Biodegradation of thiophene by cometabolism in a biofilm system

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Abstract An investigation of the cometabolic degradation of thiophene in the presence of benzene has been performed in a continuous biofilm system in order to study the kinetics of this conversion. The study showed result that thiophene interferes with the metabolism of benzene: benzene is not completely mineralised in the presence of thiophene. The micro-organisms are inactivated by thiophene, its metabolites or the metabolites of benzene. For low ratios of thiophene to benzene in the inlet of the reactor, the bacteria seem to adapt to thiophene and regain part of their activity that was lost when thiophene was introduced in the system. A hypothesis for this phenomena is that toxic metabolites of thiophene are converted biologically or abiotically into compounds, which are less harmful and inhibiting to the micro-organisms. Resting cells, which have been activated previously with benzene, are able to degrade thiophene, and this conversion leads to the inactivation or the death of the cells.

Keywords Benzene; biofilm; cometabolism; inactivation; inhibition; thiophene

Introduction and background
Dalton and Stirling defined cometabolism as the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound (Dalton et al., 1982). This process has a considerable impact on the environment because it allows the removal of toxic compounds which otherwise would be persistent to biological degradation.

Cometabolism mostly finds its explanation in the unspecificity of enzymes, which are induced by the growth substrate and are able to convert the cosubstrate (or secondary substrate). When the growth substrate (also called primary substrate) and the secondary substrate are present at the same time, both compounds are competing for the same enzyme and consequently inhibiting the degradation of each other (Folsom et al., 1990; Strand et al., 1990). The conversion of the secondary substrate leads to an organic product which cannot further be transformed by the micro-organisms into metabolic intermediates used for biosynthesis or energy production (Alexander, 1994). This transformation is neither supporting growth, nor is producing energy. In order to transform the secondary substrate, the cells need an energy source which is generated during the transformation of the primary substrate.

Non-growing cells (also called resting cells) which have been activated previously by the growth substrate contains enzymes and energy sources in the form of reducing equivalents (NADH) (Oldenhuis et al., 1989; Bae et al., 1995) or storage compounds (Henrysson et al., 1993). These cells are able to degrade the cosubstrate. However, since no regeneration of the reducing power takes place during the conversion of the latter, this degradation process leads to the depletion of storage compounds and reducing equivalents and the cells are inactivated.

The cosubstrate may be toxic to the organism, whereas the transformation product is not. However, very often the cometabolic activity results in the production of toxic compounds from relatively non-toxic substrates (Dalton et al., 1982; Wackett et al., 1988). If there are no other organisms present which are able to degrade the metabolites of the cosubstrate, the transformation products are accumulating in the system.

Dyreborg et al. (1998) reported on the cometabolic degradation of thiophene with benzene as primary substrate in suspended batch cultures. Thiophene is a toxic creosote
compound present in old coal gasification sites. The boiling point of thiophene and benzene are very similar (80.1°C for thiophene and 84.2°C for benzene). As a consequence, these two compounds are very difficult to separate by distillation. Thiophene is therefore present in the crude benzene fraction of coal tar distillate. The investigations in batch culture of Dyreborg et al. (1998) showed that thiophene cannot be degraded as sole carbon source by micro-organisms originating from a creosote contaminated site and that thiophene is not supporting growth of these bacteria. The degradation of thiophene and benzene in binary system was characterised by competitive inhibition. Further, it was shown that the cometabolic degradation of thiophene with benzene as primary substrate is only taking place when the concentration ratio of thiophene to benzene is lower than a critical threshold value. This critical value varied between 10 and 20 mg thiophene/mg benzene. The reason for this phenomenon was not identified.

The purpose of this work was to investigate the cometabolic degradation of benzene and thiophene in a continuous biofilm system, in particular in regards to the inactivation of the bacteria and the formation of metabolites.

Materials and methods
The experimental work was carried out in an annular rotating reactor of Plexiglas, a so-called rototorque with a volume of 0.923 l and a surface area of 0.16 m². The system was operated continuously with a flow of 4 l/h, which results in a hydraulic retention time of approximately 14 minutes. The system includes a recirculation pump, which ensures a complete mixing of the reactor. The experimental set-up is represented in Figure 1.

Growth medium and chemicals. The mineral medium contained the following compounds: 155 mg/l PO₄³⁻ –P, 20 mg/l K⁺, 1 mg/l NO₃⁻ –N, 2 mg/l Ca²⁺, 2 mg/l Mg²⁺, 8 mg/l SO₄²⁻ plus trace-elements (Schlegel, 1992); benzene, purity >99% and thiophene, purity >99% were purchased from Merck, Darmstadt, Germany. The growth medium was saturated with atmospheric oxygen using an air pump and a diffuser. The pH was kept at 7 by means of the phosphate buffer and the temperature was kept at 25°C.

The bacteria inoculum was an enrichment culture which was obtained originally from a creosote contaminated site situated near Fredensborg, Denmark.

During the investigation the benzene and thiophene concentrations were measured by gas chromatography with a GC-14A Shimadzu after extraction from the water phase with pentane. The oxygen concentration was monitored on-line with an oxygen probe. The organic metabolites were determined as Non Volatile Organic Carbon (NVOC) and the total CO₂ production was measured as Total Inorganic Carbon (TIC) with a Total Organic Carbon Analyser Model 700 from O.I. Corporation, College Station, Texas.

The first part of the experiment was the establishment of the biofilm in the reactor by feeding the system with benzene as sole carbon source. At the time of inoculation, no carbon source was present in the reactor and the reactor was operated as a batch. After three days of starvation of the bacteria, the reactor was fed with mineral medium with a flow of 4 l/h containing 5 mg/l of benzene as sole carbon substrate. When the biofilm thickness reached approximately 100 µm, step changes of the inlet concentrations of benzene and thiophene were performed in order to investigate the metabolite formation from these two compounds. Dynamic experiments involving thiophene (see Table 1) were later performed in order to investigate the kinetics of the cometabolic degradation of thiophene with benzene as primary substrate.

Results and discussion
Metabolite formation from benzene and thiophene
In order to investigate the metabolite formation from benzene and thiophene, step changes in upward direction of the benzene or thiophene inlet concentrations were performed.
The step changes of the benzene concentration were conducted while no thiophene was present in the reactor. These experiments showed that benzene was completely mineralised to CO₂ and the yield coefficient of biomass from benzene was calculated as 0.52 g biomass COD/g benzene COD.

Step changes of the thiophene inlet concentration were performed at constant benzene inlet concentration for three different concentrations of benzene (2, 5.15 and 9.5 gCOD/m³). For each of these steps the concentration of benzene, thiophene, TIC and NVOC were measured at the steady state and the degradation, respectively production rates of these compounds were calculated.

**Table 1** Step changes of the thiophene and benzene inlet concentrations during the dynamic experiment

<table>
<thead>
<tr>
<th>Step</th>
<th>Concentration before the step (mg/l)</th>
<th>T/B after the step (mg/mg)</th>
<th>Concentration after the step (mg/l)</th>
<th>T/B after the step (mg/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 thiophene ↑ 3.0 0.00</td>
<td>0.35 0.14</td>
<td>2.5 0.35 0.14</td>
<td></td>
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</tr>
<tr>
<td>2 thiophene ↓ 3.0 2.50</td>
<td>0.83 3.0 1.50 0.50</td>
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<tr>
<td>3 thiophene ↓ 3.0 1.50 0.50 2.5 0.00 0.00</td>
<td></td>
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<tr>
<td>4 thiophene ↓ 2.5 0.00 0.00 2.5 0.35 0.14</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 thiophene ↑ 2.5 0.35 0.14 2.5 0.60 0.24</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>6 thiophene ↑ 2.5 0.60 0.24 2.5 1.10 0.40</td>
<td></td>
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</tr>
<tr>
<td>7 thiophene ↑ 2.5 1.10 0.40 2.5 2.00 0.80</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 thiophene ↑ 1.7 1.50 0.88 0.0 1.50 0.23</td>
<td></td>
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</tr>
<tr>
<td>9 thiophene ↑ 1.8 0.00 0.00 1.6 3.00 1.87</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 thiophene ↑ 1.4 0.00 0.00 1.4 2.10 1.50</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>11 thiophene ↑ 1.9 0.00 0.00 1.9 0.55 0.29</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>12 thiophene ↑ 1.9 0.00 0.00 1.9 1.20 0.63</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>13 benzene ↓ 1.7 1.50 0.88 0.0 1.50 0.23</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>14 benzene ↓ 3.6 0.70 0.19 0.0 0.70 0.23</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>15 benzene ↓ 0.0 0.70 0.19 0.0 0.70 0.23</td>
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</table>

* T/B is the ratio of the concentrations of thiophene to benzene in the reactor inlet.
In Figure 2 the production of TIC per unit of benzene is plotted versus the degradation rate of thiophene for these step changes. It can be seen that the production of TIC per unit of benzene removed decreased as the degradation of thiophene increased. This shows that the degradation of thiophene has an influence on the metabolism of benzene. Since an increase of the yield of biomass production on benzene cannot be an explanation for this decreased CO₂ production, this indicates that part of the benzene is transformed into metabolites.

According to Andersen et al. (1998), all the thiophene transformed is retrieved in the NVOC. So for 1 mole of thiophene degraded, 4 moles of NVOC-C should be obtained. However, from Figure 3 which shows the production rate of NVOC versus the degradation rate of thiophene for the step changes described above, it appears that for 1 mole of thiophene degraded approximately 5 moles of NVOC-C were produced. This is supporting the hypothesis that benzene is not completely mineralised when thiophene is present and that part of the benzene is transformed into metabolites.

Benzene is transformed into biomass, CO₂, and NVOC and thiophene is transformed only into NVOC (Andersen, S., 1998). Considering a constant yield coefficient, the mass balances for carbon and COD results in a consumption of 3.42 moles of oxygen per mole of benzene-NVOC and a consumption of 1.58 moles of O₂ per mole of thiophene-NVOC produced.

**Inactivation of the micro-organisms**

The dynamic experiments 1 to 12 (see Table 1) showed evidence of inactivation of the bacteria by thiophene or its metabolites. During the first experiments (step 1 to 3, Table 1; Figure 4), the biofilm reactor was fed with 3 mg/l of benzene and the concentration of thiophene was increased from 0 to 2.5 mg/l (T/B=0.83). The thiophene and benzene concentrations in the reactor reached the inlet thiophene and benzene concentrations first 24 hours.
after the step, which is about 100 times the residence time. Since there was no sorption of thiophene nor benzene in the reactor, this indicates that both compounds were partly degraded. The degradation rates of benzene and thiophene decreased with increasing time and stopped 24 hours after step 1. 26 hours after step 1 the concentration of thiophene in the inlet was decreased from 2.5 mg/l to 1.5 mg/l (step 2) without any decrease of the concentration of benzene and thiophene in the reactor. 48 hours after step 1, thiophene was completely eliminated from the inlet (step 3). The degradation rate of benzene increased but did not recover to the level of degradation reached before these experiments. This indicates that part of the biomass was inactivated by thiophene or its metabolites.

The next step changes of the thiophene concentration in the inlet were introduced (steps 4 to 7, see Table 1) after recovery of the biomass activity by feeding the reactor with benzene as sole carbon source during several days. During these experiments, the thiophene and benzene concentrations reached a steady state after each step change of thiophene. For step 7, the T/B$_{\text{inlet}}$ ratio (T/B=0.8) after the step had almost the same value as for step 1 (T/B=0.83). During step 1 the bacteria were completely inactivated after the step. During step 7 the system reached a steady state at which the bacteria degraded 25 mg thiophene/h/m$^2$ biofilm and 37.5 mg benzene/h/m$^2$ biofilm. The difference between both steps was the magnitude of the concentration change: during step 1, the T/B$_{\text{inlet}}$ ratio was increased from 0 to 0.83, while during step 7, the T/B$_{\text{inlet}}$ ratio was only increased from 0.4 to 0.8. The magnitude of the step seems to have an influence on the response of the bacteria.

This phenomena was further investigated by the study of the steps 8 to 12 (Table 1). The concentration of thiophene and benzene for the five step changes of the thiophene inlet concentration are presented in Figures 5 and 6 normed against the inlet concentration of thiophene, respectively benzene. No thiophene was present in the reactor for at least one day before each of the step changes.

For each of the step changes, thiophene was degraded as soon as it entered the reactor. After each step change, the concentration of benzene as well as the concentration of thiophene in the reactor increased with duration of the experiment. This means that the degradation rates of both compounds decreased. Thiophene or its degradation products inhibited or inactivated the bacteria. The higher the ratio of concentrations of thiophene to benzene was in the inlet of the reactor, the faster the degradation rates of benzene and thiophene decreased, and the faster the inhibition or inactivation took place. For ratios above 1.5 mg thiophene/mg benzene, the inhibition or inactivation of the micro-organisms was complete. Few hours after each step thiophene was completely removed from the inlet and the degradation of benzene increased instantly. However, the micro-organisms did not recover to the level of activity reached before the thiophene concentration step in upward direction.
same phenomenon was observed after step 3. This indicates that part of the bacteria were inactivated and the rest were only inhibited.

The inactivation might be explained by the depletion of reducing equivalents. These reducing equivalents are regenerated during the degradation of the primary substrate and used during the degradation of primary and secondary substrates (Bae et al., 1995). Due to competition with the cosubstrate thiophene (Folsom et al., 1990), a smaller amount of benzene was converted, which lead to a diminished regeneration of reducing power until a steady state or a complete stop of degradation was reached.

The inactivation might also be due to killing of the bacteria, but from this study it is not possible to distinguish between the part of the bacteria which have been killed and the part of the bacteria that only have been inactivated.

Adaptation to the degradation of thiophene

For lower inlet ratios of the concentration of thiophene and benzene (0.29; 0.63) the degradation rates of benzene and thiophene decreased after the addition of thiophene but increased after a certain time and reached a steady state. An explanation for this phenomenon could be that the degradation of thiophene first lead to metabolites which inhibited or inactivated the micro-organisms and that subsequently the toxic metabolites were converted biologically or abiotically into compounds, which were less inhibiting for the bacteria. When the step change of thiophene concentration was large compared to the benzene concentration in the inlet, the inactivation by the thiophene metabolites probably got faster than their further conversion and this lead to the inactivation of the micro-organisms. An investigation of the metabolites of thiophene and benzene is underway to confirm this hypothesis.

Degradation of thiophene by resting cells

Figures 7 and 8 show the concentration of thiophene over time after a step change of benzene from 1.7 mg/l and 3.6 mg/l, to 0 mg/l (step 13 and 14, Table 1). After these steps, it was expected...
that the degradation of thiophene would stop immediately since there was no primary substrate to support its degradation. The thiophene concentration in the bulk should reach the thiophene concentration in the inlet after approximately 50 minutes according to the mass balance calculations. After benzene was removed from the inlet, the thiophene degradation stopped first after 3.5 hours for step 13 and after 90 hours for step 14. For this period after the disappearance of benzene from the inlet, the bacteria still degraded thiophene. The length of this period seems to depend on the concentration level of thiophene and benzene before the step.

In order to degrade the secondary substrate, the micro-organisms need enzymes and reducing equivalents or energy sources which are induced by the primary substrate (Oldenhuis et al., 1989; Folsom et al., 1990; Strand et al., 1990; Bae et al., 1995). When benzene was removed from the medium, the enzymes and reducing power or storage compounds were still present in the system and this may explain the degradation of thiophene by the resting cells. The decrease of the degradation rate of thiophene with time could be due to the depletion of enzymes, reducing power or storage compounds.

**Competitive inhibition between benzene and thiophene**

The first part of Figure 7 and 8 which is highlighted shows that the degradation rate of thiophene increased immediately after the removal of benzene from the inlet. This illustrates the competitive inhibition between benzene and thiophene. However, the degradation rate only increased for a short period, i.e. a few minutes for step 13 up to one hour for step 14.

**Regeneration of the cometabolic activity**

Figure 9 shows the reactivation of the bacteria (step 15, Table 1) after step 14 by the addition of benzene in the inlet at a concentration of 3 mg/l, while thiophene was kept constant at 0.7 mg/l. The bacteria started instantly to degrade a small amount of benzene (0.3 mg/l).
The degradation rate of benzene increased then over time until the bulk concentration of benzene reached 15 mg/l. The micro-organisms also degraded thiophene, but the degradation of this latter was detectable first 24 hours after the benzene step. Four days after the step in benzene concentration, the cometabolic activity of the bacteria was completely regenerated.

**Conclusion**

The investigation of the cometabolic degradation of thiophene with benzene as the primary substrate in a continuous biofilm reactor gave information on the metabolite formation from benzene and thiophene and on some of the processes involved in this metabolism.

In the absence of thiophene, benzene is metabolised to biomass and CO₂. The introduction of thiophene interferes with the metabolism of the benzene degradation in such a way that part of the degraded benzene is not completely mineralised, but transformed into organic metabolites.

The experiments indicated the inactivation of the micro-organisms by thiophene or its transformation products. With an increasing ratio of the concentrations of thiophene to benzene in the reactor inlet, the inactivation of the micro-organisms got faster. For low ratios of these inlet concentrations, the bacteria regained part of their activity after a certain time of inactivation. This adaptation behaviour could be an evidence for the biological or abiotic conversion of toxic metabolites of thiophene or benzene into compounds, which are less harmful to the bacteria.

Resting cells, which previously have been activated with benzene, were able to degrade thiophene. However, the conversion of thiophene lead to the inactivation of the cells. Their reactivation and the regeneration of the cometabolic activity could be obtained by the addition of the growth substrate benzene.

**References**


