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Activity of Selected Microorganisms and Mixture in BTX Biodegradation

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Abstract

This paper presents the behaviour of selected bacteria, the mixture of, and indigenous to municipal sewage microorganisms, in biodegradation of benzene, toluene, o-xylene and p-xylene (BTX). Apart from an expected different behaviour under aerobic and anaerobic conditions the difference in degradation rate obtained for the different microorganisms tested, suppositions on practical application of the results were made. It was found that the mixture of microorganisms present in municipal sewage can be advantageously used in biodegradation of aromatic hydrocarbons under aerobic conditions. Opposite conclusions could be drawn from the experiments performed in anaerobic conditions where selected specific bacteria have been much more active. The observations made, of toluene biosynthesis under specific anaerobic conditions seem to be totally new and unique.

Keywords: aerobic and anaerobic BTX biodegradation, wastewater microorganisms

Introduction

Aromatic hydrocarbons (AHC) have for many years drawn the attention of investigators because of their detrimental impact on water ecosystems resulting in a threat to humans, as the users of water.

Behaviour in the process of biodegradation under aerobic and/or anaerobic conditions seems to be of great practical importance. In general biodegradation takes place as a result of biological activity of specific species of bacteria widely present in the natural environment.

Aerobic conditions permit a rapid and complete mineralization of hydrocarbons. Catabolic pathways of volatile aromatic hydrocarbon degradation by aerobic microorganisms can vary distinctively as far as chemical reactions and the end products [4, 8, 11, 15] which are later incorporated in the metabolic pathways as sources of carbon and energy.

When oxygen becomes exhausted by aerobic organisms the degradation processes continue under anaerobic conditions. Essential for further transformation in the anaerobic environment are the presence of such electron and hydrogen acceptors as nitrates, sulphates, carbon dioxide and trivalent iron. Studies were carried out with mixed populations or defined mixed cultures, as well as with isolated bacteria strains from soil, sludge, sewage or water [3, 5, 6, 7, 9, 14, 16].

The processes of aromatic hydrocarbon biodegradation have been studied extensively and are described in the literature [1, 2, 9]

There are, however, no reports on hydrocarbon transformation in the process of waste water treatment leading to synthesis of such compounds. An extensive search of literature has turned up only one publication of Juttner and Henatsch [10] in which they implicate the possibility of toluene production as a result of the biological process.

In previous research [12, 13, 17] the biosynthesis of toluene was demonstrated to take place due to the biological activity of a consortia of bacteria indigenous to municipal sewage.

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Experimental Procedures

For each tested aromatic hydrocarbon, six samples of 500 ml each, composed of 1/4 raw sewage sludge from primary clarifiers and 3/4 biologically treated municipal sewage containing undefined - indigenous microorganisms, were spiked with known amounts of benzene, toluene, o-xylene and p-xylene (BTX). The tested aromatics were added in a methyl alcohol solution to ensure fast and complete mixing with reactor content. The exact concentration was determined analytically. Although identical amounts of tested BTX's as aforementioned have been added the results have shown a slight variation. It was the objective to get a concentration of 500 μg/dm^3 of toluene, p-xylene and o-xylene. Benzene was added in a higher amount, because it is usually present in the environment in higher concentration, but also to examine the effects of different concentrations. The measured values at the beginning of the experiments have been shown in Tables 1 and 2.

Table 1. Concentrations of aromatics at the beginning of investigations in aerobic conditions.

Microorganisms	Concentration [μg/dm^3]			
	Benzene	Toluene	p-Xylene	o-Xylene
A. sobria	1267	558	474	467
B. stearothermophilus	1264	455	433	486
E. sakazaki	1370	620	658	723
P. aeruginosa	950	443	405	456
S. lentus	1008	429	464	457
Mixture of microorganisms	1267	496	442	488
Microorganisms of indigenous	1238	595	377	414

Table 2. Concentrations of aromatics at the beginning of investigations in anaerobic conditions.

Microorganisms	Concentration [μg/dm^3]			
	Benzene	Toluene	p-Xylene	o-Xylene
A. sobria	1082	477	379	416
E. sakazaki	1149	523	448	452
P. aeruginosa	1302	601	518	527
S. lentus	1235	546	445	493
Mixture of microorganisms	1158	557	465	508
Microorganisms of indigenous	993	397	422	327

In three reactors (Erlenmeyer flasks) for each tested hydrocarbon, the process of transformation was run in aerobic conditions, in the other three samples anaerobic conditions were maintained. Anaerobic conditions have been assured in reactors covered with black paper and tightly sealed. The flasks have been fixed on a laboratory shaker for continuous mixing. The only contact to the surrounding environment, in the anaerobic tests, was by a syringe needle through which produced gas could escape. Seeded with respective culture (named later in this paper) samples, they were allowed to multiply and consume all eventual present dissolved oxygen. Two different methods of dissolved oxygen measurement were used to assure that the anaerobic conditions have been reached. Afterwards tested aromatics have been added.

The flasks in the aerobic conditions were supplied with a controlled amount of air pressure to maintain dissolved oxygen on the level of 2.0 mg O_2/dm^3, also before the addition of aromatics. Dissolved oxygen was measured with a do probe. The results of hydrocarbon degradation in aerobic conditions given in this paper, are values from which the amount of BTX stripped to the atmosphere have been subtracted. Experiments were carried out at room temperature of about 20°C. For each experiment in aerobic and anaerobic conditions there was a parallel control sample with sewage sludge and treated sewage without BTX.

Erlenmeyer flasks with the aforementioned mixture of raw sewage sludge and biologically treated sewage, were sterilized for 30 minutes in an autoclave at a temperature of 112°C, and a pressure of 150 kPa. After such a procedure samples from all of the sterile flasks (66 flasks in total) were tested for the effectiveness of the process on enriched agar plates.

The microorganisms applied in the experiments under aerobic conditions were: *Aeromonas sobria, Bacillus stearothermophilus, Enterobacter sakazaki, Pseudomonas aeruginosa and Staphylococcus lentus.* Investigations with a mixture of all of these bacteria were also done. Studies in anaerobic conditions were performed with, *Aeromonas sobria, Enterobacter sakazaki, Pseudomonas aeruginosa and Staphylococcus lentus,* and a mixture of these bacteria.

Colonies of selected bacteria were cultivated in a thermostat at 27°C on blood agar spread plates. Grown cultures were washed out with 1 ml of physiological salt solution, and added to the sterile mixture of sludge and sewage. The inoculated material was incubated for 9 days in order to multiplicate the number of bacteria. In case of anaerobic reactors the preliminary period was also required for complete dissolved oxygen depletion.

To the prepared reactors, according to the aforementioned procedure, benzene, toluene, o-xylene and p-xylene were added in identical amounts to what was added in the test with indigenous microorganisms.

The content of BTX was analyzed with a Hewlett Packard 6890 series gas chromatograph, on a capillary column, equipped with a purge and trap array. Each measurement was repeated three times and results have been periodically checked with reference samples.

Results

The investigated bacterial cultures have shown to be active in BTX degradation in aerobic and anaerobic conditions.

The most intensive decomposition of tested aromatic hydrocarbons in aerobic conditions was stated for indigenous to municipal sewage microorganisms. Total degradation of benzene was found in the second day of incubation Toluene was decomposed within three days and o-xylene and p-xylene in four days. (Fig 1.) As described under methods used the results do not include amount stripped to the air.

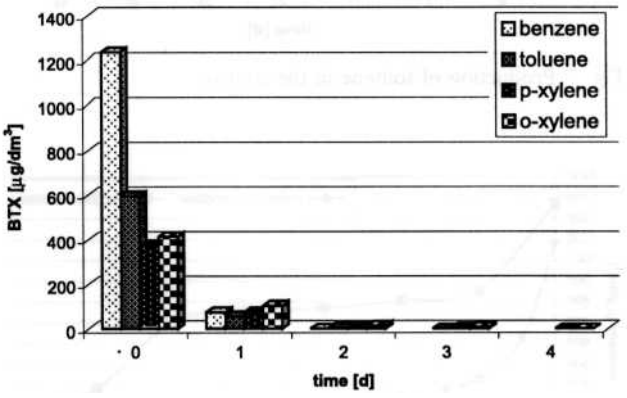
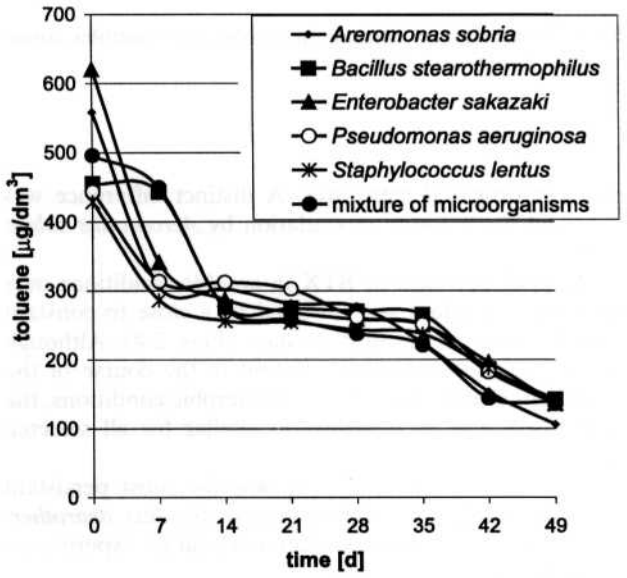


Fig.l. BTX biodegradation by indigenous microorganisms in aerobic conditions.

Fig. 2. Toluene biodegradation by selected microorganisms and the mixture of these microorganisms in aerobic conditions.



In aerobic conditions isolated bacteria *Aeromonas sobria, Bacillus steareothermophilus, Enterobacter sakzaki, Pseudomonas aeruginosa, Staphylococcus lentus* as well as the mixture of these cultures have also been able to decompose BTXs, however with a much slower rate. In the case of toluene a rapid degradation within the first 7 days of incubation occurred as a result of the activity of *Aeromonas sobria, Enterobacter sakzaki, Pseudomonas aeruginosa and Staphylococcus lentus.* For *Bacillus steareothermophilus* a lag phase was observed (Fig. 2).

In the following days of the experiment, between 7 and 35 days the degradation of BTX was linear and

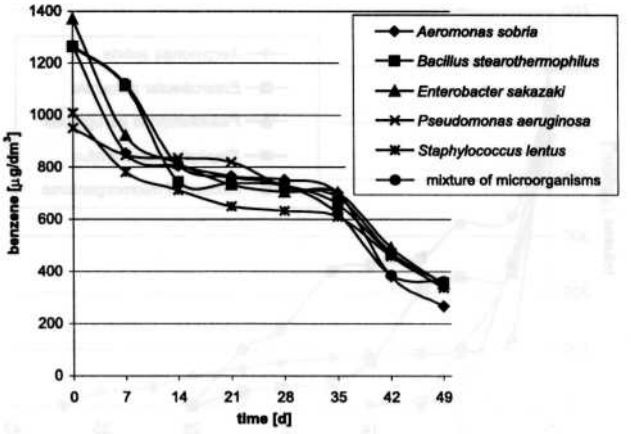


Fig. 3. Benzene biodegradation by selected microorganisms and mixture of microorganisms in aerobic conditions.

rather slow. During the last 14 days of experiments (day 35 to 49) an increase in the degradation was observed. The changes with time were very similar for o-xylene and p-xylene. The measured changes in concentration were presented in Figs. 2, 3 and 4.

The selected cultures of bacteria as well as the mixture of these bacteria used in our experiments have also been active in anaerobic conditions. Each of the selected bacteria has shown a different activity in aromates degradation. An example for toluene was given in Fig 5.

The most active was *Aeromonas sobria, Pseudomonas aeruginosa.* The population of *Staphylococcus lentus* transformed toluene within 14 days. Much slower degradation was found for the mixture of *Aeromonas sobria, Bacillus steareothermophilus, Enterobacter sakzaki, Pseudomonas aeruginosa and Staphylococcus lentus,* where degradation was completed after only 28 days.

Specific - indigenous to municipal sewage microorganisms have been shown to be able to synthesize toluene under anaerobic conditions. During the first phase

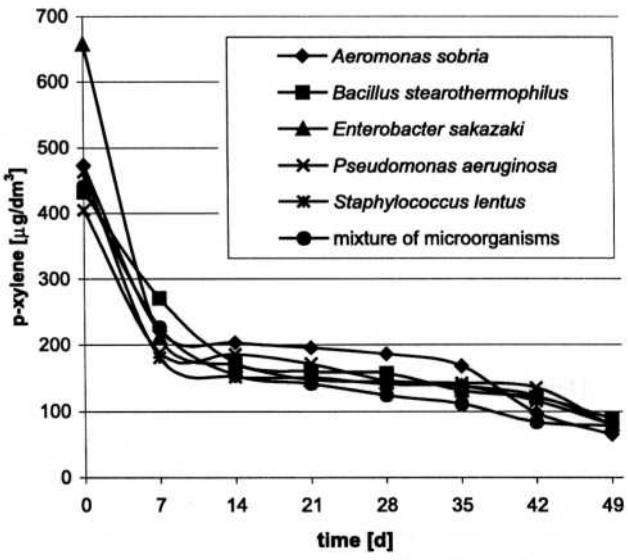


Fig. 4. p-Xylene biodegradation by selected microorganisms and mixture of microorganisms in aerobic conditions.

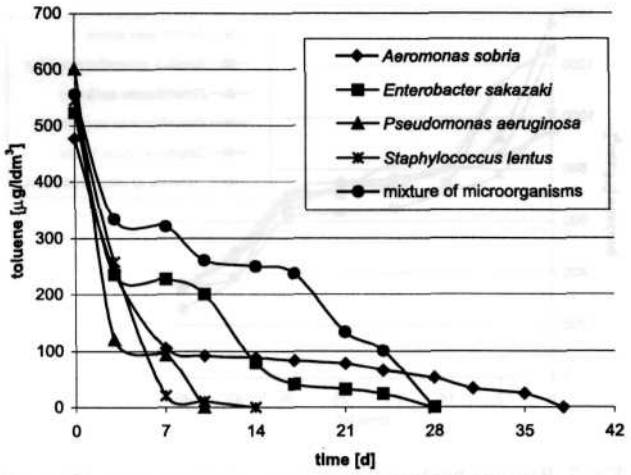


Fig. 5. Toluene biodegradation by selected microorganisms and mixture of microorganisms in anaerobic conditions.

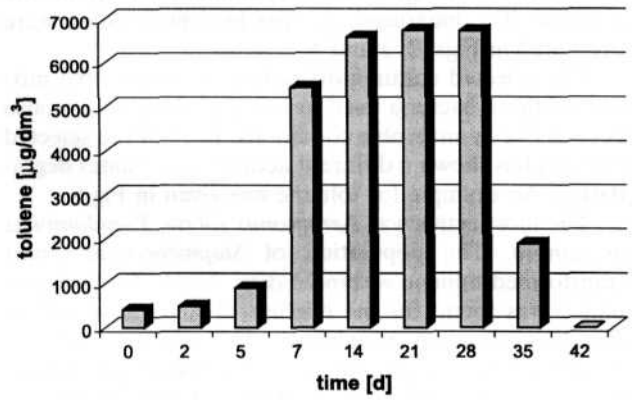


Fig. 6. Production of toluene under anaerobic conditions by the population of indigenous to municipal sewage microorganisms.

of digestion a relatively rapid increase of toluene was measured which was followed by an even faster rate of degradation.

The results given in Fig. 6 were obtained for a sample to which 500 µg/l of toluene was added. As can be seen from Fig. 7, in the control reactor without addition of any aromatic hydrocarbon, no initial seed of toluene was required to start the synthesis.

Discussion

Degradation of aromatic hydrocarbons, benzene, toluene and xylenes, was shown to be possible, both in aerobic and anaerobic conditions. Selected bacteria behaved differently in the different electron donor situations. A higher rate of biodegradation was found under anaerobic conditions in comparison to aerobic environments. The higher degradation rate under anaerobic conditions could be seen from the comparison of Figs. 2 and 6 for toluene. The different behaviour is also evident for

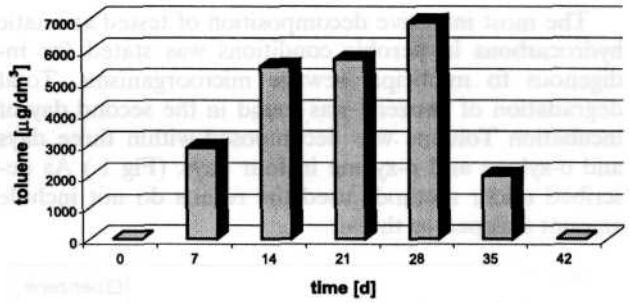


Fig. 7. Production of toluene in the control.

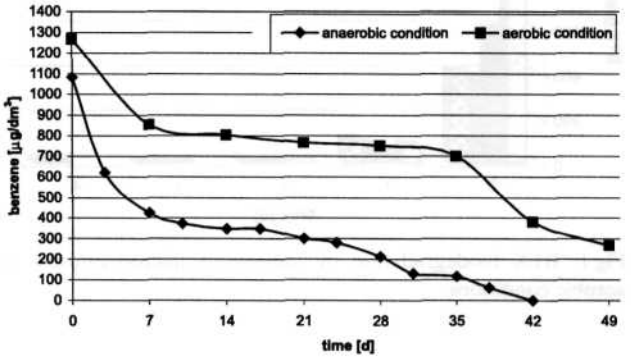


Fig.8. Degradation of benzene in aerobic and anaerobic conditions by *Aeromonas sobria.*

other investigated substrates. A distinct difference was observed for benzene degradation by *Aeromonas sobria* (Fig. 8.).

A rapid decrease of BTX in aerobic conditions over the first seven days was followed by a close to constant slow decrease in the next 28 days (Figs. 2-4). Although the degradation rate was different in the course of the investigated period of 49 days for aerobic conditions, the final effect was to some extent similar for all selected bacteria.

It was found that benzene was the most persistent substrate for biodegradation (except *Bacillus stearothermophilus*). Most easily biodegradable in all experiments was p-xylene.

However, there was a distinct difference in the first days of incubation (Fig. 9). Comparing results it appears that the bacteria exhibiting the highest rate of degradation was usually *Enterobacter sakazaki.* The degradation rate shown in Fig. 9 is based on assumption of a first order reaction rate at the beginning and therefore is a simple arithmetic value as a result of dividing of the amount degraded over the time of the first seven days of incubation. While the calculated degradation rate is only a rough simplification, it is described here as assumed degradation.

Although *Enterobacter sakazaki* was also present in the mixture of the tested bacteria the effect of degradation was controlled by the bacteria having the lowest affinity of enzymes to BTX, in this case mainly by *Bacillus stearothermophilus.*

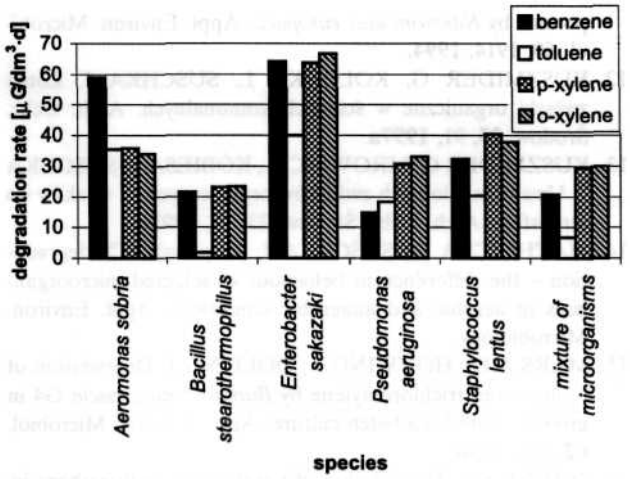


Fig. 9. Calculated degradation rate for aerobic conditions by the species of microorganisms tested.

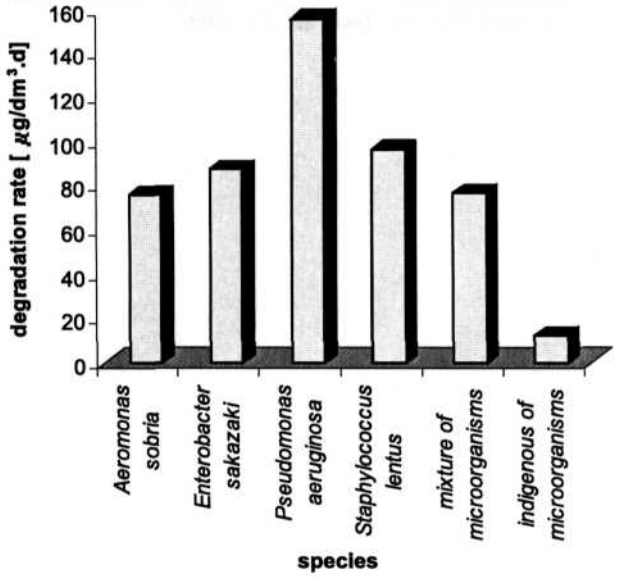


Fig. 10. Degradation of o-xylene in anaerobic environment.

Based on the presented results it can be concluded that seeking for a final result of biodegradation, when the time factor is not of primary importance, there is no need to search for specific bacteria. If, however, the time factor is important, specific microorganisms of higher affinity can be of great value.

If aromatic hydrocarbons are present in municipal sewage (which is often the case) we demonstrated that the indigenous sewage bacteria have the highest ability for fast biodegradation of these compounds. All investigated aromatic hydrocarbons degraded almost completely within two days. Probable enrichment of the microcosm in remediation efforts of contaminated with aromatic hydrocarbons soils, with present in municipal sewage microorganisms can have a positive effect on the overall biodegradation rate.

In contrast, for anaerobic conditions microorganisms indigenous to municipal sewage have been found to be less active than selected bacteria or the mixture of these bacteria. As an example the assumed degradation rate for three days of incubation was given in Fig. 10 for o-xylene.

Similar to aerobic condition the degradation rate by the mixture of selected bacteria was controlled by the species showing the lowest rate. In anaerobic condition *Pseoudomonas aeruginosa* was for all tested aromatic hydrocarbons the most active bacteria with a distinctly higher biodegradation rate. It was concluded that for anaerobic conditions the search for specific bacteria is much more justified than in the case of aerobic conditions.

What has not been reported previously in the literature was the demonstrated biosynthesis of toluene by microorganisms indigenous to municipal sewage (Figs. 6 and 7). High concentrations of toluene have been found before in the supernatant of anaerobicaly digested sewage sludge [12]. The presented investigations have proved that those found under real conditions at several municipal treatment plants toluene does not originate from external sources but can be synthesised in biological processes. As shown in Figures 6 and 7, there was a period of synthesis and degradation. The change from biosynthesis to biodegradation was found to be related to pH, or more precisely to acidic (the measured value of pH was 5.9) and methanogenic (with a pH of 7.1) phase of anaerobic digestion.

Conclusions

1. As demonstrated previously by other researchers, biodegradation of BTX occurs both in aerobic and anaerobic conditions. What has not been proved before, was the relatively higher biodegradation rate under anaerobic in comparison to aerobic conditions exerted by selected bacteria.
2. Indigenous to municipal sewage consortia of micro organisms have shown a much higher ability of biode gradation under aerobic conditions. In contrast, under anaerobic conditions the search for specific microorgan isms was demonstrated to be justified.
3. The biosynthesis of toluene under anaerobic condi tions is an original observation and more investigations are required to better explain this phenomenon.

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