Degradation of Cypermethrin by indigenous bacteria in local industrial, beech- and spruce-forest soil

Joakim Engblom

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School of Business and Engineering

Supervisor: Per Woin
Abstract

Soil from local beech-forest, spruce-forest and an industrial area was taken. Control- and test-microcosms containing 150 ml soil were spiked with cypermethrin 0.4 mg/ml soil. Cypermethrin residues were extracted on day seven and 14.

Cyclohexane and deionized water was utilized in multiple step extraction processes. Samples were analyzed in a Gas Chromatograph (GC) with electron capture detector (ECD).

Concentration values for the samples were highest for beech-forest soil and lower for the other two soil-types. Statistical differences in concentrations between control- and test-microcosms for each soil-type on day seven and day 14 were evaluated with Mann Whitney U tests. Significant result was only found in the industrial 14-day group. The small amounts of cypermethrin in the extracts could not only be ascribed to a bacterial degradation process. Used insecticide has a high bonding affinity for particles and is sequestered in soil.

Keywords: Pyrethroids; Cypermethrin; Soil-bacteria, Beech-forest soil; Spruce-forest soil; Industrial soil; Microcosms

Introduction

Soil- and marine-bacteria versatility

Bacteria are versatile organisms, which inhabit every biotope on earth. They adapt fast to environmental perturbations. Biostimulation is a remediation method, which has been applied on several occasions, the contaminated sites are supplemented with inorganic nutrients and indigenous bacteria degrade the toxicant. Another procedure is to add bacteria known to degrade the special xenobiotic present in a location targeted for remediation. This method is called bioaugmentation. During oil spill bioremediation in marine mesocosms it was found that the bacteria Alcanivorax plays a crucial role in the degradation of hydrocarbons (Cappello et al., 2006). Even in extremely cold places bacteria are an active part of the soil. Indigenous bacteria to the Antarctic have the ability to degrade components of the special fuel mixture Special Antarctic Blend (Snape et al., 2004).

Bacteria strains from the volcano island of Santorini were isolated and tested for their ability to degrade crude oil. Of 150 thermophilic bacteria nine genetically related to the Geobacillus species and one to the Bacillus species were able to grow in liquid cultures with crude oil as the carbon source. These bacteria had capacity to degrade long chain alkanes. (Meintanis et al., 2006)

Bacillus sphaericus JG-A12 are able to accumulate metals such as Cu, Pb, Al, U, Cd and precious metals. Some of these metals are stored in the cell’s protein layer (Pollmann et al., 2006).

Synthetic pyrethroids, toxicity and ecotoxicity

Synthetic pyrethroids (SP) are based on the molecule pyrethrin that is derived from special Chrysanthemum species (Grant et al., 2001). Cypermethrin (CM) is a neurotoxicant pyrethroid that is persistent in soil and sediment (for structure see fig 1).
The compound is very toxic to aquatic organisms. Freshwater fish (*Channa punctatus*) exposed to cypermethrin has low levels of red blood cells and proteins in the blood (Saxena et al., 2002). The invertebrate biodiversity of exposed limnic environments are severely skewed (Friberg-Jensen et al., 2002). It has been suggested that pesticides are one of the factors for the worldwide decline in amphibian populations. Tadpoles are sensitive to low doses of this insecticide; it causes deformities, behavioral abnormalities like twisting and uncoordinated swimming. The growth process is also inhibited (Greulich et al., 2004). Cypermethrin has a low bioconcentration factor hence it is not considered harmful for top-predators, but results from direct dose studies shows that it is harmful for the test organisms. Studies on rabbits have shown that high levels of this chemical are neurotoxic and cause ataxia, excessive salivation, choreoathetosis, tremors and convulsions (Khanna et al., 2002). Cypermethrin affects the voltage dependent sodium channel and ATPase in neuronal membranes. It binds to nuclear-DNA, which leads to destabilization and unwinding (Patel et al., 2006). Studies on mouse hepatocytes have shown that some of the metabolites of this insecticide could form DNA-monoadducts and DNA interstrand crosslinks. DNA-interferences like these could be carcinogenic (Cui et al., 2006).

The most common reported reversible symptoms in exposed humans are sensations of burning, tingling, itching, or numbness, paraesthesia and irritations of skin and respiratory tract. Pyrethroids have fast metabolism rate in the human body, the central esterbond is cleaved and the alcoholic products are oxidized to carboxylic acids (Hardt and Angerer, 2002). In the same study metabolites from pyrethroids were investigated in workers handling these pesticides and the conclusion was that none had been exposed to amounts above acceptable daily intake (ADI). Some of the metabolites were also found in the control groups, which would imply that they had been indirectly exposed to pyrethroids (Hardt and Angerer, 2002). A commonly used spraying technique is the use of lance. The workers carry the tubes with pesticides and apply it on the foliage via a lance. In such an exposed environment it is extremely important to use proper protective measures (Choi et al., 2005).

![Molecular structure of Cypermethrin.](image)

**Cypermethrin, soil and bacteria**

The bioavailability for cypermethrin in soil is dependent on the amount of organic material, temperature, pH and the silicate composition. In a sorption-study on corundum,
quartz, montmorillonite and kaolinite with the pesticides lamda-cyhalotrin, cypermethrin, deltamethrin and fenvalerate it was shown that corundum had the highest bonding affinity and montmorillonite, kaolite the lowest. Cypermethrin had the lowest bonding affinity of these four pesticides (Oudou Chaaieri and Bruun Hansen, 2002). Conventional extraction procedures can be time consuming but a new method involving the use of ultrasound improves extraction time and lessens the consumption of solvents. Liquid from the extraction procedure is chromatographed without ensuing clean-up steps and analysis time is shortened (Babic et al., 1998).

Cypermethrin is co-metabolized by bacteria in soil. In vitro studies have shown two soil-bacteria that are able to degrade this insecticide; they are members of the genera *Pseudomonas* and *Serratia*. Genetic analyzes showed that these bacteria were specifically related to *Serratia plymuthica* and the *Pseudomonas* intragenic cluster (Grant et al., 2001). A synthetic pyrethroid is often a mixture of different isomers. Bacteria preferentially degrade some enantiomers over others (Liu et al., 2005).

Two years ago, a lot of spruce-forest fell during the storm “Gudrun” in the southern parts of Sweden. This caused intensive logging. Bark beetles infested stacked timber. In order to reduce the damage caused by them the insecticide cypermethrin is being used in certain timber-traps in conjunction with pheromones. The traps are built of short spruce-logs and stacked so that as much as possible of the bark surface will be accessible by the beetle. The logs are being sprayed with cypermethrin; special pheromone traps will be hung on one of the logs (Lindén, 2007). At present there are other alternatives discussed and evaluated but this method is used to some extent.

The aim of this study is to investigate different soil-bacteria’s inherent capabilities to degrade the insecticide cypermethrin. Experiments are performed in the original soil instead of as in a majority of pesticide degradation studies, nutrient-broth spiked with the chemical in question. Though in vitro, natural conditions are copied to an extent. Parameters like temperature, pH and humidity are controlled.

### Materials and methods

#### Soils and bacterial activity

Soil was taken from three different localities; beech-, spruce forest and urban industrial area (see fig 2). Topsoil was removed and a part of the layer below was extracted. Root-rests and leaves were removed. An overview of bacterial activity was performed; 5 g of each soil-type was put in 250 ml of nutrient-broth (Merck) and was shaken at 75 rpm overnight in room temperature 21 °C. A small amount of each bacterial suspension was applied to agar-plates (Merck), which were incubated for 24 hours at 37 °C (see fig 3 a, b and c).
Treatment and insecticide application

Cyperplus (cypermethrin conc., 100 g/l) was acquired from Granngården Halmstad in a 500 ml container.

The different soils were rinsed with 0.25 ml nutrient-enriched water per ml soil (recipe from Bushnell Haas), pH 7.0 (Grant and Daniell, 2002). Three different
suspensions were obtained, one for each locality. Indigenous soil bacteria were extracted during the washing processes. Soil was spread on a 95% ethanol washed plastic sheaths. Cypermethrin was applied at 0,4 mg per ml soil. Cyperplus-emulsion was mixed with deionized water to a CM-concentration of 19,35 g/l. The suspension was applied to the soil on the plastic sheaths by spraying. Soil for the controls were autoclaved for 35 min at 121 °C in order to kill all soil native bacteria, the application-process for the pesticide was the same as for the tests.

Microcosms

Microcosms were made of plastic jars with ventilation holes (the degradation processes studied were primarily aerobic). 150 ml cypermethrin spiked soil was put in each jar. 4,8 ml of water from the rinsing processes were reapplied to each beech- and spruce-forest microcosm. The rinse-water volume for industrial soil containers were 2,0 ml. By adding nutrient enriched water from the washing procedures it was assured that viable (not affected by the shock-treatment of cypermethrin application) bacteria were present in the test soils. The reused rinse-water for the control containers had been autoclaved (121 °C, 20 min). Control microcosm contained sterilized soil hence no bacterial activity. The soil in the test microcosms was not subjected to the autoclave treatment so bacterial processes should have continued unhindered. The containers were kept at constant temperature 21,5 ± 1,0 °C in the dark. Soil was kept moist by application of 4,0 ml of autoclaved (121 °C, 20 min) tap water (pH 7,0 with HCl) to the jars containing spruce- and beech-forest soil every day. Applied daily water volume for industrial soil microcosms were 2,0 ml. Initial humidity for the latter soil-type was higher than for the two forest-soils.

Extraction procedures

Pesticide residues were extracted on days 7 and 14 by suspending each soil sample (150 ml) in 30 ml deionized water and 10 ml of cyclohexane and shaken at 150 rpm for one hour. Soil, water and hexane were applied to 500 ml centrifuge-tubes; additional 15 ml of deionized water was used to cleanse soil residues from the flasks used in the shaking-process this volume was also added to the 500 ml tubes. The half-liter containers were centrifuged at 2000 g for 5 minutes. The liquid-phase was poured into 50 ml tubes and centrifuged for 5 minutes. The cyclohexane-phase was extracted and applied to glass-tubes for storage until Gas Chromatography (GC) analysis. Small amounts of particles were suspended in the solvent after the third extraction-step for industrial soil, an additional clean-up step had to be performed, the extracted hexane from the 50 ml tubes were applied to smaller Falcon-tubes and centrifuged at 2000g for 5 min, the supernatant was put into glass-tubes waiting for analysis.
Analyses

1.0 µl of the extracts was analyzed with a Varian GP-3800 gas chromatograph with an electron capture detector (GC-ECD). The GC parameters were: splitless injector 250 ºC, oven 60 ºC (2 min), 100 ºC/min to 170 ºC (1 min), 170 ºC (1 min), 10 ºC/min to 295 ºC (12.5 min), 295 ºC (6 min), detector 350 ºC; carrier gas was helium.

Before GC-analysis the samples had to be diluted, generally a 1000 times, on some occasions more depending on the cypermethrin concentration.

Calculations and chromatograms

Cypermethrin is a blend of eight isomers. In GC analysis there are four peaks each one representing a pair of cis- or trans-isomers (Jin and Webster, 1997). In the below chromatogram two major peaks are seen and hints of two others in between (see fig 4). Most chromatograms only contained the two major peaks; all isomers of the analyzed compound were included in these.

![Chromatogram](image)

Fig 4. Chromatogram from a seven-day beech-forest sample. The X-axis displays the retention time for the different compounds. The signal strength is shown on the Y-axis. The peaks before the marker TI-ON are from the solvent and are not registered. The two major peaks at around 15 min and the hints of peaks in between are from Cypermethrin isomers. The small peak at 13 min was not identified.

To be able to calculate the amount of Cypermethrin in the extracted samples the following formula was used. A is the area of the peaks in the chromatograms for the insecticide analyzed.
\[ \begin{align*}
V_{\text{soil}} & = 150 \text{ ml} \\
A_{\text{soil}} & = 43733 \cdot 5 \mu V / \text{min} \\
c_{\text{soil}} & = 1,0 \cdot 10^{-6} \text{ mg/ml} \\
k_{\text{dilution}} & : \text{ dilution factor} \\
c_{\text{sample}} & = \frac{V_{\text{extracted}}}{V_{\text{soil}}} \times \frac{V_{\text{sample/diluted}}}{V_{\text{soil}}} \times \frac{A_{\text{sample}}}{A_{\text{soil}}} \times \frac{c_{\text{soil}}}{c_{\text{soil}}} \times k_{\text{dilution}} \\
\Rightarrow c_{\text{sample}} & = \frac{V_{\text{extracted}}}{V_{\text{soil}}} \times \frac{A_{\text{sample}}}{A_{\text{soil}}} \times \frac{c_{\text{soil}}}{c_{\text{soil}}} \times k_{\text{dilution}}
\end{align*} \]

The dilution-factor differed for each sample, but it was generally 1000.

Statistics

There was one control-group (autoclaved) and one test-group for each soil-type on days seven and 14. All groups contained seven replicates. The total sum of all samples is 84.

The results were evaluated with a Mann-Whitney U test in SPSS 13.0 for Windows. Mann Whitney U tests were used to test for differences between control- and test-groups on day seven and 14 for each soil-type, a total of 6 tests. Differences between soil-types were not statistically evaluated because of big differences in homogeneity of variances.

Results

During visual inspections of the agar-plates from beech- and spruce-forest soil two main colony-forming bacteria was found. The first type had a whitish transparent hue and the second was of a more yellow characteristic (see fig 3a and b). Bacteria-cultures from industrial-soil were also composed of two main colony-forming strains; like in them from beech- and spruce-forest one was whitish and transparent. The other had a saturated yellow-white color and was only native to industrial-soil (see fig 3c).

Fig 3a. Bacteria-culture from beech-forest soil. Unknown mycorrhiza is seen at the top left edge of the densest population of bacteria-colonies.

Fig 3b. Bacteria-culture from spruce-forest soil. Mycorrhiza from an undetermined species of fungi is seen at the lower edge of the most closely spaced population of
Mould was found in a majority of the control-microcosms for beech- and spruce-forest soil after five days. In industrial soil only about half of the controls showed had any mould growth. This phenomenon could be an opportunistic effect. During autoclaving the soil-native microorganisms are killed and foreign sporangium could invade and start to grow. Or the sterilization time was not long enough to kill more resilient already existing sporangium.

During pesticide extraction from industrial-soil the cyclohexane was colored black, this was probably due to crude-oil residues. The chromatograms from the analysis in the gas chromatograph (GC) did not show any peaks that could give a hint of what these unknown components could be. To be able to analyze a molecule it must contain a halogen in order for the electron capture detector (ECD) to detect it.

Calculated concentrations had a large spread (see fig 5 a, b, c and d). The diagrams display the mean values with 95% confidence intervals. Cypermethrin concentrations for the beech-forest sample groups are not shown in the same diagram due to large variations.

Fig 3c. Bacteria-culture from industrial soil.
Fig 5 a – d. The colored bars displays mean values for the extracted cypermethrin (CM) concentrations for each soil type on days seven and 14. T-bars indicate 95% confidence intervals. The days below the bars indicates the time lapse between application of insecticide and extraction. Observe the scales on the y-axes they are in µg cypermethrin / ml soil except in figure c where µg is mg.
According to the Mann Whitney U test there was only one significant result, which was in the 14-day industrial soil group (see table 2). There is also an indication of an effect in the group for day seven spruce-forest soil, though not significant.

Table 2. Output from Mann Whitney U test. Grouping variable was treatment (sterilized or natural).

<table>
<thead>
<tr>
<th></th>
<th>Conc7dbeech</th>
<th>Conc14dbeech</th>
<th>Conc7dspruce</th>
<th>Conc14dspruce</th>
<th>Conc7dind</th>
<th>Conc14dind</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mann-Whitney U</td>
<td>22,000</td>
<td>21,000</td>
<td>10,000</td>
<td>16,000</td>
<td>20,000</td>
<td>8,000</td>
</tr>
<tr>
<td>Z</td>
<td>-0.319</td>
<td>-0.447</td>
<td>-1.853</td>
<td>-1.086</td>
<td>-0.575</td>
<td>-2.108</td>
</tr>
<tr>
<td>Asymp.Sig (2-tailed)</td>
<td>0.749</td>
<td>0.655</td>
<td>0.064</td>
<td>0.277</td>
<td>0.565</td>
<td>0.035</td>
</tr>
<tr>
<td>Exact Sig. [2*(1-tailed Sig.)]</td>
<td>0.805(a)</td>
<td>0.710(a)</td>
<td>0.073(a)</td>
<td>0.318(a)</td>
<td>0.620(a)</td>
<td>0.038(a)</td>
</tr>
</tbody>
</table>

a Not corrected for ties.

The intra-divergence in the CM-concentration values in some series warranted a log-transformed (base 10) display in order to be able to compare them graphically (see fig 6 a,b and c).

Fig 6a. Concentration values for beech-forest soil, logarithm-transformed (base 10).

Fig 6b. Logarithm-transformed (base 10) concentration values for spruce-forest.
Bacteria are pervasive and highly adaptable. In most cases natural bacteria are able to degrade pollutants if nutrients are applied to contaminated sites but in some cases that is not enough, specialized bacteria has to be added. Wetlands, natural or constructed, contain large amounts of nutrients hence substantial aerobic and anaerobic bacterial activity and a potential for degradation of xenobiotics. A combination of different bacterial strains is optimal for rendering petroleum components innocuous in wetlands (Shuhong et al., 2006). In soil nutrients and toxicants are not as bioavailable as in water, thus bioremediation of contaminated soil by indigenous bacteria take a long time.

Mann-Whitney U tests were used to evaluate the data. Other tests have also been tried. Resulting P-values for Levene’s test for equality of variances during t-tests with the concentration values were 0.005 for the beech-forest day-seven group. Another low significance number for equality of variances was in the industrial soil day-seven group (P=0.095). The data was log-transformed (base 10) and was tried in a new t-test. The test for equality of variances showed that the test and control groups for the 14-day samples for spruce-forest did not have equal variance P was 0.002 (P>0.05 if the variance is the same). Also the 14 day industrial soil samples had a low significance figure for equality of variances P=0.083, although not critical but low. Two-way ANOVAs were also used in a set of trials; fixed factors were spot and treatment (control or test). Dependent
variables were the concentrations for day seven and 14. The values were analyzed both as they were and log-transformed. The significance value in all Levene’s test of equality of error variances was well below 0.05. It was deemed appropriate to use the Mann-Whitney U tests for statistical analysis. Due to large differences in variances in the concentrations of the extracted cypermethrin between the different soil-types an inter soil-type comparison was not practicable. Considering the disparities in organic and particle content a statistical evaluation would not have rendered any useful data. The most important factors in the differences between the soil-types are the internal particle size distribution, their crystalline structure that is dependent on chemical composition. Two particle-types of the same material and same mass but different sizes have different surface areas; the smaller ones have a larger total surface area thus a better binding-potential for cypermethrin than their larger counterparts.

Pesticides have different affinities for different organic material and silicate-particles. Bioavailability decreases as the toxicants affinity for soil-particles increases. (Chaaieri Oudou and Bruun Hansen, 2002)

Considering the initial concentration of the insecticide of 0.4 mg per ml soil, the low values of cypermethrin in the extracted samples cannot solely depend on microbiological activity, another mechanism has to be involved. CM molecules have a high affinity for particles and are sequestered in soil. In retrospect the utilized extraction procedure was not a sufficient method for capturing cypermethrin molecules. Ultrasound-extraction in one or several steps would be optimal as described by Babic et al., 1998. Another novel approach is the use of microwaves in the extraction procedure (Esteve-Turillas et al., 2004). This procedure should be used with caution; too much effect increases the heat and might disrupt the molecules.

A computerized irrigation system for the microcosms would be optimal compared to the manual daily watering procedure used. By specifying a certain moisture value in percent for the soil-containers the computer would control the application of water and an almost exact moisture-level would be achieved and maintained. Level conditions are beneficial for soil-bacteria.

Resolution in a chromatogram from a GC is dependent on the temperature program, pressure in the column and the flow of gas mixture. Optimal resolution is achieved by manipulating these parameters. Some of the chromatograms showed a hint of more than the for these analyses two characteristic cypermethrin peaks. Further configuration changes of the GC for better separation of the peaks were not considered necessary because it was the total amount of the insecticide that was analyzed.

If the detection-limit for the analyzed compounds is relatively high, then or if the experimenter wishes to increase the accuracy of the analysis then the sample can be fortified with the chemical in question. Jin and Webster (1998) used this procedure for testing the amounts of cypermethrin in soil, elm bark and litter. Their method was not implemented in this investigation; the samples contained so large quantities of CM that they had to be diluted before analysis.

A possible explanation for the significant result obtained in the 14 day industrial-soil case could be that the indigenous bacteria has been exposed to xenobiotics and are better adapted through natural selection to cope with different chemical contaminants.

Bacteria from the genera *Pseudomonas* and *Serratia* were found in all soil-samples in the Grant et al., 2002 study. Identification of the soil-native bacteria in this survey was
not performed but *Serratia* and *Pseudomonas* were probably present in all soil-samples. Comparison with the Grant et al., 2002 report, the significant effect in the 14-day industrial soil group could tentatively be ascribed to *Pseudomonas fluorescens* and *Serratia plymuthica* activity.

Mould was present in all spruce- and beech-forest control microcosms on day seven; it is a known fact that certain fungi are potent at rendering some xenobiotics innocuous. Certain strains of mould fungi are able to degrade imidazolium-based wood preservatives and quaternary ammonium compounds (Zabielska-Matejuk and Czaczyk, 2006). By studying the last mentioned report, some strains of mould fungi might be endowed with the mechanisms for cypermethrin-degradation, but this remains uncorroborated.

Statistical outcome from the spruce-forest day seven, though not significant, gave an indication that a bacterial degradation process might be active. Another highly speculative alternative is that the disparity might be caused by a special strain of mould fungi.

Bacteria capable of converting particular xenobiotics into harmless products might also be able to transform structurally similar molecules. A mixed bacterial culture habituated to and grown in crude oil containing medium is capable of degrading 2,4,6-trinitrotoluene (TNT) with low production of metabolites (Popesku et al., 2006).

Parameters pertaining to experimental procedures that should be altered in similar studies in the future are autoclaving time, extraction method and the GC sample injection technique. The sterilization time for soil samples ought to be at least 2 –3 h at 121 °C. For comments about extraction see earlier part of the discussion. It would be preferable to use an auto-injector when injecting the samples into the GC instead of the manual process practiced here. This would ensure that the applied volumes would be exactly the same in all analyses.

Also after spray-application of the insecticide onto the soil a shaking step in a suitable glass-container should be implemented to make sure that the cypermethrin is perfectly equally distributed throughout the soil-matrix, although the used procedure is good but by adding this extra step would make it even better.

Soil-bacteria should be collected from different spots and incubated in nutrient-broth for 24h. By applying pure cultures and nutrients to different matrices with identical properties spiked with cypermethrin, a comparison of degradation efficiency between the populations would be possible.

This study indicates that new contaminants to a particular spot do not have to be of similar molecular-structure as older already present pollutants, for indigenous soil-bacteria to be able to transform them into harmless metabolites. It seems that bacteria exposed and acclimatized to hazardous chemicals are more resilient and better at dealing with new toxicants. More surveys have to be performed before making any final conclusions.

An approach is to procure bacteria from localities notorious for high levels of pollutants, grow pure cultures and test their capabilities for degradation of different xenobiotics and store them. This procedure could be utilized for building an arsenal of bacteria capable of combating almost any hazardous anthropogenic compound. Taking modern genetic engineering into consideration there are virtually no limits for what bacteria will be able carry out.
Presumably all bacteria have a capacity to degrade some xenobiotics. For example we would probably discover that bacteria native to the human body have inherent enzyme-systems able of transforming some dangerous compounds. A notion not so far out based on all reports of bacterial versatility, but it remains a thought until more research has been done.

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