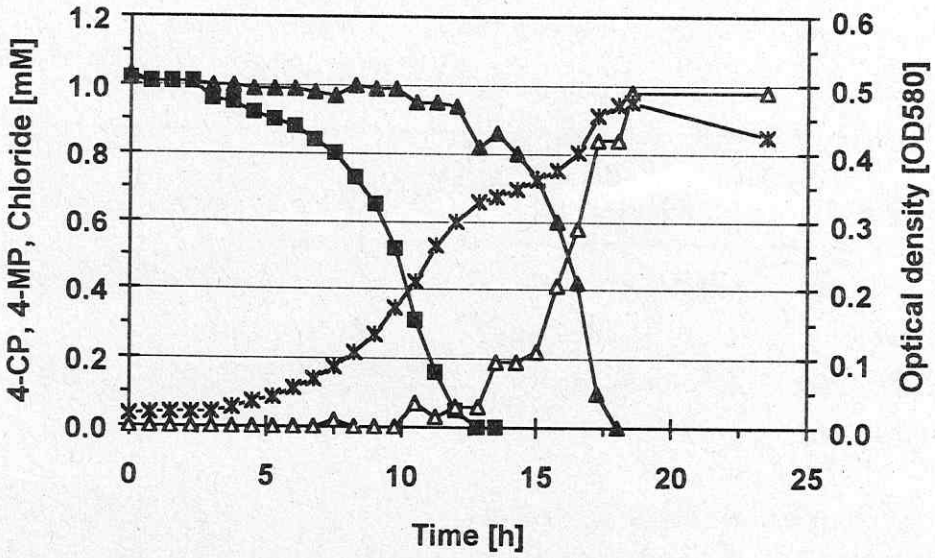

Fig. 5. Successive degradation of 4-methylphenol (4-MP) and 4-chlorophenol (4-CP) by strain JH5. ■, 4-methylphenol; ♦, 4-chlorophenol; Δ, chloride; *, cell density

The mixed culture has a broader degradation potential than the pure cultures of strain JH1 and JH5 (Figure 4). The mixed culture mineralized all single compounds completely because of the additive degradation abilities of both strains. All substrate mixtures could be degraded at least partially by the mixed culture.

Strain JH1 did not degrade 2-MP as single substrates but in mixture with the chlorophenols the compound was transformed partially by co-metabolism. Since the catechol 2,3-dioxygenase is inactivated by chlorocatechols the strain metabolized all substrate mixture by the *ortho* pathway. Consequently methyllactones accumulated almost stoichiometrically which strain JH1 could not degrade further. The medium of some assays turned pink or brown, indicating that catechols accumulated which easily be oxidized and polymerized as described elsewhere [7, 9].

Strain JH5 degraded completely all methylphenols as single substrates. In mixture with 2-CP and 3-CP the degradation of 2-MP and 3-MP via the *meta* cleavage pathway was inhibited because the formed 3-chlorocatechol inactivated the catechol-2,3-dioxygenase. The *ortho* pathway is not available in strain JH5. 4-MP was mineralized in all substrate mixtures completely via the protocatechuate pathway. Mixtures of 4-CP and all isomeric methylphenols were degraded completely because strain JH5 uses the *meta* cleavage pathway for the mineralization of 4-CP and 4-CC does not inactivate the *meta* cleaving dioxygenase.

The mixed culture shows a higher degradation ability than the additive abilities of strain JH1 and JH5. Apart from 2-methyllactone the methyllactones were degraded by an interaction of the members of the mixed culture or by the activity of other strains. The 2-CP/3-MP mixture which could not be mineralized by strain JH1 and JH5 was degraded by the mixed culture. However, the chloride release is not complete indicating that a partial misrouting of 3-chlorocatechol in the *meta* cleavage pathway could not be prevented. In general, the results

show that natural communities are able to mineralize incompatible substrate mixtures by the addition and the interaction of the degradation abilities of different organisms.

Activity	Substrate	I t = 0 h	II t = 1.5 h	III t = 5.5 h
Methylhydroxylase [mU/mg protein]	4-Methylphenol	0	40	146
Phenolhydroxylase [mU/mg protein]	4-Chlorophenol	310	192	69
Oxygen consumption [nmol O_2/(min·mg protein)]	4-OH-benzaldehyde	33	222	227
	4-OH-benzoic acid	30	77	102
	Protocatechuate	0	22	51

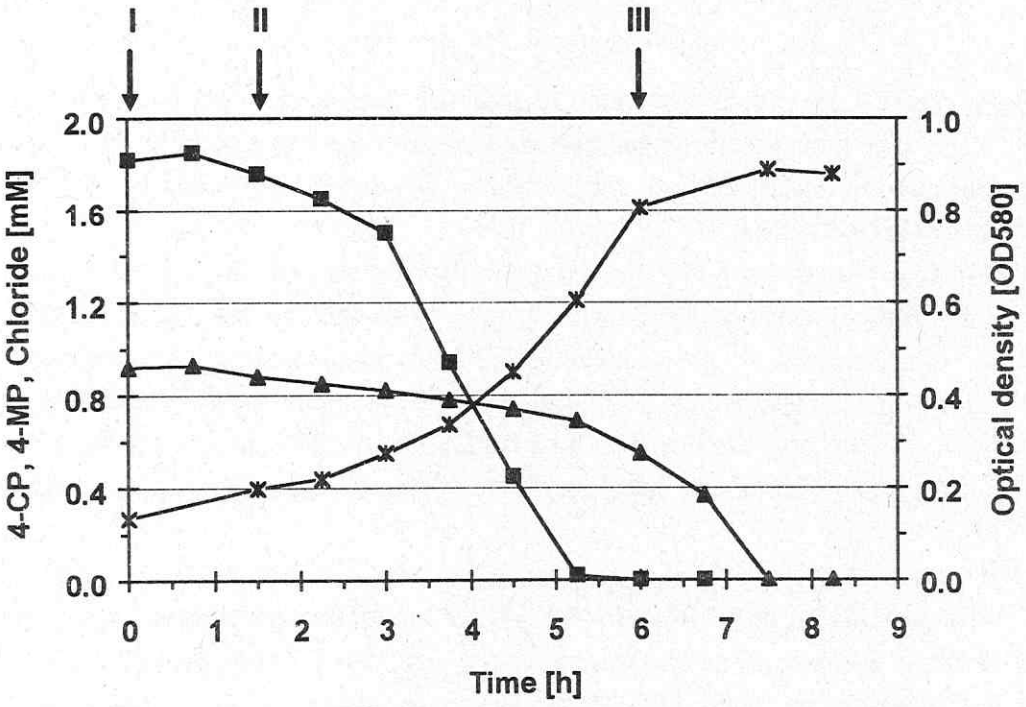

Fig. 6. Degradation of 4-chlorophenol (4-CP) and 4-methylphenol (4-MP) by cells of strain JH5 grown on 4-CP to the late exponential growth phase and enzymatic activities during the degradation. ■, 4-methylphenol; ♦, 4-chlorophenol; *, cell density

3.4. Kinetic studies on degradation of 4-CP and 4-MP in mixture

The course of degradation was studied for some substrate mixtures. The degradation of 4-CP and 4-MP by the mixed culture and also by strain JH5 which determines obviously the mixed culture at this substrate mixture showed a special feature (Figure 5). The substrates were

degraded successively accompanied by a diauxic growth. First 4-MP was mineralized exclusively and not until after a further lag-phase the degradation of 4-CP and the release of chloride started. The successive degradation was observed at different substrate concentrations and inocula of all growth phases. In contrast, mixtures of the other methylphenols and 4-CP were degraded simultaneously.

To explain the diauxic growth further experiments were conducted. The degradation of 4-CP and 4-MP by 4-CP grown cells was studied and the activities of the methylhydroxylase and phenolhydroxylase were measured during the degradation (Figure 6). At the beginning the 4-CP grown cells showed a phenolhydroxylase activity whereas a methylhydroxylase was not induced. 1.5 h after the addition of 4-MP and 4-CP a methylhydroxylase was induced and the phenolhydroxylase activity was reduced. During the following fast degradation of 4-MP the activity of the methylhydroxylase increased and the activity of the phenolhydroxylase decreased further. The enzymes which catalyze the further degradation of 4-MP were also induced as shown by the increasing oxygen consumptions with the metabolites during the experiment. After the complete degradation of 4-MP 4-CP was transformed fast. The results indicate that the degradation of 4-MP by the protocatechuate pathway is preferred and induction of the methylhydroxylase occurred whenever 4-MP is present in mixtures. The formation of the phenolhydroxylase for the turnover of 4-CP is repressed during the transformation of 4-MP. The proposed regulation was confirmed by the same successive degradation of other substrate mixtures which are degraded by the *meta* catechol and protocatechuate pathway as 3-MP and 4-MP. Besides 4-MP 4-hydroxybenzoic acid caused the same repression of the 4-CP breakdown.

Acknowledgments

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References

[1] Arensdorf, J. J., and D. D. Focht (1994) Formation of chlorocatechol *meta* cleavage products by *Pseudomonas* during metabolism of monochlorobiphenyls. Appl. Environ. Microbiol. 60: 2884-2889.

[2] Arensdorf, J. J., and D. D. Focht (1995) A *meta* cleavage pathway for 4-chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepacia* P166. Appl. Environ. Microbiol. 61: 443-447.

[3] Bae, H.S., J.M. Lee, Y.B.Kim and S.-T. Lee (1996) Biodegradation of the mixtures of 4-chlorophenol and phenol by *Comamonas testosteroni* CPW301. Biodegradation 7: 463-469.

[4] Bae, H.S., J.M. Lee, and S.-T. Lee (1997) Biodegradation of the mixture of 2,4,6-trichlorophenol, 4-chlorophenol, and phenol by a defined mixed culture. J. Gen. Appl. Microbiol. 43: 97-103.

[5] Bartels, I., H.-J. Knackmuss, and W. Reineke (1984) Suicide inactivation of catechol 2,3-dioxygenase from *Pseudomonas putida* mt-2 by 3-halocatechols. Appl. Environ. Microbiol. 47: 500-505.

[6] Dagley, S. (1978) Pathways for the utilization of organic growth substrates, p 305-388. *In* I.C. Gunsalus (ed.), The bacteria, Vol VI, Bacterial diversity. Academic Press, New York.

[7] Fava, F., D. Di Gioia, C. Romagnoli, L. Marchetti, and D. Mares. 1993. Biosynthesis and cytoplasmic accumulation of a chlorinated catechol pigment during 3-chlorobenzoate aerobic co-metabolism in *Pseudomonas fluorescens*. Arch Microbiol. 160: 350-357.

[8] Feigel, B.J., and H.-J. Knackmuss (1993) Syntrophic interactions during degradation of 4-aminobenzenesulfonic acid by a two species bacterial culture. Arch. Microbiol. 159: 124-130.

[9] Haller, H.D., and R.K. Finn (1979) Biodegradation of 3-chlorobenzoate and formation of black colour in the presence and absence of benzoate. Eur. J. Appl. Microbiol. Biotechnol. 8: 191-205.

[10] Havel, J., and W. Reineke (1993) Microbial degradation of chlorinated acetophenones. Appl. Environ. Microbiol. 59: 2706-2712.

[11] Hess, T.F., K. Schmidt, J.Silverstein, and B. Howe (1990) Supplemental substrate enhancement of 2,4-dinitrophenol mineralization by a bacterial consortium. Appl. Environ. Microbiol. 56: 1551-1558.

[12] Hollender, J., W. Dott, and J. Hopp (1994) Regulation of chloro- and methylphenol degradation in *Comamonas testosteroni* JH5. Appl. Environ. Microbiol. 60: 2330-2338.

[13] Hollender, J., J. Hopp, and W. Dott (1997) Degradation of 4-chlorophenol via the *meta* cleavage pathway by *Comamonas testosteroni* JH5. Appl. Environ. Microbiol. 63: 4567-4572.

[14] Kersten, P. J., P. J. Chapman, and S. Dagley (1985) Enzymatic release of halogens or methanol from some substituted protocatechuic acids. J. Bacteriol. 162: 693-697.

[15] Klecka, G. M., and D. T. Gibson (1981) Inhibition of catechol 2,3-dioxygenase from *Pseudomonas putida* by 3-chlorocatechol. Appl. Environ. Microbiol. 41: 1159-1165.

[16] Knackmuss, H.-J. (1984) Biochemistry and practical implications of organohalide degradation, p. 687-693. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. American Society for Microbiology, Washington, D.C.

[17] Konopka, A. (1993) Isolation and characterization of a subsurface bacterium that degrades aniline and methylanilines. FEMS Microbiol. Lett. 111: 93-100.

[18] Mars, A.E., T. Kasberg, S. R. Kaschabek, M H.vanAgteren, D.B. Janssen, and W. Reineke (1997) Microbial degradation of chloroaromatics: use of the *meta*-cleavage pathway for mineralization of chlorobenzene. J. Bacteriol. 179: 4530-4537.

[19] Menke, B., and H.-J. Rehm (1992) Degradation of mixtures of monochlorophenols and phenol as substrates for free and immobilized cells of *Alcaligenes* sp. 17-2. Appl. Microbiol. Biotechnol. 37: 655-661.

[20] Schmidt, E. (1987) Response of a chlorophenols degrading mixed culture to changing loads of phenol, chlorophenol and cresols. Appl. Microbiol. Biotechnol 27: 94-99.

[21] Taeger, K., H.-J. Knackmuss, and E. Schmidt (1988) Biodegradability of mixtures of chloro- and methylsubstituted aromatics. Simultaneous degradation of 3-chlorobenzoate and 3-methylbenzoate. Appl. Microbiol. Biotechnol. 28: 603-608.

[22] Wieser, M., J. Eberspächer, B. Vogler, and F. Lingens (1994) Metabolism of 4-chlorophenol by *Azotobacter* sp. GP1: structure of the *meta* cleavage product of 4-chlorocatechol. FEMS Microbiol. Lett. 116: 73-78.

Molecular biology of chlorophenol degradation catalyzed by a bacterium of the *Rhizobiaceae* group

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Abstract

The Gram-negative bacterium strain S1 is able to biodegrade chlorinated phenols by the modified *ortho*-pathway. Analysis of the 16S rDNA sequence revealed its phylogenetic position within the α-subgroup of the *Proteobacteria* intermediate to the genera *Phyllobacterium*, *Mesorhizobium* and *Sinorhizobium*. The gene *clpB*, which codes for the 2,4-dichlorophenol hydroxylase was localized on a 4.5 kb-fragment of *Pst*I-digested total DNA and was subsequently sequenced. Fingerprint motifs for binding of the ADP moieties of FAD and NADH, respectively and a third conserved region reported to be involved in FAD binding were identified. The amino acid sequences derived from *clpB* and the *tfdB* gene of *Ralstonia eutropha* JMP 134 (pJP4) exhibited 75 % identical positions. Upstream of *clpB*, the gene *clpC* and the C-terminal part of an open reading frame *clpR* were indicated, which, on the basis of sequence homologies, should encode a chlorocatechol 1,2-dioxygenase and a LysR-type regulator protein. In the downstream region of *clpB* the divergently transcribed gene *clpF* coding for a putative maleylacetate reductase was localized. The arrangement of these degradative genes of strain S1 differs from that of the chlorocatechol catabolic operon found on plasmid pJP4, indicating a separate way of gene recruitment during evolution of the catabolic pathway.

1. Introduction

The biodegradation of 2,4-dichlorophenol (2,4-DCP) was often studied in context with the microbial utilization of the herbicide 2,4-dichlorophenoxyacetate (2,4-D) [e.g. 1]. A great number of bacteria of different phylogenetic origin has been described to degrade 2,4-D, e.g. several members of the *Proteobacteria* like *Xanthobacter* sp. (α-subclass [2]), *Burkholderia cepacia* CSV90 (β-subclass [3]) and *Pseudomonas putida* PaW85 (γ-subclass [4]). Regulation and function of the enzymes of the 2,4-D degradation pathway as well as the localization of the respective genes on the plasmid pJP4 and their sequences were described in detail in a bacterium of the β-subclass, *Ralstonia eutropha* strain JMP 134 [1, 5]. The genes are grouped in three operons: *tfdA* (2-oxoglutarate dependent dioxygenase), *tfdB* (2,4-dichlorophenol hydroxylase) and the chlorocatechol catabolic operon encoding a chlorocatechol 1,2-dioxygenase (*tfdC*), chloromuconate cycloisomerase (*tfdD*), dienelactone hydrolase (*tfdE*) and

(chloro)maleylacetate reductase (*tfdF*), which convert the chlorocatechol into 3-oxoadipate. The expression of all three operons is positively regulated by the TfdR protein, which is encoded by the identical *tfdR and tfdS* genes [6]. A similar organization and a high overall sequence homology of the chlorocatechol catabolic operons and the regulatory genes were found on plasmids pP51 and pAC27 of the chlorobenzene degrading *Pseudomonas* sp. strain P51 and a chlorobenzoate degrading *Pseudomonas putida*, respectively [7].

Recent work [8] indicated, that members of the α-subdivision possess a high potential to degrade 2,4-D, but the degradative genes exhibited only low similarity to the canonical *tfd* genes of the plasmid pJP4.

We have isolated a chlorophenol-utilizing bacterium (strain S1) from activated sludge of a herbicide factory (Schwarzheide, Germany), which was assigned to the α-subclass of the *Proteobacteria* by chemotaxonomic traits [9]. This bacterium is unable to utilize 2,4-D and 4-chloro-2-methylphenoxyacetate, respectively, but can grow with different chlorophenols as sole carbon source. Here we describe its phylogenetic position within the α-subclass on the basis of the 16S rDNA sequence. The sequence and organization of part of the genes responsible for biodegradation of 2,4-dichlorophenol in strain S1 are presented.

2. Materials and methods

2.1. Strains, growth conditions and phenotypic characterization

The strains S1 and S4 (both isolated from activated sludge [9]) and the reference strains *S. meliloti* (DSM 30135^T) and *S. fredii* (DSM 5851^T) were grown on nutrient broth I (Sifin GmbH, Berlin) at 30°C. *Ralstonia eutropha* JMP134 (pJP4) (DSM 4058) was grown in mineral medium [1] with 100 mg 2,4-D ml^{-1}. To study biodegradation, cells were harvested, resuspended in mineral medium and incubated with 0.2 mM of the respective chlorophenol [9]. Carbon assimilation tests were done with the Biotype 100-kit (bioMerieux, Lyon).

2.2. Analysis of the 16S ribosomal DNA

The 16S rDNA was amplified by PCR from a single colony of strain S1 by using the primers fD1 and rP2 [10]. The amplified product was purified by electrophoresis on a 0,8 % agarose gel, cut with *Bam*HI and *Sal*I and cloned into plasmid vector pBluescript SK^+. Sequencing was performed with primers as described elsewhere [11, 12].

2.3. DNA manipulations, hybridization and sequencing

DNA from strain S1 was prepared by the CTAB-method [13] and was completely digested with *Eco*RI and *Pst*I, respectively, and restriction fragment sizes were selected on a sucrose gradient [14]. Appropriate fractions of the *Eco*RI and *Pst*I digestions were ligated into pUC19

and pBluescript SK+, respectively, and plasmids were transformed into *E. coli* XL2blue. The plasmids pCP1 and pCP2 comprising a 3.5 kb *Eco*RI and a 4.8 kb *Pst*I fragment, respectively, were identified to code for genes of the degradation pathway by Southern blot analyses performed with the DIG-system (Boehringer, Mannheim). DNA probes homologous to the *tfd* genes were amplified by PCR using total DNA of *Ralstonia eutropha* JMP 134 (pJP4) as template and detected by chemiluminescence. Hybridizations were carried out in standard hybridization buffer at 54 °C. Following hybridization, blots were washed at low stringency (first: room temperature, 2 % SSC, 0.1 % SDS; second: 54 °C, 2 % SSC, 0.1 % SDS). Small- and large-scale plasmid preparations were carried out by ion exchange chromatography with tip20/100 columns (Quiagen, Hilden). Nucleotide sequences of the inserts of pCP1, pCP2 and further subclones were determined using the AutoRead-sequencing kit (Pharmacia, Freiburg), synthetic oligonucleotide primers and the automated laser fluorescence sequencer (ALF, Pharmacia Feiburg).

2.4. Sequence analyses

Computer analyses of nucleotide and amino acid sequences were performed using the databases EMBL, SWISSPROT and GenBank. The PC/Gene software (IntelliGenetics, Oxford) and the Clustal W program was used for pairwaise and multiple sequence alignments. Phylogenetic analyses were performed with the PHYLIP phylogeny inference package using the programs DNADIST and NEIGHBOR. PROTDIST, FITCH and NEIGHBOR were used to construct dendrograms from protein sequences.

2.5. Heterologous expression of the 2,4-dichlorophenol hydroxylase of strain S1 in *E. coli*

The *clpB* gene was amplified by PCR, cloned into pBluescript SK^+ (pY1) and transformed into *E. coli* K38 pGP1-2. Expression was studied using the T7-RNA polymerase/promoter system [15] and methods established for the investigation of the 2,4-dichlorophenol hydroxylase of strain S1 [16].

3. Results and discussion

3.1. Taxonomic description of strain S1

By chemotaxonomic properties, strain S1 has been classified as a member of the α-2 subgroup of the *Proteobacteria* [9]. Fatty acid analysis revealed *Ochrobactrum anthropi* as the most similar organism with a low similarity index (0.366). For differentiation of the strains S1 and S4 from the putatively related genera *Ochrobactrum* and *Sinorhizobium*, the capability to utilize different chlorophenols and aromatic compounds and a range of carbohydrates, organic acids and amines was compared (Table 1). Biodegradation of chlorophenols was restricted to

strains S1 and S4. Compared with the reference strains, only a limited number of carbohydrates and organic acids, but several amino acids and amines served as carbon sources for S1 and S4. Table 1 summarizes the utilization pattern of aromatic compounds and lists those substrates, which were utilized by the isolates, but not or only by one of the reference strains. Sequencing of almost the complete 16S-rRNA gene of strain S1 revealed that the strain branches within the *Rhizobiaceae* group close to the genera *Phyllobacterium*, *Mesorhizobium* and *Sinorhizobium*. Within the α subclass of the *Proteobacteria* only a few genera have been described to harbour strains that metabolize chlorophenols or 2,4-D. Most of them belong to *Sphingomonas* (e.g. [17]). Recently members of the *Bradyrhizobium* group were isolated from pristine soils, which exhibited slow growth with 2,4-D [18]. Our findings support the potential of this phylogenetic group, which might be underestimated in the past.

Table 1. Features that differentiate strains S1 and S4 from *S. meliloti*, *S. fredii* and *O. anthropi*.

Substrate	S1/S4	*S. meliloti*	*S. fredii*	*O. anthropi*[1]
2,4-DCP[2]	+	-	-	-
4-Chloro-2-methylphenol[2]	+	-	-	n.d.
4-Chlorophenol[2]	+	-	-	-
Protocatechuate	-	+	+	n.r.
p-Hydroxybenzoate	-	-	-	+
Benzoate	-	-	-	-
L-Tryptophan	+	-	-	-
Putrescine	+	-	+	-
Glutarate	+	-	-	+
D,L-5-Amino-n-valerate	+	-	-	n.r.
Malonate	+	-	+	-

[1]Data were taken from Holmes et al. [19]. Results were obtained from Biotype-100 carbon assimilation tests and [2]biodegradation tests. n.d.: not determined; n.r.: not reported; +: Biodegradation of the chlorophenols occurred within 10 h, growth was observed within 4 days; -: Biodegradation of the chlorophenols did not occur over 3 days, no growth occured within 4 days.

3.2. Nucleotide sequence analysis and expression of the clpB gene

Sequence analysis of the 6. kb *Pst*I-*Eco*RI DNA fragment from strain S1 cloned in pCP1 and pCP2 demonstrated the presence of four open reading frames probably connected to chlorophenol degradation. Based on the sequence homology to known proteins the identified genes coded for 2,4-dichlorophenol hydroxylase (*clpB*), chlorocatechol 1,2-dioxygenase (*clpC*), maleylacetate reductase (*clpF*) and for about 75% of the LysR type regulatory gene *clpR'* (Figure 1).

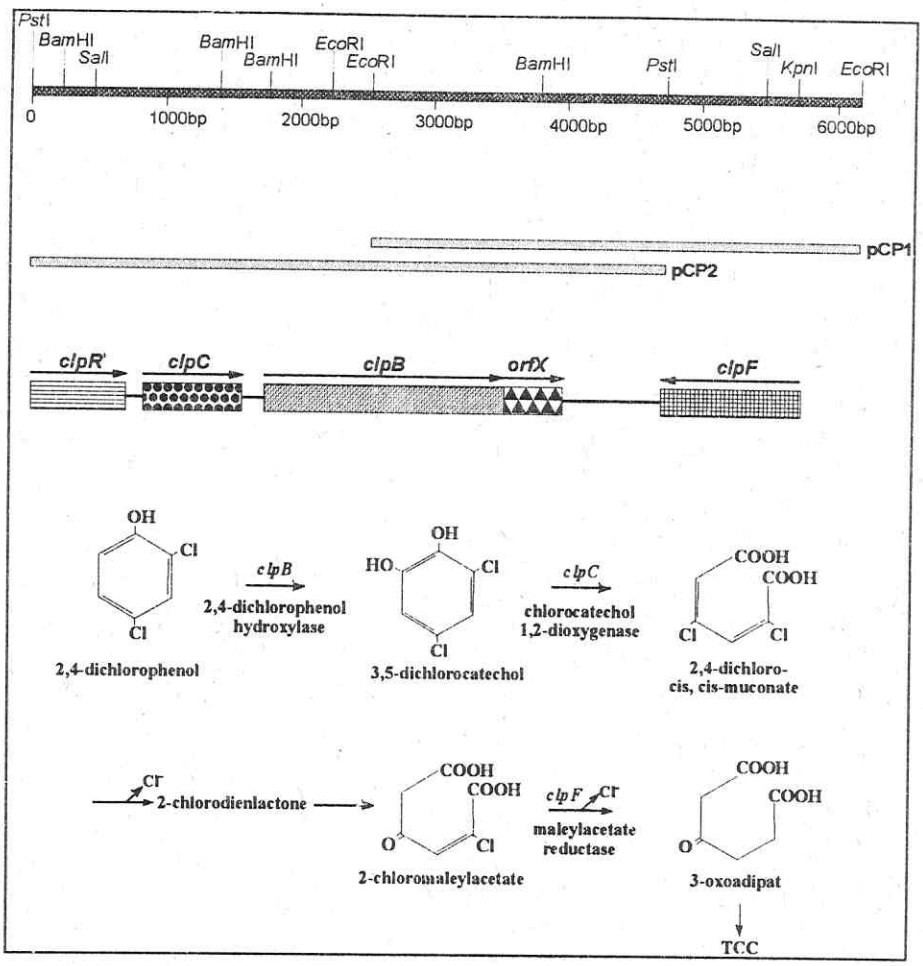

FIG. 1. Restriction map of the 6.2 kb *Pst*I-*Eco*RI DNA fragment of strain S1 and the organization of the genes which encode several enzymes of the chlorocatechol metabolism: *clpB*, 2,4-dichlorophenol hydroxylase; *clpC*, putative chlorocatechol 1,2-dioxygenase; *clpF*, putative maleylacetate reductase; *clpR'*, fragment of a LysR type regulatory protein. *orfX*: gene function unknown. The lower part shows the conversion steps mediated by the appropriate gene product.

The amino acid sequence deduced from *clpB* exhibits significant homology to the 2,4-dichlorophenol hydroxylase derived from *tfdB* of *R. eutropha* JMP134 (pJP4) (75 % identity) [5] and *Pseudomonas* sp. pEST4011 (63 % identity) [20], respectively. The expression of *clpB* cloned on pY1 was studied in *E. coli*. A protein of about 65 kDa was expressed using the T7 RNA polymerase/promoter system. 2,4-Dichlorophenol was converted by cell free extracts of this recombinant strain. The deduced amino acid sequence of *clpC* exhibited 45-47% identity to several chlorocatechol dioxygenases. A dendrogramm was constructed to determine the evolutionary position of protein ClpC, by using known sequences of catechol and chlorocatechol dioxygenases of gram-positive and gram-negative bacteria [21]. ClpC formed a cluster together with the chlorocatechol dioxygenases of gram-negative bacteria, but

represented a separate sublineage. Comparision of ClpF with sequences of (chloro)maleyl-acetate reductases encoded e.g. by pJP4 and pP51 revealed an identity of 46-56%.

The order of the chlorocatechol degradative genes on the 6.2 kb DNA fragment of strain S1 differed significantly from the structure of the chlorocatechol degradative operons of the plasmids pJP4, pP51 and pAC27. Additionally, the genes *clpC* and *clpF* are not localized in one operon. Those findings confirm observations of Fulthorpe et al. [8] that 2,4-D degrading bacteria contain mosaics of catabolic genes, recruited from different origins.

References

[1] Pieper, D.H., Engesser, K.-H. and Knackmuss, H.-J. (1989) Regulation of catabolic pathways of phenoxyacetic acids and phenols in *Alcaligenes eutrophus* JMP 134. Arch. Microbiol. 151, 365-371

[2] Dietzelmüller, G., Loidl, M. and Streichsbier, F. (1989) Isolation and characterization of a 2,4-dichlorophenoxacetic acid-degrading soil bacterium. Appl. Microbiol. Biotechnol. 31, 93-96

[3] Bhat, M.A., Tsuda, M., Horiike, K., Nozaki, M., Vaidyanathan, C.S. and Nakazawa, T. (1994) Identification and characterization of a plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. Appl. Environ. Microbiol. 60, 307-312

[4] Mäe, A.A., Marits, R.O., Ausmees, N.R., Koiv, V.M. and Heinaru, A.L. (1993) Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localization of catabolic genes. J. Gen. Microbiol. 139, 3165-3170

[5] Perkins, E.J., Gordon, M.P., Caceres, O. and Lurquin, P.F. (1990) Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. 172, 2351-2359

[6] Leveau, J.H.J. and van der Meer, J.R. (1996) The *tfdR* gene product can successfully take over the role of the insertion element-inactivated TfdT protein as a transcriptional activator of the *tfdCDEF* gene cluster, which encodes chlorocatechol degradation in *Ralstonia eutropha* JMP134(pJP4). J. Bacteriol. 178, 6824-6823

[7] Schlömann, M. (1994) Evolution of chlorocatechol catabolic pathways. Biodegradation 5, 301-321

[8] Fulthorpe, R.R., McGowan, C., Maltseva, O.V., Holben, W.E. and Tiedje, J.M. (1995) 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61, 3274-3281

[9] Lechner, U., Baumbach, R., Becker, D., Kitunen, V., Auling, G. and Salkinoja-Salonen, M. (1995) Degradation of 4-chloro-2-methylphenol by an activated sludge isolate and its taxonomic description. Biodegradation 6, 83-92

[10] Weisburg, W.G., Barns, S.B., Pelletier, D.A. and Lane, D.J. (1991) 16S Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697-703

[11] Snel, J., Blok, H.J., Kengen, H.M.P., Ludwig, W., Poelma, F.G.J., Koopman, J.P. and Akkermans, A.D.L. (1994) Phylogenetic characterization of *Clostridium* related

segmented filamentous bacteria in mice based on 16S ribosomal RNA analysis. System. Appl. Microbiol. 17, 172-179

[12] Sawada, H., Ieki, H, Oyaizu, H and Matsumoto, S. (1993) Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. Int. J. Syst. Bacteriol. 43, 694-702

[13] Ausubel, F.M., Brent, R., Kingston, R.E, Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) Current protocols in molecular biology. John Wiley and Sons, Inc.

[14] Luthe, D.S. (1983) A simple technique for the preparation and storage of sucrose gradients. Anal. Biochem. 135, 230-232

[15] Tabor, S. and Richardson, C. C.(1985) A bacteriophage T7 RNA polymerase/promoter-system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82, 1074-1078

[16] Makdessi and Lechner (1997) Purification and characterization of 2,4-dichlorophenol hydroxylase isolated from a bacterium of the α-2 subgroup of the *Proteobacteria*. FEMS Microbiol. Lett. 157, 95-101

[17] Nohynek, L.J., Suhonen, E.L., Nurmiaho-Lassila, E.-L., Hantula, J. and Salkinoja-Salonen, M. (1995) Description of four pentachlorophenol-degrading bacterial strains as *Sphingomonas chlorophenolica* sp. nov. System. Appl. Microbiol. 18, 527-538

[18] Kamagata, Y., Fulthorpe, R.R., Tamura, K., Takami, H., Forney, L.J. and Tiedje, J.M. (1997) Pristine environments harbor a new group of oligotrophic 2,4-dichlorophenoxyacetic acid-degrading bacteria. Appl. Environ. Microbiol. 63, 2266-2272

[19] Holmes, B., Popoff, M., Kiredijan, M. and Kersters, K. (1988) *Ochrobactrum anthropi* gen. Nov., sp. nov. From human clinical specimens and previously known as group Vd. Int. J. Syst. Bacteriol. 38: 406-416

[20] Koiv, V., Marits, R., and Heinaru, A. (1996) Sequence analysis of the 2,4-dichlorophenol hydroxylase gene *tfdB* and 3,5-dichlorocatechol 1,2-dioxygenase gene *tfdC* of 2,4-dichlorophenoxyacetic acid-degrading plasmid pEST4011. Gene 174, 293-297

[21] Eulberg, D., Golovleva, L.A., and Schlömann, M. (1997) Characterization of catechol catabolic genes from *Rhodococcus erythropolis* 1CP. J. Bacteriol. 179, 370-381

Convergent evolution of chlorocatechol catabolism – a problem for the design of functional gene probes

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Abstract

Functional gene probes may be useful for the assessment of the catabolic potential of microbial communities, since they do not require an isolation of degradative strains and thus are not subject to the biases inherent to those procedures. In order to give meaningful results, however, the probes have to be sufficiently specific for the catabolic pathway in question, and they also have to take into account the diversity of the respective genes. Many chloroaromatic compounds are degraded via chlorocatechols as intermediates. The known chlorocatechol catabolic pathways of proteobacteria appear to have evolved from the same origin. The corresponding pathway of *Rhodococcus opacus* 1CP, in contrast, has evolved independently by functionally convergent evolution. Thus, for molecular studies on the occurrence of catabolic pathways in microbial communities, separate probes have to be designed for proteobacterial and rhodococcal genes.

1. Functional gene probes – potential uses and general requirements

In industrial sewage treatment plants the situation sometimes occurs that pollutants are not mineralized under the given conditions although, in principle, they are biodegradable. In such situations, knowledge about the occurrence and frequency of strains capable of degrading the compound might be useful, for example to adjust the pollutant load to the number of degrading microorganisms. Likewise, if such chemicals have accidentally entered soil or water, information on the occurrence and frequency of degraders in the respective microbial community may provide an important basis for decisions about remediation strategies, e. g. about the feasibility of an *in situ* bioremediation or the necessity to augment the native community with proven laboratory strains.

A classical approach to investigate the occurrence of degradative strains is their isolation using the pollutant as carbon, nitrogen, or sulfur source, or as electron acceptor. If relatively high numbers of such strains are present in the system, direct plating may be used for their quantification. However, since many bacteria so far cannot be isolated, and since pollutants additionally may have toxic effects on bacteria, isolation methods will tend to underestimate the numbers of degradative strains considerably.

Molecular methods provide an opportunity to investigate the occurrence of certain genes in DNA isolated from whole microbial communities [1 - 3]. They thus avoid the problems resulting from the isolation of bacterial strains. The molecular ecology of bacteria is most advanced with rRNA- or rDNA-directed probes, which allow an investigation of the taxonomic structure of communities not altered by laboratory manipulations. By hybridization with fluorescence-labeled rRNA-directed probes, single cells may be assigned to larger or smaller taxa [4]. Polymerase chain reaction (PCR) experiments combined with temperature gradient or denaturing gradient gel electrophoresis make it possible to analyse the taxonomic diversity of communities [5 - 7]. However, with respect to the catabolic capabilities rDNA probes will yield only very limited insight. This is due to the facts that (i) for many taxa the degradative potential for pollutants is unknown, and that (ii) special degradative capabilities are often encoded by broad-host-range plasmids and may only be found in some strains of a given taxon.

The limitations of rDNA-directed probes may be overcome by the use of functional gene probes. In order to obtain meaningful results with probes directed towards catabolic genes, several requirements must be met: To avoid wrong positive results, the probe has to be specific for the respective catabolic pathway. Genes for enzymes with a role in various degradative routes thus are not suited as targets. In addition, it is desirable to know the most closely related proteins with a different function. In order to minimize wrong negative results, the diversity of catabolic genes has to be taken into account, which is considerably greater than that of rDNA. Since PCR strategies can focus on particularly well conserved short segments, they are potentially better suited in this respect than hybridization procedures. However, possible inhibition of PCR reactions, especially with DNA from soil communities, may present a major problem [8 - 10], while another important issue is the design of adequate PCR primers. Overall, the use of functional probes requires a considerable knowledge about the evolution of the genes which constitute the respective pathway.

2. Convergent evolution of chlorocatechol pathways

Chloroaromatic compounds, in general, due to the persistence and toxicity of some of them, represent a class of chemicals of major environmental concern [11]. However, quite a number of them can, under favorable conditions, be completely degraded and utilized as carbon source by specific bacteria. In many of the known cases, the chloroaromatic growth substrates are degraded via *ortho*-cleavage of chlorocatechols [12]. Usually, four enzymes are necessary to convert chlorocatechols to 3-oxoadipate, a common intermediate of catabolic pathways for non-halogenated compounds: chlorocatechol 1,2-dioxygenase, chloromuconate cyclo-isomerase, dienelactone hydrolase, and maleylacetate reductase (Figure 1). The former three enzymes catalyze reactions analogous to those of the catechol branch of the 3-oxoadipate pathway, while the fourth enzyme catalyzes a reaction not occurring in that pathway.

The genes of chlorocatechol pathways are usually located on catabolic plasmids, such as pAC27 from the 3-chlorobenzoate utilizing strain *Pseudomonas putida* AC866 [13], pJP4 of the 2,4-dichlorophenoxyacetate catabolizing bacterium *Ralstonia eutropha* (*Alcaligenes*

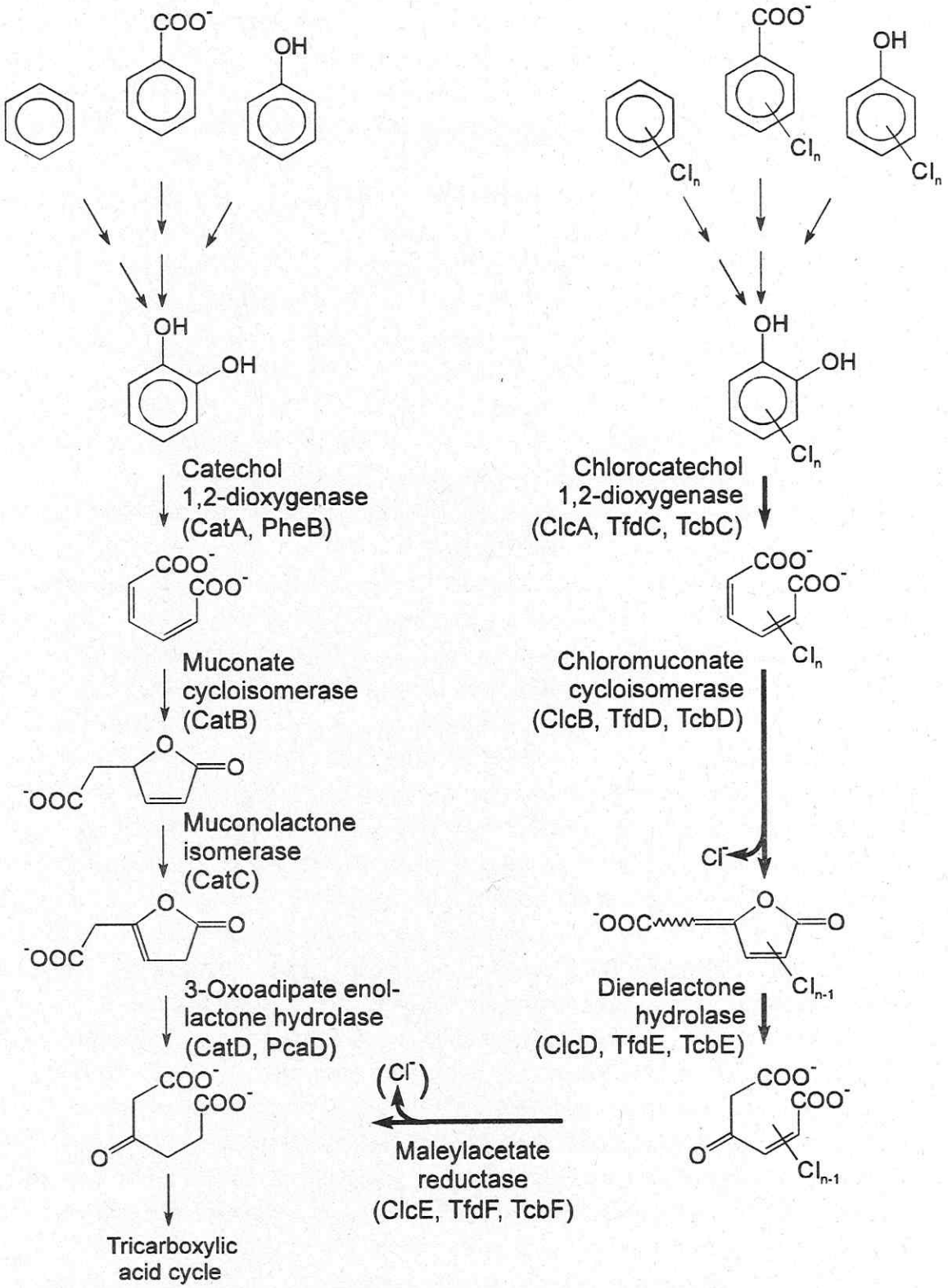

Fig. 1. Catabolic pathways for aromatic compounds via catechol and for chloroaromatic compounds via chlorocatechols. In addition to enzyme names, their designations as gene products are given.

eutrophus) JMP134 [14], and pP51 from the 1,2,4-trichlorobenzene degrader *Pseudomonas* sp. P51 [15]. Sequencing of the archetypal chlorocatechol gene clusters from these plasmids revealed that the chlorocatechol 1,2-dioxygenases and the chloromuconate cycloisomerases are homologous to catechol 1,2-dioxygenases or muconate cycloisomerases, respectively [16 - 18]. Moreover, the chlorocatechol 1,2-dioxygenases were shown to be more closely related to each other than to the catechol 1,2-dioxygenases. Similarly, the chloromuconate cycloisomerases form a group of closely related enzymes which excludes the muconate cycloisomerases. These results, together with the quite conserved operon structures, allowed the conclusion that the archetypal chlorocatechol gene clusters originated from a common origin, more recent than the common origin shared with catechol catabolic gene clusters [19]. The chlorocatechol genes of various other strains have been shown by hybridization to be closely related to those of pAC27, pJP4 (module 1), or pP51 [20 - 24], while some others, under the conditions employed, did not hybridize [20, 22, 23, 25]. The fact that most known chlorocatechol gene clusters appear to belong to the same group of related pathways suggests that there should be a limited number of such groups in natural communities.

While the chlorocatechol 1,2-dioxygenase and the chloromuconate cycloisomerase of the archetypal chlorocatechol gene clusters diverged from the corresponding enzymes of the catechol pathway, the dienelactone hydrolase and the maleylacetate reductase were apparently recruited into these gene clusters from some other genetic contexts than catechol degradation. In the case of the reductase this is obvious, since no such reaction occurs in the catechol branch of the 3-oxoadipate pathway. Maleylacetate reductases have, however, been shown to play a role in pathways via hydroquinol or hydroxyhydroquinol as ring cleavage substrates [26 - 29]. The dienelactone hydrolases apparently share with the 3-oxoadipate enol-lactone hydrolases of the catechol and the protocatechuate pathways the α/β-hydrolase fold [30, 31], but their sequences are so different that they must have diverged much earlier than the catechol and chlorocatechol 1,2-dioxygenases or the muconate and chloromuconate cycloisomerases [19]. Recently the characterization of a second chlorocatechol gene cluster on pJP4 and of a gene cluster on pEST4011 revealed dienelactone hydrolases which are related to each other, but only distantly related to those encoded on pAC27, pJP4 (module 1), and pP51. Biochemical characterization of the Gram-positive, chlorophenols utilizing strain *Rhodococcus opacus* (*erythropolis*) 1CP has previously revealed unusual properties of the chlorocatechol catabolic enzymes [32 - 34]. Especially intriguing were the similarities between the rhodococcal muconate and chloromuconate cycloisomerase which were not shared by the respective proteobacterial counterparts [34]. This led to the hypothesis that the adaptation to the conversion of chlorinated catechols developed independently in proteobacteria and in Gram-positive bacteria. By sequencing of catechol as well as chlorocatechol catabolic genes from *R. opacus* 1CP, this hypothesis was more recently shown to be correct [35, 36]. In dendrograms of the 1,2-dioxygenases and cycloisomerases the rhodococcal chlorocatechol enzymes clearly represent different branches than their proteobacterial counterparts (Figures 2A,B). Thus, based on a sequence divergence, a functionally convergent evolution of chlorocatechol catabolism appears to have taken place. The observation that the rhodococcal dienelactone hydolase is relatively closely related to the dienelactone hydrolases of pEST4011 and pJP4 (module 2) does not disprove this conclusion, since the evolutionary distance is still

greater between these proteins than between pairs of muconate and chloromuconate cycloisomerases (Figure 2C).

3. Perspectives for the molecular detection of chlorocatechol genes in microbial communities

As a consequence of the convergent evolution of chlorocatechol pathways it will probably not be possible to design universal hybridization probes or universal PCR primers for this pathway. Such primers would have to be so unspecific that they would at least give hybridization signals or PCR products, respectively, also with catechol catabolic genes. More specific probes, directed, for example, at only the proteobacterial genes will fail to indicate the presence of the rhodoccal genes. This qualification does apply to a number of ecological studies performed with pJP4-derived probes [e.g. 23, 24, 37]. Thus, for attempts to indicate the whole chlorocatechol catabolic potential of a community, the different evolutionary lines of the pathway will have to be investigated by using different primer pairs.

The dienelactone hydrolase genes and the maleylacetate reductase genes will, in the near future, not be useful for molecular studies on the occurrence of chlorocatechol pathways, since they do not yet meet the criteria developed above. The dienelactone hydrolases have apparently been recruited several times into chlorocatechol pathways from unknown other metabolic contexts. The maleylacetate reductases are even known to be functional in various pathways. Primers directed towards the hydrolase or reductase genes may therefore not be specific for chlorocatechol catabolism. The chlorocatechol 1,2-dioxygenases and chloromuconate cycloisomerases, in contrast, according to present knowledge, are functional only in chlorocatechol degradation. And since the evolutionary origin of these enzymes is known to be in the dioxygenases and cycloisomerases of the catechol pathway, relatively closely related genes with a different function are known which can be taken into account for primer design.

A major challenge for the ambitious aim to predict the chlorocatechol catabolic potential of communities by molecular methods is certainly that the diversity of the genes has to be accounted for. The genetic diversity of chlorocatechol pathways functional in Gram-positive bacteria is completely unknown, since only one gene cluster has so far been characterized. From proteobacteria, several sequences are available, which allow to define relatively conserved regions. In the near future, primers for chloromuconate cycloisomerase genes will be tested with strains which are known to harbor a chlorocatechol pathway, in order to see whether the primers designed so far are useful for indicating the presence of the respective genes. Certainly the database needs to be increased. However, it appears possible that, in a few years, molecular methods may render it possible to gain additional insight into the catabolic potential of communities.

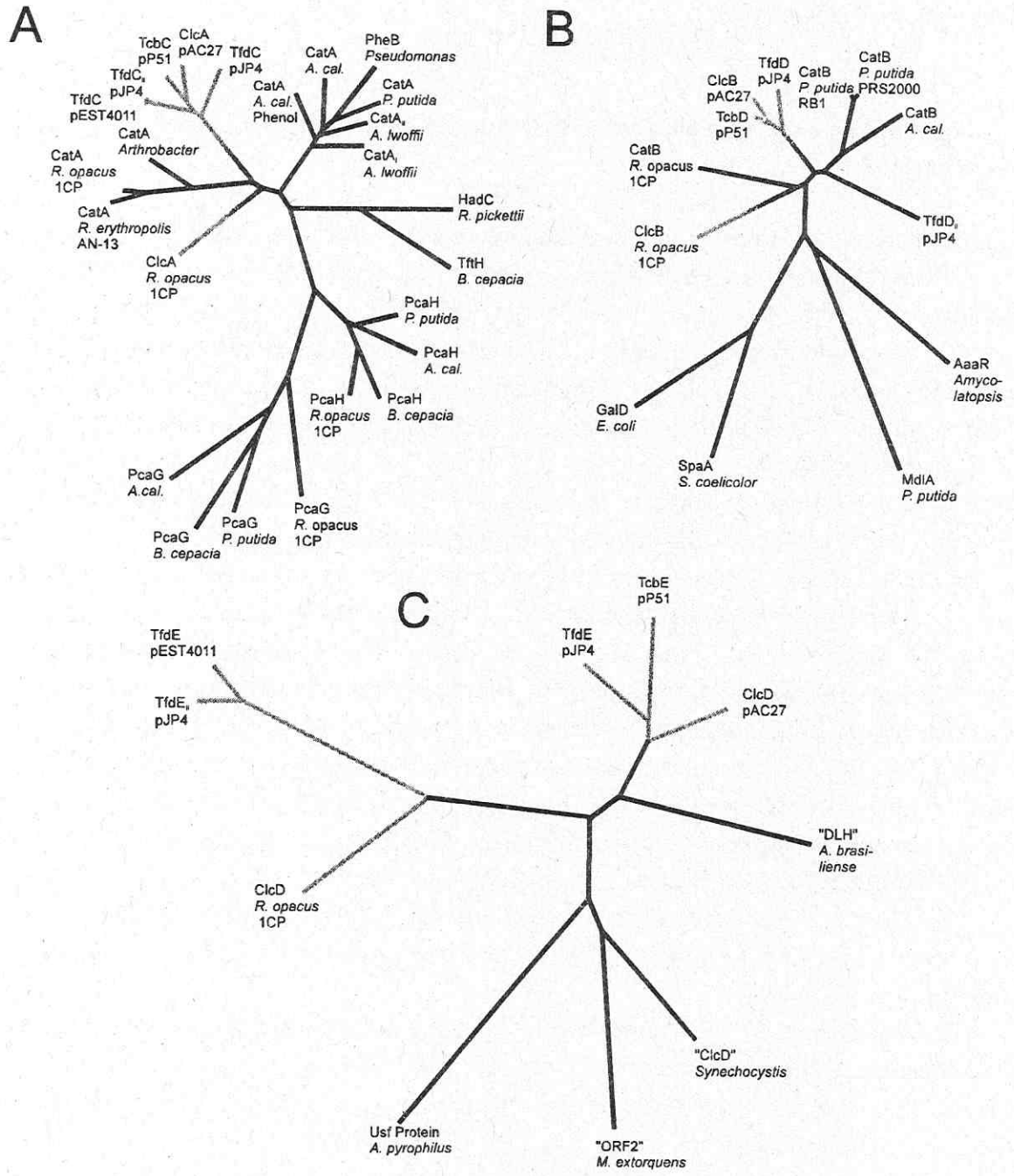

Fig. 2. Dendrograms showing the relatedness of catechol and chlorocatechol 1,2-dioxygenases (A), muconate and chloromuconate cycloisomerases (B), and dienelactone hydrolases (C). Proteins are referred to as gene products. Gray branches represent enzymes involved in chlorocatechol catabolic pathways, black branches enzymes of catechol pathways and proteins used as outgroups. Branch lengths are drawn to the same scale. The dendrograms have been taken from reference [36], with dendrogram A having been updated by addition of two new sequences [38].

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References

[1] Sayler, G.S. and Layton, A.C. (1990) Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. 44, 625-648.

[2] Steffan, J.R. and Atlas, R.M. (1991) Polymerase chain reaction: applications in environmental microbiology. Annu. Rev. Microbiol. 45, 137-161.

[3] Knaebel, D.B. and Crawford, R.L. (1995) Extraction and purification of microbial DNA from petroleum-contaminated soils and detection of low numbers of toluene, octane and pesticide degraders by multiplex polymerase chain reaction and Southern analysis. Mol. Ecol. 4, 579-591.

[4] Amann, R.I., Ludwig, W. and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143-169.

[5] Ferris, M.J., Muyzer, G. and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. 62, 340-346.

[6] Muyzer, G., Hottenträger, S., Teske, A. and Wawer, C. (1996) Denaturing gradient gel electrophoresis of PCR-amplified 16S rRNA – a new molecular approach to analyse the genetic diversity of mixed microbial communities. Molecular Microbial Ecology Manual 3.4.4, 1-23.

[7] Stoner, D.L., Browning, C.K., Bulmer, D.K., Ward, T.E. and MacDonell, M.T. (1996) Direct 5S rRNA assay for monitoring mixed-culture bioprocesses. Appl. Environ. Microbiol. 62, 1969-1976.

[8] Picard, C., Ponsonnet, C., Paget, E., Nesme, X. and Simonet, P. (1992) Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. Environ. Microbiol. 58, 2717-2722.

[9] Young, C.C., Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R. and Hinton, S.M. (1993) Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl. Environ. Microbiol. 59, 1972-1974.

[10] Volossiouk, T., Robb, E.J. and Nazar, R.N. (1995) Direct DNA extraction for PCR-mediated assays of soil organisms. Appl. Environ. Microbiol. 61, 3972-3976.

[11] Ghisalba, O. (1983) Chemical wastes and their biodegradation – an overview. Experientia 39, 1247-1257.

[12] Häggblom, M.M. (1992) Microbial breakdown of halogenated aromatic pesticides and related compounds. FEMS Microbiol. Rev. 103, 29-72.

[13] Chatterjee, D.K. and Chakrabarty, A.M. (1982) Genetic rearrangements in plasmids specifying total degradation of chlorinated benzoic acids. Mol. Gen. Genet. 188, 279-285.

[14] Don, R.H., Weightman, A.J., Knackmuss, H.-J. and Timmis, K.N. (1985) Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP134(pJP4). J. Bacteriol. 161, 85-90.

[15] van der Meer, J.R., van Neerven, A.R.W., de Vries, E.J., de Vos, W.M. and Zehnder, A.J.B. (1991) Cloning and characterization of plasmid-encoded genes for the degradation of 1,2-dichloro-, 1,4-dichloro-, and 1,2,4-trichlorobenzene of *Pseudomonas* sp. strain P51. J. Bacteriol. 173, 6-15.

[16] Frantz, B. and Chakrabarty, A.M. (1987) Organization and nucleotide sequence determination of a gene cluster involved in 3-chlorocatechol degradation. Proc. Natl. Acad. Sci. USA 84, 4460-4464.

[17] Perkins, E.J., Gordon, M.P., Caceres, O. and Lurquin, P.F. (1990) Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. 172, 2351-2359.

[18] van der Meer, J.R., Eggen, R.I.L., Zehnder, A.J.B., de Vos, W.M. (1991) Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: evidence for specialization of catechol 1,2-dioxygenases for chlorinated substrates. J. Bacteriol. 173, 2425-2434.

[19] Schlömann, M. (1994) Evolution of chlorocatechol catabolic pathways. Conclusions to be drawn from comparisons of lactone hydrolases. Biodegradation 5, 301-321.

[20] Amy, P.S., Schulke, J.W., Frazier, L.M. and Seidler, R.J. (1985) Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. 49, 1237-1245.

[21] Mäe, A.A., Marits, R.O., Ausmees, N.R., Kõiv, V.M. and Heinaru, A.L. (1993) Characterization of a new 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011: physical map and localization of catabolic genes. J. Gen. Microbiol. 139, 3165-3170

[22] Ka, J.O., Holben, W.E. and Tiedje, J.M. (1994) Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading bacteria isolated from 2,4-D-treated field soils. Appl. Environ. Microbiol. 60, 1106-1115.

[23] Fulthorpe, R.R., McGowan, C., Maltseva, O.V., Holben, W.E. and Tiedje, J.M. (1995) 2,4-Dichlorophenoxyacetic acid-degrading bacteria contain mosaics of catabolic genes. Appl. Environ. Microbiol. 61, 3274-3281.

[24] Top, E.M., Holben, W.E. and Forney, L.J. (1995) Characterization of diverse 2,4-dichlorophenoxyacetic acid-degradative plasmids islolated from soil by complementation. Appl. Environ. Microbiol. 61, 1691-1698.

[25] Holben, W.E., Schroeter, B.M., Calabrese, V.G.M., Olsen, R.H., Kukor, J.K., Biederbeck, V.O., Smith, A.E. and Tiedje, J.M. (1992) Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. Appl. Environ. Microbiol. 58, 3941-3948.

[26] Chapman, P.J. and Ribbons, D.W. (1976) Metabolism of resorcinylic compounds by bacteria: alternative pathways for resorcinol catabolism in *Pseudomonas putida*. J. Bacteriol. 125, 985-998.

[27] Spain, J.C. and Gibson, D.T. (1991) Pathway for biodegradation of *p*-nitrophenol in a *Moraxella* sp. Appl. Environ. Microbiol. 57, 812-819.

[28] Stolz, A. and Knackmuss, H.-J. (1993) Degradation of 2,4-dihydroxybenzoate by *Pseudomonas* sp. BN9. FEMS Microbiol. Lett. 108, 219-224.

[29] Bang, B.-W. and Zylstra, G.J. (1997) Cloning and sequencing of the hydroquinone 1,2-dioxygenase, g-hydroxymuconic semialdehyde dehydrogenase, maleylacetate reductase genes from *Pseudomonas fluorescens* ENV2030. Abstr. Annu. Meet. Am. Soc. Microbiol., abstr. Q-383, p. 519.

[30] Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussman, J.L., Verschueren, K.H.G. and Goldman, A. (1992) The α/β hydrolase fold. Protein Eng. 5, 197-211.

[31] Hartnett, G.B. and Ornston, L.N. (1994) Acquisition of apparent DNA slippage structures during extensive evolutionary divergence of *pcaD* and *catD* genes encoding identical catalytic activities in *Acinetobacter calcoaceticus*. Gene 142, 23-29.

[32] Maltseva, O.V., Solyanikova, I.P. and Golovleva, L.A. (1994) Chlorocatechol 1,2-dioxygenase from *Rhodococcus erythropolis* 1CP. Kinetic and immunochemical comparison with analogous enzymes from Gram-negative strains. Eur. J. Biochem. 226, 1053-1061.

[33] Maltseva, O.V., Solyanikova, I.P., Golovleva, L.A., Schlömann, M. and Knackmuss, H.-J. (1994) Dienelactone hydrolase from *Rhodococcus erythropolis* 1CP: purification and properties. Arch. Microbiol. 162, 368-374.

[34] Solyanikova, I.P., Maltseva, O.V., Vollmer, M.D., Golovleva, L.A. and Schlömann, M. (1995) Characterization of muconate and chloromuconate cycloisomerase from *Rhodococcus erythropolis* 1CP: indications for functionally convergent evolution among bacterial cycloisomerases. J. Bacteriol. 177, 2821-2826.

[35] Eulberg, D., Golovleva, L.A. and Schlömann, M. (1997) Characterization of catechol catabolic genes from *Rhodococcus erythropolis* 1CP. J. Bacteriol. 179, 370-381.

[36] Eulberg, D., Kourbatova, E.M., Golovleva, L.A. and Schlömann, M. Evolutionary relationship between chlorocatechol catabolic enzymes from *Rhodococcus opacus* 1CP and their counterparts in proteobacteria: sequence divergence and functional convergence. J. Bacteriol., in press.

[37] Ka, J.O., Holben, W.E. and Tiedje, J.M. (1994) Use of gene probes to aid in recovery and identification of functionally dominant 2,4-dichlorophenoxyacetic acid-degrading populations in soil. Appl. Environ. Microbiol. 60, 1116-1120.

[38] Kim, S.I., Leem, S.-H., Choi, J.-S., Chung, Y.H., Kim, S., Park, Y.-M., Park, Y.K., Lee, Y.N. and Ha, K.-S. (1997) Cloning and characterization of two *catA* genes in *Acinetobacter lwoffii* K24. J. Bacteriol. 179, 5226-5232.

Investigation of PAH degrading bacteria from wastewater and other habitats by molecular and cultural techniques

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Abstract

Bacteria with the ability to degrade polyaromatic hydrocarbons like phenanthrene and naphthalene were isolated from different sites. The strains were analyzed for their taxonomic position using different identification methods, such as 16S rRNA typing, fatty acid methylester analysis and biochemical tests in microplates. The isolates were examined for the presence of an initial PAH dioxygenase and a catechol 2,3-dioxygenase using a PCR-based approach. Oligonucleotide probes for the detection of these genes were developed on the basis of existing and newly retrieved sequences. These probes should be useful in subsequent investigations to detect potential for PAH degradation in environmental samples directly, avoiding the need for prior cultivation.

1. Introduction

Polyaromatic hydrocarbons (PAH) are widespread environmental contaminants [1] containing two or more condensed benzene rings. They are formed during incomplete combustion of a variety of materials such as fossil fuels and organic compounds. The increase in anthropogenic industrial activities resulted in an increase of the PAH-concentration in the environment during the last century. Because of the genotoxicity of several PAHs and their metabolites there is a growing interest to develop technologies to detoxify PAH-contaminated wastes.

PAHs like many other xenobiotic compounds can be degraded biologically by certain bacteria [2, 3]. These organisms are not all closely related but belong to different phylogenetic groups. Some members of these groups possess capabilities for PAH degradation since this depends on the presence of distinct enzymes, namely dioxygenases. These dioxygenases catalyze keysteps within the breakdown of PAHs. The first important enzyme is the initial PAH dioxygenase, whereas the activity of the catechol 2,3-dioxygenase completes the PAH-degradation, catalyzing the breakdown of single aromatic rings. The genes for the dioxygenases can be used as targets for specific detection of bacteria containing metabolic trait.

2. Materials and methods

2.1. Bacterial strains

The following bacterial strains were used as reference organisms in this study: *Pseudomonas putida* OUS 82, *Sphingomonas yanoikuyae* DSM 6900, *Mycobacterium* sp. DSM 7251 and *Mycobacterium* VF1, described as PAH degrading strains [4, 5, 6]. *Pseudomonas putida* KT2440 [7] as negative control for catechol 2,3-dioxygenase amplification and *E. coli* SURE (Stratagene, Heidelberg) without any PAH degradation ability.

2.2. Characterization of sampling sites, isolation procedure and monitoring mineralization of naphthalene and phenanthrene

Aquatic samples investigated in the experiments were collected from two different waste treatment water plant and leakage water from a waste disposal site. Soil samples were taken from a parking place and a tar oil contaminated soil. The samples were used for selective enrichment of naphthalene- and phenanthrene-degrading bacteria.

Mixed liquor and bulk soil samples from terrestric sites were collected in sterilized jars. Serial dilutions of these samples were prepared with 0,9 % NaCl solution. The soil samples were previously shaken with sodium pyrophosphate solution (2,0 g/l). 25 ml of a basal mineral medium described by Kästner et al. [3] was enriched with 0,05 % naphthalene or phenanthrene as the sole source of carbon and energy and inoculated with an aliquot of the dilutions. The samples were cultivated aerobically at a temperature of 25 °C. Single naphthalene- or phenanthrene-degrading strains were isolated by repeated streaking of individual colonies on solid R2A-medium (Oxoid, Wesel, FRG) and checked for metabolization of PAH. The mineralization of the PAHs was quantitatively examined by gaschromatographic determination of the remaining PAHs in the medium during five days. The increase of optical density was measured simultaneously.

2.3. Identification of PAH-degrading isolates

The isolates were identified by whole cell hybridization with rRNA-targeted oligonucleotides [8], determination of fatty acid patterns using the MIDI system [9], as well as by morphological appearance and 90 physiological tests performed in microplates [10].

2.4. Microbial community analysis

The composition of microbial community was examined by staining the cells with DAPI (4′,6′-Diamidino-2-phenylindol-dihydrochlorid-dilactat) and in situ hybridization with different rRNA targeted oligonucleotides (EUB [11], PS [12], ACI, COM [13]).

2.5. DNA preparation and analysis

Bacterial reference strains and isolates were grown in yeast-extract/trypton-medium up to an optical density (600 nm) of maximally 1.0. Bacterial DNA was purified using standard procedures [14].

2.6. PCR primer and internal oligonucleotide probe design and PCR amplification

Primers for amplification of fragments of the initial PAH dioxygenase and catechol 2,3-dioxygenase and internal oligonucleotide probes were selected using the Lasergene software (DNASTAR, Inc., Madison, WI, USA). For catechol 2,3-dioxygenase amplification additonally a pair of primers described by Wikström et al. [15] was used. PCR amplification was performed using standard protocols [16] with different annealing temperatures according to the particular primers. PCR fragments were separated on 1.0 % agarose gels, stained with ethidiumbromide and transferred on positively charged nylonmembranes (Amersham, Buckinghamshire, UK).

2.7. Cloning and sequencing of PCR fragments

PCR fragments were ligated with the vector Bluescript II SK and transformed into *E. coli* DH5α as described previously [16]. Recombinant plasmids were isolated by QIAprep-Spin-Mini Kit as described by the manufacturer (Qiagen, Hilden). The cloned PCR fragments were sequenced by cycle sequencing using a GATC 1500 direct blotter according to the manufacturers instructions (GATC, Konstanz, FRG).

2.8. Labelling and hybridization of nucleic acids

Oligonucleotides with about 20 nucleotides were labelled with peroxidase [17]. Southern blots were hybridized according to the different oligonucleotides with variable stringency. Using enhanced chemiluminescence the bound probes are detected as described by the manufacturer (Amersham, Buckinghamshire, UK).

3. Results and discussion

3.1. Isolates with phenanthrene and/or naphthalene degradation ability

From the wastewater sludge of the Giessen plant, 14 isolates with phenanthrene or naphthalene degradation capabilities were isolated (Table 1). Four of them were identified as *Pseudomonas fluorescens*, three as *Acidovorax* sp., two as *Comamonas testosteroni*, and the other five PAH

degraders were identified as *Pseudomonas putida, P. mendocina, Comamonas terrigena, Rhodococcus erythropolis,* and *Sphingomonas* sp.. The three *Acidovorax* strains are able to degrade phenanthrene as sole source of carbon and energy whereas the other isolates metabolized naphthalene instead. The other two aquatic isolates, a *Sphingomonas* sp. and a *Rhodococcus rhodochrous* strain, also metabolize only naphthalene. The two terrestric isolates, a *Pseudomonas fluorescens* strain and an alpha-proteobacterium, on the other hand are able to degrade both substances. The isolated PAH-degraders belong to a heterogenous assemblage of different bacterial groups; strains of the alpha-, beta- and gamma subdivision of Proteobacteria as well as Gram positive bacteria with high GC content of DNA are able to degrade PAHs.

Table 1. Sampling sites and taxonomic affiliation of the isolated bacteria with naphthalene and/ or phenanthrene degrading ability

Sample sites	Isolate	Degradation ability
Wastewater plant, Giessen	*Acidovorax* sp. (isolate E)	phenanthrene
	Acidovorax sp. (isolate E10)	phenanthrene
	Acidovorax sp. (isolate V10)	phenanthrene
	Pseudomonas fluorescens (isolate F9)	naphthalene
	Pseudomonas fluorescens (isolate H9)	naphthalene
	Pseudomonas fluorescens (isolate T9)	naphthalene
	Pseudomonas fluorescens (isolate A10)	naphthalene
	Pseudomonas putida (isolate F)	naphthalene
	Pseudomonas mendocina (isolate L7)	naphthalene
	Comamonas testosteroni (isolate G)	naphthalene
	Comamonas testosteroni (isolate H)	naphthalene
	Comamonas terrigena (isolate M7)	naphthalene
	Rhodococcus erythropolis (isolate G10)	naphthalene
	Sphingomonas sp. (isolate Y9)	naphthalene
Waste disposal site, leakage water, Reiskirchen	*Sphingomonas* sp. (isolate E3)	naphthalene
Industrial wastewater plant, Frankfurt	*Rhodococcus rhodochrous* (isolate B1)	naphthalene
Soil from parking place, Giessen	*Pseudomonas fluorescens* (isolate C4)	naphthalene, phenanthrene
Tar oil contaminated soil, Hulin	α-Proteobacterium (isolate 3)	naphthalene, phenanthrene

3.2. Analysis of the microbial community of the wastewater sewage

Since the highest number of isolates could be obtained from the municipal sewage plant a sample from this habitat were examined more thoroughly for the inhabiting populations. Total

cell counts of the sample using the DNA-binding dye DAPI were determined as 1,9 x 10^8 cells ml^{-1} mixed liquor. Differentiation of the microbial community was achieved by in situ hybridization with rRNA-targeted oligonucleotide probes. This approach allows cultivation-independent detection of metabolic active cells in activated sludge. A general probe detecting all bacteria (EUB) as well as probes specific for genera to which the isolates from this sample belong were used. The proportions of the hybridized cells relative to the DAPI stain were estimated. With probe EUB approximately 70 % of DAPI-stained cells yielded hybridization signals. This value is in the normal range for activated sludge samples [8]. With probes specific for the genera *Pseudomonas*, *Comamonas*, and *Acidovorax* between 1 and 5 % of the DAPI-stained cells showed hybridization signals. These values show that the three genera including most of the isolates from the municipal sludge comprise an important part of the active microbial community.

3.3. Degradation and growth kinetics of isolates during phenanthrene and naphthalene metabolization

The degradation of phenanthrene by different isolates provided similar results. The phenanthrene content shared approximately a decrease proportional with time. After five days about 60 % of the original concentration was metabolized. During this time period the optical density increased continuously. In Figure 1 the phenanthrene disappearance and the increase of optical density of isolate E (*Acidovorax sp.*) and isolate C4 (*P. fluorescens*) is shown. In contrast to phenanthrene degradation the metabolization of naphthalene is faster; after two days about 80 % are degraded. The kinetic of naphthalene degradation and the increase of optical density of isolate T9 (*P. fluorescens*) and isolate G10 (*R. erythropolis*) are exemplary shown in Figure 2. It is striking that the cell growth of the two Gram-positive *Rhodococcus* strains with naphthalene as carbon source are very slow.

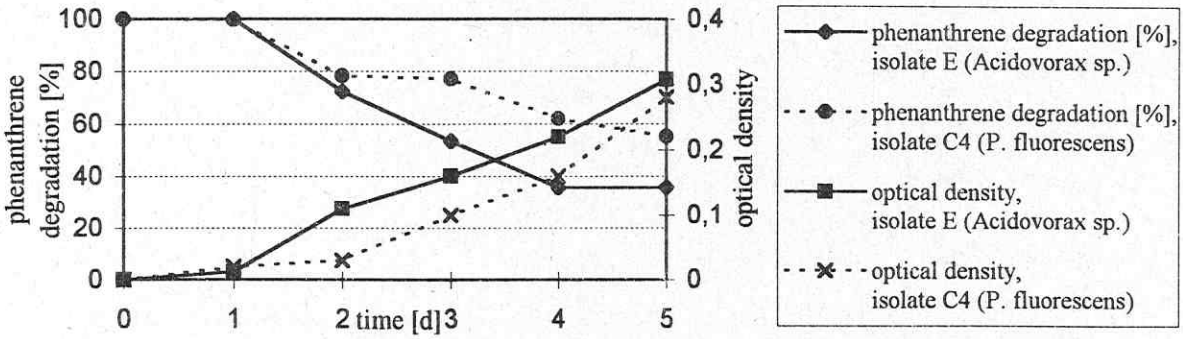

Fig. 1. Gaschromatographically determined phenanthrene degradation by isolate E (*Acidovorax* sp.) and by isolate C4 (*P. fluorescens*) and the photometrically measured optical density of the culture over a period of five days.

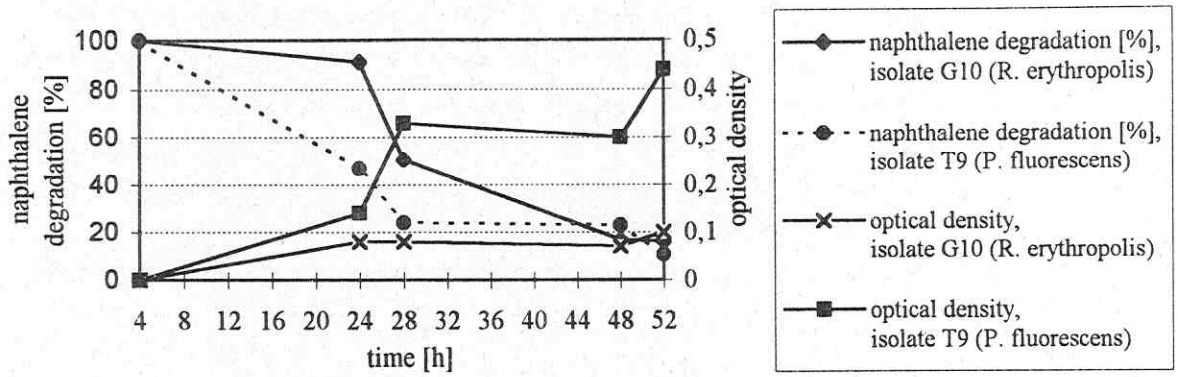

Fig. 2. Gaschromatographically determined naphthalene degradation by isolate T9 (*P. fluorescens*) and by isolate G10 (*R. erythropolis*) and the photometrically measured optical density of the culture over a period of 52 hours.

Both the isolated bacteria C4 (*P. fluorescens*) and F (*P. putida*) and the reference strains which were the positive controls belonging to the genus *Pseudomonas* all yielded unique PCR-amplified DNA-fragments of one predicted size. Isolated strains not belonging to the genus *Pseudomonas* but showing the ability to degrade naphthalene and/or phenanthrene however, showed either quite different PCR-fragment patterns or weak fragment signals of the predicted size.

In summary, it is possible to amplify DNA fragments of the genus *Pseudomonas* which are able to degrade naphthalene and/or phenanthrene with the naphthalene dioxygenase primers constructed. On the other hand initial enzymes of non-*Pseudomonas* strains containing nah/phe-dioxygenases are quite different from the *Pseudomonas* naphthalene dioxygenases.

In order to investigate the use of primers specific for nah/phe-dioxygenase of strains not belonging to the genus *Pseudomonas*, it is necessary to acquire more sequence information about their initial dioxygenases. This could be achieved by sequencing amplified PCR fragments and/or the cloning and sequencing of selected initial nah/phe-dioxygenases of bacterial isolates.

3.5. Catechol 2,3-dioxygenase

The catechol 2,3-dioxygenase catalyzes the extradiol cleavage of catechol, an important intermediate of the degradation of aromatic and polyaromatic compounds. It consists of four identical subunits with about 320 aminoacids and one ferrous ion per subunit [19]. 20 DNA sequences of catechol 2,3 dioxygenase genes are already published. 12 of the 20 gene sequences show a homology among 45.0 % (*S. yanoikuyae* B1 and a *Bacillus subtilis* strain) and 90.5 % (*P. putida* 35x and *P. putida* H). This group of homologous sequences is divided into two subgroups, on the one hand eight different *Pseudomonas* strains, one *Bacillus subtilis* and one *Alcaligenes* sp. KF711 strain with sequence similarities of at least 73 % (named "*Pseudomonas*"-group) and on the other hand two *Sphingomonas* sp. with 76.5 % similarity

(named "*Sphingomonas*" group). The similarities of these subgroups among each other are among 51 and 45 %. In comparison, the other eight sequences are only about 30 % or less similar. The two *R. rhodochrous* sequences for example are only 25.4 % homologous. Amplification primers and probes for detection of this gene are initially based on the alignment of these homologous sequences. A pair of primers published by Wikström et al. [15] was tested for its potential to amplify gene fragments of the catechol 2,3-dioxygenase from the environmental isolates. Table 2 shows the results of PCR amplification with this primer pair.

Table 2. Results of PCR reaction with a published pair of primers. The length of amplified fragment is expected to be about 700 bp. The identity of the amplificates was checked by hybridization with an internal oligonucleotide.

Bacterial strains	Amplificate
Reference strains	
P. putida OUS82	yes
Mycobacterium sp. DSM 7251	no
Mycobacterium VF1	no
S. yanoikuyae DSM 6900	yes
P. putida KT2440	no
E. coli SURE	no
Environmental isolates	
Acidovorax sp. (isolate E)	no
Acidovorax sp. (isolate E10)	no
Acidovorax sp. (isolate V10)	no
P. fluorescens (isolate F9)	yes
P. fluorescens (isolate H9)	yes
P. fluorescens (isolate T9)	yes
P. fluorescens (isolate A10)	yes
P. putida (isolate F)	yes
P. mendocina (isolate L7)	no
C. testosteroni (isolate G)	no
C. testosteroni (isolate H)	no
C. terrigena (isolate M7)	no
R. erythropolis (isolate G10)	no
Sphingomonas sp. (isolate Y9)	yes
Sphingomonas sp. (isolate E3)	yes
R. rhodochrous (isolate B1)	no
P. fluorescens (isolate C4)	yes
α-Proteobacterium (isolate 3)	no

Results show that the *Pseudomonas* and *Sphingomonas* isolates are able to degrade naphthalene and/or phenanthrene, own a catechol 2,3-dioxygenase and that their respective

genes are recordable with the described pair of primers. The negative results could either be due to low homology of the genes or a complete lack of a catechol 2,3-dioxygenase.

The partial sequencing of PCR amplificates from different strains and isolates established the affiliation to either the "*Pseudomonas*"group or "*Sphingomonas*" group. Isolate F a *P. putida* strain and isolate C4 a *P. fluorescens* strain belong to the "*Pseudomonas*" group with about 75 % similarity to *P. putida* P35 x. On the other hand, the partial sequences of isolate E3 a *Sphingomonas* sp.strain and the reference strain *S. yanoikuyae* DSM 6900 are more than 75 % similar to the catechol 2,3-dioxygenase sequence of *S. yanoikuyae* B1. The further sequencing of the amplified PCR-fragments of the other isolates will establish if all catechol 2,3-dioxygenase sequences of the *Pseudomonas* isolates belong to the "*Pseudomonas*"-group and those of the *Sphingomonas* isolates to the "*Sphingomonas*"-*group.*

The development of oligonucleotides for the detection of catechol 2,3-dioxygenase is based on the alignment of published sequences and the additionally obtained sequence information. One probe should be used for detection of the genes of the "*Pseudomonas*"-group and the other probe should detect the corresponding sequences belonging to "*Sphingomonas*"-group. The specificities of these probes were tested by hybridization of the separated and blotted PCR-fragments of the different strains with both probes. While the amplified fragments of *S. yanoikuyae* DSM 6900, *Sphingomonas* sp. (isolate E3) and *Sphingomonas* sp. (isolate Y9) can be detected with the "*Sphingomonas*"-probe, the PCR-amplificates of the *Pseudomonas* strains (*P. putida* OUS 82, isolate F, C4, F9, H9 and T9) showed no hybridization signals, as expected. The PCR fragments of different *Pseudomonas* strains, *P. putida* (OUS82 and isolate F), *P. fluorescens* (isolate C4, F9, H9, T9 and A10) and *P. mendocina* (isolate L7) can be detected using the "*Pseudomonas*" probe, but the PCR-amplificates of the *Sphingomonas* strains (DSM 6900, isolate E3 and Y9) showed no hybridization signals. The two probes seem to be suitable for specific detection of catechol 2,3-dioxgenase sequences belonging to "*Pseudomonas*"- or "*Sphingomonas*"-group.

In future the developed nucleic acid probes should be used for the detection of the initial PAH dioxygenase and catechol 2,3-dioxygenase sequences in environmental samples to monitor the potential for degradation of PAHs without previous cultivation.

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References

[1] Jacob, J., Grimmer, G. and Hildebrandt, A. (1997) Long-term decline of atmospheric and marine pollution by polycyclic aromatic hydrocarbons (PAHS) in Germany. Chemosphere 34, 2099-2108

[2] Heitkamp, M.E. and Cerniglia, C.E. (1988) Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment between an oil field. Appl. Environ. Microbiol. 54, 1612-1614

[3] Kästner, M., Breuer-Jammali, M. and Mahro, B. (1994) Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl. Microbiol. Biotechnol. 41, 267-273

[4] Takizawa, N., Kaida, N., Torigoe, S., Moritani, T., Sawada, T., Satoh, S., Kiyohara, H. (1994) Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydogenase in *Pseudomonas putida* OUS82. J. Bacteriol. 176, 2444-2449

[5] Gibson, D.T., Roberts, R.L., Wells, M.C. and Kobal, V.M. (1972) Oxidation of biphenyl by a *Beijerinckia* species. Biochem. Biophys. Res. Com. 50, 211-219

[6] Kelley, I., Freeman, J.P., Evans, F.E. and Cerniglia, C.E. (1993) Identification of metabolites from degradation of fluoranthene by *Mycobacterium* sp. strain PYR-1. Appl. Environ. Microbiol. 59, 800-806

[7] Franklin, F.C., Bagdasarian, H.M., Bagdasarian, M.M. and Timmis, K.N. (1981) Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of genes for the entire regulated aromatic ring meta cleavage pathway. Proc. Natl. Acad. Sci. 78, 7458-7462

[8] Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Reviews 59, 143-169

[9] Kämpfer, P., Erhart, R., Beimfohr, C., Böhringer, J., Wagner, M. and Amann, R. (1996) Characterization of bacterial communities from activated sludge: Culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probe. Microbiol. Ecol. 32, 101-121

[10] Kämpfer, P., Steiof, M., and Dott, W. (1991) Microbiological charactrization of a fuel-oil contaminated site including numerical identification of heterotrophic water and soil bacteria. Microbiol. Ecol. 21, 227-251

[11] Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990): Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environm. Microbiol. 56, 1919-1925

[12] Schleifer, K.-H., Amann, R., Ludwig, W., Rothemund, C., Springer, N. and Dorn, S. (1992) Nucleic acid probes for the identification and *in situ* detection of pseudomonads. *In*: E. Galli, S. Silver, and B. Witholt (eds.). Pseudomonas: Molecular Biology and Biotechnology. American Society for Microbiology, Washington. p. 127-134

[13] Amann, R., Ludwig, W., Schulze, R., Spring, S., Moore, E. and Schleifer, K.-H. (1996) rRNA-targeted oligonucleotide probes for the identification of genuine and former pseudomonads. System. Appl. Microbiol. 19, 501-509

[14] Wilson, K. (1994) Preparation of genomic DNA from bacteria. *In*: Chanda, V.B. (ed.), Current Protocols in Molecular Biology, John Wiley & Sons Inc., NY, Unit 2.4, pp. 2.4.1-2.4.5

[15] Wikström, P., Wiklund, A., Anderson, A.C. and Forsman, M. (1996) DNA recovery and PCR quantification of catechol 2,3-dioxygenase genes from different soil types. J. Biotechnol. 52, 107-120

[16] Sambrook, J., Fritsch, E.F. and Maniatis, T.(1989) Molecular cloning: a laboratory manual. 2. Auflage. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

[17] Wagner, M., Amann, R., Lemmer, H. and Schleifer, K.H. (1993) Probing activated sludge with proteobacteria-specific oligonucleotides: inadequacy of culture-dependent methods for describing microbial community structure. Appl. Environ. Microbiol. 59, 1520-1525

[18] Simon, M.J., Osslund, T.D., Saunders, R., Ensley, D.B., Suggs, S., Harcourt, A., Suen, W.C., Cruden, D.L., Gibson, D.D. and Zylstra, G.J. (1993) Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene 127, 31-37

[19] Harayama, S. and Rekik, M. (1989) Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. J. Biological Chemistry 264, 15328-15333

- Strategies to enhance biodegradation -

Use of special bacterial cultures to improve the biological efficiency of a trickling filter

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Abstract

To improve the biological efficiency of an old overloaded, functional disturbed trickling filter of a waste water treatment plant with inhibitants of the catchment area and population equivalents of 4500 p.e. special bacterial preparations were added. According to the operation instructions of the manufacturer a defined dosage of two preparations was given into the feed channel of the trickling filter over a period of about 4 months. The influence on the degradation of the organic load and the efficiency of nitrification were monitored over a period of about 2 years. The COD-concentration of the trickling filter effluent could be reduced from an arithmetical value of 123 mg/l before dosage to 56 mg/l after treatment. This increased degradation rate was maintained without further addition of bacterial suspension. The proposed goal to guarantee the control value of COD in the effluent of the plant with a sufficient safety factor could be achieved up to the date the plant was closed. Beyond that also the efficiency of nitrification could be increased significantly. Related to the whole time of monitored period the additional cost of treating one m^3 of waste water amounted to only about 0,007 DM/m^3.

1. Introduction

The RUHRVERBAND as one of the major water associations in North Rhine-Westphalia ensures that the river Ruhr has enough water for the supply of about 5 million people throughout the year. Besides this, it checks the Ruhr river's water quality. The RV plans, constructs and operates waste water treatment plants. One of its goals is to develop effective and environmental friendly solutions to increase the biological efficiency of the purification process.

One project in this field was to improve the biological efficiency of an old overloaded trickling filter of a waste water treatment plant with inhabitants of the catchment area and

population equivalents of 4500 p.e. As a result of an accident with latex contaminated industrial waste water the hydraulic conditions and oxygen supply of the trickling filter dramatically decreased due to the obstructed filter medium. Therefore the control value of COD in the effluent of the plant of 140 mg/l could not be hold continuously. The practical solution had to be realised in a short time with low finical and personnel investment because the plant was to be closed in the near future (meanwhile realised).

In this case we investigated whether the addition of special bacterial cultures could improve the productivity of the trickling filter immediately up to the scheduled closing date. Two commercial lyophilised bacterial products available on the market containing different strains of obligate aerobic and facultative anaerobic organisms with special ability to decompose a variety of organic compounds like fats, oils, hydrocarbons and residues from the chemical industry including components of varnish and paint were used. In addition, these mixed cultures should be able to reduce the biofilm consisting of dead cells and polysaccharolytic slime. To support these processes special enzymes and non-ionic surfactants are contained in the chosen products.

2. Materials and methods

According to the operating instructions of the manufacturer the immobilized bacterial preparations (fixed on bruised grain) were resuspended with about 20 l of drinking water. Afterwards this suspension was aerated in 100 l of pre-settled waste water adding blood- and bone-meal as nutrients. This activated microbial suspension was given into the feed channel of the trickling filter (TF) for about 1 hour. The addition of the product was carried out over a period of about 4 months (April to August 1993). Initially (April/May) an amount of 700 g lyophilised preparation A and B were added daily. Later on the daily dosage was reduced to 300 g of each preparation per day.

The influence of the added special bacterial preparation was monitored by the parameters temperature (TF- effluent), $COD_{orig.}$ (TF- influent), $COD_{sett.}$ (TF- effluent), settleable solids, NO_3-N and the biological efficiency of COD degradation.

3. Results and discussion

Figure 1 shows the course of the COD-concentration of the waste water in- and effluent of the trickling filter during the entire investigation period of about 4 years. These results indicate that the organic load of the waste water influent ranged from 30 to 750 mg/l COD caused considerably by rainfalls. After an industrial accident the biological efficiency decreased. Consequently the organic load of the effluent increased. The control value laid down in the certificate of official approval (140 mg/l COD) could not be kept consequently before adding the bacterial preparation. Values up to 185 mg/l COD could be observed.

As expected, no measurable effects were observed immediately after the beginning of addition. On the contrary the increase of COD-concentration observed in the effluent

continued up to values of 218 mg/l. However, after a lag-phase of about 1 month a continuous, significant decrease in organic load (COD) was noted. Stable values in the range about 50 mg/l COD were obtained. This effect was maintained without further addition of bacterial suspension up to spring of 1995 when the plant was closed.

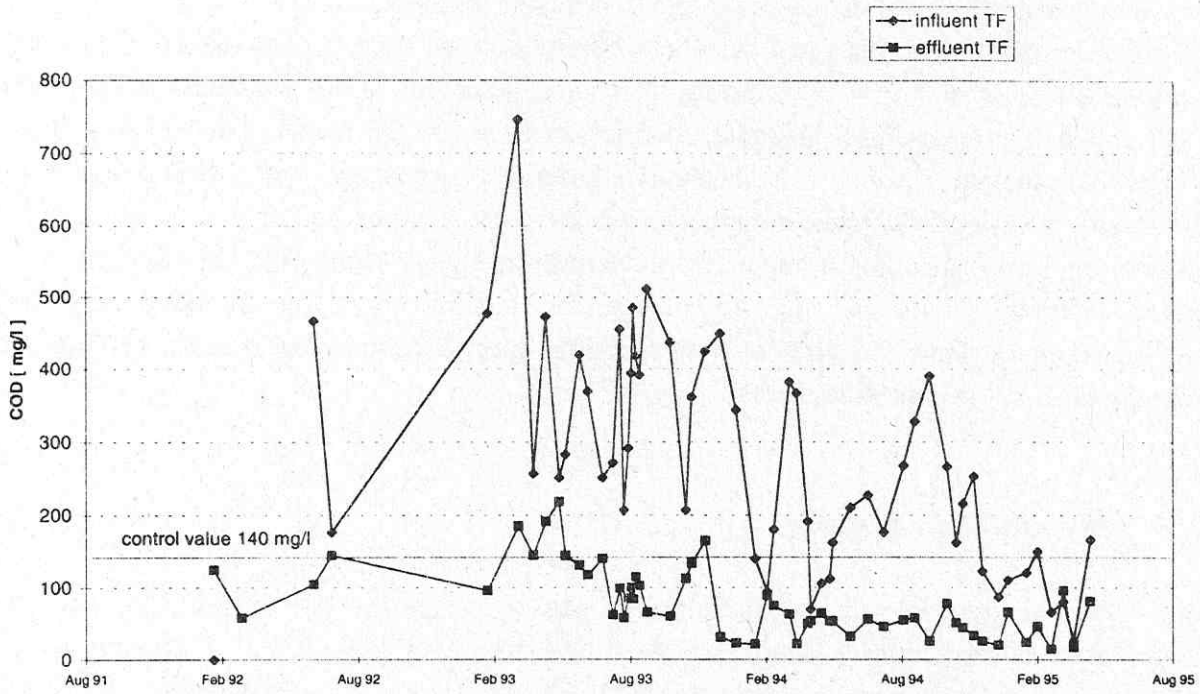

Fig. 1. COD-concentration in the influent and effluent of the trickling filter (TF)

The percentage of biological efficiency of the trickling filter with regard to COD is shown in Figure 2. In comparison with Figure 1 there is obviously a good correlation between the decrease in the biological efficiency and increase in COD-concentration during the period from 1992 to spring 1993 (before addition). A reduction in efficiency from values of 75 to 30 % could be observed. The addition of the special bacterial suspension resulted in a continuous increase in the efficiency which became stable at values of about 80 %. This situation could be kept stable beyond the period of dosage except winter months with temperatures below 10 °C (see Figure 3).

It is generally known that the bacterial activity is reduced with decreasing temperature. During our experiment we observed a similar temperature effect.

The influence of temperature on the activity of microorganisms is calculated by Tucek [1] according following equation:

$$K_t = K_{20} \times 1{,}047^{T-20}$$

where

K_t = activity coefficient of given temperature (T)
K_{20} = activity coefficient at temperature of 20 °C
T = temperature in °C

Consequently there is an activity change of 4,7 % per degree of temperature change.

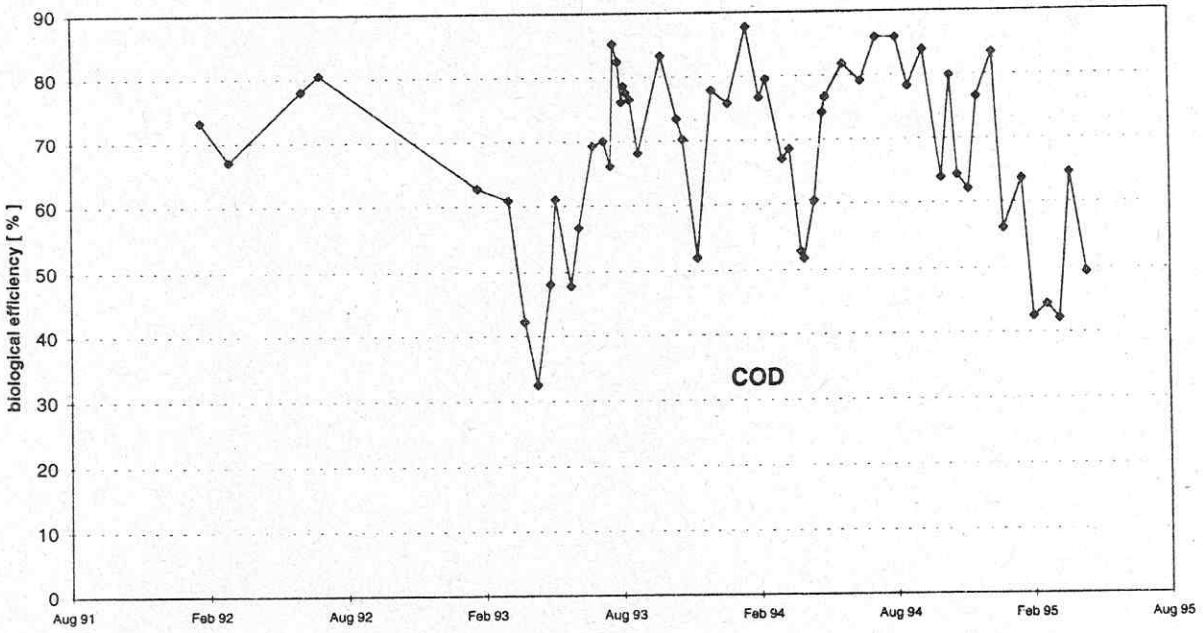

Fig. 2. Biological efficiency of the trickling filter (TF)

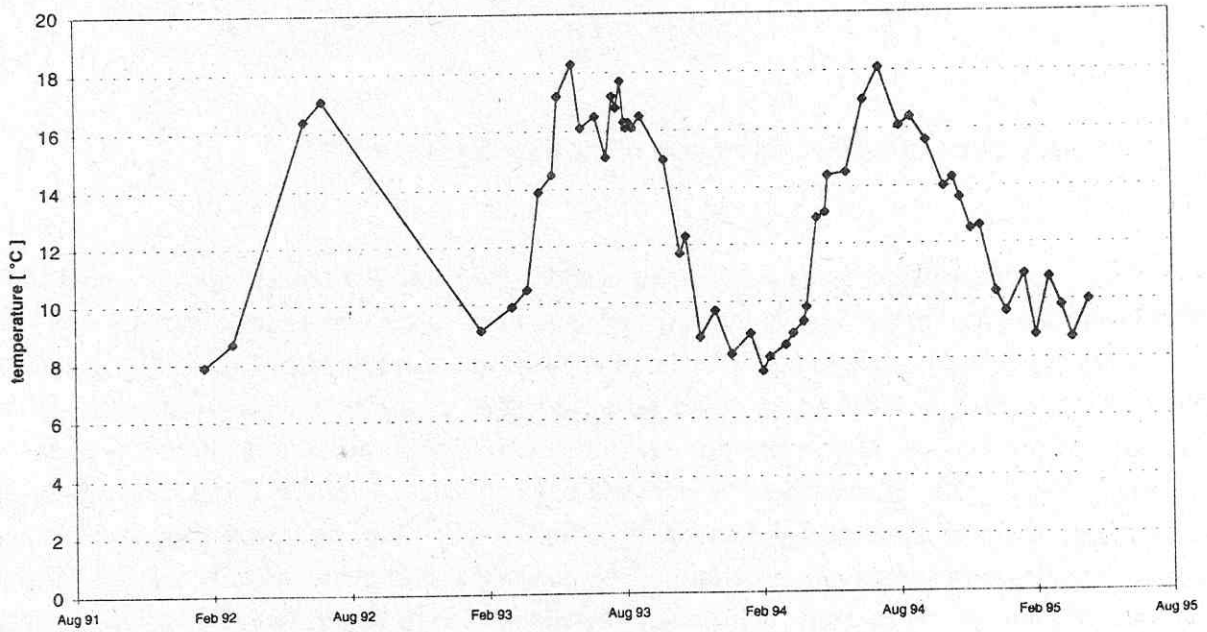

Fig. 3. Temperature in the effluent of the trickling filter (TF)

A decrease in biological efficiency in the same dimension was reported by Weston [2] and Imhoff [3]. Our results confirmed this. The efficiency of the biological activity of the trickling filter depends on the seasonal temperature fluctuation (compare Figure 2 and Figure 3). The activity decreased about 40 to 50 % when the water temperature dropped from 17 to 9 °C. In

the case of a temperature difference of 10°C following the equation of Tucek a reduced activity in the same dimension could be calculated. Subsequently when the waste water temperature increased in the summer months of 1994 the level of 80 % biological efficiency was reached again, although the dosage of bacterial suspension had been stopped at the end of August 1993. It follows that the reduction in the biological efficiency is strictly correlated with the decreased waste water temperature.

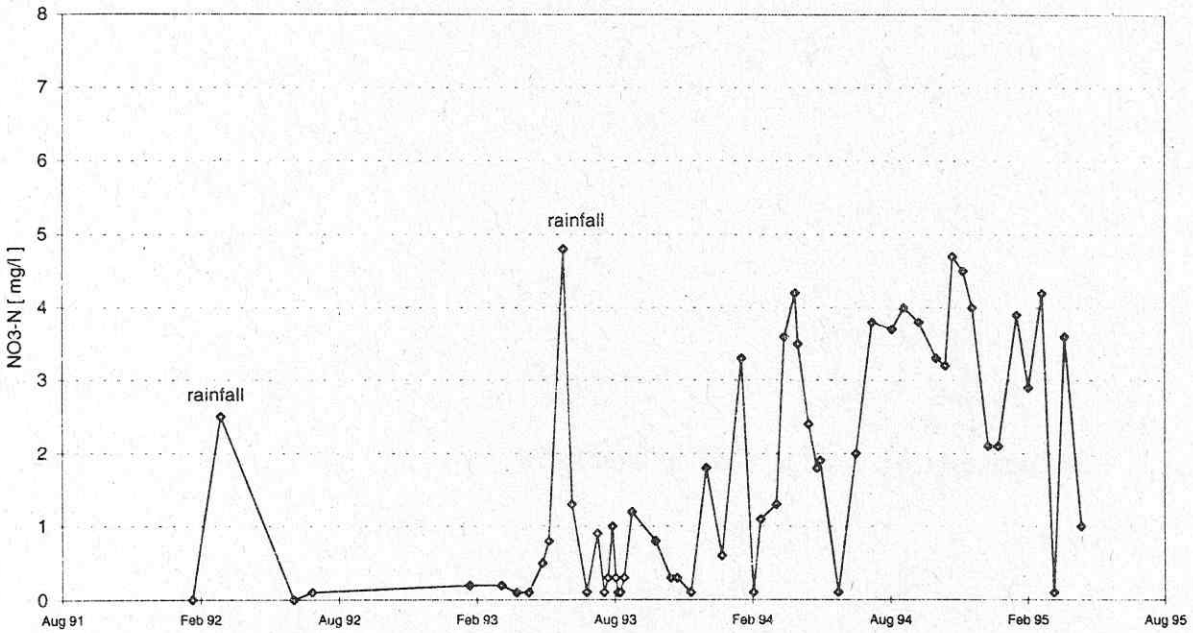

Fig. 4. NO_3-N concentration in the effluent of the trickling filter (TF)

It was not possible to make a statement whether the improved effluent quality could have been maintained for further time because the running of the plant was stopped in April 1995.

As to the NO_3-N- concentration in the effluent of the trickling filter from 1992 to 1995 a significant increase in nitrification could be observed in connection with the dosage of the biological preparations. Before dosage the measured concentration was normally about < 1,0 mg/l NO_3-N, after dosage the concentration reached values between 2 and 4 mg/l NO_3-N. That means the postulated partial degradation of inactive biofilms, polysaccharolytic slims and other organic compounds on account of the microbial enzymatic activity of the special biological preparation occurred. This partial degradation led to better hydraulic conditions and thus to more effective oxygen supply inside the trickling filter. This resulted in a higher activity of the activated sludge particularly of the nitrificants.

The increased solid material outflow observed in the effluent of the trickling filter after dosage of the preparation proved the effect of the partial purification of the filter material (Figure 5). Normally the arithmetical value of the settleable solids in the effluent amounted to about 1,0 ml/l. With growing effective duration of the biological preparation the content of

settleable solids increased and reached its maximum arithmetical value at 1,7 ml/l in September 1993. In the following time the content of settleable solids turned back to the initial level.

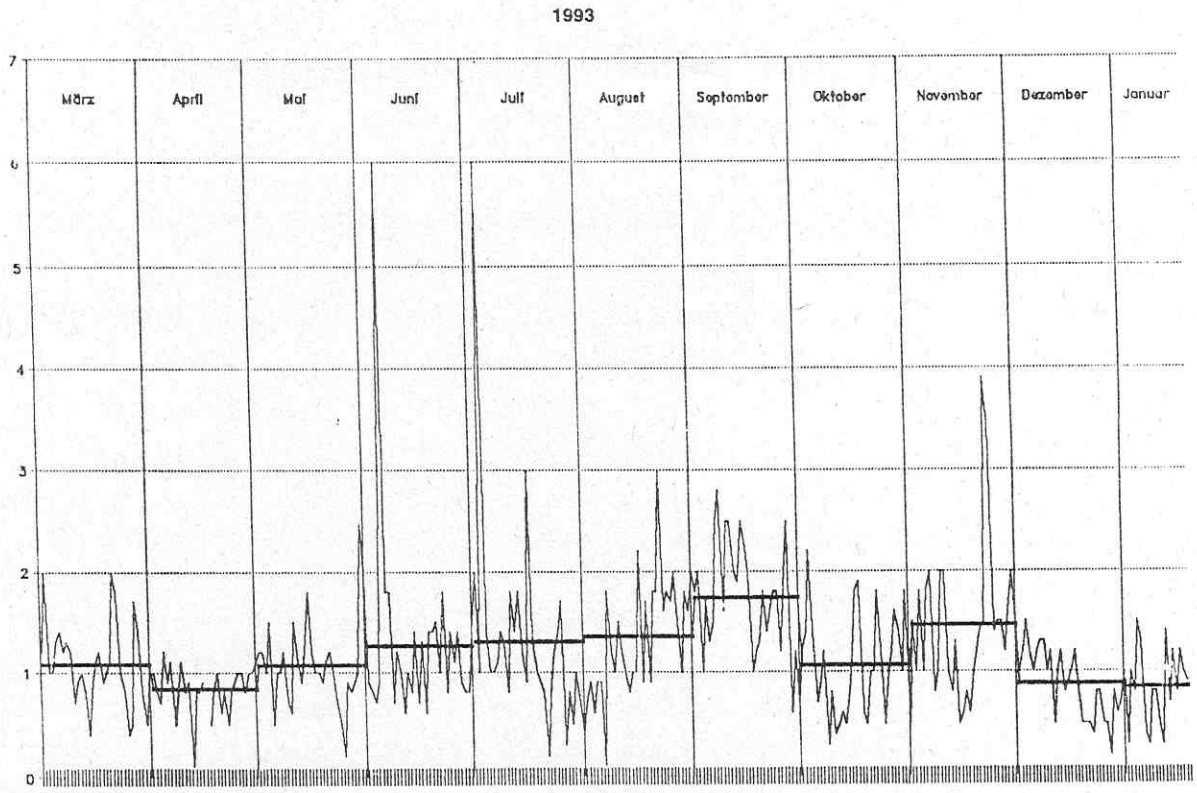

Fig. 5. Settleable solids (ml/l) in the effluent of the trickling filter (TF) during the influence of added bacteria (special preparation)
(— arithmetical mean value)

References

[1] Tucek, D. (1971) Unified basic design of biological aerobic treatment processes. Water Research 5, 647 - 680

[2] Weston, A. (1963) Fundamentals of aerobic biological treatment of waste water. Public Works, 77 - 83

[3] Imhoff, K. (1993) Taschenbuch der Stadtentwässerung. R.Oldenbourg Verlag, München, Wien

Monitoring of non-genetically engineered *Pseudomonas putida* strains after release in an aquatic ecosystem

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Abstract

Monitoring strategies were developed to track non-genetically engineered *Pseudomonas putida* strains in an aquatic ecosystem. The strain E1 was used for four years for the biodegradation of phenolic compounds in industrial waste water in Põlva, Estonia. In this study the strain E2 was used which is a non-carbenicillin-resistant variant of the strain E1. Both strains have a deletion of approximately 34 kb in the TOL plasmid pWW0 which served for discrimination from indigenous bacteria by molecular techniques. Other targets used for PCR and hybridization were the *xylE* gene and a sequence located in the left hand of the transposon Tn*4652* of the TOL plasmid. Detection of the released strain was possible only to 32 hours after release. It is assumed that the released strains did not survive in the aquatic ecosystem mainly due to the high dilution rate. The combination of cultivation on selective media and molecular techniques proved useful for tracking *Pseudomonas putida* strain E2 in an aquatic environment.

1. Introduction

Specific methods for detection and monitoring of microorganisms used for bioremediation and in agriculture are needed to investigate the fate of the released microorganism or to study its possible impact on the ecosystem [3, 4, 18]. The majority of studies performed to date deal with the monitoring of genetically engineered microorganisms (GEMs) [7, 15]. Consequently, due to the strong restrictions existing for working with recombinant organisms, nearly all of these studies have been undertaken in contained conditions, mostly in microcosms [7, 10]. In contrast, studying non-recombinant microorganisms is not restricted to laboratory microcosms, and hence can be performed under natural conditions. However, monitoring of non-recombinant organisms, which have no artificial marker, may prove to be more difficult [16]. Concerning the specific detection of non-recombinant bacteria used for bioremediation, immunological methods with monoclonal antibodies [3] as well as molecular, i.e., genetic, techniques [16], have been applied.

In a timber processing plant at Põlva, Estonia, 50 km from Tartu, the non-genetically engineered *Pseudomonas putida* E1 was used for degradation of phenolic waste waters over a period of four years. After decontamination, the treated water together with the biomass was then transferred to the municipal sewage plant and hence released into the aquatic ecosystem. Because it is also the intention to use this strain for biodegradation of phenolic contaminations at a different site in the north-east region of Estonia, at Kohtla-Järve, there existed a need to study the survival of the released bacteria in the aquatic ecosystem.

The objective of this study was to develop detection methods for this non-engineered strain with particular regard to molecular techniques. To track the released bacteria we applied a combination of cultivation on selective media and genotypic methods. Concerning the latter methods, naturally occurring sequences were chosen as targets for gene probes and PCR. Due to the fact that the release of the biomass occurred at irregular intervals (depending on the effluent of waste water), a controlled release experiment was performed to look for the strain immediately after release.

2. Material and Methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *Pseudomonas putida* E1 represents a segregant of the strain E0 which was generated by co-cultivation of the strains *Pseudomonas putida* AC783 and *Pseudomonas putida* PaW160 at the Institute of Molecular and Cell Biology in Tartu, Estonia. It harbours two plasmids, a larger one of about 83 kb which carries the *meta*-cleavage operon, and a smaller one of about 13 kb on which the carbenicillin resistance is thought to be encoded. It grows well on phenol, *m*-toluate, and Na-benzoate and exhibits good decontamination capabilities of phenolic waste water under non-optimized conditions. The strain *Pseudomonas putida* E2 used in this study is a non-carbenicillin-resistant variant of the strain *Pseudomonas putida* E1 lacking the small (13 kb) plasmid. The strains were maintained on M9 medium [8] supplemented with trace elements [1] and with 5 mM phenol. To evaluate the antibiotic resistances, Luria Broth agar (Sigma, Deisenhofen, Germany) supplemented with streptomycin 1000 µg/ml (LB/Sm) or carbenicillin 1000 µg/ml (LB/Cb) was used. Strain *Pseudomonas putida* PaW160 [18] was used as a positive control for the TOL deletion amplification product.

2.2. Target-sequences for gene probes and primers

Due to the fact that the strains *P.putida* E1 and E2 respectively harbour a plasmid something similar to the TOL plasmid pWW0, three sequences, known as components of the pWW0, were chosen as targets for both PCR amplification and gene probe hybridization. Of these, one was the *xylE* sequence (EMBL Data Library, accession number J01845), and the others were situated at the right-hand (*RH*) sequence (accession number X83686), and the left-hand (*LH*) sequences (not known to date, see *Results and Discussion*) of the transposon Tn*4652*. On the basis of the known sequences of *xylE* and RH of Tn*4652*, the following primers for PCR amplification of the target-se-

quences were selected using the PC Gene program (IntelliGenetics, Inc., USA): *xylE* primers: E1: 5'-TCAAGGTTGTGGATG-AGGATGC-3', E2: 5'-AGAACACTTCGTTGCGGTTACC-3'; *RH* primers of Tn*4652*: RH1: 5'-TAGCGGAGGCATTGCCGGACATGAC-3', RH2: 5'-TATTCGA-GAGGCCGTGGCTTGCTGG-3'. By using these primer pairs the PCR amplification should result in products of 594 bp (*xylE*) and 329 bp (*RH*).

Table 1. *Pseudomonas putida* strains used in this study

Strain	Plasmid	Characteristics[a]	Notes	Reference	Source
PaW85	none	$Phe^-Xyl^-Tol^-$	Tn*4652* in chromosome	[2]	P. Broda (UMIST, Manchester, UK)
PaW160	pWW0-160	$Phe^-Xyl^+Tol^+$		[18]	P. Broda
AC783	none	$Phe^-Xyl^-Tol^-$ Sm^rAde^-*catA*$^-$	Strain of A.M.Chakrabarty	[14]	I Starovoitov (Puschino, Russia)
E0	pWW0-160	$Phe^+Xyl^+Tol^+$ Sm^r*catA*	Spontaneous prototrophic transconjugant of AC783 from the cross PaW160 x AC783		This study
E1	$pWW0^{mut90}$	$Phe^+Xyl^-Tol^+$ Sm^rCb^r*catA*$^-$	Segregant of E0 having 34-kb deletion in plasmid		This study
E2	$pWW0^{mut90}$	$Phe^+Xyl^-Tol^+$ Sm^rCb^r*catA*$^-$	Non-carbenicillin resistant derivative of E1		This study

[a] $Phe^+Xyl^+Tol^+$, ability to grow on phenol, *m*-xylene, *m*-toluate as sole carbon sources; Sm^rCb^r, resistance to streptomycin and carbenicillin; Ade, requirement for adenine; *catA*$^-$, mutation in catechol 1,2-dioxygenase gene

2.3. PCR conditions and colony hybridization

Amplification was performed in a total volume of 50 ml using the UNO-Thermoblock (Biometra, Göttingen, Germany). The concentrations of the components in the reaction mixture were as follows: 10mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton X-100, 0.4 mM each of the primers, and 0.2 mM each of the four nucleotides. The temperature program started with lysis at 98°C for 10 min, followed by addition of 0.5 U of polymerase (Primezyme, Biometra, Göttingen, Germany) at 72°C. Thermal cycling began with denaturation at 93°C for 1 min, followed by annealing at a temperature which depended on the primer pairs (60°C for *xylE*, 53°C for LH, and 68°C for RH) for 1 min, and extension at 72°C for 1 min. 35 cycles were performed. We applied a final extension step at 72°C for 10 min. The PCR products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

To perform colony hybridization, cells from colonies grown on different selective media were dotted with a toothpick onto the uncharged nylon membranes Nytran NY 13 N (pore size 0.45 mm; Schleicher & Schuell, Dassel, Germany) which were placed on R2A (Difco) agar media followed by 24 hours growth at 30°C. Labeling of the PCR-generated gene probes with DIG DNA labeling and detection kit (Boehringer, Mannheim, Germany) and the subsequent hybridization procedure were performed in accordance with the Boehringer protocol.

2.4. Membrane filtration

To simulate the tracking of the released bacteria under natural conditions, a membrane filtration experiment was performed. Fixed numbers of the strain E2 were added to 20 ml river water samples from the River Elster in Leipzig. The cell number of the suspension of the strain E2 used for inoculation was determined by the AODC (Acridine Orange Direct Count) method [5] and by determination of the CFUs on different media (R2A-agar; Luria Broth agar supplemented with streptomycin 1000 µg/ml; M9 medium [8] containing 5 mM phenol). The inoculated samples were filtered through nylon membranes (Nytran NY 13 N, pore size 0,45 mm) which were subsequently placed on selective agar media. After 24 hours' growth on LB/Sm or 48 hours growth on minimal medium containing 5 mM phenol at 30°C, a replica was made. Colony hybridization was performed according to some protocols given in literature.

2.5. Release experiment

Due to the fact that the strain E1 had been used for the degradation of phenolic wastes by the factory for four years, it might be expected that these bacteria could be isolated at or near the point of discharge. However, E1 cells could not be detected in water samples taken from the aquatic ecosystem at different points. Consequently, we decided to perform a controlled release experiment after using the strain E2 for the decontamination of phenolic waste waters in the tank at the timber processing plant.

The fermentation of the biomass which was needed for decontamination was performed separately. In total, 220 l of biomass with an OD_{580}=1.0 was prepared. The biomass was added to 6000 liters of phenolic waste water which were contained in a subterranean fermentation tank.
The temperature in the tank was 11.5°C and the pH 7.3. The content of the tank was weakly aerated (approximately 20 l/min) for 5 days and transferred through the municipal sewage pipes system to the sewage station. The average length of time the waste water spent in the cleaning station was approximately 4 hours. To monitor the bacteria, samples were taken from the tank before and after addition of biomass both on the second and fifth day, immediately before release. After releasing the biomass into the sewage pipes system, samples were taken at 8, 32, and 104 hours at two different points of the aquatic ecosystem. Sampling sites were located at the sewage station, and on the River Orajõgi, 3 km downstream from the sewage cleaning station. Samples of 1 l were collected in sterile bottles and placed on ice for transport to the laboratory where analysis began not more than 4 hours after the samples had been taken. Different volumes of water samples were plated onto selective agar plates. Phenol, *m*-toluate, Na-benzoate, and LB/Sm were used as

selective media. Grown colonies were analyzed by colony hybridization using the *xylE* gene probe. Positive colonies were tested by PCR using the *xylE* primer set. Final verification was made using *LH* and *RH* primer sets.

3. Results and discussion

As verified by restriction analysis using restrictases *HindIII, EcoRI* and *XhoI*, the larger plasmid in the strains E1 and E2 respectively, designated by us as pWW0^{mut90}, is a 34-kb deletion derivative of the TOL plasmid pWW0 and encompasses the whole upper TOL plasmid operon *xylCAB*, the right hand (*RH*) of the transposon Tn*4652* and adjoining nucleotide sequences up to tra-genes in the original pWW0 plasmid. Due to the deletion of the transposase gene *tnpA*, the transposon Tn*4652* should not be able to act as a transposon and hence not detract from the stability of these strains. This deletion gives the strains E2 and E1 a unique "positive" marker making it distinguishable from other strains with homologous sequences.

Due to the fact the left hand nucleotide sequences were not available to date, we partially determined the sequences of this region by the dideoxy method [12]. On the basis of these results two 20-nucleotide sequences were chosen which were used as primer pairs for PCR amplification generating a DNA fragment approximately 720 bp in length: *LH* primers of Tn*4652*: LH1: 5'-AGTGTT-CGACGATGGTCTCG-3', LH2: 5'-GCTTTCCCTGTGTATCAACG-3'. The amplified fragment hybridized exclusively to *P. putida* strains with this Tn*4652* left hand sequence and not to any of the strains lacking this transposon.

To test the sensitivity and specificity of the detection methods in a model experiment, we combined membrane filtration with colony hybridization and the PCR. We were able to detect the number of cells that had been added to 20 ml of river water at concentrations as low as 2 cells/20 ml. However, this high sensitivity of detection was only possible due to the low numbers of phenol-degrading and streptomycin-resistant bacteria among the indigenous flora. The limitation is dictated by the number of colonies which can grow on the membrane placed on the selective agar media.

In the release experiment, we used the more conventional and laborious strategy of cultivation and subsequent application of molecular techniques to overcome the high abundance of indigenous bacteria and assumed low numbers of strain E2. This enabled us to ensure that rare events of released cells could be detected. From 2405 colonies, which were isolated from different sampling points using selective media, 51 hybridized with the *xylE* gene probe. However, only 2 isolates - one each in samples taken from the sewage station 8 and 32 h after release and no isolates from all other samples - could be verified as being identical with the released strain by the PCR amplification with *xylE*, *LH*, and *RH* primers (Table 2).

This was possible because the deletion of the *RH* fragment allowed us to differentiate between the released strain and indigenous strains that were supposed to have high homology to the *xylE* sequence of our strain. However, we did not find TOL plasmid carrying bacteria other than E2 in the aquatic ecosystem. From this we have no evidence of transfer of plasmid pWW0^{mut90} of the strains E1 and E2 in the environment to other bacteria in Põlva region.

In evaluating the results of tracking the released strains E1 and E2 in the aquatic ecosystem near Põlva, we have to assume that the strains do not persist in nature. This may be due to not only

physiological and biological but also technological reasons. Assuming no growth, the introduction of the tank water (6 m^3; 2.0 x 10^4 CFUs/ml) into the activated sludge reactor (250-m^3 capacity) the high throughput of waste water through the sewage station (250 m^3/h), and subsequent dilution by the river water (2,400 m^3/h) lead to the sharp reduction of population size.

Table 2. Analysis of isolates of the release experiment

Sampling sites	Postrelease sampling time (hours)	Number of analyzed isolates	No. Hybridizing to the *xylE* probe	No. verified as E2 by PCR
Sewage station	8	653	21	1
	32	662	17	1
	104	270	5	-
River Orajögi	8	349	3	-
	32	255	3	-
	104	216	2	-

Therefore, it is difficult to study survival of released bacteria in open aquatic ecosystems under these conditions. Our experiments confirm that the added bacteria do not persist in nature. However, it has been demonstrated that the molecular techniques that were used were appropriate for tracking the non-genetically engineered *Pseudomonas putida* strains in the open environment.

Acknowledgments

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References

[1] Bauchop, T. and Elsden, S.R. (1960) The growth of microorganisms in relation to their energy supply. J. Gen. Microbiol. 23, 457-469

[2] Bayley, S.A., Duggleby, C.J., Worsey, M.J., Williams, P.A., Hardy, K.G. and Broda, P. (1977) Two modes of loss of the TOL function from *Pseudomonas putida* mt-2. Mol. Gen. Genet. 154, 203-204

[3] Brettar, I., Ramos-Gonzales, M.I., Ramos, J.I. and Höfle, M.G. (1994) Fate of *Pseudomonas putida* after release into lake water mesocosms: different survival mechanisms in response to environmental conditions. Microb. Ecol. 27, 99-122

[4] Doyle, J.D., Stotzky, G., McClung, G., and Hendricks, C.W. (1995) Effects of genetically engineered microorganisms on microbial populations and processes in natural habitats. Adv. Appl. Microbiol. 40, 237-287

[5] Hobbie, J.E., Daley, R.J., Jasper, S. (1977) Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33, 1225-1228

[6] Jackman, S.C., Lee, H., Trevors, J.T. (1992) Survival, detection and containment of bacteria. Microb. Releases 1, 125-154

[7] Leser, T.D., Boye, M. and Hendricksen, N.B. (1995) Survival and activity of *Pseudomonas* sp. strain B13 (FR1) in a marine microcosm determined by quantitative PCR and an rRNA-targeting probe and ist effect on the indigenous bacterioplankton. Appl. Environ. Microbiol. 61, 1201-1207

[8] Maniatis, T., Fritsch, E.F., Sambrock, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

[9] Pickup, R.W. (1991) Development of molecular methods for the detection of specific bacteria in the environment. J. General Microbiol. 137, 1009-1019

[10] Pipke, R., Wagner-Doebler, I., Timmis, K.N. and Dwyer, D.F. (1992) Survival and function of a genetically engineered pseudomonad in aquatic sediment microcosms. Appl. Environ. Microbiol. 58, 1259-1265

[11] Prosser, J.I. (1994) Molecular marker systems for detection of genetically engineered microorganisms in the environment. Microbiology 140, 5-17

[12] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467

[13] Sarand, I., Mäe, A., Vilu, R. and Heinaru, A. (1993) New derivatives of TOL plasmid pWW0. J. Gen. Microbiol. 139, 2379-2385

[14] Starovoitov, I.I. and Timkina, E.O. (1981) The biodegradative pBS2 plasmid controls the synthesis of catechol 1,2-oxygenases. Doklady Akademii Nauk SSSR 256, 196-198

[15] Steffan, R.J. and Atlas, R.M. (1988) DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. 54, 2185-2191

[16] Thiem, S.M., Krumme, M.L., Smith, R.L. and Tiedje, J.M. (1994) Use of molecular techniques to evaluate the survival of a microorganism injected into an aquifer. Appl. Environ. Microbiol. 60, 1059-1067

[17] Wand, H., Laht, T., Peters, M., Becker, P.M., Heinaru, A. and Stottmeister, U. (1997) Monitoring of biodegradative *Pseudomonas putida* strains in aquatic environments using molecular techniques. Microb. Ecol. 33, 124-133

[18] Williams, P.A. and Murray, K. (1974) Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. 120, 416-423

[19] Wilson, M. and Lindow, S.E. (1993) Release of recombinant microorganisms. Annu. Rev. Microbiol. 47, 913-944

[20] Winstanley, C., Morgan, J.A.W., Pickup, R.W. and Saunders, J.R. (1991) Use of a *xylE*marker gene to monitor survival of recombinant *Pseudomonas putida* populations in lake water by culture on nonselective media. Appl. Environ. Microbiol. 57, 1905-1913

Strategies to improve microbial degradation capacities - Reaction engineering studies on the application of mobile degradation capabilities

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Abstract

The dynamics of plasmid transfer were investigated under various conditions: in populations of suspended cells, in flocs, between cells immobilized on filters and in natural biofilms. The study was carried out to develop a strategy to augment a biodegradation process by taking advantage of natural gene transfer. The rate constants for plasmid transfer, k_T, were calculated from the kinetics of the conjugal transfer of the TOL plasmid pWWO using *Pseudomonas putida* PAW1 as the donor strain and a floc forming or a non-flocculating variant of *Pseudomonas putida* PAW 340 as the recipient. For on-line monitoring of population growth and plasmid transfer event, the parental strains and plasmid were marked with reporter enzymes. The rate constants for plasmid transfer between suspended cells and in the natural biofilm were $2.8 \cdot 10^{-15}$ and $8.0 \cdot 10^{-16}$ [ml/(cells·h)], respectively, whereas the rate constants for gene transfer during floc formation or in filter mating experiments reached higher values, i.e. $3 \cdot 10^{-11}$ and $2 \cdot 10^{-12}$ [ml/(cells·h)], respectively. Analyzing the mating conditions from a reaction engineering point of view, two process schemes for a bioaugmentation unit in a wastewater treatment plant were proposed.

1. Introduction

The degradative activity of a biodegradation process depends on the metabolic and genetic capabilities of the adapted indigenous microorganisms. Bioaugmentation is a process which attempts to improve biodegradation by direct addition of specific microorganisms to a wastewater treatment plant. The discussion and problems associated with the use of selected microorganisms, specific single strains and commercial products are controversial [1, 2]. Focusing on industrial wastewater with chemical pollutants, the examinations indicated that for the stable performance of an industrial wastewater treatment process a continuous re-inoculation needs to be applied [3, 4], due to the fact that introduced specialists cannot survive in the population of indigenous bacteria [5].

However, there are some reports dealing with the survival and functionality of genetically engineered microorganisms in wastewater treatment showing evidence that a degradative activity can be established in an activated sludge process after transfer of the genetic information to members of the indigenous bacteria [6, 7]. Since a lot of degradative genes and pathways are located on mobile conjugative plasmids, this is an approach to bioaugment biodegradation processes making use of the natural gene transfer. For an successful application of this mechanism more quantitative information on the rate of plasmid transfer particularly under different reaction conditions in a wastewater treatment process are needed. The aim of the present study was to evaluate the rate constants of the conjugative plasmid transfer reaction under different environmental conditions: for cells suspended in liquid, associated in flocs or immobilized on filters, as well as in a natural biofilm. As a model mating system the TOL plasmid was chosen.

2. Materials and Methods

2.1. Bacterial strain and media

Pseudomonas putida PAW1 (tol+) carrying the TOL plasmid pWWO was used as the donor strain [8]. Spontaneous nalidixic acid resistant mutants of the *P. putida* strain PAW 340 [8] were used as the flocculating recipient PAW 340-5 (tol^-, trp^-, Str^R, Nal^R, floc+). The non-flocculating variant (PAW 340-50, tol^-, trp^-, Str^R, Nal^R, $floc^-$) was isolated from the supernatant of a stationary batch culture after three days of sedimentation [9].

Donor and recipient (PAW 340-50) strains were labeled on their chromosome with the gene for ß-galactosidase (*lacZ)* from *E. coli* or the gene for firefly luciferase (*luc)* from *Photinus phyralis*, respectively. The transfer of TOL plasmid was visualized by complementation of the bacterial luciferase from *Vibrio harveyi* due to integrating the gene of one subunit of the enzyme, *luxB*, on the chromosome of the recipient and the other, *luxA*, on the plasmid (Figure 1). The complete genetic elements as well as the test conditions for enzyme activities are described in detail by Karle [10].

All cell numbers were estimated by selective plating as a triplet determination. Medium composition is described in [9].

2.2. Mating procedures

Matings were performed under sterile non-selective conditions in minimal salt medium with glucose as the sole carbon source. For batch matings in shaking flasks and on filters, prior the mating donor and recipients were grown separately until they reached the exponential growth phase. After harvested by centrifugation, the appropriate cell numbers estimated by a Helber counting chamber were adjusted in the mating mixture [9]. Experiments in the continuous culture were carried out using 2L glass fermentors. If not mentioned otherwise, strain PaW 340-50 was used as the recipient strain.

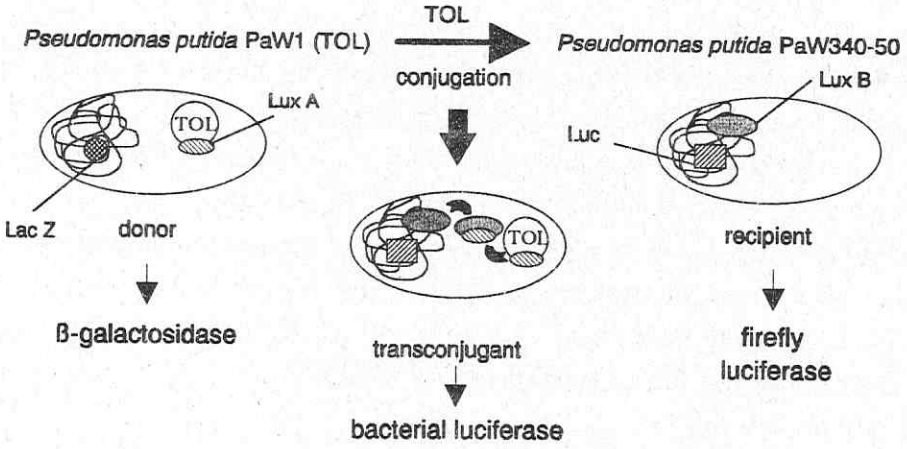


Fig. 1. Model system for conjugation experiments. Labeling of mating partners with reporter enzymes and detection of the transconjugants by enzyme complementation

2.3. Kinetic analysis

For analyzing the experimental data, the mass action model suggested by Levin, Stewart and Rice [11] was applied. It is based on two reactions, one between donor (D) and recipient (R) cells, and the other between transconjugants (T) and recipient cells.

$$D + R \xrightarrow{k_T} D + T$$

$$T + R \xrightarrow{k_T} T + T$$

Both reactions result in new transconjugants where the increase of the transconjugants is proportional to the concentration of recipients and plasmid harboring cells, as well as to the reaction rate constant k_T, which has the dimension [ml/(cells·h)] or [cm^2/(cells·h)]. The material balance equations for a batch mating experiment can be written as:

$$\frac{dN_R}{dt} = \mu_R N_R - k_T (N_D + N_T) N_R \quad (1)$$

$$\frac{dN_D}{dt} = \mu_D N_D \quad (2)$$

$$\frac{dN_T}{dt} = \underbrace{\mu_T N_T}_{growth} + \underbrace{k_T (N_D + N_T) N_R}_{conjugation} \quad (3)$$

with the specific growth rate, μ_i, the cell numbers, N_i, of D(onor), R(ecipient), T(ransconjugant), respectively.

For batch mating experiments the analytical solutions of the material balance equations were calculated, whereas the equations for the two-stage continuous cultures were solved by numerical simulation.

3. Results

3.1. Kinetics of filter mating, on-line monitoring with reporter enzymes

Filter mating was performed by inoculation of cellulose acetate filters with N_D (t = 0h) = $7.8 \cdot 10^7$ [cells/cm^2] and N_R (t = 0h) = $9.5 \cdot 10^7$ [cells/cm^2] and resuspending the cells at defined time intervals.

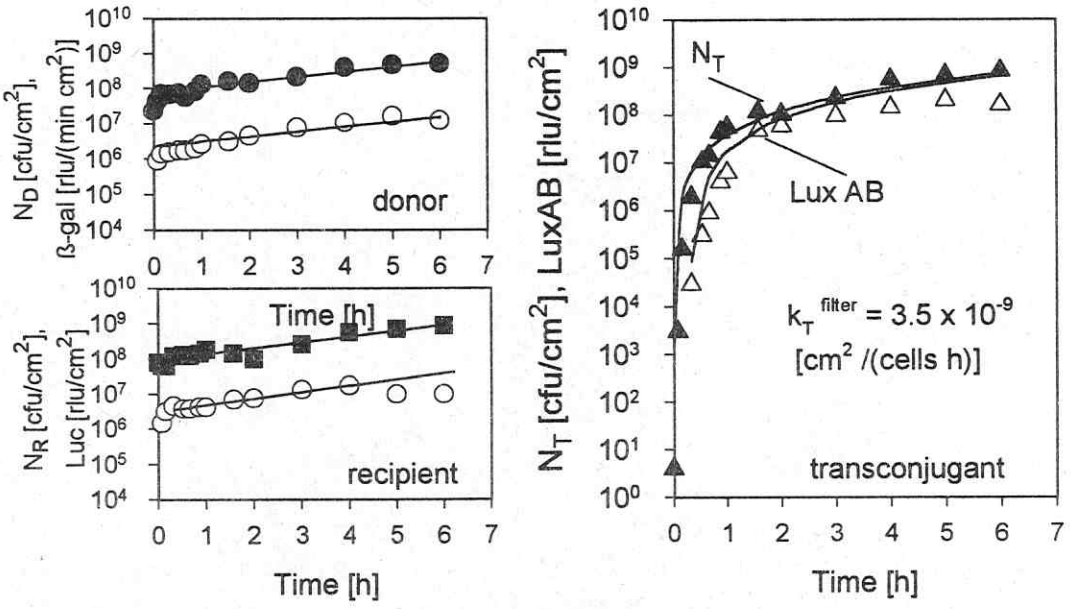


Fig. 2. Model predictions and experimental results of a filter mating. Colony forming units N_D (●), N_R (■), N_T (♦) and the corresponding reporter enzyme activities ß-galactosidase (○), firefly luciferase (luc) (□) and bacterial luciferase (luxAB) (Δ). With colony forming units, cfu, and relative light units, rfu. Estimated growth rates: μ_D = 0.33 [1/h], $\mu_{R,T}$ = 0.37 [1/h].

The growth of donor and recipients documented by colony forming units (cfu) or the reporter enzyme activity of ß-galactosidase and firefly luciferase showed a good correlation. The growth parameters μ_D and μ_R were quite the same and were estimated to be 0.33 [1/h] and 0.37 [1/h], respectively (Figure 2).

Even the spread of the plasmid in the immobilized population could be monitored by the activity of bacterial luciferase. The estimated reaction rate constant k_T^{filter} was $3.5 \cdot 10^{-9}$ [cm^2/(cells·h)], when taking into account a 15 minutes delay for enzyme expression in the transconjugants (Figure 2).

3.2. Kinetics of plasmid transfer in liquid culture and in a natural biofilm

To investigate the reaction kinetics of plasmid transfer in suspended cultures under constant reproducible physiological conditions prior to and during the mating, a two-stage continuous process was designed. Three stirred reactors were connected as illustrated in Figure 3. In the first processing stage donor and recipient cells were cultivated continuously in separate reactors.

In order to initiate a mating, the effluents were connected with the mating stage, and the dilution rate was adjusted to maintain a constant growth rate. The desired cell concentrations and other parameters such as fluid dynamic conditions could be suitably varied also.

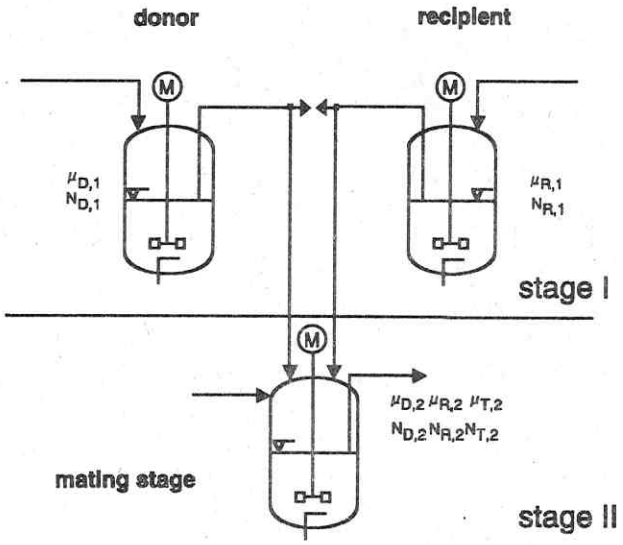


Fig. 3. Two-stage continuous culture. Conjugation takes place in the second stage

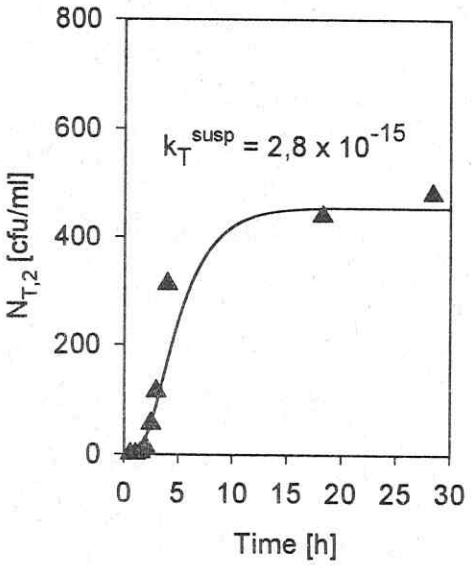


Fig. 4. Increase of the transconjugant concentration in the suspension of the mating stage after connecting the mating stage with the first stage at t= 0h. $D_{D,1} = D_{R,1} = 0.3$ [1/h], $D_2 = 0.6$ [1/h]; $N_{D,2} = 4 \cdot 10^8$ [cfu/ml], $N_{R,2} = 1.6 \cdot 10^8$ [cfu/ml]

About 10 hours after connecting the mating stage, a constant concentration of transconjugants (450 cfu/ml) could be observed. Figure 4 shows the results of one time course at 150 rpm. Comparison of experimental results and the simulation gave a reaction rate constant, k_T^{susp} , of $2.8 \cdot 10^{-15}$ [ml/(cells·h)]. The parameter was not effected by the mixing velocity.

After a process time of 30 hours, the concentration of suspended transconjugants in the reactor started to increase again reaching a constant value of 3000 cfu/ml (data not shown). A thin biofilm on the glas wall of the reactor was isolated containing five times more tansconjugants as estimated in the total liquid volume of the reactor. These results indicate that plasmid transfer occurred also in this natural biofilm. When assuming a maximal growth rate of the cells growing in the biofilm of $\mu_{film} = 1/10\ \mu_{liquid} = 0.003$ [1/h], the reactions rate constant k_T^{film} was calculated to be 10^{-11} [cm^2/(cells·h)].

3.3. Kinetics of plasmid transfer in a population of floc forming bacteria

To compare the spread of the plasmid between suspended single cells and flocs, batch matings in shaking flasks were performed with the flocculating recipient strain PaW 340-5 and the non-flocculating mutant PaW 340-50.

Figure 5 shows the rapid increase of transconjugants in the population. The calculated rate constant k_T^{floc} for the plasmid transfer to the flocculating recipient was $3 \cdot 10^{-11}$ [ml/(cells·h)]. This high rate constant was observed during an initial period of floc formation, however, the mathematical approach of the simple collision could not describe the reduced plasmid transfer when flocs had grown to a bigger size.

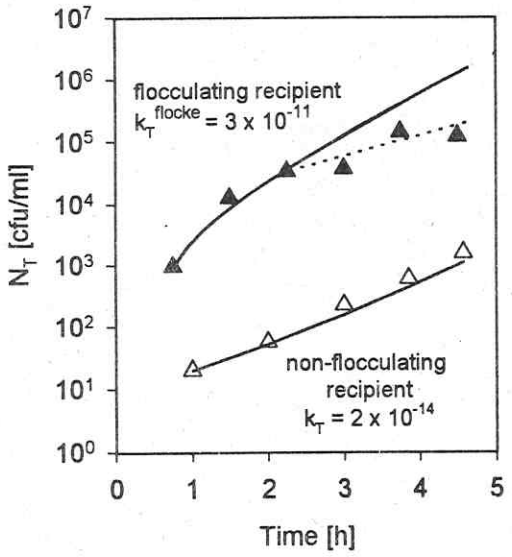


Fig. 5. Model predictions and experimental results of batch matings in shaking flasks with the flocculating recipient PaW 340-5 (♦) and the non-flocculating recipient PaW 340-50 (Δ). $N_D(t = 0) = 7.5 \cdot 10^6$ [cfu/ml], $N_R\ (t = 0) = 8.2 \cdot 10^6$ [cfu/ml], $\mu = 0.73$ [1/h]

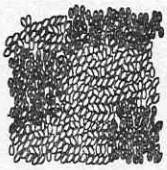
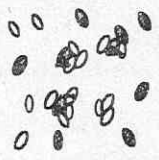
4. Discussion

4.1. Analysis of the mating conditions

A better comparison of the results obtained within this study, if the rate constants were transformed with respect to the volume of the active biomass (Table 1). For an reaction engineering interpretation of the rate constants, the mating conditions were analyzed as illustrated in Table 1. Two aspects are influencing the spread of a plasmid within a population.

The first is the mobility of the mating partners in the population, which determines the probability of the contact between donor and potential recipient cells. The second is the efficiency of this contact to form a mating pair and to trigger the plasmid transfer. The lowest rate constants of about 10^{-15} [ml/(cells·h)] were observed with single suspended cells and in the natural biofilm.

Table 1. Reaction rate constants k_T with respect to the volume of the active biophase

mating conditions		rate constant $[\frac{ml}{cells\ h}]$
filter		$k_T^{filter} = 2 \times 10^{-12}$
suspension		$k_T^{susp} = 1.5 \times 10^{-15}$
biofilm		$k_T^{biofilm} = 8 \times 10^{-16}$
flocculation		$k_T^{floc} = 3 \times 10^{-11}$

While the efficiency of contacts is limiting the mating in suspension at turbulent fluid dynamic conditions, the spread of genetic information in the biofilm is impaired due to mobility restrictions. Very high rate constants can be reached in systems that guarantee frequent contact as in the phase of floc formation combined with the stabilization of this contact in the floc. The

rate constants were higher by four orders of magnitudes for the latter systems compared to the other systems used. Similar results could be obtained in the filter mating experiment, where the immobilized donor and recipient cells were statistically spread on the filter.

4.2. Process design

For the realization of a bioaugmentation process by increasing the gene pool within the population of a wastewater treatment process, two process schemes can be derived from the requirements of effective conjugation. Both run in parallel with the existing activated sludge process unit and produce the indigenous transconjugants needed to deal with mineralization problems. Figure 6a illustrates the combination of a side stream reactor for flocculation.

After shear treating the indigenous bacteria, they are mixed with the donor strain and left for flocculation and plasmid transfer. The alternative configuration incorporates a fixed bed or trickling filter to co-immobilize donor strains and indigenous bacteria. The resulting transconjugants can be released by backwashing cycles (Figure 6b).

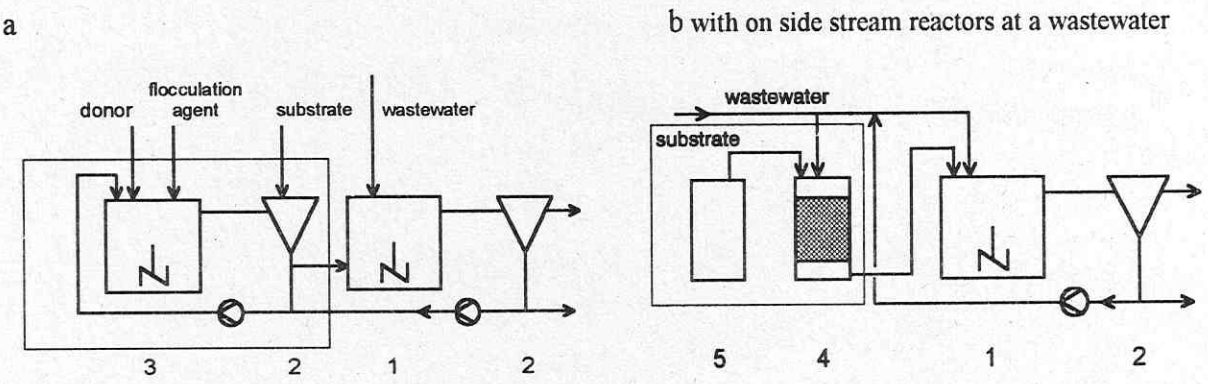


Fig. 6. Process design of bioaugmentation units treatment plant. 1 activated sludge unit, 2 sedimentation unit, 3 fermentor for shear treatment and mixing, 4 fixed bed with immobilized bacteria, 5 selective substrate tank

References

[1] Stephenson, D. and Stephenson, T. (1992) Bioaugmentation for enhancing biological wastewater treatment. Biotech. Adv. 10, 549-559

[2] Huban, C.M. and Plowma, R.D. (1997) Bioaugmentation: put microbes to work. Chemical Engineering, March 97, 74-84

[3] Fujita, M., Ike, M. and Uesugi, K. (1994) Operation parameters affecting the survival of genetically engineered microorganisms in activated sludge process. Wat. Res. 228, 1667-1672

[4] Lewandowsky, G., Salerno, S., McMullen, N., Gneiding, L. and Adamowitz, D. (1986) Biodegradation of toxic chemicals using commercial preparations. Environ. Progr. 5, No 3, 212-217

[5] Babcock, R.W. Jr., Ro, K.S., Hsieh, C.-C. and Strenstrom, M.K. (1992) Development of an off-line enricher-reactor process for activated sludge degradation of hazardous wastes. Water Environ. Res. 64, 782-791

[6] McClure, N.C., Fry, J.C. and Weightman, A.J. (1991) Survival and catabolic activity of natural and genetically engineered bacteria in a laboratory scale activated sludge unit. Appl. Environ. Microbiol. 57, 366-373

[7] Nüßlein, K., Maris, D., Timmis, K. and Dwyer, D.F. (1992) Expression and transfer of engineered catabolic pathways harbored by Pseudomonas spp. Introduced into activated sludge microcosm. Appl. Environ. Microbiol. 58, 3380-3386

[8] Bradley, D.E. & Williams, P.A. (1982) The TOL plasmid is naturally derepressed for transfer. J. Gen. Microbiol. 128, 3019-3024

[9] Dössereck, C. (1997) Reaktionstechnische Untersuchungen zur konjugativen Genübertragung beim mikrobiellen Schadstoffabbau. Fortschrittberichte VDI, Reihe 15, Nr. 184, VDI-Verlag, Düsseldorf

[10] Karle, A. Dissertation an der Universität Stuttgart, Fakultät Geo- und Biowissenschaften, Institut für Industrielle Genetik, in Vorbereitung

[11] Levin, B. R., Stewart, F. M. and Rice, V. A. (1979) The kinetics of conjugative plasmid transmission: fit of a simple mass action model. Plasmid 2, 247-260

- Ecology of polluted sites -

Autecology of wastewater organisms supports sewage treatment performance

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Abstract

Microscopic investigations of wastewater biocenoses support sewage treatment plant operation: biologically sound process optimization is possible by revealing the community structure and providing autecological data on the members of the biocenoses. Applying classical light microscopy will yield good results with conspicuous protozoa and metazoa and some success with filamentous bacteria in simple reactors. The organisms indicate concentration ranges of carbonaceous substrate, ammonia, and oxygen as well as of sludge retention times. The more sophisticated nutrient removal plants require several reactors to accomplish the various purification steps such as carbon source elimination, nitrification, denitrification, and biological phosphorus removal. To assess the impact of physiologically important members of the bacteria community in these systems light microscopy is insufficient and thus inadequate. The outcome by applying cultivation after light microscopy will yield satisfactory results with conspicuous bacteria, where you know what to grow and to physiologically characterize. A satisfactory description of the community structure of non-conspicuous bacteria is not possible before applying further steps such as nucleic acid staining techniques combined with fluorescent microscopy which will be a widespread tool in the near future. Providing autecological data on relevant wastewater bacteria will then make sewage treatment consulting much more powerful than it is today.

1. Biological wastewater treatment - history and today's problems

Biological wastewater treatment takes advantage of the degradation processes known from surface waters. In case of the activated sludge process the degradation of organic substances in the wastewater is performed by suspended biomass. Purification is accelerated by means of aeration and made quite efficient by recycling the biomass after a sludge separation step. In case of trickling filters the biomass is bound to a substratum such as lava. Both treatment systems have the advantage of being confined to a short reaction time and a small area

compared to self purification in the adjacent surface waters. These types of aerobic wastewater treatment have been used since the beginning of the century. The sludge load in carbonaceous substrate eliminating systems used to be quite high with F/M-ratios of around 0.5 to 1 kg BOD/(kg·d) (BOD = biological oxygen demand measured as oxygen consumption of e.g. an activated sludge community in a 5 days batch test ("BOD_5")). Effluent values were around 20 to 30 mg L^{-1} BOD accompanied with high amounts of ammonia exceeding 20 mg L^{-1}.

In the eighties the eutrophication factors ammonia and phosphorus were discussed as to be eliminated during the treatment process. As a consequence phosphorus had to be precipitated and nitrification had to be established in the system [1]. In order to guarantee a stable nitrifying bacteria population mean residence time of the sludge had to be prolonged from 1 to 2 days up to at least 8 days. Thus sludge load had to be diminished to around 0.15 kg BOD/(kg·d). Effluent quality with respect to carbonaceous substrate was good with BOD values below 10 mg L^{-1}. Nitrate-N in secondary effluents of municipal wastewater used then to be in the range of 20 to 30 mg L^{-1}.

Carbonaceous substrate degradation was performed for many years in completely mixed one step reactors. With the introduction of nitrification cascade, plug flow, or two-stage performance was introduced. It was quite an easy task to run and control these technologically simple systems.

After the 2nd North Sea Protection Conference in 1987 Germany agreed to diminish the input of phosphorus and nitrogen to the North Sea by 50% until 1995 [2]. Because of nitrate being a major eutrophication factor in marine systems total inorganic nitrogen in secondary effluents was confined by legislation to 18 mg L^{-1} in treatment plants exceeding 5.000 population equivalents (pe). 1 mg L^{-1} of total phosphorus has to be met in plants exceeding 100.000 pe. This is the reason why these "nutrient removal plants" are about to be upgraded by denitrification steps and, partly, by enhanced biological phosphorus removal (EBPR). Denitrification requires the introduction of an anoxic zone, biological phosphorus removal empirically turned out to be enhanced by introducing an anaerobic step.

Problems in biological treatment of wastewater, in particular in nutrient removal plants, predominantly are consisting in the control of the different biochemical purification steps. Besides this every once in a while sludge separation problems in the secondary clarifiers have hampered efficient performance during the long history of aerobic wastewater treatment. Since the most common process to separate the organisms from the clarified water is sedimentation two major phenomena have caused problems ever since: sludge bulking and sludge scumming. The first phenomenon means a bulky structure of the sludge ending up in a voluminous sludge leading to sludge losses into the secondary effluent in the worst case. Sludge scumming means that part of the sludge separates properly, another part will float to the secondary clarifiers surface forming scum or dense sludge blankets which hamper oxygen input and sludge circulation.

2. Microscopic investigation of wastewater biocenoses

2.1. Conspicuous organisms - classical light microscopy

Performance of the biological wastewater treatment process has commonly been monitored by measurement of chemical and physical parameters such as oxygen concentration, temperature, pH value, organic load as BOD and COD concentration in the influent, and system efficiency determined as BOD and COD elimination in the process (COD = chemical oxygen demand, determined as chemical oxidation of organics by potassium dichromate). This is valid for both carbonaceous substrate elimination and nitrification processes as well as for nutrient removal plants with denitrification and enhanced biological phosphorus removal.

Very early in wastewater treatment history in addition to physical-chemical measurements microscopic investigations on the structure of the (waste)water-borne biocenoses have been started. The prominent aim of revealing the biocenosis structure was to gain a better understanding of the processes underlying wastewater purification. These early studies laid the foundations for the famous work of Liebmann, Bick, Curds and many others who summarized the knowledge on the biology of organisms in polluted water [e.g. 3, 4, 5]. Field experiences together with autecological data were used to define the optimal growth conditions for different organisms and thus to describe their indicator value in the so-called saprobial index system. The restrictions of light microscopy led to a strong bias towards protozoa and metazoa on one hand, and filamentous bacteria and fungi on the other side, since these groups are conspicuous organisms and can therefore be quite easily differentiated. Meanwhile a large database of field data and autecological data on protozoa is available [e.g 6, 7, 8, 9, 10]. The prevailing processes where indicator organisms have satisfactorily been assessed are carbonaceous substrate eliminating and nitrification systems.

As mentioned above both bulking and scumming have been known as common sludge separation problems since a long time. They are in most cases caused by the excessive growth of filamentous sludge organisms such as filamentous bacteria and fungi. 1975 Eikelboom summarized some morphological data on these microscopically conspicuous bacteria and fungi after a comprehensive survey of sludge biocenoses [11]. These investigations contributed to a dichotomic identification key which is since then used in practical wastewater treatment performance monitoring [12, 13]. Because of the difficulties to isolate and cultivate these filamentous organisms autecological data are available for only a few of them. Nevertheless there are valuable hints on selection factors via thorough bench scale and field investigations on the occurrence and frequency of these organisms in activated sludges [for reviews see 14, 15, 16, 17, 18].

2.2. Non-conspicuous organisms - cultivation and fluorescence microscopy

Classical light microscopy is restricted to conspicuous organisms. The structure of non-filamentous bacterial populations in the biocenosis can therefore not be properly defined by this method. Since non-filamentous bacteria play the major role in the degradation processes and

build up the major part of the biomass in the biocenosis their investigation was started by applying classical microbiological methods including isolation from the biocenosis, cultivation, and physiological characterization [19, 20, 21, 22]. This concept led to a bias towards bacteria such as enterobacteria, aeromonads, pseudomonads etc. being easily culturable at the conditions offered, which in most cases were characterized by high substrate and salt concentrations. In contrast, today's knowledge gained by molecular techniques showed that cultivation will lead to a warped description of community structures because of any culture condition being prone to bias and because of bacteria existing which are obviously viable but not (yet) culturable (VBNC). Up to now in most wastewater communities we are not able to properly describe more than at best 10% of the existing populations. Since we do not yet know who are the major actors in the process autecological data on wastewater bacteria gained by classical isolation and cultivation in general are not useful to reflect realistic field conditions. It is just now that we start to overcome these problems by means of gene probe and fluorescent labeling techniques [for reviews see 23, 24].

3. Autecology of wastewater organisms helps to support wastewater treatment

3.1. Carbon source elimination and nitrifying systems

High load systems with an F/M ratio over 0.7 kg BOD/(kg dry weight · d) are characterized by a low retention time of the sludge and its organisms. This is the reason why in these systems only those microorganisms can exist whose generation time is equal or shorter than the given sludge retention time. A common community of microorganisms in such a system consists of high numbers of free-swimming bacteria as well as of numerous flagellates and amoeba, both osmo- and phagotrophic. Organisms with a complex organization and a longer generation time than a few hours will be outcompeted in this system. The knowledge on autecological data such as oxygen requirement or else ammonia tolerance of the indicator organisms found support process performance by supplying redundant information on the environmental conditions in the reactors. Thus microscopy is able to confirm or disprove the physico-chemical measurements of the field conditions.

On the other hand, a low loaded nitrifying system with an F/M ratio of 0.1 kg BOD/(kg·d) with a sludge retention time of 8 to 10 days will promote a highly diverse community with bacteria being aggregated in the so-called sludge flocs and a vast number of various protozoa and metazoa being involved. Besides stalked ciliates such as members of the genera *Vorticella* and *Epistylis,* which indicate oxygen concentrations exceeding 2 mg L^{-1}, we will find crawling or free-swimming ciliates such as *Euplotes* or *Litonotus* species. The introduction of such predators indicates a stable system with long retention times, as does the occurrence of metazoa such as rotifers and oligochetes with generation times over 10 days. The practical use of monitoring sludge age indicators is again to gain redundant information on biomass retention time and performance efficacy such as BOD elimination and nitrification efficiency.

3.2. Sludge separation problems by sludge bulking and scumming

Another example for sludge organisms autecology supporting sewage treatment performance is the monitoring of the filamentous bacteria community during - or better before - a sludge separation problem. Filaments of the Eikelboom type 021N are prone to proliferate when easily degradable substrate is available in high amounts which often comes along with a deficiency of nitrogen and/or phosphorus resources. This is the case e.g. during wine or vegetable processing campaigns and often leads to severe bulking problems. By using autecological data which indicate 021N 's preference for acetate as a substrate over many other carbon sources troubleshooting with a biological background is possible [25, 26]. The introduction of a high loaded zone preceding the aeration step, a so-called selector, will outcompete 021N by enhanced substrate adsorption and degradation by floc forming bacteria. Avoiding long retention times in the primary clarifier which promote acetate production by primary sludge hydrolysis will also help to prevent 021N bulking. Moreover, as 021N is known to perform high rates of ammonia uptake [25] and is thus competitive at nitrogen-limited conditions dosage of nitrogen sources will provide an additional remedy for curing 021N bulking problems.

Scum formation in wastewater treatment often comes along with an excessive growth of nocardioform actinomycetes. Numerous autecological data are available for these organisms [27, 28, 29, 30]. Thus biologically sound consulting is easy in this case. Actinomycetes are known to produce a strongly hydrophobic cell surface in particular when fed hydrophobic substrate such as long chain fatty acids or alkanes. Therefore their proliferation as well as their hydrophobicity can be effectively decreased by preventing hydrophobic substrate to enter the biological treatment step. Pretreatment of respective industry wastewaters, an effective grease trapping, and removal of actinomycete-enriched scum layers will promptly cure actinomycete-borne foaming problems. Moreover, decreasing sludge age by increasing the F/M ratio with respect to readily degradable substrate will outcompete the slow-growing actinomycetes.

3.3. Nutrient removal systems with denitrification

Our investigations on the denitrification capacity of a methanol-fed sandfilter system will serve as an example for biologically well-founded optimization of denitrification [31]. Classical physiological characterization studies on denitrifiers isolated from the biofilms situated on the carrier material in the sandfilter supported the idea of 4 main genera being involved in denitrification: *Paracoccus, Hyphomicrobium, Hydrogenophaga,* and *Comamonas.* Both taxonomy and quantification was confirmed by fluorescently labeled rRNA-targeted nucleic acid probes [32]. In addition to that the in situ-distribution of the denitrifiers could be resolved. With these results together with autecological studies from the literature we were able to conclude the following practical hints for running the sand filter [33]: because of the pH optimum of paracocci and hyphomicrobia being in the neutral to light alkaline milieu thick biofilms promoting low pH values by acid production and CO_2 trapping have strictly to be avoided. Therefore quartz sand with its thin biofilms turned out to be advantageous over

expanded shale as a carrier material. Furthermore dosage of methanol as an external carbon source may be recommended not to exceed a ratio of 2.5 kg methanol per kg nitrate-$N_{eliminated}$ since aut-ecological data suggested this ratio to be optimal for the denitrifiers involved.

3.4. Nutrient removal systems with enhanced biological phosphorus removal (EBPR)

From classical cultivation studies on EBPR activated sludges members of the genus *Acinetobacter* were supposed to play a major role in biological phosphorus removal [34]. As mentioned above in the case of EBPR again staining techniques showed that cultivation on selective media leads to a population shift from the in situ-community to a cultivation dependent bias [35, 36]. The high concentration of carbonaceous substrate and salts selected for *Acinetobacter* strains, bacteria of the γ-subclass of the proteobacteria. In contrast to the results of cultivation studies, the important populations in situ determined by nucleic acid staining techniques turned out to be members of the β-subclass of proteobacteria. Overestimating non-realistic EBPR-populations has led to the awkward situation that most simulation models applied for EBPR design today are derived from kinetic data on *Acinetobacter* [37]. Since this is not the major EBPR actor the models are not able to predict realistic performance. These studies yield an example where overestimation of the "wrong" populations will strongly influence sewage treatment operation in a non-desired way.

4. Conclusions and outlook

In the case of protozoa and metazoa as well as many filamentous bacteria classical light microscopy has turned out to be a useful tool to describe the community structure of wastewater-borne biocenoses. By applying autecological data on the indicator organisms to wastewater treatment performance the biologist will gain redundant information on the process conditions and is in turn able to give hints how to select for organisms desired in the process or else to avoid non-desired biocenoses. Thus the engineer is provided with valuable information on process optimization.

In case of non-conspicuous bacteria community structure has to be resolved by introducing further investigation steps. An important molecular method which will be commonly applied in the near future is to stain bacteria by hybridizing their genes with fluorescently labeled oligonucleotides. Classical light microscopy will then not only be applied as such but will be complemented by fluorescent microscopy. This technique will enable us to not only qualify but also quantify the most relevant actors of wastewater degradation in situ. With this knowledge we can go back to the classical microbiological methods of isolation and physiological characterization of the bacteria involved. Autecology will then - as is common practice with filaments and protozoa - be able to support process optimization by promoting selection and support of bacteria communities desired in the process.

Nevertheless there are some problems left even with the detection and identification of filamentous bacteria and protozoa. Filaments might either be hidden or disintegrated in single

cells. Small amoeba, flagellates, and ciliates might be hidden in dense floc or biofilm matrices. Modern fluorescent gene labeling techniques will therefore be applied to both prokaryotic and eukaryotic organisms in the near future [38, 39].

Autecology data up to now were a major tool to characterize the members of wastewater biocenoses and to define their indicator value. In addition to that new aspects are revealed by current research where the description of bacteria community structure of different sludges has been supplemented by in situ feeding tests with radiolabeled substrates such as acetate or long chain fatty acids [40, 41]. This will lead to a first understanding of not only the structure but also some functional relations in a sludge biocenosis, e. g. the "bottom up" regulation of the organisms by environmental factors in situ.

Another step will be a proper description of predator-prey relations between members of the community. After having been neglected for many years current research is again dealing with these aspects of "top down" control of freshwater bacteria communities [42, 43].

All these pieces of a puzzle will finally lead to an assessment of the synecological function of a wastewater biocenosis. The performance of wastewater treatment will then be supported by biological analyses in a much more efficient way than is possible today.

References

[1] Erste Allgemeine Verwaltungsvorschrift über Mindestanforderungen an das Einleiten von Abwasser in Gewässer - 1. AbwasserVwV vom 9.11.1988, GMBI (1988), 602

[2] 2nd Int. North Sea Conference - 24 - 25 Nov 1987, declaration of the ministers. London (1987)

[3] Liebmann, H. (1962) Handbuch der Frischwasser- und Abwasser-Biologie. Oldenbourg Verlag, München

[4] Bick, H. (1972) Ciliated Protozoa. World Health Organization, Geneva

[5] Curds, C.R. and Hawkes, H.A. (1975) Ecological aspects of used-water treatment. Vol. 1: The organisms and their ecology. Academic Press, London

[6] Foissner, W., Blatterer, H., Berger, H. and Kohmann, F. (1991) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Bd.I: Cyrtophorida, Oligotrichida, Hypotrichida, Colpodea. Informationsberichte des Bayer. Landesamts für Wasserwirtschaft, Heft 1/91, 478 pp.

[7] Foissner, W., Berger, H. and Kohmann, F. (1992) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Bd.II: Peritrichia, Heterotrichida, Odontostomatida. Informationsberichte des Bayer. Landesamts für Wasserwirtschaft, Heft 5/92, 502 pp.

[8] Foissner, W., Berger, H. and Kohmann, F. (1994) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Bd.III: Hymenostomata, Prostomatida, Nassulida. Informationsberichte des Bayer. Landesamts für Wasserwirtschaft, Heft 1/94, 548 pp.

[9] Foissner, W., Berger, H., Blatterer, H. and Kohmann, F. (1995) Taxonomische und ökologische Revision der Ciliaten des Saprobiensystems. Bd.IV: Gymnostomatea,

Loxodes, Suctoria. Informationsberichte des Bayer. Landesamts für Wasserwirtschaft, Heft 1/95, 540 pp.

[10] Patterson, D.J. and Hedley, S. (1992) Free-living freshwater protozoa. Wolfe Publishing Ltd., London

[11] Eikelboom, D.H. (1975) Filamentous organisms observed in activated sludge. Wat. Res. 9, 365-388

[12] Eikelboom, D.H. and van Buijsen, H.J.J. (1983) Handbuch für die mikroskopische Schlammuntersuchung. Hirthammer, München

[13] Jenkins, D., Richard, M.G. and Daigger, G.T. (1993) Manual on the causes and control of activated sludge bulking and foaming. Lewis Publishers, 2nd ed., Boca Raton, USA.

[14] Lemmer, H. (1992) Fadenförmige Mikroorganismen aus belebtem Schlamm. Vorkommen - Biologie - Bekämpfung. ATV-Dokumentation- und Schriftenreihe aus Wissenschaft und Praxis, Band 30, Hennef

[15] Seviour, E.M., Williams, C., DeGrey, B., Soddell, J.A., Seviour, R.J. and Lindrea, K.C. (1994) Studies on filamentous bacteria from Australian activated sludge plants. Wat. Res. 28, 2335-2342

[16] Kämpfer, P., Weltin, D., Hoffmeister, D. and Dott, W. (1995) Growth requirement of filamentous bacteria isolated from bulking and scumming sludge. Wat. Res. 29, 1585-1588

[17] Lemmer, H. (1996) Biologische Ursachen von Schaum und Schwimmschlamm in Belebungsanlagen sowie mögliche Gegenmaßnahmen. *In*: Ökologie der Abwasserorganismen (Lemmer, H., Griebe, T., Flemming, H.-C. Hrsg.), pp 259 - 271. Springer Verlag Heidelberg

[18] Lemmer, H. (1996) Ursachen und Bekämpfung von Blähschlamm. *In*: Ökologie der Abwasserorganismen (Lemmer, H., Griebe, T., Flemming, H.-C. Hrsg.), pp 247 - 257. Springer Verlag Heidelberg

[19] Prakasam, T.B.S. and Dondero, N.C. (1967) Aerobic heterotrophic populations of sewage and activated sludge. I. Enumeration. Appl. Microbiol. 15, 461-467

[20] Pike, E.B. and Curds, C.R. (1971) The microbial ecology of the activated sludge process. In Microbial aspects of pollution (G. Sykes and F.A. Skinner, eds.), pp. 123-147. Academic Press, London, UK

[21] Benedict, R.G. and Carlson, D.A. (1971) Aerobic heterotrophic bacteria in activated sludge. Wat. Res. 5, 1023-1030

[22] Kavanaugh, R.G. and Randall, C.W. (1994) Bacterial populations in a biological nutrient removal plant. Wat. Sci. Technol. 29, 25-34

[23] Amann, R., Glöckner, F.-O. and Neef, A. (1997) Modern methods in subsurface microbiology: in situ identification of microorganisms with nucleic acid probes. FEMS Microbiology Reviews 20, 191-200

[24] Amann, R., Lemmer, H. and Wagner M. (1998) Monitoring the community structure of wastewater treatment plants: a comparison of old and new techniques. FEMS Microbiology Reviews, in press

[25] Richard, M.G., Shimizu, G.P., Jenkins, D. (1985) The growth physiology of he filamentous organism Type 021N and its significance to activated sludge bulking. J. Wat. Pollut. Control Fed. 57, 1152-1162

[26] Williams, T. M., Unz, R. F. (1985) Filamentous sulfur bacteria of activated sludge: Characterization of *Thiothrix, Beggiatoa* and Eikelboom 021N strains. Appl. Environ. Microbiol. 49, 887 - 898

[27] Lemmer, H. and Kroppenstedt, R.M. (1984) Chemotaxonomy and physiology of some actinomycetes isolated from scumming activated sludge. System. Appl. Microbiol. 5, 124-135

[28] Lemmer, H. and Baumann, M. (1988) Scum actinomycetes in sewage treatment plants. Part 2. The effect of hydrophobic substrate. Wat. Res. 22, 761-763

[29] Lemmer, H., Ries, H. and Baumann, M. (1991) Selection and growth strategies of filamentous microorganisms in bulking and scumming activated sludge. *In:* Biological Approach to Sewage Treatment Process: Current Status and Perspectives (Madoni, P. ed.) Luigi Bazzucchi Center, Perugia, Italien, 151-155

[30] Lemmer, H., Lind, G., Schade, M. and Ziegelmayer, B. (1997) Autecology of scum producing bacteria. Proc. 2nd Int. Conf. Microorganisms in Activated Sludge, 21.-23.7.97, Berkeley, CA

[31] Lemmer, H., Zaglauer, A., Neef, A., Meier, H. and Amann, R. (1997) Denitrification in a methanol-fed fixed bed reactor. Part 2: Composition and ecology of the bacteria community in the biofilms. Wat. Res. 31, 1903-1908

[32] Neef, A., Zaglauer, A., Meier, H., Amann, R., Lemmer, H., Schleifer, K.-H.: In situ population analysis in a denitrifying sand filter: Conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. Appl. Environ. Microbiol. 62, 4329 - 4339, 1996

[33] Lemmer, H., Zaglauer, A. and Metzner, G. (1997) Denitrification in a methanol-fed fixed bed reactor. Part 1: Physico-chemical and biological characterization supports optimal operation. Wat. Res. 31, 1897-1902

[34] Fuhs, G.W., Chen, M. (1975) Microbiological basis of phosphate removal in the activated sludge process for the treatment of wastewater. Microb. Ecol. 2, 119-138

[35] Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schleifer, K.-H. (1994) Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792-800

[36] Wedi, D., Wagner, M., Amann, R., Erhart, R., Lemmer, H. and Wilderer, P.A. (1995) In-situ-Nachweis der untergeordneten Rolle von Bakterien der Gattung *Acinetobacter* bei der erhöhten biologischen P-Elimination. Abwasser Special I Phosphor-Elimination, GWF Wasser-Abwasser 136/13, 17-23

[37] Gujer, W., Henze, M., Mino, T., Matsuo, T., Wentzel, M.C. and Marais, G.v.R. (1995) The activated sludge model No.2: Biological phosphorus removal. Wat. Sci. Techn. 31, 13-23

[38] Wagner, M., Amann, R., Kämpfer, P., Aßmus, B., Hartmann, A., Hutzler, P., Springer, N. and Schleifer, K.-H. (1994) Identification and in situ detection of Gram-negative filamentous bacteria in activated sludge. System. Appl. Microbiol. 17, 405-417

[39] Lim, E.L., Caron, D.A. and DeLong, E.F. (1996) Development and field application of a quantitative method for examining natural assemblages of protists with oligonucleotide probes. Appl. Environ. Microbiol. 62, 1416-1423

[40] Andreasen, K., Nielsen, P. H. (1997) In situ characterization of substrate uptake by *Microthrix parvicella* using microautoradiography. Proc. 2nd Int. Conf. on Microorganisms in Activated Sludge and Biofilm Processes. July 21 - 23, 1997, Berkeley, California

[41] Nielsen, P. H. , Andreasen, K., Wagner, M., Blackall, L.L., Lemmer, H., Seviour, R.J.: Variability of Type 021N in activated sludge as determined by in situ substrate uptake pattern and in situ hybridization with fluorescent rRNA targeted probes. Proc. 2nd Int. Conf. on Microorganisms in Activated Sludge and Biofilm Processes. July 21 - 23, 1997, Berkeley, California

[42] Jürgens, K. and Güde, H. (1994) The potential importance of grazing-resistant bacteria in planktonic systems. Mar. Ecol. Prog. Ser. 112, 169-188

[43] Cech, J.S., Hartman, P. and Macek, M. (1994) Bacteria and protozoa population dynamics in biological phosphate removal systems. Wat. Sci. Tech. 29, 109-117

Functional characterization of biofilm biocenoses of fixed bed reactors for advanced wastewater treatment

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Abstract

As the performance of biological fixed bed reactors for wastewater treatment is significantly affected by the spatial distribution of biomass and its activity and the biotic structure of microbial consortia, these parameters were in the center of interest in this study. Methods applied for the examination of heterogeneity of biofilm communities along the reactor depth were discussed. Results obtained from two identical but differently operated membrane biofilm reactors were compared. Different biomass parameters showed that continuous flow operation resulted in a stratified growth along the direction of flow. Different proportions of specialized microorganisms in different reactor depths were made evident for both investigated types of biofilm reactors by the comparison of catechol-1,2-dioxygenase activity and general heterotrophic activity in terms of enzymatic hydrolysis of FDA. Thus this approach allows a functional characterization of (biofilm) biocenoses from reactors for wastewater treatment. Using fluorescent rRNA-targeted oligonucleotide probes on biofilm samples, the diversity of bacterial biocenoses in the two biofilm reactors was examined. However, the composition of microbial communities at the level of main groups of *Eubacteria* did not significantly differ from the communities found in other wastewater treatment systems. Consequently, there is a need of more specific probes and functional markers to obtain more detailed information on the population dynamics of microbial films.

1. Introduction

Biological wastewater treatment exploits the capacity of microorganisms to carry out different biochemical reactions which result in the purification of wastewater. As a prerequisite, a biocenosis with the capacity to degrade organic substances contained in sewage and industrial effluents has to evolve and be maintained in a bioreactor. Biological fixed bed reactors meet this requirement by retaining the microorganisms in the reactor in the form of a biofilm. In such a biofilm reactor even slowly-growing bacteria, i.e. nitrifiers or xenobiotic-degrading bacteria, may propagate and persist independently of the hydraulic load. The reactor performance is supposed to be significantly influenced by the longitudinal distribution of biomass, its activity and the population dynamics of biofilm biocenoses. Therefore, by analyzing biofilm dynamics

due to changes of operational parameters, it is intended to control biological fixed bed reactors performance (see Lazarova and Manem [1], for example).

This contribution discusses some methods applied to characterize biofilm biocenoses of fixed bed reactors for advanced wastewater treatment. To study the influence of different operation strategies on reactor performance, we compared two identical membrane biofilm reactors, one operated discontinuously as Sequencing Batch Biofilm Reactor (SBBR) (as introduced by Wilderer [2]), the other with continuous flow (Continuous Flow Biofilm Reactor - CFBR) examining the elimination of chlorophenols which serve as a model for poorly degradable xenobiotics. As the reactor design and the mode of operation are supposed to affect biofilm dynamics, biofilm samples were taken from both reactors and from different sections of these reactors. Differences between the biofilm biocenoses were to be shown by the quantification of biomass, by the estimation of microbial activities and by the investigation of the composition of microbial communities.

2. Material and methods

2.1. Experimental set-up

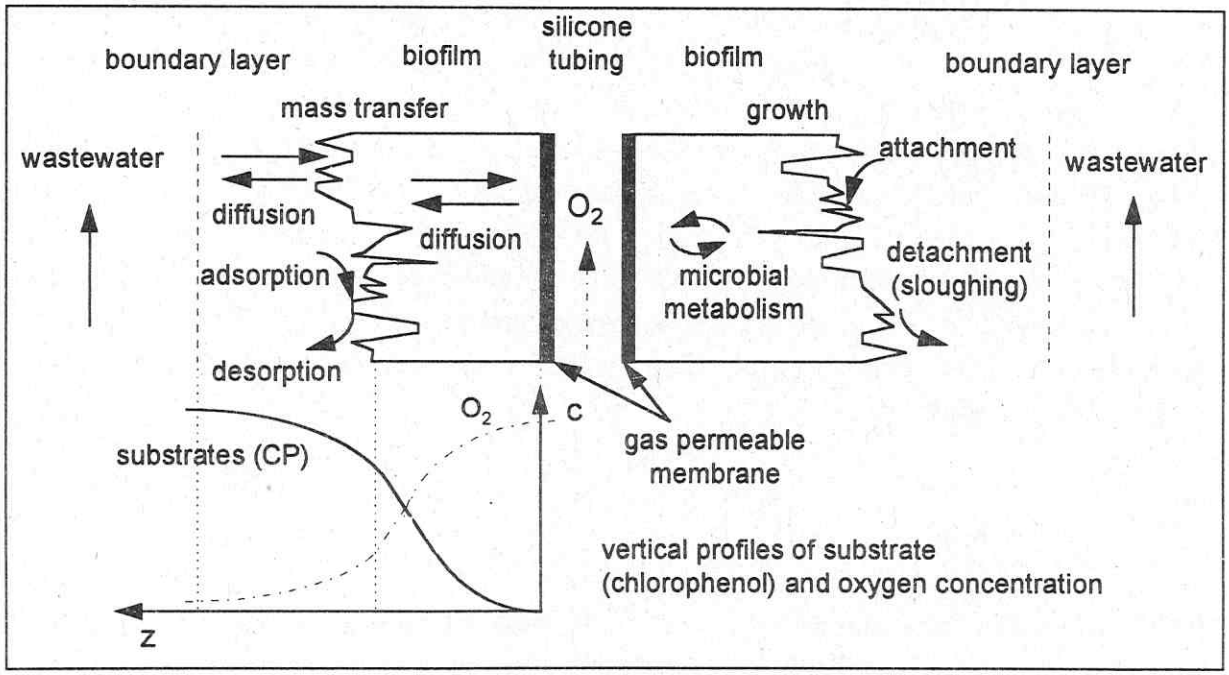

Fig. 1. Schematic representation of biofilm grown on gas permeable silicon tubings, of concentration gradients across the biofilm, and of main processes significant to substrate utilization by biofilm organisms. (The thickness of the biofilm is superelevated as to the diameter of the silicone tubing.)

The laboratory plant, which has been previously described [3, 4] consisted of two identical cylindrical reactors with a length of 2 m and an inner diameter of 0.11 m. As the inner axis (made from polyethylene) had a helical form with 68 coils, the total distance of flow was about

15 m. Thus the flow characteristic was in full accordance with a plug-flow reactor. Six oxygen-permeable silicone tubings laid out in parallel served as carrier material for the biofilm and supplied the microorganisms with oxygen from the inside. Figure 1 characterizes the "inverted" biofilm on the tubing and the concentration gradients across the membrane-biofilm system. To prevent compression, the tubing was sheathed by a screen of polyester wire. In addition to the analysis of influent and effluent, it was possible to take water samples at five different heights of reactor.

The reactors were fed with the effluent from a municipal activated sludge plant to which 4-monochlorophenol (4MCP) at influent concentrations between 10 and 80 mg l^{-1} was added. The wastewater passed through the CFBR in upflow direction. In order to have a standard of comparison, the residence time of the water in the CFBR of 6 hours corresponded to the length of one cycle of the SBBR which included a fill, a reaction and a draw period. The SBBR was filled from the bottom to the top within 0.5 hour. After circulating the water during the reaction period of 5 hours the reactor was drained against the direction of filling for 0.5 hour.

2.2. Removal of biofilm samples and preparation for biochemical investigation

For the investigation of membrane-grown wastewater biofilms presented in this study, slices of silicone tubings of 20 to 50 mm length were cut out after draining the reactor and removed from the reactor through the sampling ports. Prior to resuspension of biofilm matter in phosphate buffer, the biofilm was removed from the tubing mechanically (for investigation of eukaryotes) or by sonication in a sonic water bath and subsequent scraping (for biochemical and microbiological investigations).

Besides the microscopic enumeration of microorganisms in biofilm samples after staining with DAPI (4,6-diamidino-2-phenyl-indol-dihydrochloride) [5, 6], cellular components like DNA or total protein were used as biomass parameters. As a first step, these substances have to be extracted from the cells. Therefore, the following procedure was applicated to membrane grown biofilm samples from the laboratory plant.

- Washing of biomass with phosphate buffer (0.05 M, pH 7.0), centrifugation at 20,000 g at 4°C for 10 min (using high speed centrifuge 3K30, SIGMA, Osterode, Germany)
- Sonication of the resuspended pellets by an ultrasonic desintegrator (Sonopuls HD 60, Bandelin electronic, Berlin, Germany) with 100 % power for 6 * 30 seconds (kept at 0°C by icewater)
- Removal of cell debris and other particular material by centrifugation at 25,000 g at 4°C for 20 min

2.3. Biochemical methods

The content of DNA in the supernatant was determined by use of bisbenzimide Hoechst 33258 according to Obst [7]. The reaction mixture (3 ml) contained: 50 to 200 µl of supernatant, citrate buffer (0.17 M, pH 7.0), 10 µg ml^{-1} bisbenzimide. Fluorescence was measured at an

excitation wavelength of 345 nm and at an emission wavelength of 455 nm against a blank containing buffer and bisbenzimide using a spectrofluorometer (RF 5001-PC, Shimadzu Europa GmbH, Duisburg, Germany). Proteins were detected in aliquotes of the cell free extracts by Lowry's method according to standard manuals [8].

For characterization of the metabolic potential of biofilm samples, enzyme assays were performed concerning both general microbial activity and activity of more specific enzymes. As a marker of general heterotrophic activity, the release of fluorescein by hydrolysis of fluorescein diacetate (FDA) catalyzed by extracellular esterases were determined according to the protocol published by Obst [7]. Homogenized, but not sonicated biofilm samples were diluted with phosphate buffer (0.06 M, pH 7.6) and, after addition of 100 µl of FDA (2 mg ml^{-1}), incubated for one hour at 20 °C in a rotating mixer. The released fluorescein was measured photometrically at 490 nm (UV/VIS spectrophotometer U-2000, Hitachi Ltd., Tokyo, Japan) in the supernatant after centrifugation at about 6,000 g for 10 min against a blank containing buffer and FDA.

To estimate the activity of microorganisms which are able to degrade (chloro-)aromatics, we measured the conversion of (chloro-)catechols to (chloro-)muconic acid by the catechol-1,2-dioxygenase. This enzyme is involved in the chlorophenol degradation [9, 10] and has been determined according to an assay described by Reineke and Knackmuss [11]. The reaction mixture contained (in 3 ml): 100 µmol Tris-HCl buffer (pH 8.0), 1 µmol of catechol or of the chloro-substituted analog, 4 µmol EDTA, and 100 to 200 µl of cell free extract (corresponding to a protein content from approximately 10 to 300 µg) according to the previously described procedure. Conversion of catechols to muconic acids was followed by the increase of absorbance at 260 nm at a UV/VIS spectrophotometer (U-2000, Hitachi Ltd., Tokyo, Japan). The molar absorption coefficients (ε) available from literature [12] and the observed protein content were used for the calculation of the conversion rate in $nmol*h^{-1}*mg^{-1}$ protein.

2.4. Investigation of microbial communities

In cultivation experiments with aliquotes of resuspended biofilms, the colony-forming units (CFU) developing on a standard medium (according to the German standard methods - DEV: 10.0 g peptone, 10.0 g meat extract, 5.0 g NaCl, 10.0 g agar, add. 1000 ml) and on a mineral medium (containing up to 80 $mg*l^{-1}$ 4MCP as sole carbon source) were counted.

The direct detection and identification of microorganisms in the biofilm samples by means of fluorescently labelled, rRNA-targeted oligonucleotide probes was applied to avoid the disadvantages of cultivation methods (low percentage of cultivable cells, selection pressure of media). This technique became the most popular method to study biodiversity of complex microbial communities and protocols were published by many authors [13, 14, 15, 16]. More detailed information on the hybridization procedure applicated to the homogenized, resuspended biofilm samples are given by Röske et al. [5]. The oligonucleotide probes which have been used to examine the taxonomical composition of the biofilm samples are listed in Table 1. For the determination of the total cell number, slides with immobilized cells were stained with DAPI (1 $\mu g*ml^{-1}$) subsequently to the *in situ* hybridization. After imbedding in Citifluor AF1

(Citifluor Ltd., London, UK), at first the hybridized cells were counted, subsequently those stained with DAPI, at a epifluorescence microscope (Jenalumar, Carl Zeiss, Germany).

Table 1. Oligonucleotide probe data

Probe	Specifity	Sequence 5′-3′	rRNA target	Applied stringency (formamid concn [%])	Reference
EUB338	*Eubacteria*	GCTGCCTCCCGTAGGAGT	16S	0	13
NON338	negative control	CGACGGAGGGCATCCTCA	-	0	13
ALF1b	Alpha subclass of *Proteobacteria*	CGTTCGYTCTGAGCCAG	16S	20	16
BET42a	Beta subclass of *Proteobacteria*	GCCTTCCCACTTCGTTT	23S	35	16
GAM42a	Gamma subclass of *Proteobacteria*	GCCTTCCCACATCGTTT	23S	35	16
HGC69a	Gram-positive Bacteria with high G+C-content	TATAGTTACCACCGCCGT	23S	20	17
SRB385	sulfate reducing Bacteria	CGGCGTCGCTGCGTCAGG	16S	35	18
CF319a+b	*Cytophaga-Flavobacterium*	TGGTCCGTVTCTCAGTAC	16S	20	17
NEU23a	*Nitrosomonas* sp.	CCCCTCTGCTGCACTCTA	16S	40	19
GAM42a	unlabelled competitor for BET42a	GCCTTCCCACATCGTTT	23S	35	16
BET42a	unlabelled competitor for GAM42a	GCCTTCCCACTTCGTTT	23S	35	16
CTE	unlabelled competitor for NEU23a	TTCCATCCCCCTCTGCCG			19

3. Results and discussion

3.1. Quantification of biofilm biomass

The use of cellular components as biomass parameter has to meet the requirements of (i) an ubiquitous presence in microbial cells, (ii) a relatively constant content regardless of the species and the physiological state, and (iii) that it is characteristic of living cells [1]. Determination of

DNA seems to be well suitable as such a parameter [7]. As can be seen from Figure 2, longitudinal gradients of the DNA content of biofilm extracts were in good agreement with the distribution of biomass, which has been calculated from the number and volume of bacterial and eukaryote cells (see reference [4] for detailed information).

The longitudinal distribution both of DNA content and of microscopic estimated biomass confirmed the heterogeneous microbial growth in the CFBR caused by gradients of substrate concentration according to the plug-flow conditions (Figure 2). However, there was about 30 % more biomass in the CFBR as compared to the SBBR. The lower thickness of the biofilm in the SBBR was obviously caused by the higher shearing stress. The short filling period of SBBR avoided the formation of strong longitudinal concentration gradients of substrates. Thus, the biomass was more even distributed than in the CFBR. As the reactor was drained against the direction of filling, sloughing due to the higher shearing forces during this period and the displacement of the detached biomass to the lower part of fixed bed where it was retained, also affects the biomass distribution in the SBBR.

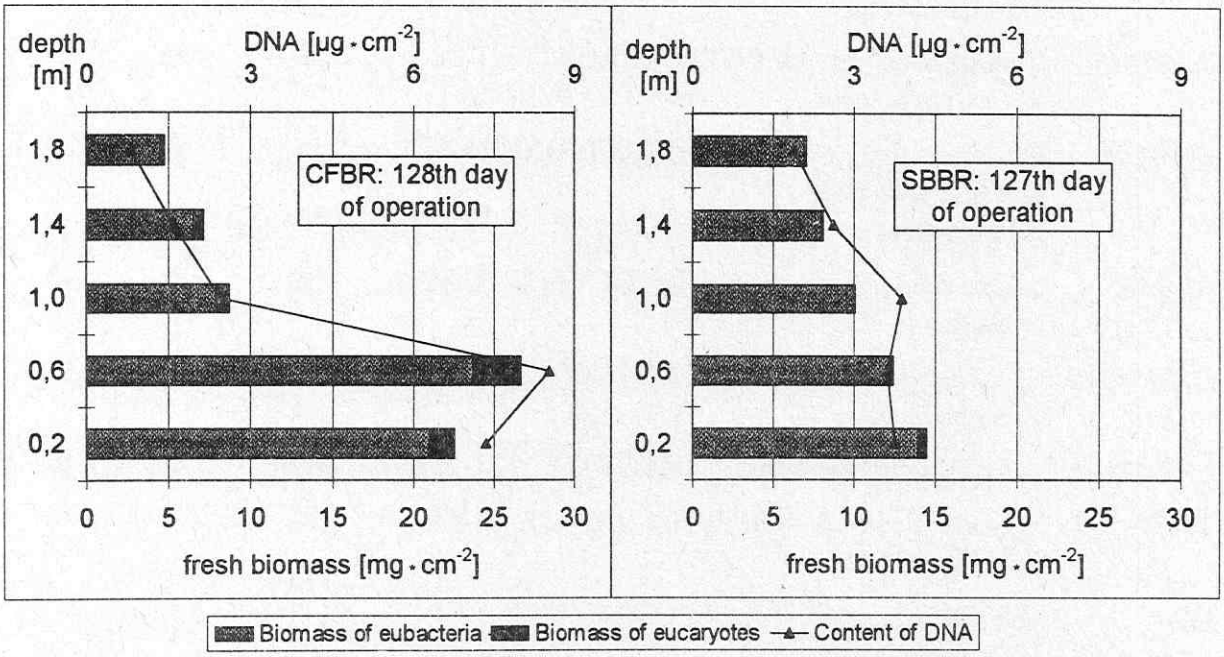

Fig. 2. Comparison of longitudinal distribution of biomass of bacteria and eukaryotes and of DNA content in the two different operated biofilm reactors with addition of 4-monochloro-phenol (4MCP) (after 127/128 days of operation).

The calculation of biomass from microscopic examination of total bacterial cell counts and eukaryotes indicates that the proto- and metazoa represented only a small percentage (almost up to 5%) of total biomass (see Figure 2). However, the biofilm reactors have to be considered as a complex ecosystem in which also food-web relationships were relevant. The interactions between bacteria and eukaryotic organisms are of particular significance to control the performance of a biofilm reactor. Grazing of the biofilm by mass growth of eukaryotes such as

oligochaetes (of the genus *Nais*, this study, see also [4]), snails [20], but also protozoa [21] may result in a decrease of reactor performance.

3.2. Estimation of biological activity

According to the substrate penetration which decreases with increasing biofilm thickness due to internal mass transfer resistance [22], it is to be supposed that the activity of biofilms does not proportionally increase to the biofilm thickness, i.e. biomass for example in terms of DNA. In case of transport limitation, an increasing part of the biofilm remains inactive. Thus, the estimation and control of active biomass becomes very important with respect to an optimization of biofilm reactor performance [1].

By means of enzymatic FDA hydrolysis it was intended to estimate the proportion of active microorganisms in different sections of the reactors, i.e. in biofilms of different thickness. An increasing proportion of inactive biomasse would be reflected by a decrease of specific enzymatic activities (related to biomass, for example protein content). However, the FDA esterase activity was shown to correlate closely with biomass parameters as total protein or the DNA content suggesting similar proportions of active biomass in both reactors at different depths (as also can be seen from Figure 3). This agrees with the close correlation of esterase activity to cell density of bacterial cultures [23] and to the ATP content [24]. Therefore it can be concluded that even in thick biofilms a considerable part of biomass kept its high heterotrophic potential. However, it has to be taken into consideration that esterases hydrolysing FDA are also produced by anaerobic bacteria [7]. Similar to the results described above, the general heterotrophic potential was more even distributed in the SBBR.

Compared to the distribution of biomass and of general heterotrophic activity, the conversion of catechol and 4-chlorocatechol by catechol-1,2-dioxygenase showed a different trend in the direction of flow in the CFBR (Figure 3). Related to the total protein, the catechol-1,2-dioxygenase activity tended to increase with reactor depth. By this observation, an accumulation of microorganisms which were well adapted to chlorinated aromatics was made evident in the upper section of the CFBR, i.e. in a part of the fixed bed with thinner biofilms. Considering the overall activities, the ratio of catechol-1,2-dioxygenase activity to FDA hydrolysis was higher in samples taken from the SBBR. From this the conclusion can be drawn that the percentage of spezialized microorganisms or their activity was higher in the SBBR. This is in good agreement with the specific elimination rates with regard to 4-chlorophenol observed during this operational period [4].

Furthermore, chlorinated analogs of catechol, especially the proposed intermediate of microbial 4MCP degradation 4-chlorocatechol, were converted up to 5 times more rapidly than unsubstituted catechol by biofilm samples from both reactors. The accelerated conversion of chlorocatechols indicates that there maybe special enzymes with higher V_{max} values and/or with a higher affinity (lower K_m values) to chlorinated substrates were synthesized by well adapted microorganisms. This assumption is in agreement with the concept of an early diverged evolution of chloroaromatic degradion pathways [25].

Although the enzyme assays do not give any information on the *in vivo* activity of microorganisms, from these results conclusions can be drawn with regard to the proportion of populations of microorganisms with a distinct metabolic potential. Thus this approach admit a functional characterization of (biofilm) biocenoses from reactors for wastewater treatment.

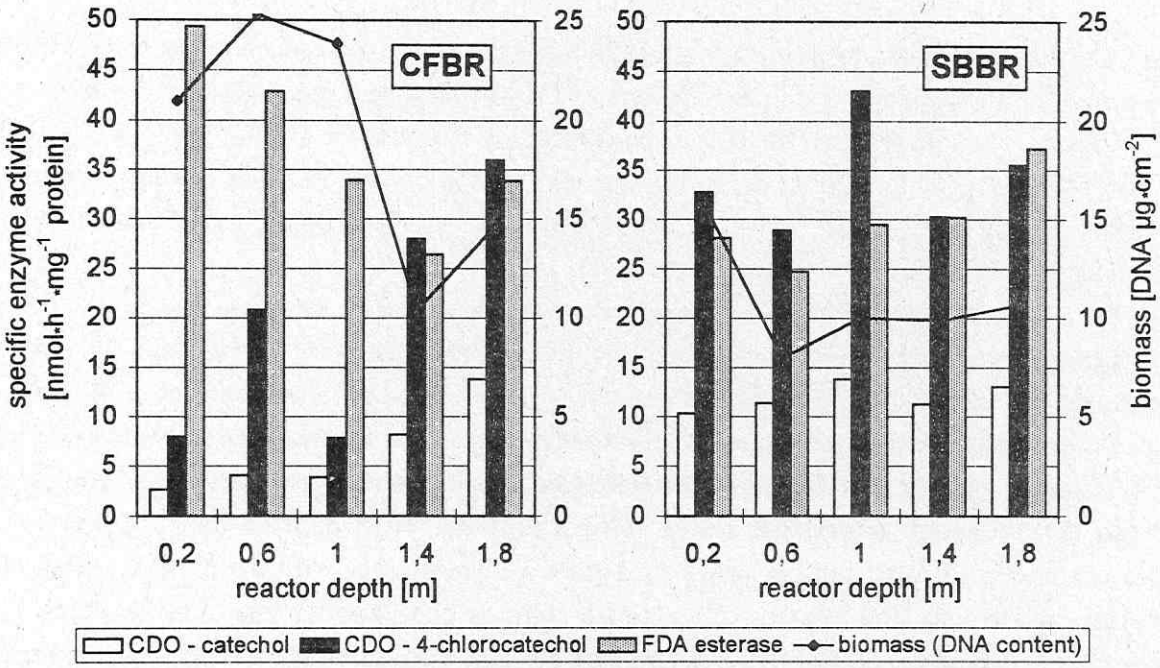

Fig. 3. General heterotrophic activity (enzymatic FDA hydrolysis) and conversion of catechol and 4-chlorocatechol, respectively, by catechol-1,2-dioxygenase (CDO) in different depths of both ractors after 50 days of operation with addition of 4-monochlorophenol compared to the distribution of biomass (in terms of DNA content).

3.3. Composition of microbial communities

As the degradative capacity of biocenoses in bioreactors for wastewater treatment depends on the composition of microbial community, it is suggested to make an attempt of identification of the individual members of the community. Cultivation techniques are well known to have severe limitations. Even by use of "optimized" media, only between 1 and 15 % of the cell counts determined by microscopic techniques are recovered [14, 15]. Moreover, there is a selective advantage for particular microbial species offered by the composition of the culture medium. This results in a shift of the population structure to this easy cultivable microorganisms whereas the most prominent species cannot be found by standard cultivation procedures [14, 15].

However, selective media can be used to enrich microorganisms with a specific metabolic potential. In order to select bacteria capable to utilize 4MCP as the only carbon source, a mineral medium with addition of 4MCP was used. The recovery from biofilm samples of both reactors was 4 % at highest of total cell count. By probing the colonies growing on this medium with group specific oligonucleotides, it was found that less than 20 % of the cells

belonged to the beta-subclass and more than 80 % to the gamma-subclass of *Proteobacteria*. From these, a *Pseudomonas* species could be identified with a specific oligonucleotide probe [26]. The capacity to decompose 4MCP has been described for pseudomonads by several authors [10, 27]. Thus, the question arises whether the 4MCP degradation in the reactors was performed by a low number of very active bacteria of the gamma-subclass of *Proteobacteria*, or whether these bacteria obtained a selective advantage by the composition of the culture medium.

The examination of the composition of microbial communities by *in situ* hybridization with oligonucleotide probes specific for main groups of bacteria (see Table 1) recovered 50 to 70 % of total cell count. As can be seen from Figure 4, the major proportion of the bacterial cells detected by means of the probes were members of the *Proteobacteria* class. The representatives of the beta subclass of *Proteobacteria* amounted to 10-50 % of the total number of bacterial cells, i.e. the total number of bacteria detectable with the EUB probe, and were the most abundant over the total length of the experimental period. This finding corresponds to the results obtained from other wastewater treatment systems [14, 15]. The microbial community in the SBBR consisted of the same groups of bacteria in similar proportions as in the biofilm from the CFBR. This indicates that the composition of the medium (i.e. the wastewater) may be more relevant to the microbial structure than the design and mode of operation of the reactor.

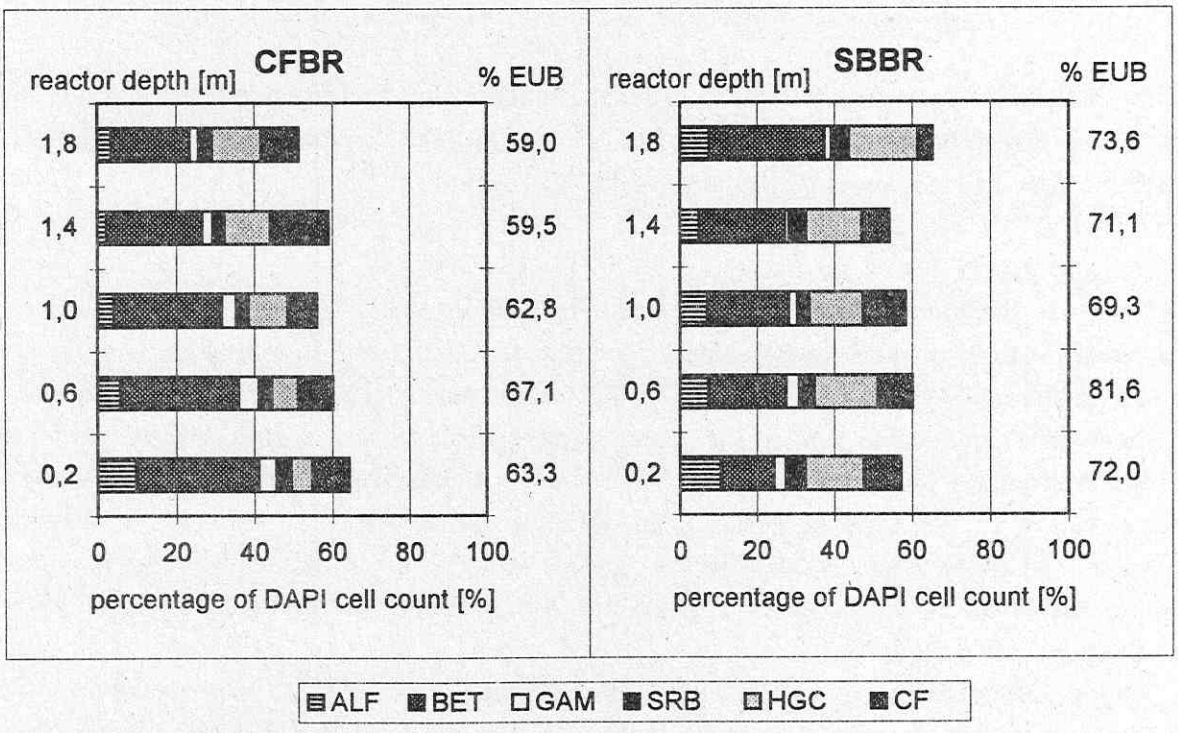

Fig. 4. Composition of the microbial film at several sampling points along the reactor depth in both biofilm reactors fed with 4MCP containing wastewater, as determined by *in situ* hybridization with oligonucleotide probes specific for the alpha-, beta-, and gamma-subclass of *Proteobacteria* (ALF, BET, GAM), for sulphate-reducing bacteria belongig to the delta-subclass (SRB), for gram-positive bacteria with high G+C content of DNA

(HGC), and for the *Cytophaga-Flavobacterium*-cluster (CF) (sampling after 308 days of operation).

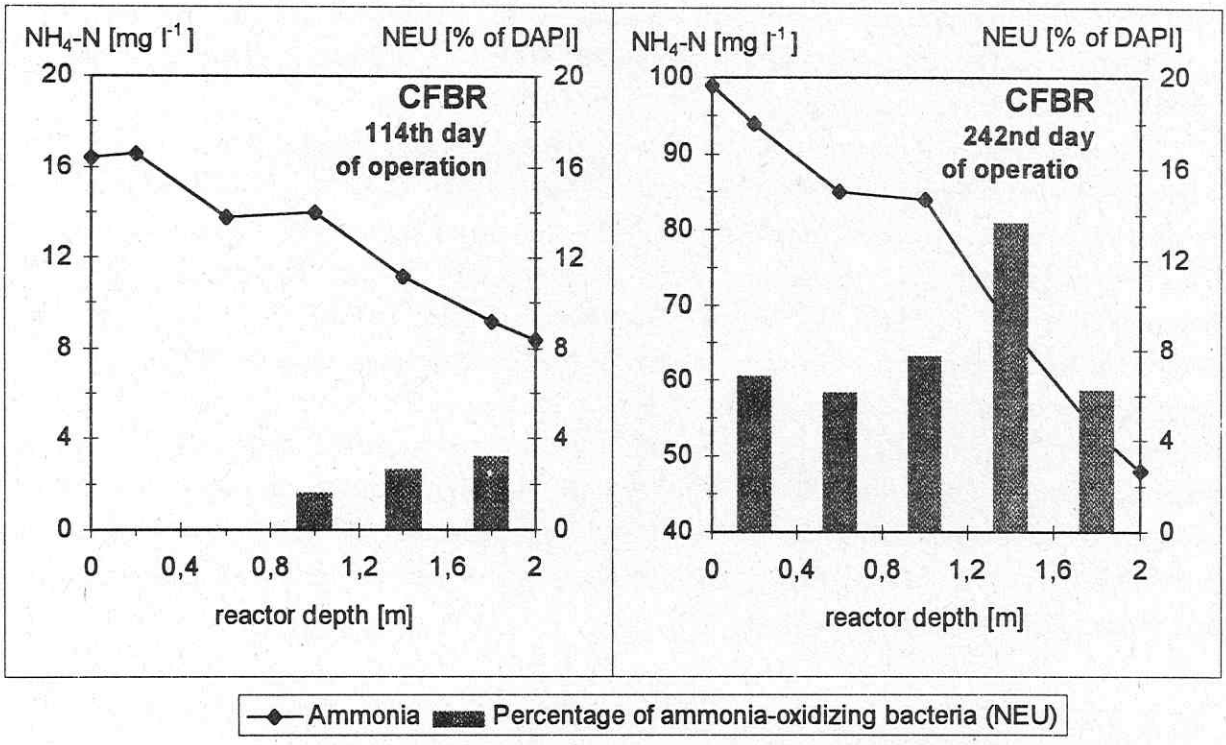

Fig. 5. Comparison between the vertical profile of ammonia concentration and the longitudinal distribution of ammonia-oxidizing bacteria (as detected by NEU oligonucleotide probe) in the CFBR in an earlier (left) and a later (right) operational period.

However, the applicated probes are specific for main groups of bacteria which comprise a very broad range of physiologically quite different taxa. Thus changes in community structure relevant to the metabolic potential of the biofilms could not detect by these probes. Possibly, the examination of biofilm samples with genus or species-specific probes will admit more detailed information on the composition of microbial communities with regard to functional aspects. However, some physiological characteristics, for example the capacity to degrade chlorophenols, are widespread over different groups of bacteria. Consequently, there is a need of functional markers for specific activities, i.e. the possibility to detect bacteria with the specific degradative sequence.

With regard to the nitrifying capacity, a more detailed information can be obtained by the use of genus-specific probes, i.e. a probe specific to *Nitrosomonas* (NEU) [19]. The presence of nitrifiers had to be expected because of the low organic load and the high ammonia concentration. While in the first months of operation the proportion of *Nitrosomonas* was below 4 % of total cell count, it increased to 6-14 % (corresponds to 10-20 % of EUB) in the following period. Correspondingly, a substantial part of the ammonia was oxidized. From Figure 5 it is evident that in the upper segment of the CFBR, where the NH_4 concentration

rapidly decreased (i.e. a high nitrification rate was observed), the proportion of *Nitrosomonas* detected with the oligonucleotide probe was highest.

4. Conclusions

In this contribution, methods for the investigation of both the spatial distribution of biomass and its activity and the population dynamics of biofilm communities from biological fixed bed reactors were discussed. These properties of a biofilm system are governed by the reactor design and the mode of operation and significantly affect the reactor performance. Thus the characterization of biofilm biocenoses can be considered as a tool for a better understanding and a more effective control of biofilm processes.

As a marker of biomass, the DNA content seems to be well suited for the estimation of spatial distribution of microbial films. However, the distribution of biomass estimated by microscopic enumeration of prokaryotes and eukaryotes was in good agreement with the longitudinal gradients of DNA content. By these parameters it becomes evident that the continuous operation of a plug-flow fixed-bed reactor results in a stratified growth.

To examine biofilm activity, biochemical assays for the determination of the activity of enzymes common among microorganisms or of enzymes involved in a specific metabolic process can be conducted to estimate the general or specific metabolic potential of biofilms respectively. Although the enzyme assays do not give any information on the *in vivo* activity of microorganisms, from these results conclusions can be drawn with regard to the proportion of populations of microorganisms with a distinct metabolic potential. For example, different proportions of specialized microorganisms in different reactor depths were made evident for both investigated types of biofilm reactors by the comparison of catechol-1,2-dioxygenase activity and general heterotrophic activity in terms of enzymatic hydrolysis of FDA. Thus this approach admit a functional characterization of (biofilm) biocenoses from reactors for wastewater treatment.

Using rRNA-targeted oligonucleotide probes for an *in situ* characterization of biofilm samples the majority of the cells detected directly by microscopic techniques could be monitored and classified, in contrast to cultivation methods in which only a small fraction were analyzed. Thus the diversity and the real composition of microbial consortia active in the biofilm reactors were reflected. However, the composition of biofilm biocenoses at the level of main groups of *Eubacteria* did not significantly differ from the communities of other wastewater treatment systems. Consequently, there is a need of more specific probes and functional markers to obtain more detailed information on the population dynamics of microbial films.

Acknowledgments

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References

[1] Lazarova, V. and Manem, J. (1995) Biofilm characterization and activity analysis in water and wastewater treatment. Wat. Res. 29, 2227-2245.

[2] Wilderer, P.A. (1992) Sequencing batch biofilm reactor technology. *In*: Harnessing biotechnology for the 21st century. (Ladisch, M.R. and Bose, A., Eds.) pp. 475-479. American Chemical Society.

[3] Wobus, A., Ulrich, S. and Röske, I. (1995) Degradation of chlorophenols by biofilms on semi-permeable membranes in two types of fixed bed reactors. Wat. Sci. Tech. 32(8), 205-212.

[4] Wobus, A. (1997) Untersuchungen zum Chlorphenolabbau in Membranbiofilmreaktoren-Vergleich zwischen kontinuierlichem und Sequencing-Batch-Betrieb. Ph.D. thesis, TU Dresden.

[5] Röske, I., Röske, K. and Uhlmann, D. (1997) Gradients in the taxonomic composition of different microbial systems: comparison between biofilms for advanced waste treatment and lake sediments. *In*: Proceedings of Second International Conference on Microorganisms in Activated Sludge and Biofilm Processes. (Jenkins, D. and Hermanowicz, S.W., Eds.) pp. 121-128.

[6] Fletcher, M. (1992) The measurement of bacterial attachement to surfaces in static systems. *In*: Biofilms - science and technology. (Melo, L.F., Bott, T.R., Fletcher, M. and Capdeville, B., Eds.), pp. 603-614. Kluwer Academic Publ., Dordrecht.

[7] Obst, U. (1995) Enzymatische Tests für die Wasseranalytik. R. Oldenbourg, München, Wien.

[8] Süßmuth, R., Eberspächer, J., Haag, R. and Springer, W. (1987) Biochemisch-mikrobiologisches Praktikum. Georg Thieme Verlag, Stuttgart, New York.

[9] Häggblom, M.M. (1990) Mechanisms of bacterial degradation and transformation of chlorinated monoaromatic compunds. J. Basic Microbiol. 30, 115-141.

[10] Knackmuss, H.-J. and Hellwig, M. (1978) Utilization and cooxidation of chlorinated phenols by *Pseudomonas* sp. B13. Arch. Microbiol. 117, 1-7.

[11] Reineke, W. and Knackmuss, H.-J. (1984) Microbial metabolism of haloaromatics: isolation and properties of a chlorobenzene-degrading bacterium. Appl. Environ. Microbiol. 47, 395-402.

[12] Dorn, E. and Knackmuss, H.-J. (1978) Chemical structure and biodegradability of halogenated aromatic compounds. Substituents effects on 1,2-dioxygenation of catechol. Biochem. J. 174, 85-94.

[13] Amann R.I., Krumholz, L. and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762-770.

[14] Manz, W., Wagner, M., Amann, R.I. and Schleifer, K.-H. (1994) *In situ* characterization of the microbial consortia active in two wastewater treatment plants. Wat. Res. 28, 1715-1723.

[15] Wagner, M., Amann, R.I., Lemmer, H. and Schleifer, K.-H. (1993) Probing activated sludge with oligonucleotides specific for proteobacteria: Inadequacy of culture-

dependent methods for describing microbial community structure. Appl. Environ. Microbiol. 59, 1520-1525.

[16] Manz, W., Amann, R.I., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: Problems and solutions. System. Appl. Microbiol. 15, 593-600.

[17] Wagner, M., Erhart, R. Manz, W., Amann, R.I., Lemmer, H., Wedi, D. and Schleifer, K.-H. (1994) Development of an rRNA-targeted oligonucleoide probe specific for the genus *Acinetobacter* and its application for *in situ* monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792-800.

[18] Amann R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919-1925.

[19] Wagner, M., Rath, G. Amann, R.I., Koops, H.-P. and Schleifer, K.-H. (1995) In situ identification of ammonia-oxidizing bacteria. System. Appl. Microbiol. 18, 251-264.

[20] Palsdottir, G. and Bishop, P.J. (1996) Nitrifying biotower upsets due to snails and their control. *In*: Proceedings of Third International IAWQ Special Conference on Biofilm Systems, 1996, Copenhagen.

[21] Lee, N.M. and Welander, T. (1994) Influence of predators on nitrification in aerobic biofilm processes. Wat. Sci. Tech. 29(7), 355-363.

[22] Harremoës, P. (1978) Biofilm kinetics. *In*: Water Pollution Microbiology, Vol. 2 (Mitchell, R., Ed.) pp. 71-111. John Wiley, New York.

[23] Schnürer, J. and Rosswall, T. (1982) Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Appl. Environ. Microbiol. 43, 1256-1261.

[24] Stubberfield, L.C.F and Shaw, P.J.A. (1990) A comparison of tetrazolium reduction and FDA-hydrolysis with other measures of microbial activity. J. Microbiol. Meth. 12: 151-162.

[25] Schlömann, M. (1992) Enzyme und Gene des Abbaus mono- und dichlorsubstituierter Brenzkatechine. Woher kommt ein neuer Abbauweg? *In*: Biologischer Abbau von Chlorkohlenwasserstoffen. (Weigert, B., Ed.) pp. 87-109. Schriftenreihe Biologische Abwasserreinigung 1, TU Berlin, Berlin.

[26] Schleifer, K.-H., Amann, R.I., Ludwig, W., Rothemund, C., Springer, N. and Dorn, S. (1992) Nucleic acid probes for the identification and *in situ detection* of pseudomonas. *In*: Pseudomonas: Molecular biology and biotechnology. (Galli, E., Silver, S., Withold, B., Eds.) pp. 127-134. American Society for Microbiology, Washington, D.C.

[27] Fava F., Armenante, P.M. and Kafkewitz, D. (1995) Aerobic degradation and dechlorination of 2-chlorophenol, 3-chlorophenol and 4-chlorophenol by a *Pseudomonas pickettii* strain. Lett. Appl. Microbiol. 21, 307-312.

The cooperation of algal and heterotrophic components in oil-polluted wastewaters

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Abstract

Practicing bioremediation in oil-polluted water ecosystems showed that usually an algal bloom was the final state of a biological succession. The algal bloom was the results of oil destruction by microbial communities, consisting of bacterial bio-preparations and the native microbiota, activated by addition of "asophos" fertilizer, a combination of nitrates and phosphorus. The blooms were caused mainly by native filamentous cyanobacteria, protococcous algae, and diatoms.

Model experiments with artificial communities consisting of *Rhodococcus* sp. and *Chlorella* sp. showed that oil degradation processes were enhanced by this treatment. The activation was probably caused by enhanced oxygen content due to the photosynthetic activity of *Chlorella* cells under these extreme conditions. Additions of algal biofilms and natural plant-algae-bacterial biocenoses in duck weed covers were used as phytoremediation technology for speeding up oil destruction in continental and estuarian pond waters.

The mechanisms of the cooperation between the organisms could be very different. In order to understand them, the studies of biocenoses of polluted waters should include an examination of the biodiversity of algae as well as the evaluation of different combinations of artificial biocenoses as prototypes of biofilters.

1. Introduction

Studies which were carried out in the Department of Microbiology of St.-Petersburg University in cooperation with the Department of Microbiological Synthesis Technology in St.-Petersburg Technological Institute have shown that combinations of oil-destructive bacterial preparations with algae or floating plants enhance the activity of microbial cenoses in the remediation of petroleum-polluted waters [1 - 3]. The use of floating plants, especially of different *Lemna* species, is the usual practice of wastewater phytoremediation in the USA [4].

The experience obtained in 1991 - 1995 allowed us to suggest that algae are the key component of the associations of organisms carrying out the self-cleaning of waters. Since 1995 this work has been continued in cooperation with Ecoprom Ltd. in the form of pilot projects.

Algae and higher plants are able to speed up the destruction of different pollutants, including petroleum [5 - 7]. The term "association" used in the description of the self-cleaning of waters implies a rather wide concept and includes nanoplankton, biofilms on water surfaces, epiphyton as well as aggregates of petroleum and microorganisms, which live in aerobic associations.

2. Methods and results

2.1. Self-cleaning of petroleum polluted water - work of biocenoses

We assume that there are two basic types of hydrocarbon oxidation. Firstly, the complete uptake and use of hydrocarbons and secondly, their transformation in intermediate products, being substrate for other organisms. The second case is well known and might explain the potential complexity of the oil-destroying biocenoses, because almost each component is necessary in the chain of the consumers of carbon and (or) energy. As a rule, these processes demand oxygen and can occur only at the surface of a biofilm. This means that photosynthetic microorganisms and macrophytes can carry out two functions. They increase the surface of a biofilm and they increase the local concentration of oxygen by light energy, thus promoting the development of the oil consumers. We were able to find some examples of such algal communities in our studies.

Most remarkable cases of algae blooms were found by us in oiltraps (Table 1), reminding of the well known case of the self-cleaning of the Arabian gulf with participation of cyanobacterial mats. The communities immobilized within mats consisted of several blue-green algae (*Phormidium, Microcoleus, Lyngbya*), diatoms, and different heterotrophic oil-degrading bacteria, actinomycetes and fungi [8 - 10].

It is well known that biocenoses which comprise different groups of organisms (algae, fungi and bacteria) are more effective in remediation, especially in the case of a toxic pollution [11]. However, it is not clear which role algal components play in these associations, i.e. in the local increase of oxygen content, in the increase of a biofilm surface, in the production of surface-active substances, in the utilization of alkans and their oxygenation [8], in a combination of these functions [10] or in other, not yet known functions of petroleum consuming communities.

Our last laboratory experiments (Table 2) show that *Chlorella* sp. strain CALU-74 enhance the process of oil degradation in different ways, depending on the alkanothrophic partner. However, the main mechanism of this action, the oxygen production by the alga, was not affected by the oil pollution. The strain CALU-74, isolated from the Siberian river Tobol, was not selected by making use of its resistance to oil-products. In situations, when we will be able to use those algal strains specially selected because of their resistance, the positive effects of the photosynthetic components could be even greater.

Table 1. Composition of photosynthetic organisms in blue-green mats (from Al Hasan et al., 1994 [8])* with additions of our observations**

Microorganisms	Saudi mats*	Kuwaiti mats*	Our observations of in 3 cases**		
			1	2	3
Cyanobacteria					
Phormidium	15 %	60 %	+++	+++	+++
Microcoleus	30 %	15 %	++	++	+++
Lyngbya	15 %	1 %	++	++	+
Oscillatoria	1 %	15 %	++		
Diatoms					
Navicula	33 %	1 %		++	+
Coscinodiscus	1 %	8 %			

*) per cent by volume of total biomass
**) in an oil trap (1), in Oredezh pond 1 (2), in Oredezh pond 2 (3);
+++ abundant; ++ common; + rare

Table 2. The efficiency of algal-bacterial consortiums in a model experiment: T=25 C, light, 750 lux, 2% oil

components of consortium		final concentrations of hydrocarbons		
algae	bacteria	mg/l	% from start level	% of control
No	No	15.0	75	100
CALU-74	No	6.5	32.5	43
No	*Rhodococcus* sp. str. 7HX	5.5	27.5	37
CALU-74	*R.* sp. 7HX	2.0	10	13
No	D-1-7	8.3	41.5	55
CALU-74	D-1-7	2.0	10	13

As the efficiency of an aerobic biocenosis is proportional to its surface, we shall discuss an aspect of the role of algae in film formation on new solid substrates:

2.2. Algae as 'pioneers' in the formation of a biofilm

The study of the formation of microbial communities has revealed that photosynthetic microorganisms are among the first during the colonization of new substrates [12, 13]. Among

the 14 genera found as colonizers of platforms, 11 were algae [14]. The colonization density was 20 times higher for algae than for heterotrophs.

In an early ecological paper [15] it was shown that components of biocenoses could be subdivided into three main groups:

- organisms with greatest activity;
- 'conformists', connected with the 1-st group;
- independent organisms.

Algae belong to the first group. They determine the development of other groups, reaching maximum densities already after 10 to 15 days. A study on the effect of a pollution on a freshwater community [16] had shown that the components most resistant to pollution were algae and some species of rotifers.

Using new, synthetic fibres with a high specific surface [17, 18] opens new prospects in designing biofilters. Heterotrophic microorganisms which destroy petroleum can be effectively immobilized on these fibers [19 - 23]. The ability of algae to colonize these surfaces should be studied. Our first data allow to suggest that additions of algae promote the biofilm formation on synthetic fibers.

2.3 Our practice of phytoremediation

For two years we have been carrying out the cleaning of a black-oil polluted pond at an electrical power station in St.-Petersburg (TEZ-5), applying phytoremediation as treatment. The surface water was heavily polluted by black oil (thickness of film reached 3 - 4 mm). Moreover, coastal vegetation was also strongly polluted. We used two kinds of bacterial preparations, "Ruden", the biomass of strain 7HX *Rodococcus* sp. (selection of Microbial Genetics Institute, Moskow) and "Olevorin", the biomass of *Acinetobacter oleovorum* (from the collection of the Protein Synthesis Institute, Moscow [24]).

The technology of the restoration and sanitation included:

(1) treatment of the water surface and pond banks by bio-preparations along the whole strech studied

(2) For intensification of oxidation process, floating biofilters with immobilized biota (algal biofilms) or with a natural cenoses (duck weed biomass) were installed

(3) chemical and microbiological analyses for the monitoring and updating of bioremediation

(4) We have shown earlier that alkanotrophs (biopreparation RUDEN) in a combination with a *Chlorella* sp. CALU-74 culture, or with a water plant (duckweed, *Lemna* sp.) performed the water remediation with better results (20 - 25%) and in shorter times [1, 3].

Table 3 shows the results of the analytical check of the petroleum contents at two periodically polluted sites (1,2) and at an output (3) of waters from the pond.

Table 3. Dynamics of the petroleum concentration in the water of a periodically polluted pond at a Heat Power Station (TEZ-5)

Points of sampling	Concentrations of black oil at dates of sampling, g/L					
	04.07.95	17.07.95	17.08.95	07.07.97	21.07.97	07.08.97
1	1.8	0.8	0.54	0.20	0.29	1.75
2	0.55	0.1	0.05	0.13	1.39	0.38
3 (output)	0.65	0.03	0.03	0.03	0.10	0.13

The remediation measures carried out for two years enabled us to reduce the dissolved oil content in the pond to the normal level. The coastal vegetation was completely cleared from black oil pollution, despite the constant inflow of petroleum into the pond. In 1997 we started a study on the algal cenosis in a ponds near the Oredezh settlement, in the Luga region of the Leningrad district. Phytoremediation was performed during summer 1997 for the last time. The river Kusinka was severely polluted by diesel fuel from a spill caused by a pipeline leakage in the Kirishi region of the Leningrad District (Table 4).

Table 4. Hydrocarbon concentration (mg/L) in the Kusinka water (in summer 1997)

Sampling sites	09.07.	15.07.	24.07.	04.08.	15.08.	22.08.	29.08.
1	110*	55.5*	80*	8.5	0.26	0.24	0.26
2	15	3.5	1.0	0.87	0.4	0.3	0.3
3	14	0.5	0.5	0.5	0.3	0.35	0.3

*) samples contained fragments of oil film

For intensification of the oxidation process stationary biofilters with an immobilized biota were established. Because of the attachment at the surface of the water body, the plants were active in photosynthesis, effective in the uptake of nutrients, and capable to accumulate heavy metals and other toxic substances. Triangles of reed mats were made to intensify the self-cleaning. The triangles were attached with cords at the edges. These mats were inhabited by colonies of microalgae, which were able to enrich the oxygen concentration in the surface layer of the water, together with duckweed which had been collected in clean ponds of the Peterhof town. To increase the production of duckweed and algae, nutrients, usually "Asofos" and dolomit powder, were added. During some months, the level of the pollution was reduced by 35 - 200 times in various parts of the river, although the leak of petroleum proceeded during all periods of our work.

3. Summary

The biological succession in an oil-degrading biota can lead to algal blooms (in the case of a surplus of nutrients). These blooms were shown to be mainly caused by native filamentous cyanobacteria, protococcous algae, and diatoms. New biotechnological methods should include specially selected associations with algae as components of biofilms and planktonic biota.

Model experiments with artificial communities consisting of *Rhodococcus* sp. and *Chlorella* sp. showed the activation of oil degradation processes. This activation was probably caused by an enhanced oxygen content due to the photosynthetic activity of *Chlorella* cells under these extreme conditions. Additions of algal biofilms and natural plant-algae-bacterial cenoses in duck weed covers situated on pond waters were used as a pilot phytoremediation technology to speed up the destruction of oil in continental and estuarian waters.

The real mechanisms of this cooperation could be very different. In order to understand them, studies on the biocenoses of polluted waters and the biodiversity of algae are needed as well as the testing of different combinations of artificial biocenoses (prototypes of biofilters).

References

[1] Kvitko K., Iankevitch M., Dmitrieva I., Safonova E., Lapunov J. (1996a) Consortiums of algae and alcanotrophic bacteria, their activity in destruction of crude oil films. Abstracts of International Conference "Ecologically safe technological processes in environmental problems solving". Irkutsk, June 18 - 22 1996, 1, 37-38 [in Russian]

[2] Kvitko K., Iankevitch M., Khadeeva V., Lizunov A., Safonova E., Dmitrieva I. (1996b) Functions of the Associations of Phototrophic and Heterotrophic Microorganisms in Processes of Soil and Waters Remediation after Oil Spills. *In*: Sustainable Development: System Analysis in Ecology, Materials of 2-nd International Conference, Sevastopol, Ukraine, September 9-12 1996, p. 55

[3] Kvitko K., Dmitrieva I., Iankevitch M., Khadeeva V., Avsukevitsch A. (1997) Photosynthetic Organizms As Novel Biotechnology Agents in the Phytoremediation of Surface Waters & Soils After Oil Spills. Abstracts of ISEB'97-MEETING, BIO-REMEDIATION, Leipzig, 24-27 Sept.1997, p. 103

[4] Reeds S.C., Midlebrooks E.J., Crites R.W. (1988) Natural Systems for Waste Management & Treatment. Mc Graw-Hill Book Company N.Y., p. 308

[5] Telitchenko M. (1972) About an opportunity of the water remediation processes management by biological methods. *In*: The theory and practice of biological self-cleaning of polluted waters. "Nauka", 20-24, [in Russian]

[6] Morozov N., Petrov G. (1972) Experience on remediation of water from petroleum in attendance of water plants. *In*: The theory and practice of biological self-cleaning of polluted waters. "Nauka", 42-46, [in Russian]

[7] Dogadina D.V. (1972) Prospects of algae use in cleaning drains of some industry. *In*: The theory and practice of biological self-cleaning of polluted waters. "Nauka", 47-49, [in Russian]

[8] Al-Hasan R, Sorkhoh N., Al-Bader D. and Radwan S. (1994) Utilization of hydrocarbons by cyanobacteria from microbial mats on oily coast of the Gulf. Appl. Microbiology and Biotechnology, 41, 615-619

[9] Sorkhoh N., Al-Hasan R., Radwan S. and Hoepner Th. (1992) Self-cleaning of the gulf. Nature (London), 359, 109

[10] Sorkhoh N., Al-Hasan R., Khanafer M. and Radwan S. (1995) Establishment of oil-degrading bacteria associated with cyanobacteria in oil polluted soils. J. Appl. Bacteriol. 78, 194-199

[11] Carpenter M., Robertson J. (1980) Marine hydrocarbon-degrading microorganisms: Community structure and biomass determination. Water Science and Technology, 20, (11-22), 433-435

[12] Cairns J.Jr. (1982) Freshwater Protozoan Communities. *In*: Microbial interaction and communities, v.1, (Bull A.T., Watkinson A.R.K. Eds.), Acad.Press, L., 249-285

[13] Cairns J.Jr., McCormick P.V. (1993) Determinants of structure in aquatic nonpelagic protozoan communities. Chin. J. Oceanol. Limnol., 11, (3), 215-228

[14] Piekarczyk R., McArdle E. (1985) Pioneer colonization and interaction of photosynthetic and heterotrophic microorganisms on an artificial substrate of polyurethane foam in E.J.Beck lake, Illinois. Transaction of Illinois Academy of Science, 78, (1-2), 81-86

[15] Ivlev V.S. (1933) Ein Versuch zur experimentellen Erforschung der Oekologie der Wasserbioeconosen . Archiv fuer Hydrobiologie, Bd. 25, 177-191

[16] Patrick R. (1949) A proposed measure of stream conditions, based on a survey of the Conestoga Basin, Lancaster County, Pennsilvania //Proceedings of The Academy of Natural Science of Philadelphia., 101, 277-341

[17] Shamolina I. (1995) Scientific bases of the technology of new biologically active fibrous materials. The Doctor of Science Dissertation Thesis, Specialities: 05.17.15 (technic. chemical fibers), 03.00.23 (biotechnology), SPb., [in Russian]

[18] Sinicin A., Rainina E., Losinskij V., Spasov S. (1994) Immobilized cells of microorganisms. A textbook. Moskow Univers. Press, 288 p. [in Russian]

[19] Iankevitch M., Khadeeva V. (1990) Prospects of bioactivated sorbents use for struggle with stand-by oil pollution in Arctic region conditions. The Kola centre of science, AS USSR, Appatits, 43 p., [in Russian]

[20] Iankevich M.I., Khadeeva V.V., Kvitko K.V. (1996) Combined biological technology of purification of the industrial waste water from the petroleum pollutions. Abstracts of III Intern. Conference "The Development of the North and Problems of Recultivation", St. Petersburg, 28-31 May 1996, p. 235

[21] Dmitrieva I., Kvitko K., Lapunov Y., Zelennikova O. (1996) Hydrocarbon oxydising microorganisms - potential components of system of the biological clean up under subarctic climate conditions. Abstracts of International Conference "Ecologically safe technological processes in environmental problems solving". Irkutsk, June 18 - 22 1996, 2, 112 - 113. [in Russian]

[22] Surzhko L., Iankevitch M., Shamolina.I. et al. (1993) Immobilisation of Yeast Cultures of the *Candida* genera on caprone cords. *In*: Guarding of the environment from pollution by industrial waste. *In*: Interuniversity collection of scientific works. SPb, SPbTI., pp.124-129, [in Russian]

[23] Surzhko L., Finkelshtein I., Baskunov B., Iankevitch M., Yakovlev V., L.Golovleva (1994) Utilisation of petroleum in ground and water by microorganism cells. Microbiology, 64, (3), 393-398. [in Russian]

[24] Biryukov V., Bitteeva M., Chtcheblykine I. (1997) Bioremediation of Oil Pollutions. Abstracts of ISEB'97-MEETING, BIOREMEDIATION, Leipzig, p. 103

Algal components of oil-polluted water ecosystems

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Abstract

Algal communities inhabiting two oil-polluted ponds were studied over one vegetation season. A total of 51 species of microscopic and one macroscopic algae were found. The attached algal communities were dominated by blue-green algae (Cyanobacteria). The biofilms covering the bottoms of the ponds and submerged objects comprised mainly several species of *Phormidium*, *Microcoleus* and *Lyngbya*.

The flora of diatom algae was rather poor and indicated a severe pollution. The dominant algae in the phytoplankton were several species of *Euglenophyta*, *Cryptophyta* and small-sized coccoid *Chlorophyta*, characteristic for highly polluted and hypertrophic water bodies.

1. Introduction

Photosynthetic organisms play an important role in the self-purification of polluted waters. It is essential to reveal the composition of the phototrophic communities inhabiting these waters. The algae and cyanobacteria living under constant pollution stress are well adapted to these conditions and can be used in artificial devices designed for sewage treatment. Unlike the communities of waters polluted by domestic and agricultural wastes, the algocenoses of oil-polluted waters are still poorly studied. The purpose of this paper is to describe the species composition of eukaryotic algae and cyanobacteria of the artificial ponds which have been heavily polluted by oil products for a long time.

2. Material and methods

The ponds studied are situated near St. Petersburg (Luga region of the Leningrad District, North-Western Russia). During the last 20 years these ponds have been used as collectors of oil products coming from the oil-tanks. The surface area of the pond N1 is 600 m^2, the surface area of the pond N2 is 1200 m^2. The mean water depth is 2 m. The water is slightly acid (pH 5.0-6.0) and highly enriched by mineral salts and organic material (bacterial preparations and duckweed), which were added to these water bodies in order to promote the biological degradation of hydrocarbons. The concentrations of the dissolved hydrocarbons in the water

varied from 0.3 to 0.5 mg/l. The banks and bottoms of the ponds were also heavily polluted by oil products. In July they were especially abundantly covered by the black oil films.

Samples of the phytoplankton and attached algae were collected in April, August and October 1997. The phytoplankton samples were taken from the water surface in the central part of the ponds, attached algae were collected at several sites from the bottom, higher water vegetation, and from other submerged objects. Floating pieces of the oil film with visible attached algae were also collected. The samples were fixed with formaline and ethanol. A total of 38 samples were studied. The algae were identified under a Leitz light microscope.

3. Results and discussion

A total of 51 species of microscopic algae and cyanobacteria were recorded in the water bodies studied (Table 1). Most of the species belonged to the class *Bacillariophyceae* (15 species) and division *Chlorophyta* (15 species). Cyanobacteria (*Cyanophyta*) were also quite numerous (11 species). Other species belonging to the algal class *Xanthophyceae*, divisions *Cryptophyta*, *Dinophyta* and *Euglenophyta*. One macroscopic alga *Chara vulgaris* from the division *Charophyta* was also found in the both ponds.

Cyanobacteria (blue-green algae) were the most abundant components of the attached algal communities. *Phormidium ambiguum* which is a very common species of soils and shallow waters of the temperate zone, formed algal mats on the bottom of ponds and on submerged objects. This species was especially abundant in April, but it was almost absent in August, after the bottoms and banks of the ponds had been severely polluted by the oil products in July. *Phormidium ambiguum* appeared again in October, but was not as abundant as in the spring. *Lyngbya* cf. *versicolor* also formed algal mats and these mats often were composed of both *Phormidium ambiguum* and *Lyngbya* cf. *versicolor*. Sometimes *Lyngbya aestuarii* was involved in the formation of these mats too. In August and October thin mats formed by *Microcoleus* spp. were found in the pond N2.

The blue-green mats were found even on the floating oil film on the surface of ponds and on the wet soil near the water. Cyanobacteria are known to be tolerant to the hydrocarbon pollution of soils [7]. It was shown experimentally that species of *Phormidium* and *Microcoleus* growing on oil-polluted marine coasts were able to utilize hydrocarbons [1]. It can be the case in freshwater too, especially when taking into account that the same genera of cyanobacteria were found in the ponds studied.

Diatoms were not abundant in the ponds. The most stable component of the algal communities was *Rhopalodia gibba*, which is characteristic for slightly alkaline waters. Some other diatom species as *Diatoma tenuis*, *Epithemia turgida* and *Synedra ulna* were also indicating relatively high water mineralization and pollution. The species of the genus *Eunotia* and *Tabellaria flocculosa* were totally absent in the ponds studied. This absence can be attributed to the severe pollution since these algae are extremely common in the area, especially in the small water bodies with slightly acid water. As the result, the overall composition of the diatom flora pointed to the higher pH values (alkaline waters) than those indicated by chemical analyses.

TABLE 1. List of algal species found in the oil-polluted ponds and their abundance (+ - rare, ++ - common, +++ - very abundant)

Algal taxa	Pond N 1			Pond N 2		
	Apr	Aug	Oct	Apr	Aug	Oct
1	2	3	4	5	6	7
Cyanophyta (Cyanobacteria)						
Anabaena sp.		+				
Aphanizomenon flos-aquae (L.)Ralfs					+	
Lyngbya aestuarii (Mert.) Liebm.	+	++	++	+		
Lyngbya cf. *versicolor* (Wartm.) Gom.	++	+			+	+
Lyngbya kossinskajae Elenk.				+		
Lyngbya limnetica Lemm.	++					
Microcoleus delicatulus W. et G.S.West					+++	+++
Microcoleus cf. *paludosus* (Kutz.) Gom.					+	
Phormidium ambiguum Gom.	+++		+	+++	+	+++
Phormidium tenue (Menegh.) Gom.				+		
Schizothrix fragilis (Kutz.) Gom.	+			+		
Heterocontophyta						
Bacillariophyceae						
Achnanthes sp.	+					+
Caloneis sp.	+					
Cymbella sp.	+					
Diatoma tenuis Ag.	+					
Epithemia turgida (Ehr.) Kutz.						++
Fragilaria sp.		+				
Gomphonema parvulum Kutz.				+	+	
Nitzschia palea (Kutz.) W. Smith	++			+		+
Navicula sp.	++	+	+			+
Rhopalodia gibba (Ehr.) O.Muller	++	+	++	+	++	+
Pinnularia microstauron (Ehr.) Cleve	++		+	+	+	+
Surirella angusta Kutz.	+			+		
Synedra acus Kutz.		+			+	
Synedra ulna (Nitzsch) Ehr.					+	+
Synedra sp.		+	+	+		+
Xanthophyceae						
Ophiocytium cohleare A.Br.	+					
Tribonema affine West	++	++	++			
Vaucheria sp.		+	++			
Cryptophyta						
Cryptomonas erosa Ehr.			++			
Dinophyta						
Glenodinium sp.			++	+	+	
Eugelnophyta						
Astasia curvata Klebs				++		
Euglena acus Ehr.	+++	+	+	+		
Phacus sp.	+	+				
Trachelomonas planktonica Swir.					+	
Trachelomonas volvocinopsis Swir.	+			+	+	

TABLE 1. (continued)

Algal taxa	Pond N 1			Pond N 2		
	Apr	Aug	Oct	Apr	Aug	Oct
1	2	3	4	5	6	7
Chlorophyta						
Carteria sp.				+		
Chlamydomonas sp.	++			+		
Chlorella sp.	+			+	+	
Crucigenia quadrata Morr.				+		
Dictyosphaerium pulchellum Wood		+				
Microthamnion strictissimum var. *macrocystis* Schmidle	+					
Monoraphidium griffithii (Berk.) Kom.-Legn.	+			++	+	++
Monoraphidium contortum (Thur.) Kom.-Legn.		+		+	+	
Scenedesmus acutus Meyen	++			++	++	++
Scenedesmus ellipticus Corda	+					
Scenedesmus obliquus (Turp.) Kutz.	+			+	+	
Scenedesmus quadricauda (Turp.) Breb.		+			+	
Sphaerocystis polycocca Korsch.	+					
Spirogyra sp.	+	++		+	+++	+
Stigeoclonium sp.	+					

The total number of recorded diatom species was extremely low for this type of water bodies. It has been noted earlier that diatoms can not tolerate a strong oil pollution. Zimonina [7] found their species numbers decreasing on the oil-polluted soils of the Vozeiskoye oil field. Diatom species richness was significantly lower in the lakes polluted by drilling mud compared to the undisturbed lakes of the Vorkuta tundra [4].

The *Xanthophyceae* were found only in the pond N1. *Tribonema affine* was rather abundant in the plankton and epiphyton. This species is very common in lakes and rivers of the Leningrad District and can tolerate a severe pollution. *Vaucheria* sp. was found mainly on the damp soil around the pond where it formed thick felts. *Xanthophyceae* as well as *Bacillariophyceae* are known as sensitive to the oil pollution [3]. They totally disappeared from soil polluted by crude oil and only several species were able to persist on the drilling mud pollution [2].

Euglenoid algae played an important role in the communities of ponds studied. In spring one of the most abundant algal species was *Euglena acus* (Euglenophyta). Its vegetative cells and cysts were found at all sites. This species is known as an indicator of beta-mesosaprobic conditions and as other euglenoid algae is inclined to heterotrophy. *Euglena* cells and cysts almost disappeared in the summer, but in the pond N2 species of the other euglenoid alga *Trachelomonas* appeared in August.

Among the filamentous green algae, only *Spirogyra* sp. was constantly present in the ponds. The small-celled chlorococcoid green algae were abundant in the plankton, especially in

the spring. They kept dominance until October in the plankton community of the larger pond (N2), but disappeared completely by this time from the smaller pond (N1). Here they were substituted by species of *Cryptophyta* and *Dinophyta*, probably because of the decreasing water temperature. A similar phytoplankton composition has been recorded in an oil-polluted lake in the Bolshezemelskaya tundra [6]. In the spring there was an outbreak of euglenoid algae. The summer phytoplankton was dominated by small *Chlorococcaceae* and *Cryptomonas erosa* (*Cryptophyta*). All these algae are characteristic of organically polluted waters and are pollution indicators.

However, not one species of *Desmidiales* which are the commonest algae in the area was recorded in the ponds studied. The high ratio of *Chlorococcales* to *Desmidiales* is known to indicate a high trophic status of the waters [5], but the total absence of desmids is an extremely rare case and is certainly caused by the oil contamination.

4. Conclusions

The algal flora studied was composed of the species common for the area, all of them are often found in soils and waters of north-west Russia. Most of the species are known to tolerate high degrees of pollution.

The total number of species was rather low for this type of water bodies. Especially striking was the low variety of diatoms in contrast to the relatively high number of cyanobacteria species which are normally found in the ponds of this area. The total absence of *Desmidiales*, *Chrysophyceae* and some diatoms (*Eunotia* spp.) is probably a special feature of the oil polluted sites.

The most abundant attached algae were *Cyanophyta* (cyanobacteria) which obviously tolerated the oil contamination well and possibly were able to utilize hydrocarbons. Plankton communities were dominated by species of *Euglenophyta*, *Chlorococcales* and *Cryptophyta*, which are characteristic of hypertrophic and polluted water bodies.

References

[1] Al Hasan, R.H., Sorkhoh, N.A., Al Bader, D., Radwan, S.S. (1994) Utilization of hydrocarbons by cyanobacteria from microbial mats on oily coasts of the Gulf. Appl. Microbiol. Biotechnol. 41, 615-619

[2] Elshina, T.A. (1986) Soil algae as indicators of the industrial soil pollution. PhD thesis. St.Petersburg. [in Russian]

[3] Shtina, E.A., Nekrasova, K.A. (1988) The algae of the oil-polluted soils. *In*: Recovery of the oil-polluted soil ecosystems. Moscow, p. 57-81. [in Russian]

[4] Stenina, A.S. (1996) Diatom algae of the tundra ponds influenced by the oil bore-holes (Arkhangelsk District). *In*: Some approaches to the ecological monitoring in the areas of oil and gas exploration, extraction and transportation. Publication N 143 of the Komi Scientific Center URO RAN. Syktyvkar, p. 111-124. [in Russian]

[5] Thunmark, S. (1945) Zur Soziologie des Suesswasser-Plankton: Eine methodologisch-ökologische Studie. Folia limnolol. Scand. Bd.3., 1 - 66

[6] Trifonova, I.S. (1990) Ecology and succession of the lake phytoplankton. Leningrad, P.180. [in Russian]

[7] Zimonina, N.M. (1996) Algal communities of the disturbed territories of the Vozeiskoye oil field. *In*: Bioindication of the environmental conditions in the Vorkuta tundras. Publication N 143 of the Komi Scientific Center URO RAN. Syktyvkar, p. 121-129. [in Russian]

The collection of algae from polluted waters and soils

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Abstract

A collection of algal strains from soils and waters polluted by oil and heavy metals was created. Algal strains for the collection were selected on the basis of their resistance to heavy metals, oil components, detergents, their psychrophily and capacity to grow on a solid substance, which are the most important characteristics for the utilization of algae in biofilters. A new estimation method of strain resistance to toxicants by testing suspensions in immunological plates is described. The resistant strains belonged to the algal genera *Scenedesmus* and *Chlorella.* It was found that the resistance of algal strains to aromatic hydrocarbons usually coincided with the resistance to detergents and heavy metals. The resistant strains can be recommended for the utilization in wastewater treatment.

1. Introduction

The self-purification mechanisms of water have been studied for a long time mainly by means of descriptive hydrobiology. The limited possibility of this approach and the need of experimental studies has been noted by Vinberg [14], Skadovsky [11], Timofeeva-Ressovskaya et al. [12], and many others. The algae and higher plants were shown to be able to speed up the degradation of pollutants, for instance, oil products [3, 7 13]. Moreover, algae were shown to sustain heavy pollutions better than other groups of organisms [2, 8]. A study on algae from a biofilter revealed that species belonged to 5 divisions: *Euglenophyta, Chlorophyta, Xantophyta, Bacillariophyta and Cyanophyta*, with diatoms and green algae playing the leading role in the community [4]. It is still unclear whether algae are able to degrade oil components [1, 10].

The aim of this study was to obtain a collection of algal strains in order to use them in biofilters in the North-West of Russia. This was achieved by means of (1) culturing algal clones from sewage and soils which were polluted by oil and heavy metals, and (2) choosing those algae among isolated forms which were (a) capable to grow at low temperatures (as in the natural water bodies), (b) able to grow on a biofilter surface in the presence of pollutants, (c) resistant to oil products and detergents, and (d) resistant to heavy metals.

2. Material and methods

2.1. Algal isolates

Samples of algae were taken from several sites. The basis of the collection was formed by algae isolated from a biofilter which was used to purify wastewater severely polluted by oil products and heavy metals at the repository of toxic wastes 'Krasny Bor' situated in the Leningrad District. Some strains were obtained from an oil-polluted water body near an electric power station in the Leningrad District. Another set of algal strains was isolated from the brackish waters of the estuary of Pechora river which is only slightly polluted. The Belaya river (near Ufa city) was polluted by phenols. The algae from oil-polluted soil samples of the cities Rostov, Cherepovets and Omsk were taken in the hope to find psychrophils, which would be able to grow at low temperatures (10 - 15°C). Hence, the algae were taken from different habitats: soils, biofilters, submerged objects, sediments, phytoplankton, etc.

2.2. Media and cultivation conditions

The inorganic media N6 and Bold-Bristole medium (BBM) were used. The medium N6 had the following composition (mg/l): KNO_3 - 1000, K_2HPO_4 - 200, $MgSO_4$ - 200, $CaCl_2$ - 150, $NaHCO_3$ - 200; microelements. The composition of BBM was (mg/l): $NaNO_3$ - 250, KH_2PO_4 - 175, K_2HPO_4 - 75, $CaCl_2 \times 2H_2O$ - 25, $MgSO_4 \times 7H_2O$ - 75, NaCl - 25; microelements - 1 ml.

The medium SPA (Dry Nutrient Agar, Makhachkhala city) was used as a solid agar medium with addition of organic substances. Its composition is (g/l): sprat hydrolyzate - 17,9; NaCl - 5,9; agar - 11,2; pH = 7,2 - 7,5. In a number of cases 1/10-diluted SPA medium was taken and agar was added up to a concentration of 1,5%. Another solid medium was 1/4 BBL (originally BBL = Becton Dickinson, USA, consists of: Pancreatic Digest of Gelatin - 5,0; Beef Extract - 3,0; Agar - 15,0; pH = 6,8). It was prepared by mixing the agar-agar powder into 1 L with 5 g of dry BBL medium powder up to an agar concentration of 1,5%. To obtain BBL*medium the mineral solution of BBM medium was added to BBL medium up to a 4 times lower concentration than the standard one.

Algae were cultivated on liquid media and on media containing 1,5% agar under continuous illumination by luminiscent lamps (1500 - 2000 lux, t=25°C) in an illuminated room and thermostats (10°C and 15°C) with 1000 - 1500 lux illumination.

2.3. Methods of purification and cloning

In order to obtain pure algal cultures separate cells were isolated on the solid BBM medium and on medium N 6 by streaking. The same media with penicillin addition (500 mg/l) were used to purify cultures from bacteria. In several cases, colonies free from bacteria were isolated by means of a Pasteur pipette. To purify algal cultures from micellar fungi they were cultivated

under t = 10°C. The purity of cultures was tested by culturing them on the organic media BBL and BBL*.

2.4. Quantitative estimation of growth rates at different temperatures

Algal growth was examined at 10, 15 and 25°C and an illumination of 1500 Lux. The estimated optimal temperature was used to separate algal strains into mesophytes and psychrophytes. The biomass accrual was quantified by measuring the optical density (wave-length - 660 nm) with a Lars Ljungberg and Co. nephelometer. The cell density was calculated using a standard calibration curve in its linear part. The algal isolates were suspended in 5 ml of medium N6 and were cultivated in the test tubes (diameter 16 mm) which had been checked to not have a damaged surface.

2.5. Estimation of strain capacity to grow on biofilters

1. Capacity to form a biofilm on capron fibres.
0,25 g of synthetic fiber was put into the test tubes containing a dense algal suspension and the capacity to grow on it was estimated qualitatively. The mark "5" was given for abundant growth on all fiber surfaces.
2. Resistance to heavy metals.
The isolates from the "Krasniy Bor" repository were tested in Petri dishes on medium BBL*+penicillin with an addition of heavy metals in concentrations of 0.33 and 1.00 for $Cd(NO_3)_2$, $CdCl_2$, $ZnSO_4$, $NiCl_2$ and 0.66 and 2.00 mM for $CuSO_4$.

We used a drop test applying a 26 point replicator. The drops consisted of three times tenfold diluted cell suspension in medium. This provided survival tests of 80 - 8000 cells in a drop. *Chlorella vulgaris* CALU 157 and cultures CALU 189 and SAG 211-8c were used as control strains.

2.6. Resistance to toxic aromatic hydrocarbons

The influence of the addition of three hydrocarbon solutions was tested. The solutions contained methylnaphthalene (0.6 - 10 ppm), 1,6-dimethylnaphthalene (0.6 - 10 ppm) and phenanthren in 50% DMSO (0.06 - 1 ppm); phenol (0.03 - 0.5 ppm) was used as a standard. For the quick estimation of the resistance, we introduced a new method which allows to determine inhibiting concentrations of 4 toxicants for 16 forms at once (Fig. 1). Four immunological plates (hollow volume 200 µl) with addition of 4 different toxicants to algal suspensions can be seen on the photograph received from a scan: 16 forms were tested in 5 concentrations on each plate. The concentration was twice increased from the center to the outlying area. In this way resistant, medium resistant and sensitive strains were identified. The density of the algal cultures corresponded to 0,9 - 2,0 units of the optical density. The dilution

of the first toxicants was 1/10 and all the following dilutions were 1/2. The algae were cultivated at t = 25°C. A scanner was used to register the results of the algal culture resistance to toxicants, giving us the opportunity to get color and black and white photographs.

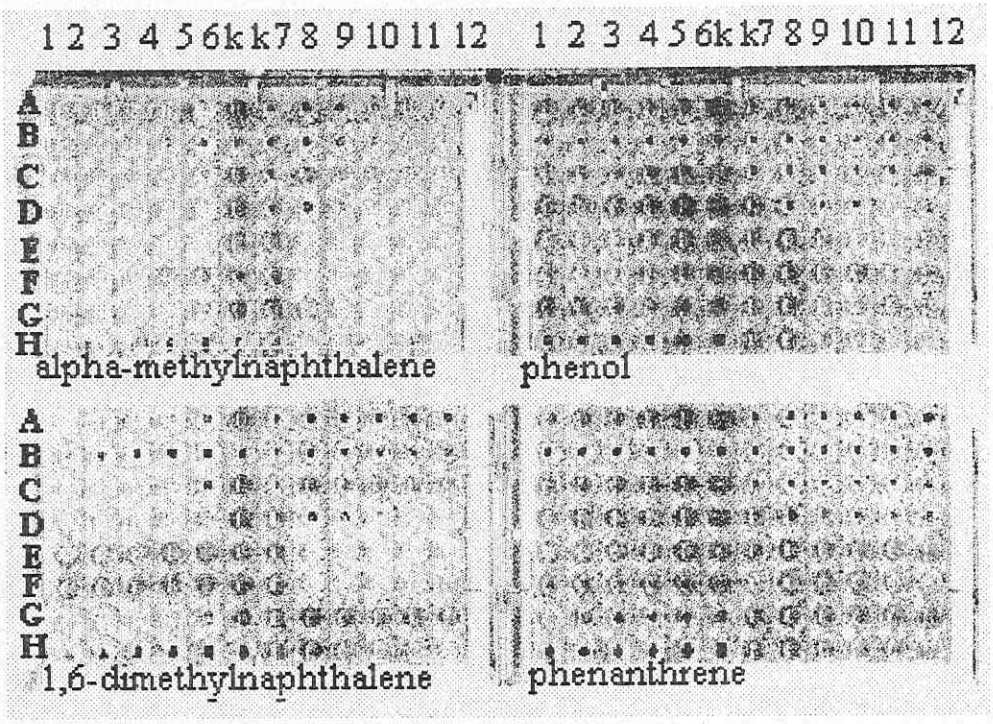

Fig. 1. Toxicity test in immunological plates

2.7. Resistance to detergents

The effect of 9 detergents was tested (two of them common soaps) - DMSO (dimethylsulfoxide), TWEEN 80, TWEEN 100, TWEEN 20, BIO Versal, SDS, LiDS, LUX (common detergent - soap powder "Lux"), detKUP (KOP Ultra plus).

The experiments were carried out in immunological plates. The algal density corresponded to 2 units of the optical density. Dilutions of the detergents were made by mixing of equal volumes of the suspensions.

It was shown experimentally that the final concentrations of the detergents were 22, 11, 5.5, 2.75, and 1.37 ppm. The plates were cultivated at t = 25°C. The results were registered by plate scanning.

3. Results and discussion

Initially 51 strains were isolated from soils and waters which were polluted with oil and heavy metals. After the duplicate strains were excluded, 28 strains were left in the collection. The algal species belonged to the divisions *Chlorophyta*, *Cyanophyta*, and the class *Xantophyceae*. The integral resistance of the strains obtained from all habitats sampled is given in Table 1.

At the first stage of the work the temperature optima of the algal strains were found out. Then the resistance of algal strains to different components of natural oils, aromatic hydrocarbons, their derivatives and detergents was studied. The minimal effective concentrations (ppm) were: methylated naphthalenes - 0.6 - 10, phenanthrene - 0.02 - 1.0, phenol - 0.03 - 0.47, TWEEN-80 and DMS - 5.5 - >22.0, SDS, LiDS and BioVersal - 1.4 - >22.0, TWEEN-100, detKUP - <1.4.

Among the algal isolates (51) from seven habitats, 10 isolates showed resistance, 8 middle resistance and 33 were sensitive to toxicants (Table 1). The proportion of sensitive strains was lowest among the isolates from polluted water bodies of the Leningrad District (17 strains). It is obvious that algal strains obtained from a severely polluted area (Krasniy Bor) were more often tolerant to toxicants than those from a relatively clean area (Pechora estuary).

Table 1. Number of strains with different levels of resistance to toxicants

Locality	Level of resistance				Number of strains
	high	medium	low	% of low	
Cherepovets	1	0	12	92	13
Rostov	1	4	3	37	8
Krasniy Bor, SPb	5	3	5	38	13
Electrical power station, SPb	1	2	1	25	4
Pechora	1	0	8	89	9
Ufa	0	0	3	100	3
Omck	1	0	0	0	1
Total	10	8	33		51

Among 10 tested algal genera, strains of *Stichococcus, Nostoc, Monodus, Scenedesmus* and *Chlorella* showed high resistance to toxicants, and strains of *Scenedesmus, Chlorella, Kirchneriella* and *Chlorhormidium* showed medium resistance (Tables 2, 3).

Strains of the genus *Chlorella* in aquatic samples (Krasny Bor, Ufa, electric power station, Pechora) and of the genera *Chlamydomonas, Gloeobotrys* (Cherepovets) and *Scenedesmus* (Rostov) in soil samples were found to be most resistant to toxicants. Among these genera *Chlorella* showed the highest, while *Gloeobotrys* and *Chlamydomonas* showed the lowest level of resistance to toxicants. The numbers of strains of other genera are too small to make any conclusions about their resistance (Tables 2, 3).

It should be pointed out that the temperature (10 - 15°C) in the Pechora estuary (ES-31, -33, -34, -37), the locality of isolation of some algae, differed from the optimal cultivation temperature which was 25°C (Table 4).

Table 2. Resistance to toxicants of algal taxa isolated from soil

algal taxa	locality	number of tested strains with different resistance levels			
		total	high	medium	low
Chlamydomonas	Cherepovets	6	0	0	6
Gloeobotrys	Cherepovets	5	0	0	5
Pleurochloris	Cherepovets	1	0	0	1
Stichococcus	Cherepovets	1	1	0	0
Scenedesmus	Rostov	4	0	3	1
Kirchneriella	Rostov	1	0	1	0
Nostoc	Rostov	1	1	0	0
Chlorella	Rostov	2	0	0	2
Monodus	Omsk	1	1	0	0

Table 3. Resistance estimation of algal taxa isolated from aquatic samples

algal taxa	locality	number of tested strains with different resistance levels			
		total	high	medium	low
Chlorhormidium	electric power station	1	0	1	0
Chlorella	Krasny Bor	13	5	3	5
Chlorella	Ufa	3	0	0	3
Chlorella	electric power station	3	1	0	2
Chlorella	Pechora	9	0	1	8

The strains resistant to aromatic hydrocarbons and their derivatives were also found to be resistant to detergents (Tables 4, 5). Strains (ES-6, -13) with high resistance to heavy metals showed also a high resistance to aromatic hydrocarbons and detergents, while strains with medium resistance to heavy metals (ES- 1, -3, -4) appeared to be sensitive to toxicants (Table 4).

The SAS-resistant mutants of *Chlorella* ES-3 were selected. One of them was retained in the collection, and its characteristics were shown to differ from those of the parent strain. Firstly, the strain switched from phototrophy to mixothrophy. Secondly, it became resistant not only to SAS, but also to aromatic hydrocarbons [9].

New isolates from oil polluted soils near Ufa and a water body in the Leningrad District were recently added to the collection. Most of them were shown to belong to the *Cyanophyta* (*Nostoc, Lyngbya, Phormidium, Anabaena*) and *Bacillariophyta* (*Navicula*).

Table 4. Characteristics of the *Chlorella* strains (phtr - phototroph, mxtr - mixotroph; +/- resistance to toxicants: phl - phenol, *a*-MN - *a*-methylnaphthalene, 1,6-DMN - 1,6 - dimethylnaphthalene, phn - phenantren, SAS - surface active substances, NT - not tested).

Code	number of strains	ecotope	trophic type	optimal temp. C	immobilization	heavy metals resist.	phl	*a*-MN	1,6-DMN	phn	SAS
ES-1,-3,4	3	water	phtr	15	good	middle	+	-	-	+	-
ES-3m-2,-6,-13	3	water	mxtr	25 and 15	bad	high	-	+	+	+	+
ES-27	1	soil	phtr	15	good	NT	+	+	+	+	+
ES-30	1	soil	phtr	15	good	NT	_	_	_	+	-
ES-41,-42	2	water	phtr	15	good	NT	-	-	-	+	-
ES-31	1	water	phtr	15	NT	NT	+	-	+	+	-
ES-33,-34,-37	3	water	phtr	25	NT	NT	-	-	-	+	-

Table 5. Characteristics of the algal taxa which were added to the collection

taxon, code	number of strains	eco-tope	opt. t	immobili-zation	resistance to hydrocarbons and their derivatives				
					phl	*a*-MN	1,6DMN	phn	SAS
Scenedesmus obliquus ES-55	1	soil	25	good	-	+	_	+	+
Sc. quadricauda	3								
ES-59		soil	15	good	-	+	-	+	+
ES-79				bad	-	-	-	+	+
ES-80				bad	-	+	-	+	+
Chlamydomonas sp.	2	soil	15	good	-	-	-	+	-
Stichococcus minor ES-19	1	soil	15	bad	+	-	+	+	+
Kirchneriella obesa ES-60	1	soil	15	bad	+	-	-	+	-/+
Chlorhormidium flaccidum ES-29	1	water	15	-	-	-	+	+	-
Nostoc sp. ES-79-2	1	soil	25	good	+	+	+	+	
Gloeobotrys chlorinus ES-67, ES-77	2	soil	15	good	-	-	-	+	-
Pleurochloris lobata ES-15	1	soil	15	good	-	-	-	-	-
Monodus coccomyxoides ES-44	1	soil	15	good	+	+	-	+	+

4. Summary

1. Psychrophytic algal isolates possessing a number of useful features, such as the capacity to attach to a solid substrate and the resistance to toxicants, which can be utilized in biofilms, were brought into culture.

2. Several algal isolates can be recommended for further tests on their usefulness as filters and degraders immobilized on biofilters.

3. The resistance of algal strains to aromatic hydrocarbons and their derivatives usually coincides with their resistance to detergents and heavy metals.

4. The minimal inhibiting concentrations ranged from 10 to 0,6 ppm for methylnaphthalene, from 1,0 to 0,02 ppm for phenanthrene, from 0,47 to 0,03 ppm for phenol; the inhibiting concentrations for detergents were higher: >22 - 5,5 ppm for TWEEN 80 and DMS, >22 - 1,4 ppm for SDS, LiDS, Bio Versal, >22 - 2,8 ppm for LUX, and >22 - 1,4 ppm for TWEEN 100, detKUP.

Acknowledgments

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References

[1] Al Hasan R.H., Sorkhoh N.A., Al-Bader D., Radwan S.S (1994) Utilisation of hydrocarbons by cyanobacteria from microbial mats on oily coast of the Gulf. Appl. Microbiol. Biotechnol. 41, 615-619

[2] Dart R.K., Stretton R.J. (1980) Microbiological Aspects of Pollution Controll. V.6, Elsevier Sci. Publ. Corp., Amster., Oxford, N.Y.

[3] Dogadina D.V. (1972) Prospects of algae use in cleaning drains of some industry. *In*: The theory and practice of biological self-cleaning of polluted waters. "Nauka", Moscow, 47-49 [in Russian]

[4] Dogadina T. V., Chukhlebova N. A. (1971) Algae of biofilms and their role in self cleaning processes. Hydrobiolology Journ., 7, (6), 56 - 60 [in Russian]

[5] Gromov B.V., Titova N.N. (1991) The CALU - collection of algal cultures of microbiological laboratory of biological Institute of St. Petersburg University. *In*: Catalogue of microalgal cultures in the collections of the USSR. M, 76-125 [in Russian]

[6] Kvitko K.V. (1974) The roles of selection and mutation processes in microbial populations.- Progress in Modern genetics, N 5, "Nauka". Moscow, 101-113

[7] Morosov N., Petrov G. (1972) Experience of remediation of water from petroleum in at attendance of water plants. *In*: The theory and practice of biological self-cleaning of polluted waters. "Nauka", Moscow, 42-46 [in Russian]

[8] Patrick R. (1949) A proposed measure of stream conditions, based on a survey of the Conestoga Basin, Lancaster County, Pennsilvania. - Proceedings of The Academy of Natural Science of Philadelphia. 101, 277-341

[9] Safonova E. Ph. (1997) Diversity of resistance to toxicants among *Chlorella* strains isolated from polluted waters. - Abstracts of the scientific conference of students and young scientists of biological faculties. Ufa, 65-66 [in Russian]

[10] Semple K.T., Cain R.B. (1996) Biodegradation of Phenols by the Alga *Ochromonas danica*. Appl. Environ. Microbiol. 1265-1273

[11] Skadovsky S. N., ed. (1961) Biocoenoses of epibioses as absorbers. (New ways of preliminary water purification for water supply). MGU Publ.House, 305 p. [in Russian].

[12] Timofeeva-Ressovskaya E. A., Agafonov B.M., Timofeev-Ressovsky N. V. (1960) On soil-biological water deactivation . Transactions of Biological Institute of the Ural branch of the USSR Academy of Science, Sverdlovsk, 13, 35-48 [in Russian]

[13] Telitchenko M. (1972) On the possibility of the water remediation by biological methods *In*: The theory and practice of biological self-cleaning of polluted waters. "Nauka", Moscow, 20-24 [in Russian]

[14] Vinberg G.G. (1972) Significance of hydrobiology in the solution of water economic problems. *In*: The theory and practice of biological self-purification of polluted waters, 7 - 12 [in Russian]

Short communications

- Polluted waters and sediments -

Nitrification in the rivers Mosel and Saar

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The evaluation of nitrification rates in the rivers Mosel and Saar in 1995 and 1996 was part of the project "Oxygen budget and biological processes in the regulated rivers Mosel and Saar". The study was initiated by the "Internationale Kommissionen zum Schutz von Mosel und Saar gegen Verunreinigung (IKSMS)" to improve the knowledge about the biological processes responsible for the deficiency of oxygen encountered during summer in the canalized sections of both rivers. Under *in situ* conditions the activity of nitrifying bacterioplankton was determined by measuring the dark ^{14}C-bicarbonate fixation with and without addition of 5 mg l^{-1} ATU (allyl-thiourea) and 10 mM chlorate, these substances were proved to be specific inhibitors for autotrophic ammonia and nitrite oxidizing bacteria. Optimal conditions (20°C, aerated, 0.3 mM ammonia supplemented) were chosen to estimate potential nitrification rates. The measured rates were in the range of those evaluated for rivers with similar ammonia concentrations (0.1 - 3.0 mg NH_4^+-N l^{-1}). Longitudinal and seasonal fluctuations of actual and potential nitrification rates were observed for both rivers. In the river Saar the longitudinal profile was characterized by increasing rates (up to 5.5 µg N l^{-1} x h^{-1}). This increase was based on an increasing addition of ammonia by the confluence of tributaries and increasing numbers of nitrifiers as determined by most probable number technique (up to 1 x 10^4 ammonia oxidizing bacteria ml^{-1} and up to 1 x 10^2 nitrite oxidizing bacteria ml^{-1}). The rates also increased from May to August due to increasing water temperatures. These results were confirmed by oxygen consumption studies (N-BOD_5, biological oxygen demand of nitrifying bacteria in 5 days) and nitrogen balance estimations. Increasing rates in the sections of the confluence of the main tributaries were also observed in the river Mosel. But due to lower ammonia concentrations the longitudinal and temporal profiles of nitrification rates showed less variation in the river Mosel than in the river Saar. Supplementation with ammonia in general resulted in higher bicarbonate fixation rates by nitrifying bacterioplankton (up to 8 µg N l^{-1} x h^{-1} in the river Saar) indicating that ammonia was a limiting factor, especially in summer when the other growth conditions were more favorable for nitrifying bacteria.

Interaction of synthetic musk compounds with river biofilms grown in rotating annular reactors

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Recently synthetic musk compounds (nitro and polycyclic substances) have been identified as a new class of ecotoxicological critical substances. In this study their interaction with lotic biofilms was investigated. For this purpose rotating annular reactors were used to grow biofilms from water of the river Elbe, Germany. To receive mature biofilms the river water served as inoculum and sole source of nutrients. The biofilm was then exposed to a cocktail of different musk compounds to study their fate in the microcosm. The hydrophobic musk compounds showed a rapid elimination from the water phase within the first few hours after spiking the system. GC-MS analyses of the biofilm demonstrated their presence within the biofilm. Multiple spiking did not result in an enrichment of the musk compounds neither in the water nor in the biofilm phase. From the results it may be suggested that the compounds are bound in a non-extractable way within the biofilm, chemically changed to more polar derivatives or that they are metabolized very rapidly. Confocal laser scanning microscopy was employed to follow the development of the interfacial community. It was found that over time the biofilm developed from a bacteria dominated film into a biofilm with bacteria and algae. The interfacial community developed from microcolonies, via a thin film into a ridged biofilm with up to 130 µm thickness. The complex biofilm architecture revealed a flow orientated structure as described for other turbulent flow systems.

Sorption and fate of selected herbicides in river biofilms

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Agricultural watersheds are heavily impacted by non-point source inputs of fertilizers and pesticides. Herbicides such as diclofop methyl and atrazine have been reported from watersheds in North America and Europe and although only 0.2 to 3% of applied herbicides reach surface waters, concentrations of 10 µg L-1 have been reported. Lotic biofilms may represent an im-

portant sink for these contaminants. In our experiments biofilms were developed in rotating annular reactors using river water as sole source of inoculum and nutrients. The herbicides diclofop methyl and atrazine were applied to one of the reactors at 1 ppb and 10 ppb while an identical reactor was held as a control. Biofilm parameters such as thickness, bacterial biomass, surface chemistry and architecture were determined using confocal scanning laser microscopy and fluorescent probes. The biofilms developed into 200 µm thick, heterogeneous, ridged structures with the highest microbial biomass in an outer 10 µm zone of the biofilm. Probing with fluor-conjugated lectins indicated a chemically heterogeneous biofilm matrix with, a variety of glycoconjugates including fucose, glucose, mannose and N-acetyl glucosamine. Analyses of herbicides in water samples was carried out using extraction and GC-MS techniques whereas biofilm samples where subjected to semi-quantitative analyses using DIP-MS/MS procedures. The concentration of atrazine and diclofop methyl in water followed the pattern of application over the 15 day time course of the experiment. Analysis of biofilm samples showed that atrazine was present in rinsed biofilm samples immediately after addition to the water, however only trace levels were found after atrazine additions were stopped. In contrast, diclofop methyl was slow to appear in the biofilm reaching maximum levels after addition of diclofop methyl to the aqueous phase was stopped. The detection of metabolites of atrazine and diclofop methyl in the biofilms during and after addition of the herbicides was suggestive of degradation in addition to sorption of the compounds. Similar results were found for both 10 ppb and 1 ppb treatments. These observations indicate that: (i) lotic biofilms play an important role in the natural remediation of herbicides in these environments; (ii) sorption to biofilms should be included in consideration of sampling, determination of half lives and environmental impacts of these and related compounds.

Structure and function of photoheterotrophic biofilms along a gradient of eutrophication in a coastal inlet of the southern Baltic Sea

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Microbial biofilms are complex associations of microorganisms immobilized at surfaces and embedded in an organic matrix secreted by the organisms. The development of microbial biofilms is the net result of transport and exchange processes between the surrounding medium and the biofilm as well as modification and decomposition processes of inorganic and organic material within the biofilm. Microbial biofilms are of a high relevance in microbial ecology and biotechnology.

Photoheterotrophic biofilms are characteristic communities colonizing rocks in the shallow brackish water of the Nordrügenschen Bodden (coastal inlets of the southern Baltic Sea, Germany), These biofilms are 1 to 2 mm thick, consisting of green algae (*Ulothrix, Urospora*), diatoms, cyanobacteria (*Calothrix*) and heterotrophic bacteria. Structure and function of biofilms revealed pronounced differences depending upon the grade of eutrophication. Organic carbon amounted to between 50 and 70% of the dry weight of the biofilms. The carbon to nitrogen (C/N) ratio at the biofilm surfaces was around 6. With increasing eutrophication the C/N ratio increased from the surface to the biofilm basis (up to about 12 at the hypertrophic location). Microprofiles of oxygen revealed that at the mesotrophic location, biofilms were oxic from the surface to the basis. With increasing eutrophication subsurface biofilm horizons became progressively anoxic. Parallel to the oxygen distribution, biofilms from the mesotrophic location showed the highest photosynthetic activity. With increasing eutrophication the importance of the heterotrophic decomposition of organic matter (as measured by hydrolytic enzyme activities) in the biofilms increased. This is in accordance to the increase in C/N ratio. At the mesotrophic, eutrophic and hypertrophic location, respiration amounted to 20, 50 and 70% of the primary production, respectively. The observations demonstrate that microbial biofilms reflect the grade of eutrophication. Measurements in the laboratory confirmed that the oxygen distribution in the biofilms was greatly influenced by light intensity and currents.

Coal-mining lakes - the ambivalent function of sulphur bacteria

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The restoration of the open-cast mining lakes is confronted with different serious difficulties, which are related to hydrological, geochemical and also microbiological processes. In many cases the biogeochemistry of sulfur compounds plays a pivotal role, which strongly influence the environmental conditions of the mining lakes. The main reactions are

(1) oxidation of pyrite, commonly associated with coal, catalyzed by bacteria like *Thiobacillus spp.* as the rate determining step. Due to the intensive oxidation large amounts of sulfate and iron (III) are formed. By this, the pH in the water column can drop to values below 4.
(2) Contrary to this acid generating process, the anaerobic reduction of sulfate to sulfide by bacteria like *Desulfovibrio spp.* generate alkalinity. Thus, sulfate reduction can effectively neutralize the acid pollution and represents an important in-lake homeostatic mechanism.

Our investigations were mainly carried out in (a) lakes of the Lusatian mining district, East Germany, experimental lake ML 111, and (b) the mining lake "Grube Johannes" in the Halle-Bitterfeld region, East Germany.

In the lake ML 111 (pH 2,7/water column, 1,200 mg SO_4^{2-} l^{-1}) we estimated high numbers of sulfur oxidizing bacteria (10^6 - 10^7 ml^{-1}, MPN-method) . The number of sulfate-reducing bacteria was likewise high (10^6 ml^{-1}, MPN-method) in the upper layers of sediments. Obviously, the intensity of the sulfate reduction (^{35}S-Sulphate-technique) was limited by the availability of electron donators, as could be shown by experiments with addition of organic substrates. This is the crucial point by this type of lakes: there is the potential for acidification and also for generation of alkalinity, and the strategy can be to supplement the sediments with suitable electron donors.

In contrast to this, in the ML Grube Johannes we have a fatal dominance of the sulfate reduction process. This mining lake was used for deposition of industrial organic waste water since 1921. At present a water column of only 1 m covers a sediment layer up to 12 m in thickness and a area of 27 hectare. The richness of organic substrates from the anaerobic breakdown of fermentable substances, e.g. cellulose, a concentration of 500 mg SO_4^{2-} l^{-1} in the water column , and pH around 7.0, respectively, cause extreme sulfide production with periods of sulfide ebullition. Therefore the remediation strategy has been the retardation of the pH and the stimulation of the aerobic sulfide-oxidizing bacteria. The number of sulfate-reducing bacteria in sediments is in the range of 10^7 ml^{-1}, whereas the number of acidophilic and neutrophilic *Thiobacilli* is in the range of 10^4 - 10^6 ml^{-1}. At present we can consider a labile balance of the contrary processes.

Summarizing our investigations we can consider the various groups of sulfur bacteria to be of very importance, but in different ways, in view of the management of the mining lakes.

Comparison of pelagic and littoral microbial degradation potentials in Lake Constance

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Lake Constance is a comparatively large (surface 540 km^2) and deep (maximum depth 254 m) prealpine lake the biotic structure and processes of which are considered to be dominated by the huge pelagic water body (49 km^3) which comprises > 99 % of the lake's volume and > 90 % of the lake's surface. Therefore, considerable knowledge has been accumulated so far on pelagic processes and structures including spatiotemporal studies of the abundance and activities of microbial communities. In contrast, much less is known with respect to the importance of littoral areas. This study is an attempt to evaluate the role of littoral water bodies and sediments for microbial degradation at the example of selected test areas.

Related to volume, the littoral water bodies exhibited during summer similar or slightly higher microbial activities as compared to values observed simultaneously for epilimnetic pe-

lagic water bodies. In contrast, volume-related microbial abundance and activities observed for littoral sediments exceeded the corresponding pelagic values by a factor of 10 to 1000. Within littoral areas, surface-related microbial degradation activities (oxygen consumption, activities of hydrolytic enzymes) were higher in sediments than in the overlying water. Among abiotic environmental factors controlling the velocity of degradation in littoral sediments, temperature and the frequent wind-induced resuspension of sediments were shown to have a decisive influence. The difference between water and sediment was most pronounced for the degradation of xenobiotic substrates as was exemplified for phenolic compounds and NTA (nitrilo-tri-acetic acid) the degradation velocity of which increased immediately when only 1 g of littoral sediments were added to test bottles containing 25 ml of pelagic or littoral water samples, respectively. These results suggest that the composition of the microbial inoculum rather than the physical structure of the environment per se (i.e. water or sediment) is ultimately decisive for the observed differences between water bodies and sediments. Sediments, however, appear to be a better place for conservation of the necessary microbial communities due to permanent inoculation by adapted populations from land (e.g. anthropogenically loaded rivers) and due to better protection against losses (e.g., grazing, viral lysis or physical transport to unfavorable areas).

Overall, the obtained results confirm on the one hand the dominance of pelagic structures and processes in degradative matter fluxes of Lake Constance. On the other hand littoral areas and especially littoral sediments were shown to be a place of intensified microbial degradative activities, which contribute over-proportionally to total degradation in the lake. Under this aspect, a buffer function at the land-water interface may be ascribed to littoral areas due to which the large water body (which is a drinking water resource) may become additionally protected against loads of undesired anthropogenic substances.

Estimation of metabolically active biomass in sediments

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As one part of the investigation program "Pollutants in suspended matter and sediments of the rivers Elbe and Oder" the following question is studied: Can the number of metabolically active bacteria (in relation to the total bacterial abundance) be used to characterize the ecological status of sediments?

Samples of fresh sandy and sluffy muds from the top sediment layer (0-10cm) were collected. In the laboratory they were diluted with isotonic sodium chloride solution and then prepared for epifluorescence microscopy. For direct enumerating of bacteria in sediments the samples were stained with DAPI (4.6-Diamidino-2-phenylindole) [1, 2] and CTC (5-Cyano-

2.3-ditolyl tetrazoliumchloride) [3, 4]. The DAPI staining method is well established for the estimation of total bacterial abundance. The number of metabolically active (respiring) bacteria can be estimated by staining with CTC. This method has been applied to sediments for the first time. Both methods were shown to be applicable to sediments and well reproducible. With respect to reproducibility the most critical step is the selection of representative subsamples for microscopy.

The methods were applied to sediments from the river Elbe under strong anthropogenic impact as well as to sediments of the river Spree being unaffected by direct anthropogenic inputs. The bacterial abundance registered ranged from 200 to 900 million cells/g (fresh weight). They showed strong (seasonal?) fluctuations in the course of the investigations. The percentage of active bacteria was not higher than 30% and in the mean as low as 20%. There was no simple correlation between the active cell numbers and the degree of anthropogenic impact. More data are nessessary to answer the question for the relation between anthropogenic impact and metabolic activity of bacteria in sediments.

References

[1] Coleman, A. W. (1980): Enhanced detection of bacteria in natural environments by fluorochrome staining of DNA. Limnol. Oceanogr. 25 (5): 948-951

[2] Porter, K. G., Feig, Y. S. (1980): The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25 (5): 943-948

[3] Schaule, G., Flemming, H.-C., Ridgway, H. F. (1993): Use of 5-Cyano-2,3-ditolyl Tetrazolium chloride for quantifying planktonic and sessile respiring bacteria in drinking water. Appl. Environ. Microbiol. 59: 3850-3857

[4] Pyle, B. H., Broadaway, S. C., McFeters, G. A. (1995): A rapid, direct method for enumerating respiring enterohemorrhagic *Escherichia coli* O157:H7 in water. Appl. Environ. Microbiol. 61: 4304-430

- Wastewater -

Substrate-dependent competition of "Bio-P"- and "G"-bacteria at the biological phosphorus removal

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The development of "G"-bacteria, which accumulate glycogen instead of polyphosphate can cause a breakdown of the enhanced biological phosphorus removal (EBPR). Carbohydrate-

containing wastewater promote the competition of "Bio-P"- and "G"-bacteria. To study the development of "G"-bacteria fresh activated phosphate accumulating and releasing sludge was cultivated in three anaerobic/aerobic sequencing batch reactors (SBRs). At the beginning of each anaerobic phase acetate, acetate/glucose and glucose were added as three different sources of carbon. After 5 anaerobic/aerobic cycles the orthophosphate dynamic showed a significant difference of the three SBRs for the following test period. The highest phosphate release was observed with acetate influence (83.5 mg/l) followed by acetate/glucose (67.2 mg/l) and glucose (40.7 mg/l). To study the effect of the substrate on the formation of the three storage compounds glycogen, polyphosphate and polyhydroxyalkanoate the accumulation and degradation during the aerobic (20 h) and anaerobic phases (4 h) were analyzed and compared. The difference of the glycogen concentrations between the two phases was with acetate addition most greatly (aerobic: 41.9 mg/g DW, anaerobic: 14.5 mg/g DW) followed by acetate/glucose (aerobic: 29.4 mg/g DW, anaerobic: 10.8 mg/g DW) and glucose (aerobic: 13.3 mg/g DW; anaerobic: 8.9 mg/g DW). In the last anaerobic cycle kinetics of acetate, orthophosphate and glucose were taken. The carbon source glucose was metabolized very fast within 20-30 min whereas acetate was transformed very slowly and not completely.

Hydrophobicity and fatty acid profile of *Acinetobacter calcoaceticus* under different nutrient conditions

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Many wastewater treatment plants go through problems with scum formation due to foam stabilization both by hydrophobic and surface active sludge ingredients. In previous investigations non-filamentous hydrophobic bacteria such as *Acinetobacter calcoaceticus* were isolated from the scum fraction. It was shown that *Acinetobacter calcoaceticus* strains growing under various carbon source conditions are able to change their emulsification capability and the hydrophobicity of their cell surface. In this study the fatty acid profile and the extent of hydrophobicity of *Acinetobacter calcoaceticus* DSM 30006^T cultivated under different nutrient conditions were investigated.

The fatty acid profile was analyzed by whole cell hydrolysates using the MIDI-system (Microbial ID Inc., Newark, Delaware, USA). Cell wall hydrophobicity was determined by microbial adhesion to hydrocarbon (MATH)-test with hexadecane. A water/hexadecane system was used to determine the emulsification capability.

The differences in the cell wall fatty acid profile of *Acinetobacter calcoaceticus* growing with 100% TSB or 100% R2A are very similar to the results gained by other authors with *Pseudomonas* strains. A significant qualitative change in the fatty acid profile was analyzed for

those cells, which were cultivated with 10% R2A. Hydrophobicity of cells grown with 100%, 50% or 25% R2A turned out to be strong with 70 to 90% of cells being adsorbed to the hexadecane phase. Very low hydrophobicity (<1%) was analyzed for the cultures grown in 100% TSB and 10% R2A. All media tested except 10% R2A led to emulsifying activity of both cells and cell-free broth (after zentrifugation). 10% R2A cultures showed little emulsification capability. In the cell-free medium of these cultures no emulsification was detected.
Further experiments will elucidate the ability of *Acinetobacter calcoaceticus* to produce extracellular polymers (EPS) and their effect on cell surface hydrophobicity.

Detergent - degrading bacteria in activated sludge

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The degradation of several tensides and builders was tested in activated sludge pilot plants with a working volume of about 8 l and in half-technical plants with a working volume of several m^3. The investigations included the anionic tensides dodecyl benzene sulphonate (DBS, linear) and tetrapropylene benzene sulphonate (TPBS, branched), the nonionic tensides alkyl(C16-C18) polyethylene glycolether (FAEO) and alkyl polyglycoside (APG), the cationic tenside alkyl(C12-C18)dimethylbenzeneammonium chloride (ADMAC), and the builder nitrilotriacetic acid (NTA). The activated sludge plants were run as low loaded nitrification plants equipped with a preceding denitrification step. Because of a higher sludge age primary degradation capacity in general was higher than found with the OECD pilot plants without nitrification/denitrification which were used for many years to assess detergent biodegradability.

Detergent - degrading saprophytic bacteria were selected and isolated from the activated sludges from both pilot and half-technical plants. The bacteria able to degrade DBS belonged to the enterobacteria and to the genus *Acinetobacter*. Media containing TPBS als sole carbon source were selective for enterobacteria and *Pseudomonas* species of the rRNA group I. FAEO was successfully degraded by bacteria belonging to the genera *Acinetobacter* and *Aeromonas*. *Agrobacterium* species as well as pseudomonads were found to successfully degrade APG. A concentration of 10 mg/l ADMAC in the medium was selective for enterobacteria and *Pseudomonas* species. At higher concentrations only pseudomonads were found. The bacteria isolated on NTA-media belonged to the enterobacteria and to the genera *Acinetobacter, Pseudomonas, Ochrobactrum, Methylobacterium,* and *Agrobacterium*.

Degradation efficiency was assessed by respiration tests. The investigations carried out with pure cultures and defined mixtures of pure cultures showed that in general the breakdown of tensides was significantly improved by addition of activated sludge. In order to define the impact of activated sludge on this improvement the isolates were incubated both with genuine

activated sludge and with autoclaved activated sludge. Many pure cultures showed only a weak response on autoclaved sludge addition with respect to degradation efficiency. This indicated that the degrading bacteria in most cases rely on the physiological activity of other sludge bacteria and not on nutrients released from the sludge into the medium. This is in agreement with the general view that - in contrast to primary degradation - it is only by synergistic co-metabolism that the complex tenside compounds are totally mineralized. Nevertheless, activated sludge tenside degradation efficiency could be improved by bioaugmentation with selected pure cultures in some cases.

- Transformation of pollutants -

Plasmid pAB96 occurring in an aquatic ecosystem encodes degradation of versatile hydrocarbons

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Out of a groundwater system, contaminated by versatile hydrocarbons since 50 years we were able to isolate different *Pseudomonas fluorescens* species capable of degradation of aliphatic as well as mono- and diaromatic hydrocarbons. By analyzing plasmid pattern of these isolates we found one single 50 kb plasmid pAB96 which was smaller than most degradative plasmids known.

We used biochemical tests for first screening, followed by PCR-techniques with primers and probes for *alk B* (encoding alkane monooxygenase), *nah Ac* (encoding naphthalene dioxygenase) and *xyl E* genes (encoding catechol 2,3-dioxygenase).

All three genes were found to be located on plasmid pAB96, that means this plasmid codes for key enzymes for xyl, alk and nah catabolic pathways and all three pathways could be located on one plasmid in a naturally bacterium. So it is the first one which encodes for degradation of versatile hydrocarbons.

At least for *meta*-pathway of pAB96 in comparison with archaetypical TOL plasmid pWW0 we found homology not only for *xyl E* but also for *xyl G* (encoding hydroxymuconic semialdehyde dehydrogenase). The third gene *xyl L* (encoding dihydroxycyclohexadiene carboxylate dehydrogenase) we investigated is a gene from *upper pathway* and was not detected. Now we are looking for location of the catabolic operon and want to find out whether pAB96 is a mini-TOL plasmid or a new one.

Phylogenetic description of a chlorophenol dechlorinating consortium by PCR amplified 16S-rDNA genes

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A mixed culture was enriched from Saale River sediment (Germany), which reductively dechlorinated 2,4,6-trichlorophenol (TCP) via 2,4-dichlorophenol (DCP) to 4-chlorophenol (CP). Pure cultures isolated from agar shake dilution series were not capable of 2,4,6-TCP dechlorination. Dechlorination activity was only maintained, if the colonies isolated consisted of more than one organism or were supplemented with cells of *Clostridium celerecrescens.* The objective was to characterize the composition of the dechlorinating mixed culture by amplification of the 16S-rDNA, restriction analyzes (ARDRA) and sequencing as well as dot blot hybridization with species-specific oligonucleotide probes. A co-culture amended with *C. celerecrescens,* which exhibited dechlorination activity, was diluted to 10^{-5} in agar shake tubes. Five morphological different colonies were isolated and 16S-rDNA genes were amplified and sequenced. The colony ZF 4 was identified as *Desulfitobacterium frappieri* with 99.2 % homology to a known sequence of this species [l]. Dechlorination activity could later be demonstrated with cells of this strain. The single colony ZF 2 harbored a 16S-rDNA gene exhibiting 93.6 % sequence homology to *Clostridium hydroxybenzoicum.* Species related to *C. hydroxybenzoicum* were reported to occur in different dechlorinating consortia. Beside *C. celerecrescens,* strains with sequence homology to *C. glycolicum and C. sporosphaeroides* were identified as members of the consortium. The diversity of the mixed culture not amended with *C. celerecrescens* was studied by ARDRA using the cell suspension as template. Twenty-three different restriction patterns were obtained from 56 clones of the 16S-rDNA library. Using species-specific oligonucleotide probes 3 restriction patterns, comprising 8 clones (14 %), were assigned to *C. hydroxybenzoicum.* Only one singular pattern corresponded to a *D. frappieri* sequence. This fact indicated, that the putative dechlorinating bacterium was present only in a small numbers within the consortium.

Reference

[1] Bouchard, B., Beaudet, R., Villemur, R., McSween, G., Lepine, F., Bisaillon, J.-G. 1996. Int. J. Syst. Bacteriol. 46, 1010-1015

Detection of chlorocatechol 1,2-dioxygenase genes in enviromental isolates by PCR and oligonucleotide probes

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Chloroaromatics are one of the most important groups of xenobiotics contaminating aquatic ecosystems. Aerobic microbial degradation of chlorinated monoaromatic compounds generally occurs via the modified *ortho* cleavage pathway with chlorocatechols as intermediates which are subsequently cleaved by chlorocatechol 1,2-dioxygenases. Detection of chlorocatechol 1,2-dioxygenase genes as a marker for the chlorocatechol degradative pathway in bacteria isolated from contaminated ecosystems may be useful for the estimation of the degradative potential of species and biocenoses. Based on the sequence of known chlorocatechol 1,2-dioxygenase genes a set of specific PCR primers and an internal oligonucleotide probe was developed. PCR primers were derived from conserved sequence motifs of chlorocatechol 1,2-dioxygenase genes *tfdC* [1, 2, 3], *clcA* [4], and *tcbC* [5]. BLAST [6] analysis revealed that the degenerated primers are specific for these genes. 11 chloroaromatics degrading bacterial strains from the DSMZ and 7 alkaliphilic bacterial strains degrading phenoxyalkanoic acid herbicides and chlorophenols [7, 8, 9] were investigated. For all test strains a specific PCR product of appropriate size could be obtained. 2,4-D degrading strain *Ralstonia eutropha* JMP134 was used as a positive control and its plasmid-free derivative JMP222 as a negative control. Specificity of the PCR products was verified by hybridization to an oligonucleotide probe. The oligonucleotide probe was derived from an internal conserved motif of the gene fragment to be amplified. BLAST analysis revealed that this motif is more evolutionary conserved among intradiol dioxygenases and therefore the degenerated oligonucleotide matches not only to chlorocatechol 1,2-dioxygenase genes but also to some catechol 1,2-dioxygenase, protocatechuate 3,4-dioxygenase and hydroxyquinol 1,2-dioxygenase genes. The probe hybridized to all PCR products of the appropriate size under stringent conditions but not to unspecific PCR products, indicating the suitability of the primer set.

References

[1] Perkins, E. J., Perkins, E. J., Gordon, M. P., Caceres, O., Lurquin, P. F. (1990) Organization and sequence analysis of the 2,4-dichlorophenol hydroxylase and dichlorocatechol oxidative operons of plasmid pJP4. J. Bacteriol. 172, 2351-2359

[2] Bhat, M. A., Tsuda, M., Horiike, K., Nozaki, M., Vaidyanathan, C. S., Nakazawa, T. (1994) Identification and characterization of a new plasmid carrying genes for degradation of 2,4-dichlorophenoxyacetate from *Pseudomonas cepacia* CSV90. Appl. Environ. Microbiol. 60, 307-312

[3] Kõiv, V., Marits, R., Heinaru, A. (1996) Sequence analysis of the 2,4-dichlorophenol hydroxylase gene *tfdB* and 3,5-dichlorocatechol 1,2-dioxygenase gene *tfdC* of 2,4-dichlorophenoxyacetic acid degrading plasmid pEST4011. Gene 174, 293-297

[4] Ghosal, D., You, I.-S. (1988) Nucleotide homology and organization of chlorocatechol oxidation genes of plasmids pJP4 and pAC27. Mol Gen Genet 211, 113-120

[5] van der Meer, J. R., Eggen, R. I. L., Zehnder, A. J. B., de Vos, W. M. (1991) Sequence analysis of the *Pseudomonas* sp. strain P51 *tcb* gene cluster, which encodes metabolism of chlorinated catechols: Evidence for specialization of catechol 1,2-dioxygenase for chlorinated substrates. J. Bacteriol. 173, 2425-2434

[6] Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403-410

[7] Hoffmann, D., Müller, R. H., Kiesel, B., Babel, W. (1996) Isolation and characterization of an alkaliphilic bacterium capable of growing on 2,4-dichlorophenoxyacetic acid and 4-chloro-2-methylphenoxyacetic acid. Acta Biotechnol.16, 121-131

[8] Ehrig, A., Müller, R. H., Babel, W. (1997) Isolation of phenoxy herbicide-degrading *Rhodoferax* species from contaminated building material. Acta Biotechnol. 17, 351-356

[9] Müller, R. H., Jorks, S., Kleinsteuber, S., Babel, W. (1998) Degradation of various chlorophenols under alkaline conditions by gramnegative bacteria closely related to *Ochrobactrum anthropi*. J. Basic Microbiol. (submitted for publication)

Metabolism studies of the degradation of 3-chlorobenzoate by the microorganisms from a suspension membrane reactor

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At the FNK research center of "Produktionsintegrierter Umweltschutz" at the University of Bremen a suspension membrane reactor (SMR) is tested as a reactor technique integrated in production processes which eliminates pollutants out of a mixture of wastewater selectively. The integrated nanofiltration enhances the dwelling time selectively and thereby the concentration of hard degradable substances. This accelerates the degradation rates by a specialized biotic community. In comparison to a conventional Airloop reactor (AIR) the higher efficiency of the SMR becomes clear especially at a short dwelling time of four hours.

For the scale-up of this reactor and for the transfer of the experimental results to other wastewater compounds it is necessary to describe the biochemical conversion processes in the SMR. Therefore the aim of these investigations was to characterize and identify the isolated bacteria from the SMR and the AIR and to explain the pathways and the kinetics of the degradation of the model pollutant 3-chlorobenzoate.

It could be pointed out that all isolated bacteria grow on 3-chlorobenzoate (1 mM) as a single carbon source and that the most isolates grow on the central metabolites catechol and protocatechuate (0.5 mM). The mixed culture of the SMR metabolizes these three substances via the *ortho* ring-cleavage. In the SMR 3-chlorobenzoate seems to be degraded via catechol or chlorocatechol with a following *ortho* ring-cleavage so that no enrichment of toxic "dead-end products" will be expected.

A pulse technique for production of adapted biomass for chlorobenzene and 1,2-dichlorobenzene degradation

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Biofiltration has been used successfully to degrade and eliminate volatile organic compounds (VOC) from different sources. Among many xenobiotic compounds, chlorobenzenes are often present in waste gases. They can be mineralized by bacteria under appropriate conditions.

Biofiltration is relatively sensitive to pollutant load shocks. This can be prevented, or the impact reduced, by treating the different types of pollutant separately, with equalizing pollutant concentration in a activated carbon column and by adding cell culture to increase biofilter capacity. This last approach was investigated during this work. We defined a strategy to cultivate a bacterial community specially adapted for the simultaneous biodegradation of chlorobenzene (CB) and 1,2-dichlorobenzene (DCB), two major pollutants.

A selection of mixed bacterial culture was taken from activated sludge and biofilters used for the treatment of gases containing halogenated aromatic compounds. For adaptation procedure CB and DCB were used as unique source of carbon.

An unconventional approach for producing the community is required in view, notably of the volatility and the toxicity of chlorobenzenes. The production takes place in a bioreactor in batch-mode, and is characterized by discontinuous oxygenation and periodic injection of chlorobenzene substrate (Figure 1).

A production cycle corresponding to several oxygenation cycles was maintained until chloride resulting from chlorobenzenes degradation attained 0.1 M or nitrogen became limiting. The number of pulses during an oxygenation cycle was determined to have oxygen in excess.

The time between two pulses should be sufficiently long to degrade all the substrates and to reduce concentration of metabolites. Indeed, it is necessary to have a residual concentration as low as possible to avoid an accumulation of intermediates and finally a growth inhibition.

After culture adaptation starting with small chlorobenzene concentrations, the pollutants were introduced with different concentration pulses: 50 µl CB/10 µL 1,2-DCB up to 200 µl CB/ 40 µl 1,2-DCB per liter.

During the culture, gas chromatographic analysis of gaseous phase had shown that chlorobenzene degradation begun before DCB utilization started. Until 200 µl CB and 40 µl DCB, these solvents were completely mineralized without accumulation of intermediates.

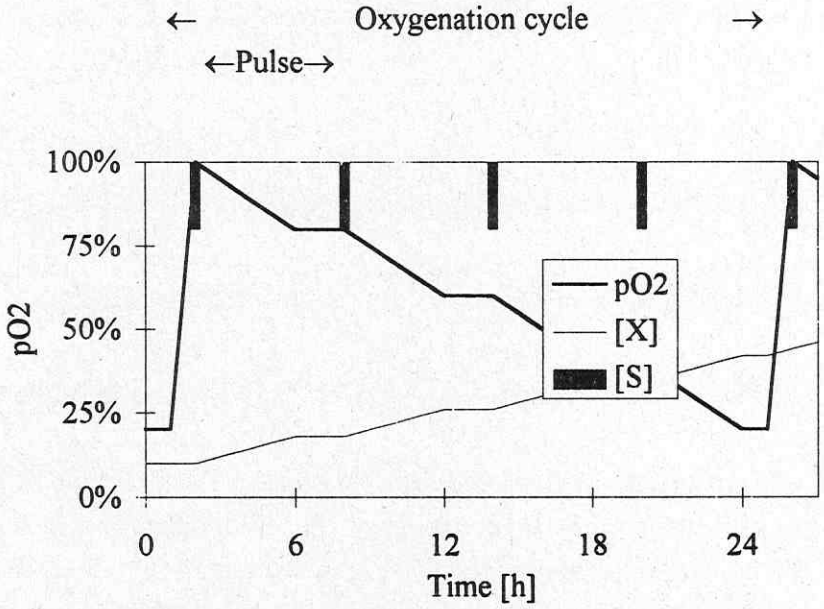

Fig. 1. Principle of the culture

Table 1. Specific degradation rate (mg pollutant/mg DW x h)

Volume injected by pulse	Q_{CB}	Q_{CB}
50 µl CB + 10 µl DCB/liter	94	13
100 µl CB + 20 µl DCB/liter	88	19
150 µl CB + 30 µl DCB/liter	121	16
200 µl CB + 40 µl DCB/liter	92	13

The maximal specific biodegradation rate was obtained with 150 µl CB and 30 µl DCB per pulse (Table 1), which is 121 and 16 mg CB/mg DW x h for CB and DCB, respectively.

Maximal biomass productivity of 51 mg MS/l x h was obtained with a feed rate of 178 mg S/h corresponding to 150µl CB/ 30µl DCB by liter of culture medium. The growth yield was 0.40 g MS/g S. Zaitsev et al. [1] reported the same yield for *Rhodococcus opacus* on CB. The maximal biomass concentration attained without decrease of productivity was 7.5 g MS/l.

This work confirms the feasibility and reliability of this batch-mode technique with sequenced substrate injection for the production of specialized biomass able to degrade chlorobenzenes. Bacterial communities adapted for volatile organic compounds removal are available for preventive injection on to industrial biofilters in case of deficiency.

Reference

[1] Zaitsev, G.M. et al. (1995). Utilisation of halogenated benzenes, phenols and benzoates by *Rhodococcus opacus* GM-14. *Appl. Environ. Microbiol.* 61, 4191-4201

Anaerobic biodegradation of fluorinated surfactants

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Fluorinated surfactants are widely used in processes in which a marked reduction of the surface-interfacial tension of water is essential. Although these detergents are produced in low amounts compared to common surfactants they may play an important role as environmental pollutants. They are known to be chemical and biological inert so that they tend to accumulate without biodegradation. That may cause damage to biological systems.

Objective of the presented investigation was to use microbial biodegradation strategies for an enhanced elimination of fluorinated surfactants in a wastewater/activated sludge treatment process. Regarding to their adsorptive properties, the compounds will even be found in the anaerobic part of this process (sludge digester).

The applied strategy was an additional supply with carbon substrates. It was used to stimulate the biodegradation of the fluorinated surfactant Fluowet OTN©.

With a modified ECETOC-test the anaerobic biodegradability of organic compounds can be determined by measuring the amount of carbon released as carbon dioxide and methane.

Fluowet OTN© was not degraded under anaerobic conditions by activated sludge from a sludge digester during the test period of 60 days. After addition of methyl alcohol or acetate, no stimulation but even inhibition of the regular methanogeneses was observed. In test vessels with formate and Fluowet OTN© the methanogeneses activity of mesophile sludge increased about 11 % in comparison to controls with formate only. Methylamine caused an extended carbon release of 9 %.

Apparently the surplus carbon derives from the non-halogenated ethoxylated part of the detergent. Because of the recalcitrance of the fluorinated part of the surfactant a defluorination and biodegradation of this component is unlikely. It is possible that primary cometabolic reactions are responsible for the transformation of Fluowet OTN© with formate or methylamine as the primary energy and/or carbon source.

Further tests will be carried out in a lab-scale digester and a direct chemical analysis will then be applied to observe the anaerobic biodegradation and to detect the metabolites released.

- Molecular detection methods -

Using PCR and gene probe techniques for determining the PAH-degradation potential of bacteria

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The first step in breaking down the aromatic ring structure of polycyclic aromatic hydrocarbons (PAH) is catalyzed by the activity of dioxygenases, enzymes comprising four different subunits arranged in a unique order [1, 2]. Bacterial isolates, which were enriched by naphthalene and/or phenanthrene as sole sources of carbon and energy were analyzed by PCR and gene probe techniques to detect the presence of initial naphthalene or phenanthrene dioxygenase. Homology comparisons of different initial dioxygenases show little similarity regarding initial naphthalene dioxygenases to benzene, toluene and biphenyl dioxygenases. Specific primer pairs for different subunits of the naphthalene-dioxygenases were derived from these sequence alignments. A gene probe (A1, 595 bp) derived via PCR was constructed using DNA from *P. putida* OUS82, a bacterial strain harboring well characterized naphthalene dioxygenase.

Both restricted chromosomal DNA *(PstI)* from reference strains and bacterial isolates were hybridized with the gene probe Al to demonstrate the presence of either homologous or heterologous naphthalene dioxygenase. Strains belonging to the genus *Pseudomonas* were probed with gene probe Al labeled with digoxigenin. Other genera were probed with Al labeled with [^{32}P]. Strong DIG-hybridization signals were detected with the reference strains *P. putida* NC1B9816, *P. putida* OUS82, *P. stutzeri* AN11, and the environmental isolates C4 *(P. fluorescens)* and F *(P. putida)*, whereas weak signals could be detected for the isolates G *(Comamonas* sp.) und H *(Comamonas* sp.). The bacterial isolate E *(Acidovorax* sp.) of the non-*Pseudomonas* genera showed signals in radioactive blot hybridization.

The sensitive PCR method with the derived primers was used to gain more information about potential naphthalene/phenanthrene dioxygenases in the environmental isolates. DNA-fragments of one predicted size could be amplified via PCR for reference strains belonging to the genus *Pseudomonas* as positive controls and the bacterial isolates C4 and F. Isolated

strains not belonging to the genus *Pseudomonas,* but able to degrade naphthalene and phenanthrene however, either had quite different PCR-fragment patterns or weak fragment signals of the predicted size. Hybridization of PCR fragments from selected strains with gene probe Al produce signals for bacterial isolate E and BI *(Rhodococcus rhodochrous). E.coli* DSM1576 often showing PCR fragments such as the positive reference strains gave no detectable signal in PCR blot hybridization.

References

[1] Kiyohara, H., S. Torigoe, N. Kaida, T. Asaki, T. Iida, H. Hayashi, and N. Takizawa. 1994. Cloning and characterization of a chromosomal gene cluster, pah, hat encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. J. Bacteriol. 176, 2439

[2] Simon, M.J., T.D. Osslund, R. Saunders, D.B. Ensley, S. Suggs, A. Harcourt, W.C. Suen, D.L. Cruden, D.D. Gibson, and G.J. Zylstra. 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene, 127, 31

Microbial diversity in a groundwater catchment area determined by Denaturing Gradient Gel Electrophoresis of PCR-amplified ribosomal DNA fragments

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The degradation of pollutants in groundwater and aquifers depends on microbiological and hydrogeochemical processes. Microorganisms influence hydrogeochemical parameters by their metabolic activities - as well as chemical and physical factors affect the microbial composition of the environment. To understand the transport and fate of anthropogenic compounds during bank filtration and artificial recharge of groundwater it is necessary to gain more information about the structure of microbial populations in these systems. Microbial diversity and population structure of oxic and anoxic groundwater as well as sediment samples were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rDNA.

Water and sediment samples were collected from a groundwater catchment area with artificial groundwater recharge near the river Ruhr in NW-Germany. In this area different characteristic chemical zones can be distinguished because of the gradual depletion of oxygen and other electron acceptors [2].

Groundwater samples were collected after 24-h pumping with a submergible pump (pump rate: 1l/min). To get sediment samples of the aquifer, the pump rate was increased up to 10-fold. The liquid and mobilized solid phase were separated by sedimentation.

Groundwater samples were filtered through 0,2 µm pore size Durapore filters (Millipore Corp.). 200 mg of the mobilized solid phase were used for the DNA extraction from sediment samples. After cell-lysis and DNA extraction, the 16S rDNA from mixed bacterial DNA was amplified by PCR with general eubacterial primers (27f GC/ 517r). To reveal the general bacterial population the amplified PCR-products, that have the same length, were separated by DGGE (denaturing gradient gel electrophoresis) on the basis of melting domain structure and nucleotid composition.

DGGE patterns from groundwater samples were compared to DGGE profiles from groundwater enrichment cultures grown under oxic and anoxic conditions (P-medium, [1]).

The DGGE pattern of the groundwater sample is very complex and differs significantly from the patterns of bacterial DNA from the groundwater enrichment cultures, that are characterized by a small number of distinct bands. These differences in the DGGE profiles indicate the small quantity of culturable microorganisms in groundwater ecosystems.

The DGGE pattern of the solid phase of the groundwater sample contains more DGGE bands than the DGGE profile of the liquid phase, reflecting a higher genetic diversity. Some bands in the DGGE pattern of the groundwater sample and the sediment sample show similar migration, supposing the same bacterial species (A). Other DGGE bands are either present in the groundwater or in the sediment sample (B).

Oxic and anoxic groundwater and sediment samples differ significantly in their DGGE profiles.

Culture-dependent approaches have been shown to isolate only 0,1-10% of the total microbial community in aquatic environments. Molecularbiological approaches are a new tool to gain more information about culturable and non-culturable microorganisms in groundwater ecosystems.

Oxic and anoxic groundwater and sediment samples show differences in their microbial population. The different hydrogeochemical zones of this groundwater catchment area are mirrored by distinct DGGE patterns indicating changes in the microbial community structure during bank filtration and artificial groundwater recharge.

References

[1] U. Schöttler, U. Schulte-Ebbert, (Hrsg.) Schadstoffe im Grundwasser - Band III, Deutsche Forschungsgemeinschaft, Bonn 1995

[2] A. Nehrkorn, Gesundh. Ing. 3, 88

[3] B. Kuhlmann, B. Eschweiler, B. Kilb, G. Preuß, E. Ziemann, U. Schöttler. Vom Wasser, 89, 205-214 (1997)

Remarks and acknowledgments

The idea of holding the *Microbiology of Polluted Aquatic Ecosystems* workshop at the UFZ, Centre for Environmental Research in Leipzig, was born during the VAAM meeting in March 1997, when the Water/Wastewater Section met. On this occasion, plans for a workshop dealing with 'career-orientated demands on microbiologists' to be held at UFZ on the 4th and 5th of June 1998, were also implemented.

The orientation of the workshop *Microbiology of Polluted Aquatic Ecosystems* was developed in talks between the microbiologists of the Departments of Environmental Microbiology, Hydrogeology, Inland Water Research and Remediation Research of the UFZ with Prof. Babel (UMB) in chair. Thank you, dear colleagues, for the heated discussions!

We were both encouraged and supported by Prof. Flemming and Prof. Szewzyk, the heads of the Water/Wastewater Section, and by Dr. Weigert of the Water Research Centre, Berlin.

The organization of the workshop was facilitated by the welcome support of Mrs. Böhme ('Public Relations' department) who quickly produced announcement and program flyers. Warm thanks must also go to Mr. Lorbeer (UMB) and the team of Mr. Färber (BA) who furnished the lecture halls, Mrs. Haufe (SAN) and Mr. Bäder-Bederski (SAN) at the registration counter and Mrs. Müller (FA) for the financial overview. Last but not least, I would like to thank Prof. Stottmeister (SAN) who released me to organize the workshop and offered the helping capacities of the Department of Remediation Research.

We obviously aroused the interest of many colleagues, because a total of 91 participants enrolled for our national workshop, with 5 of them coming from as far as Nuenen in The Netherlands, Lausanne in Switzerland or Sanct-Petersburg in Russia.

The workshop itself turned out to be an almost self-organizing event, with willing chairwomen and chairmen drawn from the audience, and volunteers always eager to project slides. Many thanks to all of you for your help in arranging the interesting and entertaining workshop we had!

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