VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

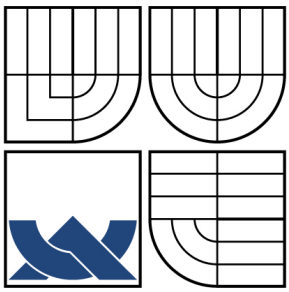
FAKULTA CHEMICKÁ
ÚSTAV CHEMIE A TECHNOLOGIE OCHRANY ŽIVOTNÍHO PROSTŘEDÍ

FACULTY OF CHEMISTRY
INSTITUTE OF CHEMISTRY AND TECHNOLOGY OF ENVIRONMENTAL PROTECTION

MICROBIAL PESTICIDE DEGRADATION IN WATER WORKS SAND FILTERS

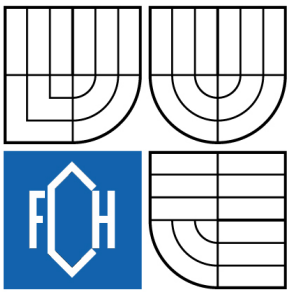
AUTOR PRÁCE — ŠÁRKA VÁCLAVKOVÁ
AUTHOR

BRNO 2009



VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY



FAKULTA CHEMICKÁ

ÚSTAV CHEMIE A TECHNOLOGIE OCHRANY ŽIVOTNÍHO PROSTŘEDÍ

FACULTY OF CHEMISTRY

INSTITUTE OF CHEMISTRY AND TECHNOLOGY OF ENVIRONMENTAL PROTECTION

MICROBIAL PESTICIDE DEGRADATION IN WATER WORKS SAND FILTERS

MICROBIAL PESTICIDE DEGRADATION IN WATER WORKS SAND FILTERS

DIPLOMOVÁ PRÁCE

DIPLOMA THESIS

AUTOR PRÁCE — ŠÁRKA VÁCLAVKOVÁ

AUTHOR

VEDOUCÍ PRÁCE — doc. Ing. JOSEF ČÁSLAVSKÝ, CSc.

SUPERVISOR

BRNO 2009

Vysoké učení technické v Brně
Fakulta chemická
Purkyňova 464/118, 61200 Brno 12

Zadání diplomové práce

Číslo diplomové práce	**FCH-DIP0217/2007** Akademický rok: **2008/2009**
Ústav	Ústav chemie a technologie ochrany životního prostředí
Student(ka)	**Šárka Václavková**
Studijní program	Chemie a technologie ochrany životního prostředí (MPCP_CHTOZP)
Studijní obor	Chemie a technologie ochrany životního prostředí (MPCO_CHTOZP)
Vedoucí diplomové práce	**doc. Ing. Josef Čáslavský, CSc.**
Konzultanti diplomové práce	

Název diplomové práce:

Microbial pesticide degradation in water works sand filters

Zadání diplomové práce:

1. Zpracování literární rešerše shrnující současný stav problematiky
2. Izolace vhodných kmenů bakterií degradujících MCPP
3. Ověření jejich vhodnosti degradací modelových roztoků MCPP
4. Identifikace degradačních cest a produktů

Termín odevzdání diplomové práce: 16.5.2008

Diplomová práce se odevzdává ve třech exemplářích na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Šárka Václavková	doc. Ing. Josef Čáslavský, CSc.	
student(ka)	Vedoucí práce	Ředitel ústavu

V Brně, dne 1.9.2007

doc. Ing. Jaromír Havlica, DrSc.
Děkan fakulty

ABSTRAKT

Předmětem této práce je izolovat aktivní bakteriální kulturu schopnou aktivovat a podporovat mineralizaci dvou zvolených polutantů: herbicidu mecoprop (MCPP) a metabolitu herbicidu dichlobenil - 2,6–dichlorobenzamidu (BAM), jež jsou v současnosti hojně identifikovány ve zdrojích pitných vod v Dánsku. Pro tuto kulturu platí předpoklad, že je izolována ze sedimentů, na nichž je přirozeně vázána, což umožní následné využití sedimentu obsahujícího tuto aktivní kulturu jako jednu z vrstev vodárenského vícevrstvého filtru. Schopnost mikroorganismů přítomných v sedimentech mineralizovat zvolené polutanty je ověřována pomocí laboratorního experimentu využívajícího tzv. „mineralizačních baněk“, tedy sterilního uzavřeného systému obsahujícího vodu, písek, vzduch a známé množství ^{14}C–značeného polutantu. Schopnost mikroorganismů mineralizovat daný polutant je pak měřena na základě množství $^{14}CO_2$ vznikajícího při mineralizaci v tomto uzavřeném systému.

Největší mineralizační potenciál byl pozorován pro mikroorganismy pocházející ze sedimentu původem z francouzského Bréville, těženého z hloubky 4,50 – 4,65 m pod povrchem. V mineralizačních baňkách obsahujících tento sediment byla pozorována cca 40 % produkce $^{14}CO_2$ během 123 denní inkubace s 28 denní fází zdržení. Zředění původního sedimentu se ukázalo jako faktor ovlivňující rychlost degradace. V žádném ze systému obsahujícím metabolit herbicidu dichobenilu BAM nebyla pozorována mineralizační aktivita.

ABSTRACT

The mineralization potential of the herbicide mecoprop and metabolite of the herbicide dichlobenil BAM has been studied. The aim of the project is to isolate active cultures able to degrade pollutants frequently identified in the sources of drinkable water in Denmark. The culture is expected to be sticked on the sediment which makes possible its future usage as a part of the waterworks sand filter. In the batch experiment, the microcosms with sediments diluted, sand and tap water have been incubated at 10°C. The [ring–U–^{14}C] labeled contaminant has been added into the systems. The evolved $^{14}CO_2$ has been measured by scintillation technique.

The highest mineralization potential has been observed for the sediment from Bréville, acquired from 4.50 – 4.65 mbs. In the batch containing this sediment, an approximate production of 40 % $^{14}CO_2$ during 123 days of incubation and a lag phase of 28 days have been reported. The dilution of the original sediment has been found to be a factor influencing the rate of biodegradation. No degradation potential has been observed in microcosms with BAM.

KLÍČOVÁ SLOVA

Mineralizace, biodegradace, pesticid, metabolit, podzemní voda, mecoprop (MCPP), dichlobenil, 2,6–dichlorobenzamid (BAM), izolace

KEY WORDS

Mineralization, biodegradation, pesticide, metabolite, groundwater, mecoprop (MCPP), dichlobenil, 2,6–dichlorobenzamide (BAM), isolation

BIBLIOGRAFICKÁ CITACE

VÁCLAVKOVÁ, Š. *Microbial pesticide degradation in water works sand filters*. Brno: Vysoké učení technické v Brně, Fakulta chemická, 2009. XY s. Vedoucí diplomové práce doc. Ing. Josef Čáslavský, CSc.

DECLARATION

I declare that the diploma thesis has been worked out by myself and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, BUT.

.....
Student's signature

ACKNOWLEDGMENT

This Master Thesis "Microbial pesticide degradation in waterworks sand filters" has been submitted as a part of the graduation as a Chemical Engineer at the Institute of Chemistry and Technology of Environmental Protection, Brno University of Technology.

The project was carried out from December 2007 to October 2008 at the Department of Environmental Engineering, Technical University of Denmark, under the supervision of Associate Professor Hans-Jørgen Albrechtsen and the technical supervising of Lene Kirstejn Jensen.

I would like to thank my "Danish" supervisors Hans-Jørgen Albrechtsen and Lene Kirstejn Jensen for helping me all the time of my project, for the numerous discussions on different issues, for answering all my questions and for their support, patient motivation and friendly teaching environment. I would like to thank also the Head of the microbiology laboratory section Mona Refstrup and the Head of the isotope laboratory section Birthe Ebert. I would like to thank also my "home university" supervisor doc. Ing. Josef Čáslavský, CSc for his patience and helpful discussions.

The last but not least thanks belong to my family and friends for their support all the time.

Šárka Václavková
Brno
January 5th, 2009

TABLE OF CONTENTS

1. INTRODUCTION

Since the second half of the 20th century, agrochemicals such as pesticides and herbicides are widely used all over Europe. The increase of pesticides usage has been announced by the need of higher food production, which increases together with the population on the Earth. It has been found later that some of the pesticides pollute the surface water by the outflow from the fields. For a relatively long period, it was expected that the layer of sediments and minerals present between the surface (the agriculturally used ground) and the underground water worldwide is a sufficient barrier against pesticide leakage into the groundwater. Later on, during the last decades of the 20th century, herbicides from the group of phenoxy acid herbicide have been frequently detected in ground water worldwide [35]. This pollution could come from landfills or agriculturally used grounds, where herbicides concentrations typically range from 10 to 250 $\mu g.L^{-1}$ [6, 23]. The concentration of mecoprop found during the pesticide research in groundwater in Western Europe showed values up to 600 $\mu g.L^{-1}$ in deep groundwater [33].

The pesticide monitoring programs have been evolving during last decade in all of the European countries. The systematic monitoring programs to localize pesticides in groundwater have been realized in Denmark since 1993 [14]. By contrast, the first monitoring of 14 pesticides has been done in Czech Republic in 2002 [11]. In 1998 the European Commission set up rules aiming to provide healthy and clean drinking water to all of the EU citizens. The quality of the drinking water has to be monitored by the Member States and reported to the European Commission at three years intervals [12].

This project is focused on two of the contaminants. The first is herbicide mecoprop (MCPP) from the group of phenoxy acid herbicide, which is one of the most frequently used groups of herbicides. The second contaminant is 2,6-dichlorobenzamide (BAM), metabolite of the herbicide dichlobenil. BAM is one of the most frequently detected contaminants in groundwater in Denmark. The usage of its mother compound dichlobenil has been banned in Denmark since 1997.

The aim of this study is to find and isolate bacterial cultures having the potential to degrade mecoprop and BAM and verify that sediments from Danish site Hvidovre and French site Bréville are able to degrade tested contaminants. This active culture is expected to be stuck on the sediment. It is expected that cultures like that can be later on used as the pesticide treating part of the waterworks sand filters.

2. THEORY

2.1 Water supply system in Denmark and in the Czech Republic, the European Union monitoring system of drinkable water quality

To secure the quality of drinking water in the whole EU the Drinking Water Directive (98/83/EC) sets the most common substances possibly found in drinking water. Each of the national legislations of the Member States of the European Union is able to regulate also other substances according to regional needs. The DWD is based on standards given by WHO (World Health Organization) guideline for drinking water, European Commission, 98/83/EC. The DWD sets the limit for individual pesticides to $0.1 \mu g.L^{-1}$ and the limit for Pesticides – Total to $0.5 \mu g.L^{-1}$, where the term 'Pesticides – Total' means the sum of all individual pesticides detected and quantified in the monitoring procedure [28].

2.1.1 The water supply system in Denmark and in the Czech Republic

99 % of the Danish drinking water is drawn from groundwater supplies. Surface water is used as a supplement to groundwater in Copenhagen only. The Danish standards for the drinking water quality are reached mostly by a simple process containing aeration followed by filtration through multilayer sand filter [3]. Additional methods are used in some of the waterworks to reach optimal drinking water quality.

The water supply system is not based only on groundwater sources in the other parts of Europe. This breeds more complicated system for the drinking water pre-treatment. In contrast with Denmark, only around 45 % of the drinking water in Czech Republic comes from underground sources [29]. The rest is taken from artificial lakes, rivers and other surface water sources. According to the differences in drinking water sources, different pre-treatment methods are used and therefore various pre-treatment procedures are used in Czech Republic. Each of the local authorities chooses their own way to reach Drinking Water Directive standards. On the other hand, there are few common denominators used in all of the drinking water pre-treatments. Those are sand filter filtration at the beginning of the pre-treatment process and final disinfection by UV-light, addition of chlorine or chloramines, or use of ozone at the end of the pre-treatment.
In the case of a water source polluted by pesticides, two ways can be considered as a treatment technique. One of them is the widely used sorption to the activated carbon filter. This method is found sufficient for a pesticide treatment. It is based on the physical properties of activated carbon. The pesticide is now bonded on the solid phase of activated carbon, but no change in the total pesticide mass happens. The disadvantage of the activated carbon sorption is the limited capacity of the activated carbon. The used activated carbon can be regenerated for example by thermic reactivation. It can be also dried and burnt. The pesticides are completely burnt into CO_2 and H_2O, and the residues can be used as a carbon source. This method is successful, but due to the limited activated carbon capacity, the energy needed for regeneration or for burning of carbon filters is relatively high. The other treatment technique for water polluted by pesticides is the biodegradation mineralization. The products of a complete mineralization are CO_2 and H_2O. If the microorganism able to degrade pesticides is stuck on the sediment, then it is possible to use this sediment as a part of the waterworks sand filter. Because no accumulation of pesticide occurs in this case, the capacity of the filter is as long as the microorganism keeps its reproduction ability unlimited. This water treatment needs less energy and the problem with pesticide contamination is solved in one system. Thus, this method is

considered sufficient and the researches led to find a culture which is possible to use as a part of waterworks sand filters is one of the widely followed topics.

2.2 Monitoring system in Denmark

In Denmark, pesticides in groundwater have been monitored for the last 15 years (beginning in 1993) and all the data are collected in the national groundwater database JUPITER. The topic centre for Danish Groundwater Monitoring is GEUS – Geological survey for Denmark and Greenland. By GEUS [14] *"In groundwater monitoring areas the percentage of well screens with pesticides or their metabolites, above and below the MAC of 0.1 μg.L^{-1} for drinking water has increased once again since 2004. One of the reasons for this is the fact that monitoring for pesticides and their metabolites now only occurs in screens with young groundwater"* The wells with content of pesticides and metabolites are closed down. *"Today the larger water works primarily abstracts drinking water from aquifers with old water. "*

During the years 2001 and 2002 the data of *"Grundvandsovervågning 2002"*are presented from two of the monitoring pesticide monitoring programs: GRUMO (national ground water monitoring) and LOOP (the wells collected water from agricultural watershed). The fact that the data from years 2001 and 2002 are the last one available in English version is the reason why those data are chosen to be presented to describe pesticide situation. The data by GEUS are collected in Table 1 and in Table 2.

Table 1 Pesticide monitoring by GEUS – yearly monitoring results

Type of well	**Details**	**Amount of pesticides/metabolites (%)**	**Amount of pesticides/metabolites over MAC (%)**
All of the wells	In 2000	21.4	6.8
	In 2001	27.2	8.5
Ground water wells	In 2000	34.8	
	In 2001	31.0*	
	Period 1990-2001		
	Depth 0-20 mbgs	50	
	Depth 60-70 mbgs	10	
Domestic wells	In 2000	50	1/3 of 50

*The most frequently detected substances are BAM, atrazin and metabolites of triazins, as well as mechlorprop and dichlorprop

Table 2 Finding of Pesticide by GEUS in yearly monitoring program [36]

	GRUMO	**LOOP**	**Water works**
Finding of pesticides/metabolites (%)	36	60	26
Finding of pesticides/metabolites in concentration > 0,1 μg.L^{-1} (%)	12	20	10

2.3 Mecoprop (MCPP), 2,6-Dichlorobenzamide (BAM) – physical chemical properties, fate in environment, removal mechanisms

2.3.1 Mecoprop (MCPP)

2.3.1.1 Mecoprop - physical chemical properties

Mecoprop is a selective hormone-type pesticide, belonging to the group of chlorphenoxyalkanoic herbicides. This pesticide is applied post emergence and it is used to control the growth of surface creeping broadleaf weeds (clovers, chickweed, ivy, plantain etc.). Mecoprop is applied on the surface of plants, where it is absorbed by the leaves of the plant and afterwards transferred to the plant roots. Here the enzyme activity and the growth of plant are affected [25]

Mecoprop is a usually mixture of two mirror stereoisomers, isomer R- and isomer S-, where the isomer R- ("mecoprop-P") possesses the herbicidal activity (Figure 1). Today is possible to order only one of the isomers. The properties of mecoprop are summarized in Table 3.

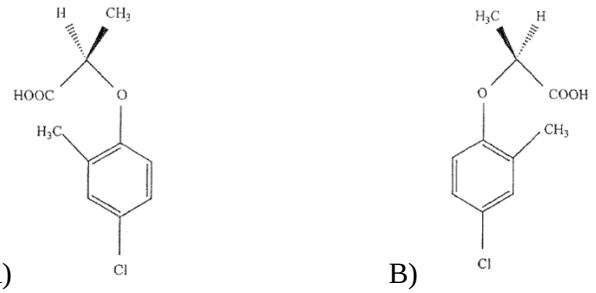


Figure 1 Structure of mecoprop: A) isomer R- , B) isomer S-

2.3.1.2 Mecoprop - toxicity

No acute toxicity for humans is reported. For tested animals, acute toxicity is low (LD_{50}=930-1210 $mg.kg^{-1}$ rats, oral exposition and LD_{50}> 4000 $mg.kg^{-1}$ rats, dermal exposition). Mecoprop is skin irritator, causes swelling and redness. Eyes also can be irritated by mecoprop which can cause cloudy visions.

Buss et al. [6] mentioned that the toxicity in aquatic ecosystems has been reported. Mecoprop is toxic for several species and fresh water bacteria. Cox [10] reported that a concentration of 17 $\mu g.L^{-1}$ kills diatoms i.e. unicellular plants which are the main food resource for aquatic animals.

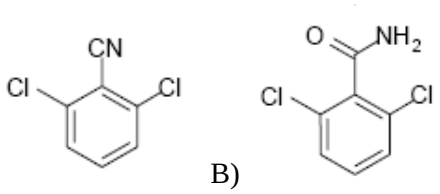
Buss et al. [6] also mentioned the toxicity to aquatic organism of the primary initial biodegradation product of mecoprop 4-chloro-2-methylphenol (4-CMP) (acute toxicity to fish LC_{50} = 2.3 – 6.6 mg/L). In most of the studies, the transformation of 4-CMP into environmental benign products is found to be a rapid complete process.

Teratogenic, mutagenic and carcinogenic effects of mecoprop are not well described by any study. Mammals are eliminating unchanged mecoprop in urine.

2.3.2 2,6-Dichlorobenzamide (BAM)

2.3.2.1 2,6-Dichlorobenzamide - physical chemical properties

BAM is the degradation product of the herbicide dichlobenil: 2, 6-dichlorobenzonitrile, formed by hydrolysis of dichlobenil [5]. The structure of both compounds is shown in the Figure 2. Dichlobenil is a worldwide used herbicide, pre-emergent herbicide, used in granules. Dichlobenil is used for killing weeds on places like railroads, roads, parking areas and other non-agricultural zones [24]. It is used just after winter on the dry soil. Dichlobenil can not be used during summer, when temperatures are higher because of its relatively high volatilization [24].



A) B)

Figure 2 Structures A) dichlobenil, B) BAM

By Clausen et al. [9] dichlobenil has a relatively high sorption distribution coefficient (K_d = 2.6-126.0 $L.kg^{-1}$) which makes the herbicide immobile in soil. In contrast BAM has a low sorption distribution coefficient (K_d = 0.10-0.93 $L.kg^{-1}$). That causes leaching of BAM through the soils to the groundwater. BAM is much more persistent and mobile than dichlobenil [14], [36]. "*The published degradation studies of dichlobenil include only topsoil" "Dichlobenil is degraded to BAM by hydrolytic reaction.* " [9]. The properties of BAM are summarized in Table 3.

2.3.2.2 2,6-Dichlorobenzamide - toxicity

BAM is slightly toxic for mammals including humans by oral route [38]. The risk of carcinogenity is lower than in the case of dichlobenil, which is classified as a member of a Group C -possible human carcinogen. BAM is not toxic for fish and aquatic organisms.

Table 3 Physical-chemical properties of mecoprop and BAM [21], [30]

CAS Number	**93-65-2**	**2008-58-4**
Chemical name (IUPAC)	2-(4-chloro-2-methyl phenoxy) propionic acid	2,6-Dichlorobenzamide
Abbreviation	MCPP	BAM
Appearance	odorless, white to light brown crystalline solid	odorless, white crystalline solid
Molecular Formula	$C_{10}H_{11}ClO_3$	$C_7H_5Cl_2NO$
Molecular Weight	214.65 $g.mol^{-1}$	190.03 $g.mol^{-1}$
Melting Point	94 - 95 °C	196 - 199 °C
Boiling Point	Decomposes before reaching boiling [37]	
Water Solubility (25°C)	734 $mg.L^{-1}$	2730 $mg.L^{-1}$
Log K_{ow} (25°C)	3.2	0.77
Vapor Pressure (20°C)	0.31 mPa	4.34 mPa
pKa (20 - 25°C)	3.78	
Henry´s Law Constant	$1.74.10^{-11}$ $atm.m^3$/ mole	$1.\ 22.10^{-9}$ $atm.m^3$/ mole
UN Classification	UN Hazard Class: 6.1	
	UN Pack Group: III	
	Symbol: Xn, N	
	R: 22-38-41-50/53 S: (2-)-13-26-37/39-60-61 Do not transport with food and feedstuffs.	

2.4 Pesticide removal process

Two processes, biodegradation and sorption, are considered in the most of the studies focused on pesticide removal processes.

2.4.1 Sorption

Sorption as a pesticide removal process is basically a combination of two actions: adsorption – the pesticide sticks to the surface of sediment particles, and absorption – diffusion of the pesticide into the pores of the sediment particles. During sorption, the contamination is transferred from one phase to another, but the total mass of contaminant in the environment is not changed.
Sorption is a process without attendance of microorganisms. The process is influenced mainly by physical and chemical properties of the pesticide and particular aquifer. Sorption can take up to 15 % of mecoprop initial concentration [9]. However sorption is not studied in this project.

2.4.2 Biodegradation

Microorganisms are able to transform many organic pollutants. Thus biotransformation plays an important role in contaminant removal processes. Those processes are carried out by different organisms such as fungi, algae, eukaryotic organisms and bacteria, where bacteria, micro-fungi and

protozoa were found in the groundwater zone [1]. The most focused group of organism studied in pesticide removal processes are bacteria.
Cheng [7] divided the removal of pesticides into the five processes: biodegradation, co-metabolism, polymerization or conjugation and accumulation. Three of those processes are considered as possible ways of biodegradation: In **mineralization** processes the pesticide is used as a substrate supporting bacterial growth. **Co-metabolism** uses transformation of pesticide by metabolic reaction, but pesticide does not support growth of bacteria. In **accumulation** the pesticide is absorbed into the microorganism. The other two processes playing role in pesticide removal are chemical processes: polymerization and conjugation or hydrolysis.

The biodegradation is the most relevant process and thus is the one discussed in most of the studies focused on MCPP removal and BAM removal. Therefore, only biodegradation is followed in this project.

Biodegradation needs the presence of microorganisms, which "*generally utilize organic contaminants as an energy source, or as an electron acceptors during degradation"* [1].
Biodegradation can cause reactions, where the final product or one of the semi finished products can be more stable or mobile in the environment or more dangerous than the mother compound. This is the case of the herbicide dichlobenil and its metabolite BAM discussed in this project. Therefore mineralization as one of the possible biodegradation pathways is suggested as the safe way of pesticide removal.

Mineralization means complete degradation, where the final products are CO_2, water and inorganic salts (Figure 3). Those products are harmless in general.

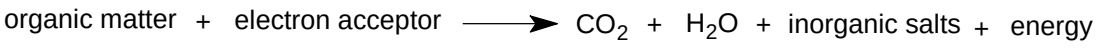


Figure 3 Short scheme of mineralization

By Nilsson et al. [26] biodegradation can take place under aerobic as well as under anaerobic conditions, although some of pesticides such as mecoprop are reported as persistent under anaerobic conditions.
The biodegradation process consists of several different phases. The first phase is the adaptation (acclimation) of the microorganisms in the environment containing contaminants. The acclimation period can occur at a significant rate (usually it is a matter of days) [20]. The acclimation period is different for each combination of the pesticide and microorganism. This period finishes with the first significant growth of microorganisms. The growth of microorganisms requires specific species of microorganisms and specific enzymes.

Different factors can influence the rate of biodegradation. Pre-exposure of the microorganisms to a contaminated environment resulted in the case of mecoprop in reduced lag time before rapid degradation [34]. Also the initial number of species able to degraded pesticide plays an important role in the time of lag phase and the rate of biodegradation.

The biodegradation of pesticides in aquifers is influenced by several abiotic factors, where the most important are pH, temperature, concentration of pesticide and redox conditions.

2.5 Fate of mecoprop and 2,6-Dichlorobenzamide in the environment

2.5.1 Fate of mecoprop in the environment

Due the water solubility and mobility of mecoprop in soils, mecoprop is able to reach low-level soils and groundwater. By Fletcher et al. [13] most of the herbicide is degraded aerobically within the topsoil soon after application. Two main mechanisms are considered as the removal routes of mecoprop from soils and ground water in the most of the studies. The first mechanism is sorption, which is possible due to presence of organic matter in sediment and partitioning between the solid and aqueous phase. Sorption is not discussed in this report. The second mechanism is degradation, in most of the cases supported by microorganisms. This project is focused on this reaction, on biodegradation.

2.5.1.1 Mecoprop biodegradation

As mentioned previously, biodegradation can take place under aerobic as well as anaerobic conditions. Nilsson et al. [26] reported mecoprop to be persistent in anaerobic conditions. By Buss et al. [6] most of the studies are focused on aerobic biodegradation of mecoprop. The half life of mecoprop in top soils is reported to be less then 25 days. Many studies show the influence of pesticide concentration, temperature, redox conditions, pre-exposure of microorganism and depth on the mecoprop biodegradation rate.

Most of the studies focused on biodegradation of mecoprop are using samples of sediments from different aquifers. The reason is the presumption, that the organism able to efficiently degrade mecoprop comes from natural aquifer and the microorganism is bonded to the aquifer sediment.

These sediments can be later on used as part of waterworks sand filter reducing the amount of mecoprop in drinking water and fill the Drinking Water Directive limits.

Buss et al [6] also stated putative chemistry of degradation process, where MCPP is used as a carbon source for growth of microorganism. The first phase of the process, the acclimation period takes place before the biodegradation process. "*This acclimation period may be the result of the time* taken for *s degradative microbial population to grow to a size that can degrade the contaminant at a clearly measurable rate, or the need for natural genetic and biochemical changes in the microorganism, or both"* [6]. Different studies show different time of the acclimation period. E.g. Heron & Christensen [40] reported a lag phase of 20 - 110 days in laboratory batch studies with sandy sediments from an unpolluted aquifer. This lag phase is followed by degradation of 50 % of mecoprop; after the second lag period the remaining mecoprop is degraded.

By Bitton & Gerba [4], cases exist, when the microbial population can preferentially degrade other substances before mecoprop (polyauxic effect).

Figure 4 shows the putative metabolic pathway of mecoprop biodegradation by Buss et al. [6] estimated in laboratory culture with soil and groundwater. Primary transformation product of mecoprop biodegradation is 4-chloro-2-methylphenol (4-CMP), which is found as toxic to aquatic organisms [6]. The further transformation of 4-CMP is done by hydroxylation at the 6-position of its ring structure and it is followed by the disconnection of the aromatic ring. In aerobic conditions is this transformation rapid. The biodegradation in anaerobic conditions is less successful and the mechanism is not fully described.

In several studies, numerous mecoprop degradators are isolated and specified. A short overview documenting the amount of degradators present in different subsurfaces and their parts found in projects where a different concentration of MCPP has been used is shown in the Table 4. By Lindberg [22] are the MCPP degradators P*roteobacteria*, where seven of the defined genes are involved in *phenoxyalcaloic* acid degradation. Those genes are not specified in this project. The aim of the project is the isolation of active bacterial culture, where the identification can be considered as a following project.

Table 4 Short overview of the isolated degradators from different subsurfaces and their parts in projects where different concentration of mecoprop is used

sediment	**No of degradators (cell/g sediment)**	**MCPP conc. Used in project**	**reference**	**Method**
Aquifer	10^6-10^7	All of bacteria	Lindberg [22]	Microscopy
Vejen aquifer	10^4 - 10^5 present only in contaminated part of aquifer (in non-polluted sediment <1cell/g sediment)	< 40 μg.L^{-1}	Lindberg [22]	MPN/method
Sjolunf landfill – narrow plume fringe	10^0 – 10^4	220 μg.L^{-1}	Tuxen et al. [35]	MPN-method
Bréville				
Topsoil	>100	1 μg.L^{-1} in the microcosm 25 μg.L^{-1} in the MPN	Lindberg [22]	Laboratory microcosm mineralization And MPN-method
Subsurface	> 14 000			
Limestone content	-			

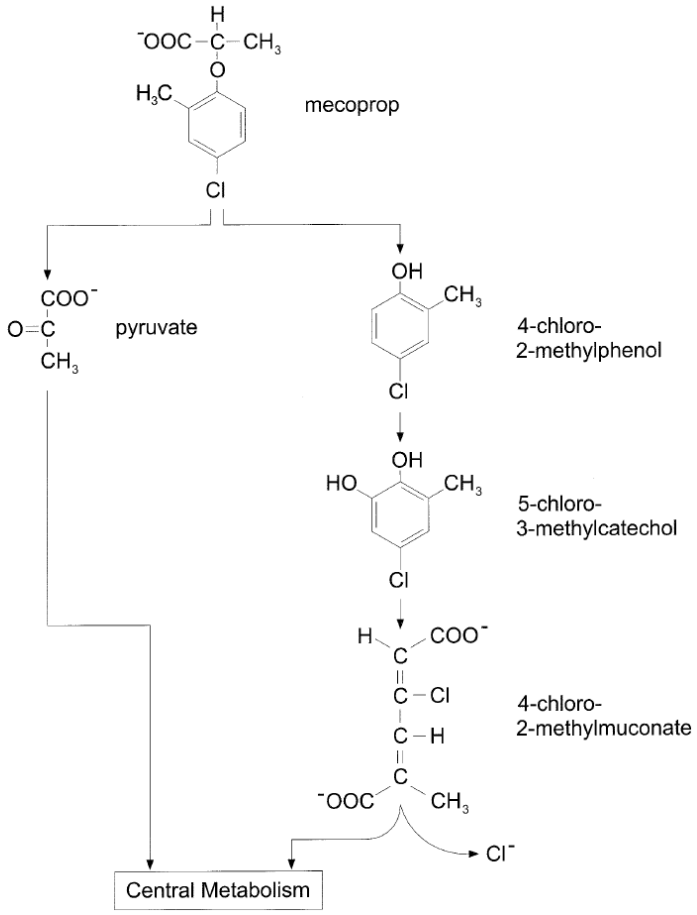


Figure 4 Buss et al. [6]:" Biodegradation pathway for mecoprop. Putative metabolic pathway based on Smith (1989), Tett et al. (1994) and Nickel et al. (1997) ".

Factors influencing rate of mecoprop biodegradation

The main factors influencing mecoprop biodegradation are: **Pesticide concentration** - most of laboratory studies are recently taken at mecoprop concentration less than 50 $\mu g.L^{-1}$. This concentration is thought to be representative of aquifer conditions. The effect of pesticide concentration has been reported many times. In some of the studies, an increasing rate of biodegradation with increasing concentration of mecoprop is reported [32]. In some other studies an opposite trend is observed, e.g. Helweg [15] describes increase in degradation rate and shorter half-life for a concentration 0.2 mg kg^{-1} then for 2 $mg.kg^{-1}$. No general attitude for a relation between mecoprop concentration and the rate of biodegradation is reported; **Temperature** - by Helweg [15] the rate of mecoprop degradation increases with the temperature (increasing factor 3.6 for each 10 °C); **Redox conditions** - the amount of nature electron acceptor such as O_2, NO_3^- etc. present in the aquifer influences a bacterial gain of energy from organic matter and thus the degradation rate. As described previously, biodegradation takes place mainly under aerobic conditions. Thus if a strong reducer is present in the system, the growth of microorganisms, thus aerobic degradation, is inhibited or stopped [1]; **Pre-exposure of microorganism to mecoprop** - the previous exposure of an aquifer to mecoprop reduces the lag time before the onset of the rapid biodegradation. Also in laboratory microcosms repeated spikes of mecoprop are degraded rapidly without any lag phase [6]; **Depth** - by Buss et al. [6] studies comparing degradation rates vertically from topsoil reported a decrease in the degradation rate with depth. The

mecoprop degradation is reported in topsoil and a slower or no degradation is reported in the unsaturated zone. E.g. Albrechtsen et al. [2] reported a decrease in degradation with depth in the unsaturated zone of a limestone at Bréville, France; **Isomer degradation** - R- and S- isomers of mecoprop have according to several studies different rates of biodegradability. However no clear attitude is reported in this problematic.

2.5.1.2 Fate of mecoprop in the environment - conclusion

Two main mechanisms are considered as possible ways of mecoprop removal processes – sorption and mineralization supported by microorganism present in the natural sediments. The biologically supported mineralization is considered as the most relevant method, as the only method by which the problem of the mecoprop pollution is solved completely. Sediments taken from different aquifers are studied, because of the presumption, that the stable active culture is bonded to the aquifer sediments. The sediment with active culture can be later on used as part of the waterworks sand filters.

2.5.2 Fate of 2,6-Dichlorobenzamide in the environment

To describe fate of metabolite BAM in the environment, it is necessary to consider also the fate of mother compound dichlobenil in the environment. The fate of dichlobenil is the factor influencing concentration and thus the fate of BAM in the environment.

2.5.2.1 Dichlobenil application - 2,6-Dichlorobenzamide pollution relationship

Due to the relatively high Henry's law constant, dichlobenil is partly evaporated from the surface and from the upper layer of topsoil [16]. This can occur in the case of application during months with higher temperature than the winter one [24]. By Clausen et al. [9] non-evaporated dichlobenil is strongly sorbed to sediment. This is possible due the relatively high value of K_d (7.4-17.4 $L.kg^{-1}$ in topsoil and 2.7-126 $L.kg^{-1}$ in clayey till sediment). However not all of the herbicide is sorbed and dichlobenil is partly degraded to its metabolite BAM. Most of the published studies are focused on the degradation in topsoil.

The degradation mechanism of dichlobenil to BAM is microbially catalyzed hydrolysis and the process is reported in topsoil and upper unsaturated zone. The degradation of dichlobenil is limited in deeper unsaturated zone and no degradation is reported in aquifers. Thus in the case of dichlobenil leaching into the groundwater source, this leaching is not increasing the level of BAM pollution in groundwater. The half-life of dichlobenil is in a range of 106-2079 days [16].

In opposite to dichlobenil, the sorption of BAM is strongly limited, due to the low K_d value, e.g. Clausen et al. [9] reported value K_d= 0.07-0.93 $L.kg^{-1}$ in topsoil and clayey till sediment. The main factor influencing sorption is change in TOC. This process is not discussed in this project.

2.5.2.2 2,6-Dichlorobenzamide biodegradation

Several studies reported the degradation of BAM as limited [8], or reported BAM as a metabolite resistant to degradation process [2]. Clausen et al. [8] reported slow but significant degradation of BAM in topsoil, upper part of unsaturated zone in sandy sediments with calculated half life 3-16 years. No degradation is reported in clayey till and in aquifers. Also degradation with pure bacteria culture was reported as unsuccessful. That limits the natural attenuation of BAM in aquifers.

Holtze [16] says that the common characteristic of most of the previous studies is that sediments used in experiments were not pre-exposed to dichlobenil or BAM.

During the last few years Holtze, Sørensen and Aamand reported several studies with rapid biodegradation of BAM in dichlobenil or BAM pre-exposed sediment [19]. In the same project, no biodegradation of BAM is reported in non-pre-exposed sediments. In pre-exposed sediments BAM degradated rapidly after a lag phase of less than 20 days in the pre-exposed soils with $T_{1/2}$ 0.5 – 4.5 days. The rapid degradation was found in pre-exposed sediment from the courtyard of a plant nursery in Hvidovre, Zeeland, Denmark. Sediment from this area is one of the sediments used in this project.

Holtze [16] reported the degradation pathway of BAM. BAM is in first step hydrolyzed to 2,6-dichlorobenzoic acid (2, 6-DCBA). This hydrolysis is observed in higher range in pre-exposed sediments, but it is possible also in non pre-exposed sediments in trace amount. This is probably due to the presence of *amidases*, which are not able to catalyze the hydrolysis without adaptation – previous pre-exposure to dichlobenil or BAM. It is necessary to mention, that 2, 6-DCBA is also a degradation product of the BAM's mothers compound dichlobenil. The study shows, that the BAM hydrolysis product 2,6-DCBA was completely mineralized to CO_2. The dechlorination of BAM to ortho-chlorobenzamide (OBAM) and its further mineralization to CO_2 was also observed during this experiment. The Figure 5 shows the complete proposed pathway of degradation of dichlobenil and thus BAM [18].

The rate of mineralization is probably affected by the initial concentration of BAM and by the type of sediment. Holtze [16] reported different rates for different initial concentrations during the degradation in clayey till topsoil, but no difference in rate for different initial concentrations during the degradation in sandy topsoil.

One of the necessary conditions of the biodegradation of BAM is the presence of microorganisms. Holtze [19] found that "*community DNA analysis of the mineralizing cultures and subsequent sequencing of dominant DNA revealed phylogenetic similarities with* Psychrobacter sp.*, or (92 %) or uncultured* γ–Proteobacteria *(97 and 98 %).*" Simonsen et al. [31] isolated for the first time BAM mineralizing bacterium, which is identified as an *Aminobacter* sp.

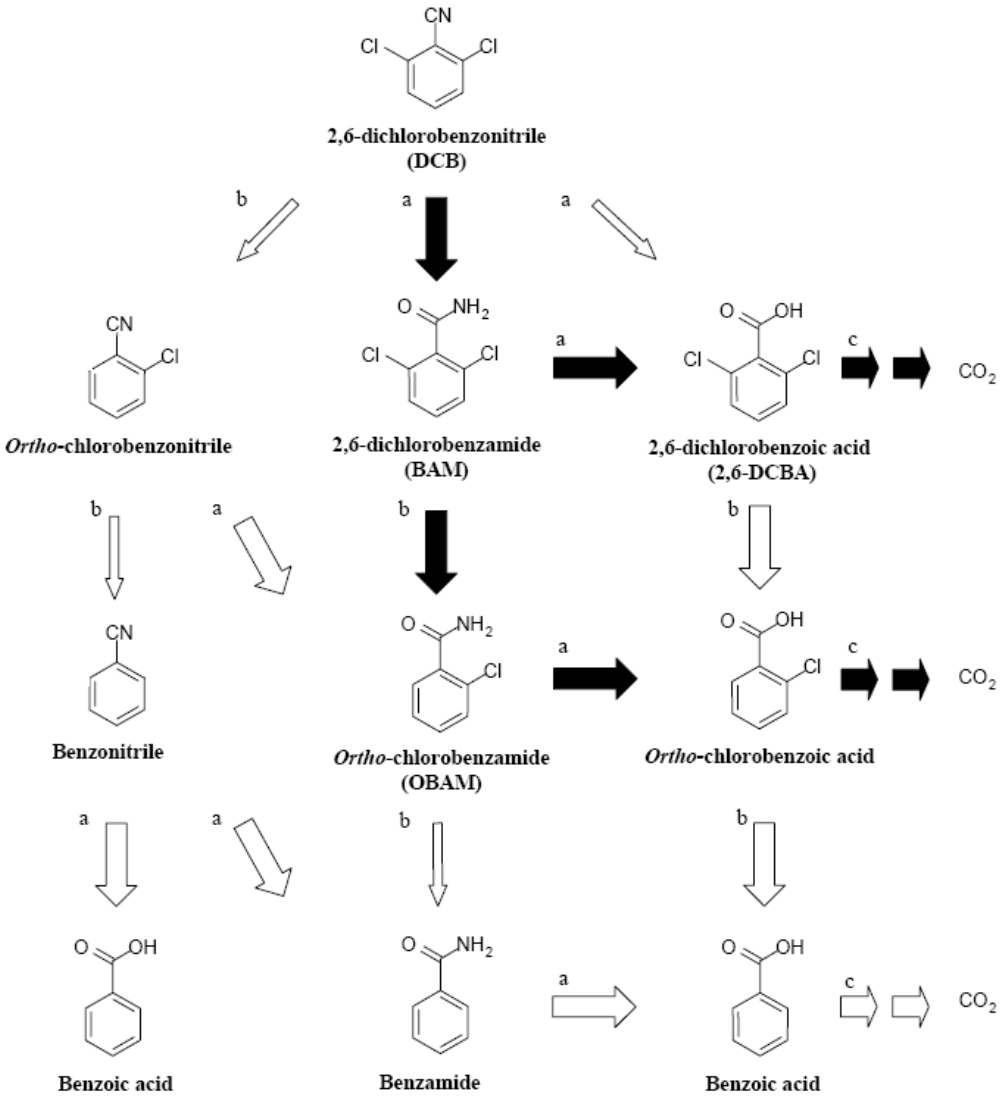


Figure 5 Proposed pathways of the dichlobenil (DCB) degradation [18] (a) Shows hydrolysis with BAM as the product, and also possible direct way to BAM degradation product 2,6-DCBA. (b) Shows dechlorination and (c) non-specified mineralization steps. Holtze: *"wide black arrows indicate pathways demonstrated the present experiment, while the white arrows indicate pathways demonstrated in other studies and white narrow arrows are based on the literature regarding analogous compounds."*

2.5.2.3 Fate of 2,6-Dichlorobenzamide in the environment - conclusion

Two main mechanisms are considered as possible ways of BAM treatment – sorption and mineralization supported by microorganism present in the natural sediments. The biologically supported mineralization is considered as the most relevant method as an only method by which the problem of the BAM pollution is solved completely. Sediments taken from different aquifers are studied, because of the presumption, that the stable active culture is bonded to the aquifer sediments. The sediment with active culture can be later on used as part of the waterworks sand filters.

3. MATERIALS & METHODS

3.1 Description of the sampling site

Sediments for this experiment were taken from mineralization incubations made by Gry Sander Janniche (2006/07), and used in the project “Preliminary attempt to isolate degrading bacteria” which is not published yet.

The reason to use sediments from the previous experiment is to confirm the theory which says that sediments containing microorganisms which are pre-exposed to a pesticide polluted environment are able to degrade this pesticide with a higher efficiency and a higher degradation rate. On the other hand there is a risk of failure of this theory, because the time from June 2007 to December 2007, when the sediments were placed in mineralization incubation bottles without any control, is giving some uncertainty. The growth of biodegradating microorganisms in this period was not checked at all.

3.1.1. Sediment for MCPP degradation – Bréville, France

The sediments used in the experiments focused on isolation of mecoprop degradators are from the French field site Bréville. Bréville is a small catchment area. The size of this area is approximately 3 km^2 and it is situated 70 km Northwest from Paris, close to Montreuil-sur-Epte, Val d´Oise, Figure 6. Bréville is an agricultural area where 50 % of the ground is used for cereals and 10 % for corn production. The pesticides atrazine, diethylatrazine, tracers of isoproturon and chlortoluron have been detected in the underground of this area.
The area is geologically a sandy aerobic aquifer overlaid by unsaturated limestone (thickness approximately 18 m) and groundwater table between 13.3 and 41.9 mbs [41]. The sediments used in this experiment are taken out from the unsaturated zone. The cores Pz17a and Pz17c where the sediments are taken from are located 4 m from each other.

Figure 6 Localization of the Bréville site

The sediment was previously used in a research. The study made by Janniche [41] shows ability to degrade mecoprop mainly in core Pz17c in depth 4.50-6.0 m, where mineralization takes place from 31 % to 0.5 %. Janniche reported no lag phase in mecoprop degradation. The rate of degradation in this sediment is reported in the first order kinetic, except for a concentration of 1 $mg.L^{-1}$, where the mineralization is faster. In the project from which sediments are transferred is reported 25 % mineralization of MCPP for sediments from core Pz17a with 1 % production of CO_2 and 32-91 % (control 7 %) mineralization for sediments from core Pz17c with CO_2 production 4-29 %(control 0 %), within 104 days . Within 178 days is reported 34 % mineralization of MCPP for sediment from core Pz17a with 3 % of CO_2 production and 44 – 96 % (control 17 %) of MCPP mineralization for sediments from core Pz17c with 5-42 % (control 0 %) production of CO_2. Appendix 1 shows a table with the results from this experiment. The characterization of the sediments, incubation mineralization and labeling in this project is shown in Table 5.

Table 5 Characterization of sediments used for isolation of mecoprop degradators

Used sediment and batch data					
Experiment labeling	**Core**	**Depth (mbs)**	**Incubation mineralization**	**Used pesticide**	**Description of texture**
1	***Pz17c***	***4.65-4.80***	3040	MCPP	Crumbly limestone (white calcarenite) and marls
2	***Pz17a***	***10.50 - 10.80***	7003	MCPP	Isn't exactly known
3	***Pz17c***	***4.50 - 4.66***	3038	MCPP	Crumbly limestone (white calcarenite) and marls
4	***Pz17c***	***4.50 - 4.65***	3035	MCPP	
5	***Pz17c***	***19.40-19.45***	7020	MCPP	Dark silty clay
6	***MCPP Control***				

3.1.1. Sediment for BAM degradation – Hvidovre, Denmark

The sediments used for this part of the project focused on the isolation of BAM biodegradators were taken from the Danish courtyard of plant nursery, Hvidovre, Zeeland, located 10 km South West from Copenhagen, Figure 7. The sediments used in this project are from two cores, taken just above and just bellow a water-bearing layer.

By Clausen et al. [9] and Holtze et al. [17], dichlobenil was used on this site frequently until 1997, before its use was forbidden in Denmark. At Hvidovre site, BAM was detected in the underlying aquifers in concentrations above the limit given by EU DWD, 0.1 $\mu g.L^{-1}$. The sediments from Hvidovre are clayey till deposits covering limestone, with significantly oxidized upper layer (2.5-6 mbs) below which the clayey till is transitioned to reduce gray material. The upper layer of soil (0-0.3 mbs) consists of broken stones and pebbles which are removed by sieving.

The same sediment was also previously used in research. Simonsen et al. [31] reported 54.8 % mineralization of BAM within 47 days in topsoil from Hvidovre and 5.6 – 35.8 % of mineralized BAM within 50 days in layer 0.7-2.0 m in sediments including Hvidovre one.

In the project from which the sediments are transferred 20 % mineralization in core HV-C and 34% mineralization in core HV-D within 35 days are reported, 20 % mineralization in sediment from HV-C and 25 % mineralization in HV-D sample are reported within 98 days and 27 % mineralization in sample from HV-C and 47 % mineralization in HV-D sample are reported within 177 days. Appendix 1 shows a table with the results from this experiment.

Figure 7 Localization of the Hvidovre site

3.2 Overview of laboratory experiments

During the last years, a description of the sediments from both sites and mineralization incubation has been done. The 1st transfer of those sediments was done by Gry Sander Janniche as a laboratory batch test. The bacterial activity was followed from February 2007 to the end of June 2007 in mecoprop degrading incubation. The BAM incubation was followed from October 2006 to the end of June 2007. In the period July - December 2007, bacteria in bottles were stored in the same conditions as those used during the following experiments.

Two main mineralization experiments, meaning two transfers of the sediment containing cultures possibly able to mineralize mecoprop, are done in the period December 2007 – May 2008. In the same period, one transfer of sediment containing cultures able to mineralize BAM is done.

The first isolation of bacterial culture mineralizing mecoprop is done at the beginning of March and after the following mineralization, the second isolation is started in the second half of April. The following mineralization takes place until the 2nd of June, when the last isolation is started. The mixture of bacteria mineralizing mecoprop is collected and frozen in the middle of June.

The first isolation of bacterial culture possibly mineralizing BAM is done at the end of April. The following mineralization takes place before the beginning of June.

In December 2007, five batches from the 1st transfer experiment containing sediments showing significant mineralization of mecoprop, and two sediments showing significant mineralization of BAM are chosen for the 2nd transfer. The goal of the 2nd transfer incubation is to determine the biodegradation potential of the chosen sediments. The biodegradation potential of the sediments used in the 2nd transfer should correspond to the biodegradation potential reported for the same sediments during the 1st transfer.

After 43 days of incubation, the 3rd transfer of biologically active suspension of sediment is done. This transfer contains only sediments able to degrade mecoprop. The bottles of the 2nd transfer are used as source bottles. Four different concentrations of mecoprop are added into the 3rd transfer incubation bottles: 0.1 $\mu g.L^{-1}$, 10 $\mu g.L^{-1}$, 50 $\mu g.L^{-1}$ and 100 $\mu g.L^{1}$. The method used for this transfer is very similar with one difference. When the batches are completed, the filtered air is used to flush out $^{14}CO_2$ the possibly present from the previous transfer. This is done to see if the chosen method is working well or if flushing can prove some positive effect on the rate of mineralization.

The 2nd transfer of sediment with culture potentially mineralizing BAM is done the same day than the 3rd transfer of mecoprop degradators. The flushing is used also in this experiment.

After 103 days of the 2nd transfer incubation, water and substrate are added. Water is added because of the low water level and the substrate is added to see the effect of repeated mecoprop pollution in the same batch with the same culture. Before this addition, 2 x 0.5 g of the sediment from the most active batch, batch No.4, is transferred into new sterilized batches. The sterilized water is added in amount to keep ratio sand: water used in all of the experiments. Finally mecoprop is added in concentration 2 $\mu g.L^{-1}$.

The active culture isolation is begun by the plating of suspension from the 2nd and the 3rd transfer batches containing sediment No.4 in different dilutions to the mecoprop polluted R2A plates. The following incubations are done by the same way as the previous mineralizations without using flushing. If the final volume of the bottle is not the same, than the ratio of water and the sand content is kept. In these bottles the sediment is not transferred. Only one colony of bacteria cultivated on the agar plate is added into the bottles as the source of biodegradating bacteria. In most of the following mineralization incubations the concentration of 2 $\mu g.L^{-1}$ is used, due to the 2nd transfer results.

The detail plan and timeline of laboratory experiments is shown in Appendix IV.

3.3 Chemicals

3.3.1 Pesticides

For biodegradation experiment, a mixture of ^{14}C-labeled and non-labeled pesticides is used. This mixture is also partly used for the isolation part of the experiment. In some of the degradator's isolation parts of experiments only non-labeled pesticides are used as described later.

3.3.1.1 Mecoprop – MCPP

The [ring – U- ^{14}C]-mecoprop with a specific activity of 23 $mCi.mMol^{-1}$ by Institute of Isotopes, Budapest, Hungary is used. The chemical purity is better than 94 %. The used solution is taken from an already prepared water stock solution with a concentration of 290 $\mu g.L^{-1}$ in water. 3.4 mL of this stock solution is filtered through a 0.2 μm hydrophilic PTFE-membrane to a sterile 10 ml batch and filled up to the guideline by sterilized demineralized water to reach a concentration close to 100 μg/L. A 50 μL sample is taken out and the amount of $DPM.mL^{-1}$ is counted by Liquid Scintilation analyzer (Packard, TRI-CARB, 1600-TR).

The non-labeled pesticide has a purity of 99.1 % (Dr. Ehrenstorfer GmbH) and a 1000 $mg.L^{-1}$ stock solution is prepared by dissolving this pesticide in a sterile 100 mL batch and filling up the to guideline by sterile demineralized water. This solution is properly mixed and filtered to a new sterile batch through 0.2 μm hydrophilic PTFE-membrane.

According to the need in specific parts of the experiment, those two stock solutions are mixed to reach the needed concentration and a sufficient value of $DPM.mL^{-1}$.

3.3.1.2 2, 4-dichlorobenzamide – BAM

The [ring – U- ^{14}C]- BAM with a specific activity of 24.2 mCi.mMol delivered by the Institute of Isotopes, Budapest, Hungary is used as the ^{14}C-labeled BAM. 1 mL of the stock solution in methanol with a concentration of 35 $\mu g.L^{-1}$ is taken and transferred through a 0.2 μm hydrophilic PTFE-membrane to a 10 ml sterile batch and the solution is flushed with a gas mixture of N_2/CO_2 to flush out the methanol. This 1 ml of solution is transferred to the stock solution of non-labeled pesticide prepared in a second sterile batch.

The non-labeled pesticide is weighted and transferred to the 10 mL sterile batch. The demineralized sterile water is added to reach a volume of 6ml and the solution is mixed. After the dissolving of all the pesticide, the solution is filtered through a 0.2 μm hydrophilic PTFE-membrane to a new sterile

batch, where 1 mL of ^{14}C-labeled pesticide is also added. This solution has a sufficient value of DPM/ml and it is used as a stock solution in the part of experiment focused on BAM degradation.

The 2nd transfer of sediment with culture potentially mineralizing BAM is done the same day as the 3rd transfer of mecoprop degradators. The flushing is used also in this experiment.

3.3.2 Other used chemicals

During the experiment, some other non-labeled chemicals are used.

In the biodegradation part of the project, three chemicals are used. To strip out the CO_2 present in the subsamples taken out from the incubation batches to count change in ^{14}C-pesticide level and $^{14}CO_2$ is used 37 % HCl p.a. (Riedel-de Haën). The $^{14}CO_2$ stripped out is in the same part of the experiment caught by NaOH, purity > 98 % (Fluka Chemika). The measure change in the ^{14}C-compounds ratio is added to the both of the phase scintillation liqueur Optilphase Lhifase (Valac).

In the isolation part of experiment, R2A (Fluka Chemika), Glycerin (Urtegaardens) and NaCl, purity > 99,5 % (Fluka Chemika) are used.

3.4 Laboratory experiments

3.4.1 Biodegradation – set up

The mineralization in the 2nd transfer is followed as a laboratory batch experiment in pre-sterilized 18 mL serum bottles and the technique is kept from the Janniche´s experiment [41]. Clean 118 mL serum bottles are filled with 30 g ww (wet weight) of sand (Dansand No.0, ρ = 1.5 g.cm^3), 60 mL of water (tap water delivered by the public network to DTU). The bottles are covered by 1 cm of rubber stick and aluminum crimp cap. All of the bottles are sterilized (autoclaving, 20 minutes in 125 ℃) to be sure that all the possible present microorganisms are killed. Once the bottles are cooled down the transfer of the suspension from the 1st transfer bottles containing active bacterial culture can take place. The bottles from the 1st experiment are gently shaken. Then the 10 mL of suspension are taken out using a sterile set of syringe and needle and transferred into the 2nd transfer bottles. Finally, 1 mL of pesticide solution containing a mixture of labeled and non-labeled pesticide with a concentration able to reach the final chosen concentration is added. The concentration which is used in the 2nd transfer bottles is 2 μg.L^{-1}. An inactive control batch is done by almost the same way, where the only difference is that the transfer of biologically active suspension is not added. Bottles are gently shaken and the subsample to estimate ^{14}C level in pesticide as well as in $^{14}CO_2$ fraction at point zero is taken out. During the incubation period, batches are stored in the dark room at a temperature of 10 ± 0.1 °C (basement storage, building 115, DTU).

3.4.2 ^{14}C analysis - method

For the following subsamplings, the batches are moved into the microbiological lab for the time necessary for subsampling. The batches are gently shaken 30 minutes before subsamples are taken. A 2 mL of suspension for measuring the ^{14}C-activity is transferred through a hydrophilic 0.2 μm PTFE-filter into a 20 mL polyethylene vial. A 6 mL vial containing 1 mL of 0,5 M NaOH is put into this 20 mL vial. Then 0.1 ml of 37 % HCl is added to the 20 mL vial in order to strip out the present CO_2 which is caught in the NaOH (in the 6 mL vial). By this way, it is possible to distinguish the ^{14}C present in pesticide form and the ^{14}C present in the mineralization product CO_2. The set of vials is stored for 48 hours in the dark. After 48 hours, the inner vial is removed and a scintillation cocktail (Optiphase "HiSafe" 3, Wallac) is added into both of polyethylene vials. All of the vials are mixed and the ^{14}C-activity is measured by Liquid scintillation analyzer (TRI-CARB, 1600-TR, Packard). The BAM mineralization batches are done by the same way.

3.4.3 Optimization of the methods

The influence of sample filtration through 2 μm hydrophilic PTFE-filter during the transfer of the subsample to a vial for ^{14}C counting on the final measured ^{14}C values is studied. The filtration is done before acidifying, during the transport of the subsample from the mother's bottle to a 20 mL testing polyethylene vial. This experiment is done at the end of the most active phase (according to the results of the 2nd transfer), during subsampling of the day 39 of the 3rd transfer. Only 6 bottles are used. The goal is to verify the method used. One of the tested samples is from a batch of the 2nd transfer – sediment 5. The other samples are taken from the 3rd transfer series of batch containing the initial mecoprop concentration 100 $\mu g.L^{-1}$.
In all cases, higher concentrations of ^{14}C-carbon are found in non-filtrated samples (see Appendix II). The difference is within range of 0–19 % in the acidified vial, in vial containing NaOH is the difference in range of 0–4 %. It is obvious, that the difference is more significant in the liquid acidified phase containing ^{14}C-mecoprop than in based phase containing $^{14}CO_2$ stripped out by acidification and caught in NaOH. Reported values are supporting the theory that bacteria are fed by pesticide. Thus mecoprop is retained on bacteria cells during the biodegradation process. This fact increases the amount of ^{14}C-carbon present in non-filtered samples. On the other hand, the reported increase of ^{14}C-carbon could be given by another process run in the biomass and could be part of the organic waste or other by-products which are produced by the bacteria during the time of incubation.

The difference of the ^{14}C activities in the 3 layers shown in the mineralization batches: In the most of the mineralization batches are shown 3 optically different layers. Therefore the sample is taken out from each of the shown layer to observe if there is some difference in ^{14}C activities in those 3 layers. This is studied in the samples of the 3rd transfer. The reason why no samples are taken from 2nd transfer which is significantly more active is the level of liquid in the batches at the time, when the layers are significant. For ^{14}C-counting batches are usually shaken 30 minutes before sub-sampling and then the suspension of small particles and the liquid part are taken out for ^{14}C-counting. In the batches which are staying some time and which are not shaken, it is possible to see really clearly three different layers in the batches – liquid layer, layer with some organic matter, which is easy to mix with liquid layer and finally sediment layer. For this experiment batches are taken from the series containing an initial concentration of mecoprop of 100 $\mu g.L^{-1}$ from the 3rd transfer.

The influence of flushing is studied by comparison of the activities in non-flushed and flushed batches.
Both of the experiment where the flushing is used show low, if any, biodegradation activity of present microorganism. The most active sediment of the 2nd and the 3rd "mecoprop" transfers, sediment No. 4, is used in the isolation part of experiment. Some of colonies growth on R2A plates polluted by mecoprop shows high mecoprop mineralization potential in following biodegradation experiment which is part of the isolation experiment. In the case of BAM transfers any biodegradation activity is not reported, thus the influence of flushing is not reported.

The flushing by filtrated air is considered as the factor negatively influencing the biodegradation and thus the mineralization rate in the batch where it is done. The flushing is probably inhibiting bacterial activity without killing the cultures. Thus it is possible to use their mineralization potential in the following non-flushed experiments.

The flushing is not used again in following experiments.

3.4.5 Isolation of the active bacterial culture

The subsample, from which the needed dilution is done to be plated on the surface of the mecoprop polluted R2A plates are taken from all of the 2nd and 3rd transfer batches containing sediment No.4. This is done with knowledge of the mineralization activities observed during the 1st and the 2nd transfer incubation. The sediments from the 3rd transfer batches are also covered in this experiment, even though less rapid mineralization is reported. The reason is hope, that the different concentrations of mecoprop or flushing at the beginning of the experiment affected the rate of mineralization.

The first part is a plate experiment, i.e. the cultivation of the bacteria present in the suspensions taken out from the 2nd and 3rd transfer bottles. As a medium for plates R2A is used. The main source of carbon supporting growth of bacteria is the pesticide. Thus to minimize the influence of R2A on the growth, the pesticide is added in a relatively high concentration. The chosen concentration is 50 mg/L. Two ways of MCPP addition are followed. In the first case, a 40 μL stock solution of MCPP with a concentration of 50 $mg.L^{-1}$ is plated to dry R2A plates. In the second case, 20 mL of stronger stock solution MCPP is added to liquid autoclaved R2A during cooling to reach a final concentration of 50 $mg.L^{-1}$ in R2A. The way used for the isolation of BAM biodegradating bacteria is the plating of 40 μL of the BAM stock solution.

Tested inoculums are transferred from the batches with a sterile syringe to a sterile eppendorf tube. 100 μL of the chosen suspension are plated on the surface of dry R2A plate in different dilutions. During all the isolations, dilutions 1x, 10x, 100x, and 1000x are used. When the inoculum is dry, the set is moved into the dark room with a temperature of 10±0.1 °C. After one week of incubation, the colonies are documented.

The second part is the new mineralization set-up, where the source of microorganism is colony isolated from the R2A plates. From the plates where it is possible to isolate one colony, this colony is taken and transferred into the new mineralization batch. 24 colonies are finally isolated and transferred. The complete provenance of transferred colonies is shown in Appendix III.
The method of preparation is kept as is described in the biodegradation experiment chapter, with the same water-to-sand ratio. The real values are 10 g of sand (Dansand No.0) and 20 mL of sterilized tap

water. Instead of the 10 mL suspension from the previous transfer, the source of microorganism is now only this one grown colony.
New mineralization incubation is started. In the case of mecoprop after significant mineralization, the isolation process is repeated with the most active bacterial culture. The 2nd series of R2A plates are done the 35th day of incubation of batches contain colonies from the 1st set of the R2A plates. After one week of incubation the colonies grown on the plates are documented. 7 colonies are chosen from the second R2A plates and transferred to the new mineralization batches. The batches are done by the same way as in the previous case. The provenance of transferred colonies is shown in Appendix III.

When the 2nd R2A plate isolation are plated, 2 mL of suspension from three of the most active batches of the 1st mineralization containing colonies from the 1st R2A series are transferred to the new batches with the same initial conditions as in the original batch.

Finally the pesticide is added in concentration 2 $\mu g.L^{-1}$ into the five active batches of the 1st incubation mineralization of batches with R2A grown colonies. This experiment is done to prove the effect of using the microorganisms pre-exposed to pesticide, meaning the repeated pesticide pollution.

Finally the most active cultures are chosen and kept for following projects. The cultures are kept by two different ways:

- Cultures are deeply frozen(-80 °C) – 250 mL of the suspension is transferred by sterile syringe into 1mL cryotubes which contain 750 mL of sterile 40 % glycerol.
- Cultures are kept in the mineralization incubation batches in the dark room with temperature 8-10 °C

In the BAM experiment the suspension from all of the batches is taken out for the isolation of bacteria. After one week of incubation, a growth of white bacteria on all the plates is shown. Thus the second mineralization batches are set-up and colonies from R2A plates are added as the source of biodegradation activity.

3.5 Data analysis

The ^{14}C-mecoprop concentration estimated during the experiments is presented as % ratio of the initial ^{14}C-mecoprop concentration. The $^{14}CO_2$ concentration is also presented as the % ratio of the initial ^{14}C-mecoprop concentration.

3.5.1 Mecoprop degradation, $^{14}CO_2$ production

The initial ^{14}C-mecoprop concentration is considered in all of the mineralizations carried out in this project as equal to 100 %.

Then the changes in the ^{14}C concentration in the batch systems are reported by two ways:

a) As the changes in the ^{14}C-pesticide level, i.e. the radioactivity of the pesticide phase. When the ^{14}C counting is done, all of the ^{14}C-carbon which is not stripped out from the subsample as $^{14}CO_2$ is considered as radioactivity belonging to ^{14}C-pesticide.
b) As the changes in $^{14}CO_2$ concentration, meaning all the $^{14}CO_2$ which is stripped out from the subsample and caught by NaOH present in the inner vial during ^{14}C counting.

The amount of evolved $^{14}CO_2$ calculated as a percentage of the radioactivity of the total amount of the initially added radioactivity is a function of the incubation time. The evolved $^{14}CO_2$ corresponds to the amount of mineralized pesticide.

In the ideal case, both ways lead to the same result. However, this is usually not valid for a real conditions laboratory experiment; the $^{14}CO_2$ production is then considered as the main parameter, used for further calculations.

3.5.2 Degradation rate, pesticide half-time

The empirical degradation rate is derived from the acquired experimental data. The degradation rates are calculated between two points: the beginning of the experiment, and the beginning of the period where the degradation stabilizes, meaning that no more $^{14}CO_2$ is produced.

The degradation rate is a mathematical function describing the change of concentration of the compound of interest in time. The degradation rate is defined by the rate law (equation (1)).
A first order rate law is expected for the rapid biodegradation. The first order rate law is expressed mathematically by the differential equation:

$$\frac{dc}{dt} = -k \cdot c \qquad (1)$$

where k [day^{-1}] is the first order kinetic constant.
If the equation is integrated from $c = c_o$ (at $t = 0$) to $c = c_t$ *(at t)* the mathematical observation of the biodegradation curve is yielded (2).

$$c_t = c_0 \cdot e^{-kt} \qquad (2)$$

By presenting the equation in a logarithmic form (3), it is easy to find the definition of the half-life of the compound i.e. the time at which the concentration of the compound equals half of the initial concentration.

$$\ln \frac{C_{Ao}}{C_A} = k \cdot t \qquad (3)$$

The half-life (d^{-1}) is independent of the concentration and it is defined (4):

$$\tau_{1/2} = \frac{\ln 2}{k} = \frac{0{,}693}{k} \qquad (4)$$

The k value is estimated graphically from the linear regression of the curve describing progress of the log of the MCPP concentration (100%-$^{14}CO_2$ production (100%)) in time of incubation. If the lag phase is present before the rapid biodegradation, then this lag phase is covered also in the k value estimation. The first point of the curve of the MCPP progress which is not covered in the k value estimation is the second point in the equilibrium part – where no more mineralization takes place.

However the $T_{1/2}$ is calculated to illustrate the ability of cultures presented in Bréville and Hvidovre sediment to degrade applied pesticides. The goal of the project is not the comparison of the degradation rate but the isolation of cultures able to degrade the chosen pesticide.

4. RESULTS AND DISCUSSION

4.1. Mecoprop mineralization in the 2nd and the 3rd transfer batches

4.1.1 Mecoprop mineralization in the 2nd transfer batches

4.1.1.1 Overview

The ^{14}C activity of the 2nd transfer after 29 days of incubation is compared with the ^{14}C activity of the 1st transfer after 32 days of incubation. The table with results from the 1st transfer measurements is in the Appendix I, the table with results from the 2nd transfer incubation is in Appendix V

The amount of ^{14}C carbon present in the acidified liquid phase, representing ^{14}C mecoprop, and the amount of ^{14}C carbon present in the basic phase, representing the $^{14}CO_2$ stripped out and caught shows that all of the sediments prove some mineralization activity within 30 days incubation. This mineralization activity is for the 2nd transfer sediments in range of 7–20 % of mineralized ^{14}C-mecoprop and 2–6 % of produced and caught $^{14}CO_2$.

The mineralization activities of the 2nd transfer have the same progression as the mineralization activities of the 1st transfer batches with lower $^{14}CO_2$ production. The most active sediment in both transfers is the sediment No. 4 and the sediment with the smallest mineralization activity is the sediment No.2.

The values of the ^{14}C-distribution after 39 days of incubation show a decrease in the ^{14}C-mecoprop concentration of 6 – 21 % in four of the batches with production of $^{14}CO_2$ of 2 - 3 %. In the last batch – batch with sediment No.4, a decrease of 45 % of the ^{14}C-mecoprop concentration and a production of $^{14}CO_2$ of 28 % is shown. According to those results, all of the sediments are covered in the 3rd transfer. The 3rd transfer is begun on the 43rd day of the 2nd transfer incubation.

The complete progress of the biodegradation activity of the bacteria present in the sediments used in the batches of the 2nd transfer is shown in the Figure 8 and in the Appendix V.

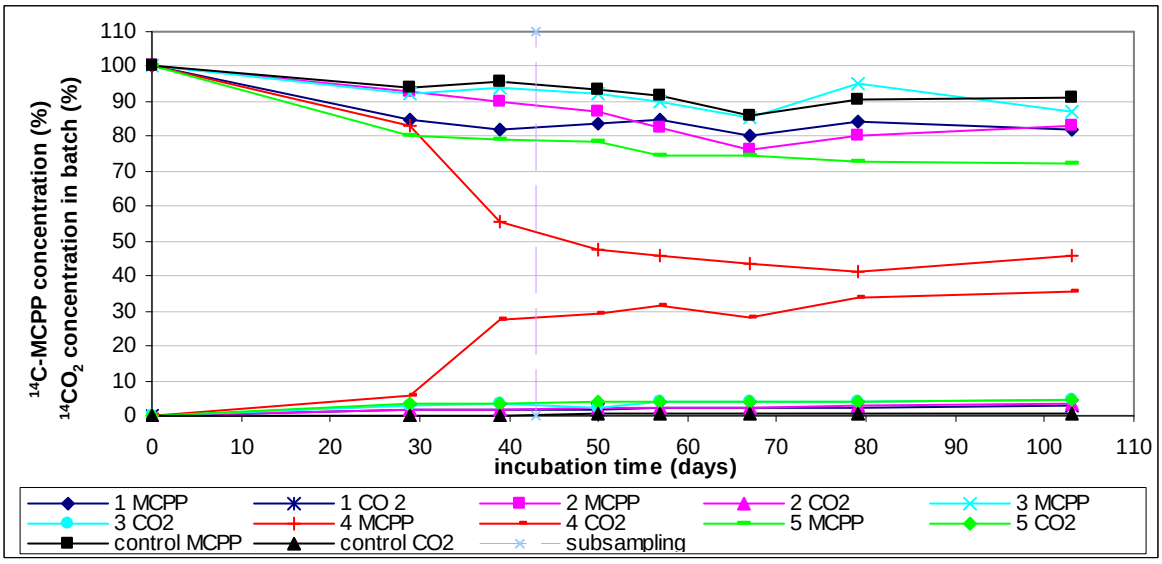


Figure 8 Complete progress of the mineralization activity in the 2nd transfer batches

4.1.1.2 Details

Two groups of sediments divided by the decrease in ^{14}C-mecoprop concentration are shown during 29 days of incubation of the 2nd transfer. The "faster" group contains sediments, 4 and 5. For the slower group of sediments, sediments 1, 2 and 3, no significant activity is shown during the first 29 days of incubation (Figure 8). In those three sediments no significant mineralization activity is shown within 103 days of incubation.

The most active group of sediments, sediments No. 4 and 5, consumes during the first 29 days 15-23 % of ^{14}C – mecoprop initial concentration with $^{14}CO_2$ production of 2-6 %, Figure 8. Consequently, the progress of the mineralization activity of the sediment No. 5 is slower. Only small, if any, changes in ^{14}C–mecoprop concentration and the $^{14}CO_2$ production are reported during the next 74 days. The mineralization activity of the sediment No. 4 shows an increase of the rate of mecoprop degradation after the first 28 days of incubation. The fastest mineralization is shown between the days 29 and 50. During this period, the ^{14}C-mecoprop concentration in batch decreases from 83 to 48 % and the $^{14}CO_2$ production increases from 6 to 30 %. The half life time of mecoprop calculated in this sediment is 231 days. During the incubation period between days 50 and 103 no significant change in the ^{14}C-mecoprop concentration and $^{14}CO_2$ production is shown. In this time, the system probably reaches the equilibrium. This assumption is supported by the graph of the $^{14}CO_2$ production (Figure 9). It is expected for the next experiments, that the most active phase is taking place between days 28 and 38 of the incubation.

In the Figure 9 comparisons of the progress ^{14}C-mecoprop concentration and $^{14}CO_2$ production for sediment No.4 in the 1st and the 2nd transfer batches is shown.

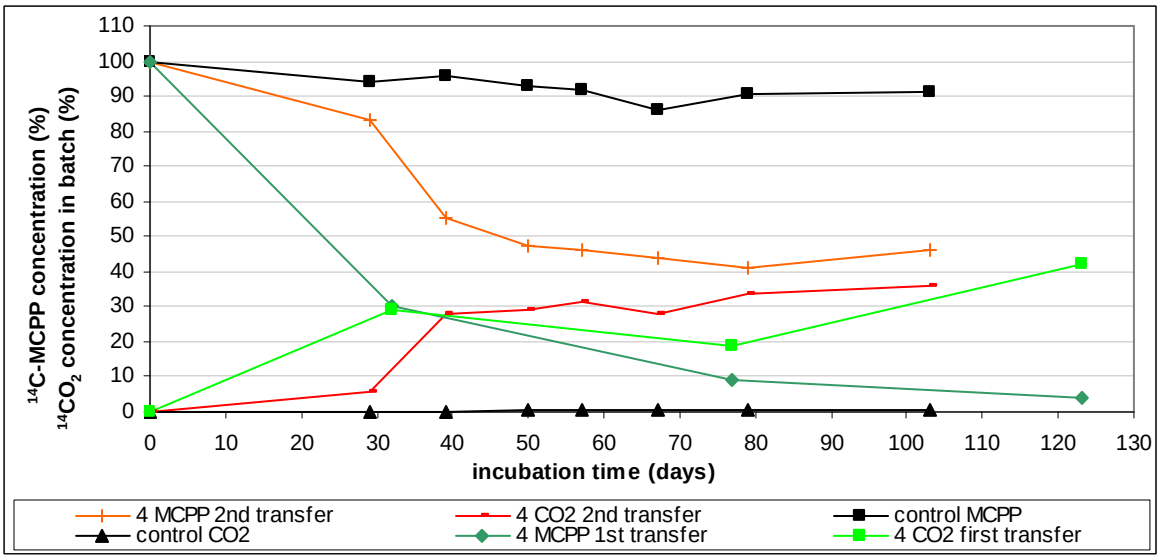


Figure 9 Detail progresses of the ^{14}C-MCPP and concentration and $^{14}CO_2$ production in the 2nd transfer batches

4.1.1.3 Biotic vs. Abiotic (control) batches

The change in ^{14}C- mecoprop is reported in both cases. In the case of abiotic control, this change is really slow and the maximum change is 9 % within 103 days of incubation. The insignificant $^{14}CO_2$ production is reported in abiotic control. The maximum $^{14}CO_2$ production is 1 %. In biotic samples, the smallest decrease in ^{14}C-mecoprop concentration is reported (13 % with a $^{14}CO_2$ production of 5%). The smallest $^{14}CO_2$ production is 3 % in the case of an 18 % decrease of ^{14}C-mecoprop concentration within 103 days. Thus the decrease of ^{14}C-mecoprop concentration in abiotic control can not be the result of non-biological mineralization.

The difference in biotic and abiotic batches is not really significant in the case of the 3rd transfer where no significant biodegradation activity is reported.

4.1.2 Mecoprop mineralization in the 3rd transfer batches

The first subsampling is done the 14th day of the incubation. No significant change in ^{14}C mecoprop concentration or $^{14}CO_2$ production is shown. After 36 days of incubation just one of the sediments, in all the three concentrations, shows more than 10 % decrease in the level of ^{14}C-mecoprop and more than 3 % increase in the concentration of $^{14}CO_2$ produced compared to its initial concentration. This active sediment is the No.4. No significant change in ^{14}C-mecoprop concentration and $^{14}CO_2$ production is reported between days 36 and 50.

Some uncertainty is given to the biodegradation activity of sediments in the 3rd transfer by the fact that one of the most significant decreases in ^{14}C-mecoprop concentration shows control batches in concentration 0,1 μg.L^{-1} and 100 μg.L^{-1} (day 36 of incubation). The degradation rates are not counted for the 3rd transfer. Complete data are shown in the Appendix VI.

In the 3rd transfer, the biggest decrease reported in ^{14}C-mecoprop concentration of 18 % is shown in the batch containing sediment No. 4 and mecoprop concentration 10 μg.L$^{-1.}$ The biggest production of $^{14}CO_2$ (24%) is reported in the batch containing the sediment No.4 and mecoprop concentration 0.1 μg.L^{-1}.

Thus the sediment No. 4 for which the biggest mineralization activity is reported in all of the three transfers is considered in comparison of the ^{14}C-concentration progress and it is the only sediment which is used in the part of the experiment focused on the isolation of active mineralizing culture.

4.1.3 Comparison of the progresses of the mineralization activities during the 1st, 2nd and in the 3rd transfer incubation- batches with diluted original sediment

The Figure 10 shows the progress of ^{14}C activities of the sediment No. 4 in the 1st, the 2nd and the 3rd transfer. The real concentration is plotted in the log scale to show the real progress. In the Appendixes I, V and VI the progress in percentage of changed ^{14}C is shown.

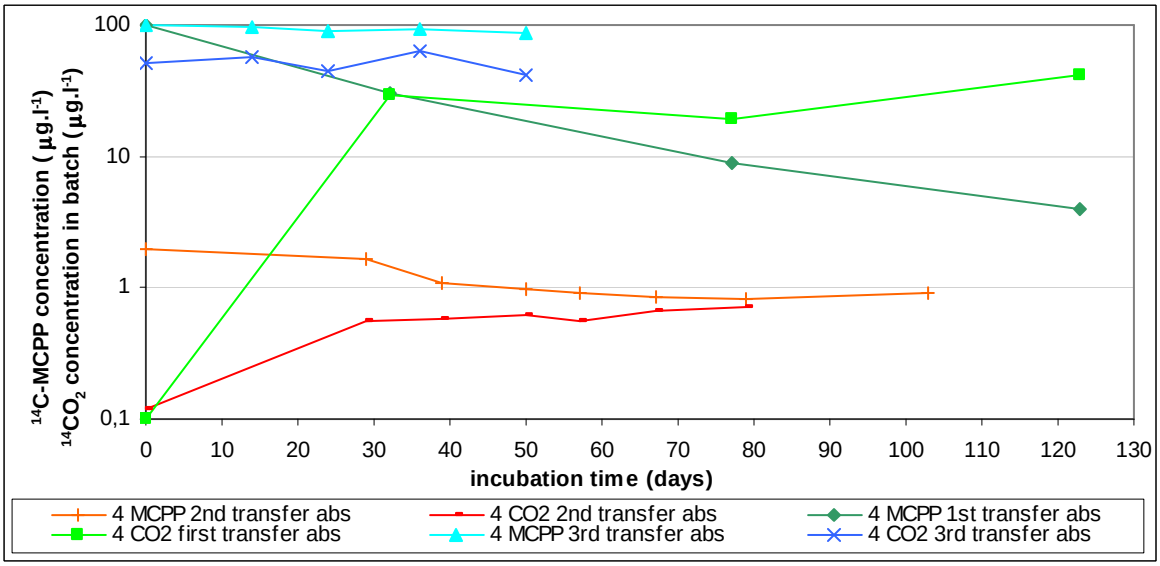


Figure 10 The progress of ^{14}C-mecoprop concentration and $^{14}CO_2$ production for the sediment No.4 during the 1st transfer incubation (initial MCPP concentration of 100 µg.L^{-1}), the 2nd transfer incubation (initial MCPP concentration of 2 µg.L^{-1}) and during the incubation of the 3rd transfer (initial concentration of MCPP of 100 µg.L^{-1}) The real concentration is plotted in the log scale to show the progress in real concentration.

In the 1st transfer batch, rapid biodegradation is shown, whereas a lag phase is shown in the 2nd transfer batch before the rapid biodegradation starts and in the 3rd transfer only small mineralization activities are reported, if any. Only 10 % of the initial amount of the ^{14}C-mecoprop is reported as remaining in the 1st transfer batch within 123 days of incubation. In the batch of the 2nd transfer, a concentration of 40-50 % of the initial ^{14}C-mecoprop concentration is reported within 103 days of incubation and finally in the 3rd transfer batch, a decrease in the concentration of ^{14}C-mecoprop of less than 20 % is reported within 50 days of incubation.

The comparison of $^{14}CO_2$ production shows that almost the same amount of $^{14}CO_2$ is produced in the 1st and the 2nd transfer batch. If we consider that the mineralized amount of mecoprop is equal to the amount of $^{14}CO_2$ produced, then the final concentration reached by the mineralization activity present in those two batches is almost the same. The culture present in the 1st transfer batches is mineralizing faster with $T_{1/2}$ of mecoprop 150 days and without any lag phase, than the culture in the second transfer, where the $T_{1/2}$ is 231 days. In the third transfer only production below 3 % of $^{14}CO_2$ is reported.
Only a part (10 mL) of the sediment suspension from the first transfer batches is used for second transfer batches, where the final volume of the batches is the same and the same process is repeated

for the 3rd transfer. That means that the culture and its natural environment are diluted 9 times with each new transfer. This can explain that the culture in the 2nd transfer batch needs more time (lag phase) to grow to the amount of bacteria able to start rapid biodegradation. The dilution connected with flushing used for preparation of the 3rd sediment is probably the reason why no biodegradation activity is reported.

It is necessary to consider that data of the first and the second transfer are not completely comparable due following reasons:

a) The concentration of substrate is changed to reach the goal of the thesis, i.e. to find active cultures degrading mecoprop in water sources. The concentration possibly present in the drinking water sources is lower than the one used in previous experiments (100 µg.L^{-1}). Thus the concentration for the 2nd transfer is chosen to be 2 µg. L^{-1}.

b) The biodegradation, thus mineralization activity of the sediments, has not been controlled during the previous six months.

4.2 Effects possibly influencing the rate of mineralization studied in the 2nd and the 3rd transfer batches

4.2.1 Effect of different mecoprop initial concentration - comparison of the progresses of the mineralization activities during the 3rd transfer incubation

The progresses of the ^{14}C-concentration for the sediment No.4 in batches with different initial concentration of mecoprop (0.1 µg.L^{-1}, 10 µg.L^{-1} and 100 µg.L^{-1}) is shown in the Figure 11.

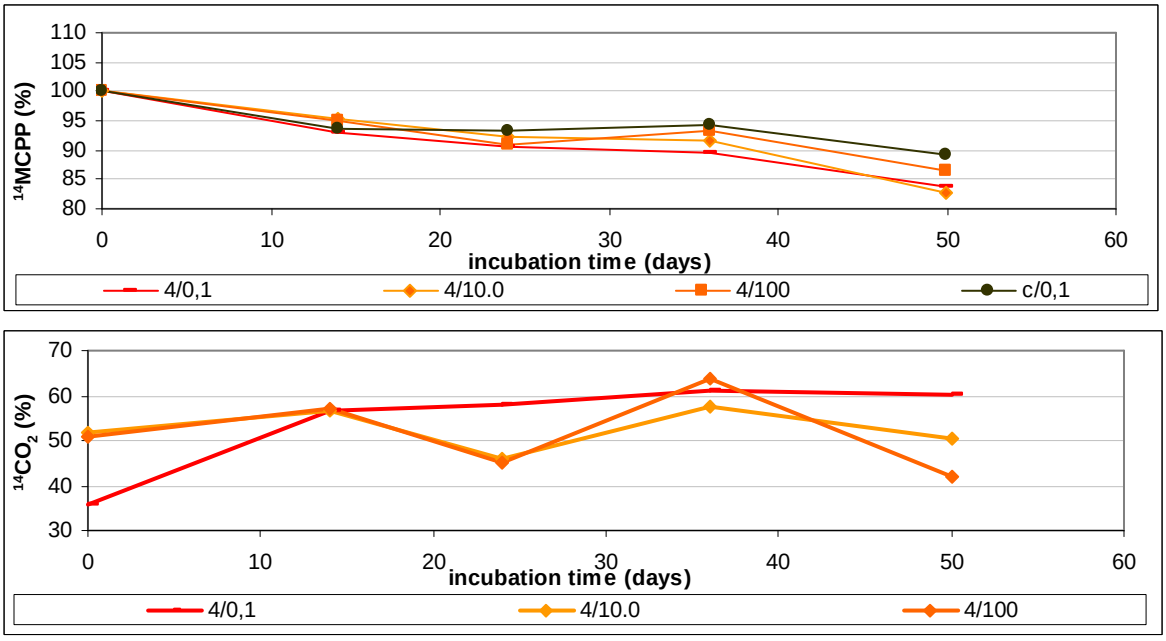


Figure 11 3rd transfer of MCPP - initial concentration 0.1, 10 and 100 mg.L^{-1} - sediment No.4

No significant difference, in between the progresses of the ^{14}C-concentreation in batches is shown. In all of the batches only small mineralization activity is shown and thus is not possible to document significant differentiation for different initial concentration of mecoprop in the batches.

4.2.2 Effect of using microorganisms pre-exposed to pesticide, repeated pesticide pollution

Comparing mineralization rates in the original 2nd transfer batches and in the same batches with repeated mecoprop pollution, no significant difference in the biodegradation progress for sediments 1, 2, 3 and 5 is shown. This could be due to already small biodegradation potential of these sediments. Thus only the most active sediment (No. 4) is considered in the results. In the original batches, a lag phase of approximately 20-28 days with mecoprop half time 231 days was observed, whereas in the same batch after repeated mecoprop pollution no lag phase is reported and the half time of mecoprop decreases to 87 days.

The progress of mineralization of the original 2nd transfer and of the 2nd transfer sediment after the incubation time and following addition of water and substrate is shown in Figure 12. Detail data of the progresses are shown in the Appendix VI.

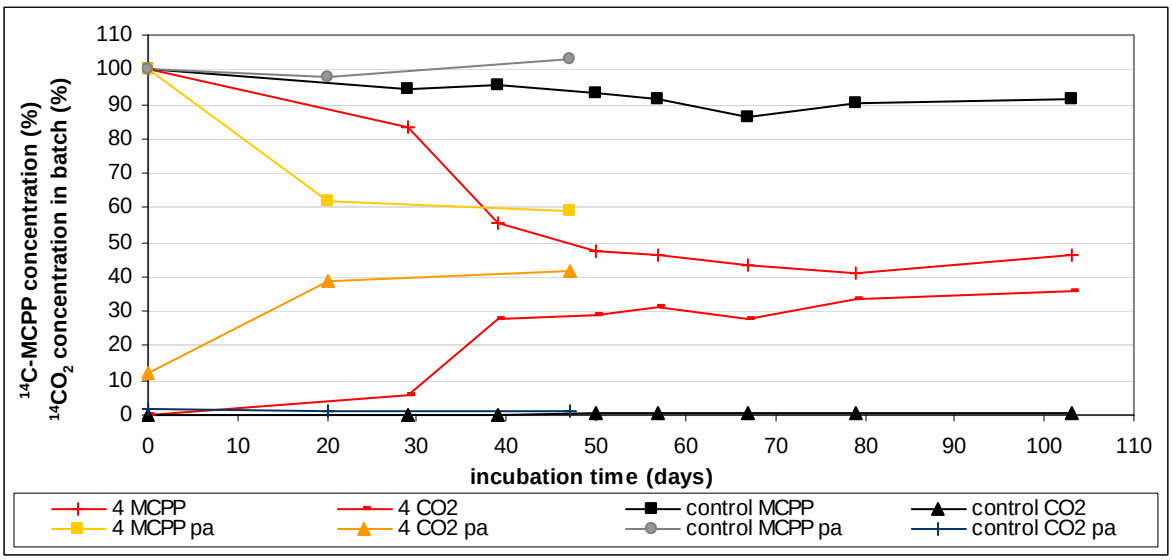


Figure 12 Effect of repeated pesticide pollution: progress of ^{14}C-mecorpop concentration and the $^{14}CO_2$ production in the original 2nd transfer batch No.4 and in the same batch, when the mecoprop pollution is repeated in the non-active phase

4.2.3 Transfer of sediment without liquid present in the mineralization incubation bottle

A significant decrease of the lag phase, compared to the original 2nd transfer batches, is shown after 47 days of incubation. The lag phase in the original 2nd transfer batches is reported approximately to 20-28 days whereas in the batches with transferred sediment, a shorter lag phase about approximately 18-23 days is reported. The lag phase in the both mineralization incubation is shorter than in the 2nd transfer case, but longer than in repeated contamination of the sediment by mecoprop. The mineralization is not as rapid as in both other cases. The final concentration of ^{14}C-mecoprop reached within approximately 50 days of incubation seems to be higher in the case of transferred sediment. The $^{14}CO_2$ production is almost the same as in the 2nd transfer case. The half life time for mecoprop is not calculated and considered in this experiment due the significant difference in ^{14}C-mecoprop concentration in batches.

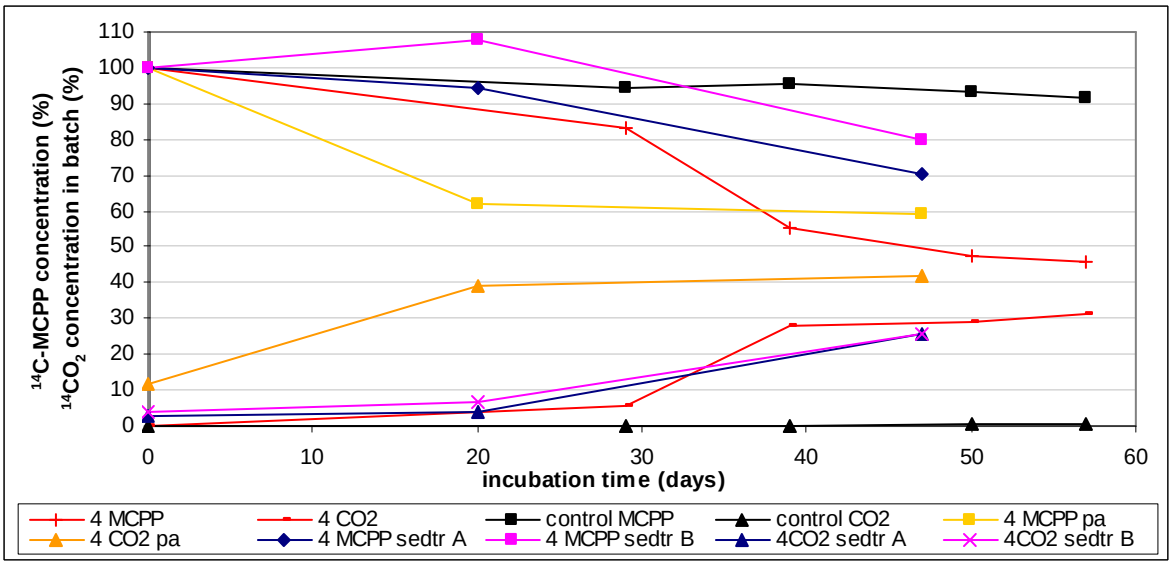


Figure 13 The progress in the ^{14}C-mecoprop concentration and $^{14}CO_2$ production in the original 2nd transfer sediment No. 4 and in the batches, where 0,5 g of this sediment where transferred and in the batches with repeated pesticide pollution

4.2.4 3 layers in biodegradation batches

The significant three layer separation in unshaken batches – liquid layer, layer with some organic matter easily mixed with a liquid layer, and finally sediment layer, is shown in the Figure 14.

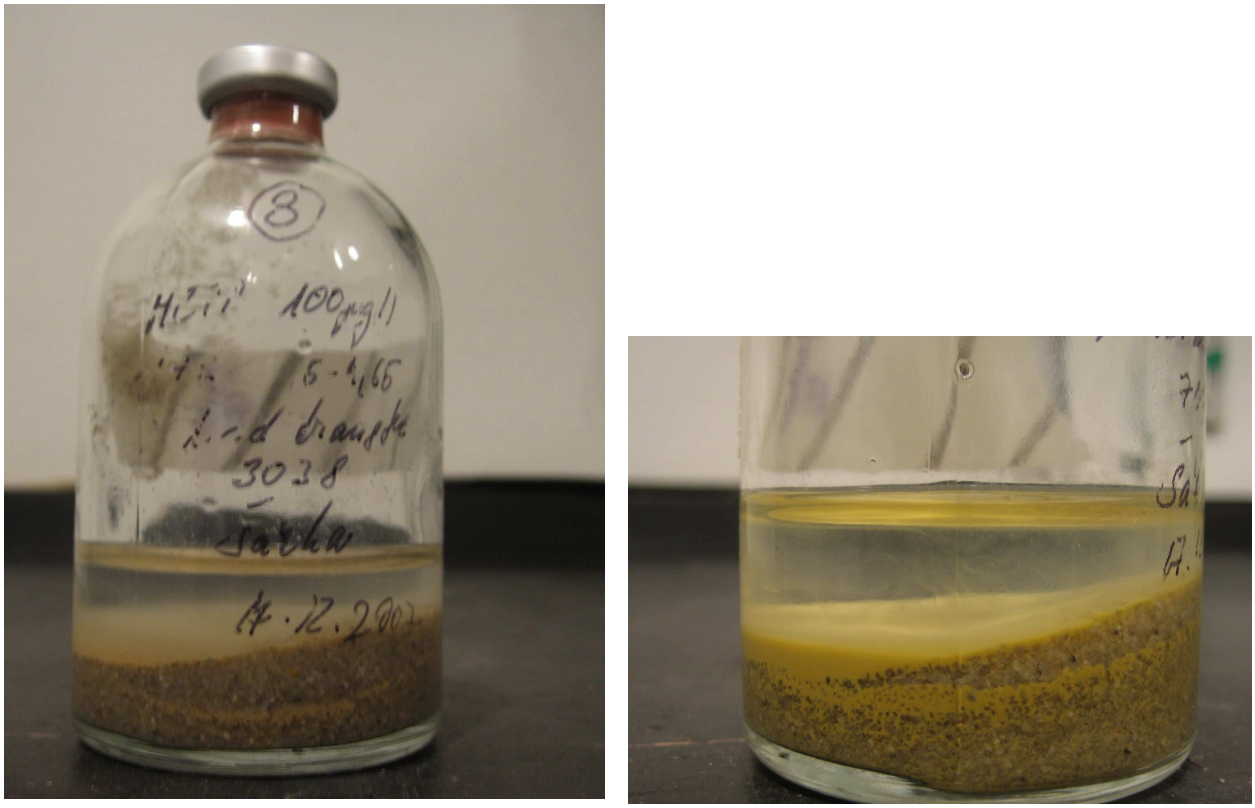


Figure 14 3layers experiment: batches with significant three layers fragmentation

The results from this experiment didn't show any significant difference in the level of ^{14}C-mecoprop concentration or $^{14}CO_2$ production in any of the phases. The results are close to each other. The interesting fact is that the highest concentration of mecoprop is usually shown in the mixture sample. This value is also higher than the average of the values in the three simply layers. The complete data are shown in the Appendix VII.

4.3 Isolation of the active bacterial culture

4.3.1 Bacterial growth on mecoprop polluted R2A plates I

After 7 days of incubation in the dark room with a temperature of 10 ± 0.1 °C, the growth of some bacterial culture is shown on the surface of all of the R2A plates excluding the control plate (no culture added).
The amount of colonies which have grown on each of the R2A plates is >60 000 CFU.ml^{-1}. No difference in the growth is reported for the R2A plates with plated mecoprop and R2A plates with supplemented R2A. The only difference that could be considered is that on the supplemented R2A, bacterial colonies are concentrated close to rim of Petri dish. Zones with no bacteria or with just few colonies are shown on the most of the plates.

Round white colonies without sharp margins are grown on all of the plates. The colonies have different sizes and three different shades of white color are shown - light white, milk white and intensive white. The colonies are flat and optically seem to be more 2D than 3D.

A significant difference is shown when using a 1 time dilution of bacterial culture, a 10 times and a 50 times (Figure 15 and Figure 16).

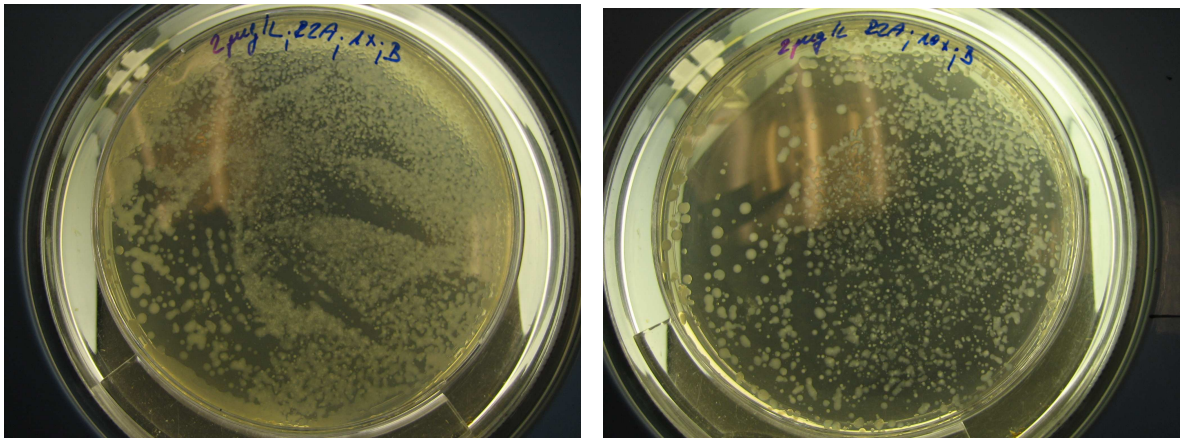


Figure 15 Documented growth on the plates of the 1st series isolation

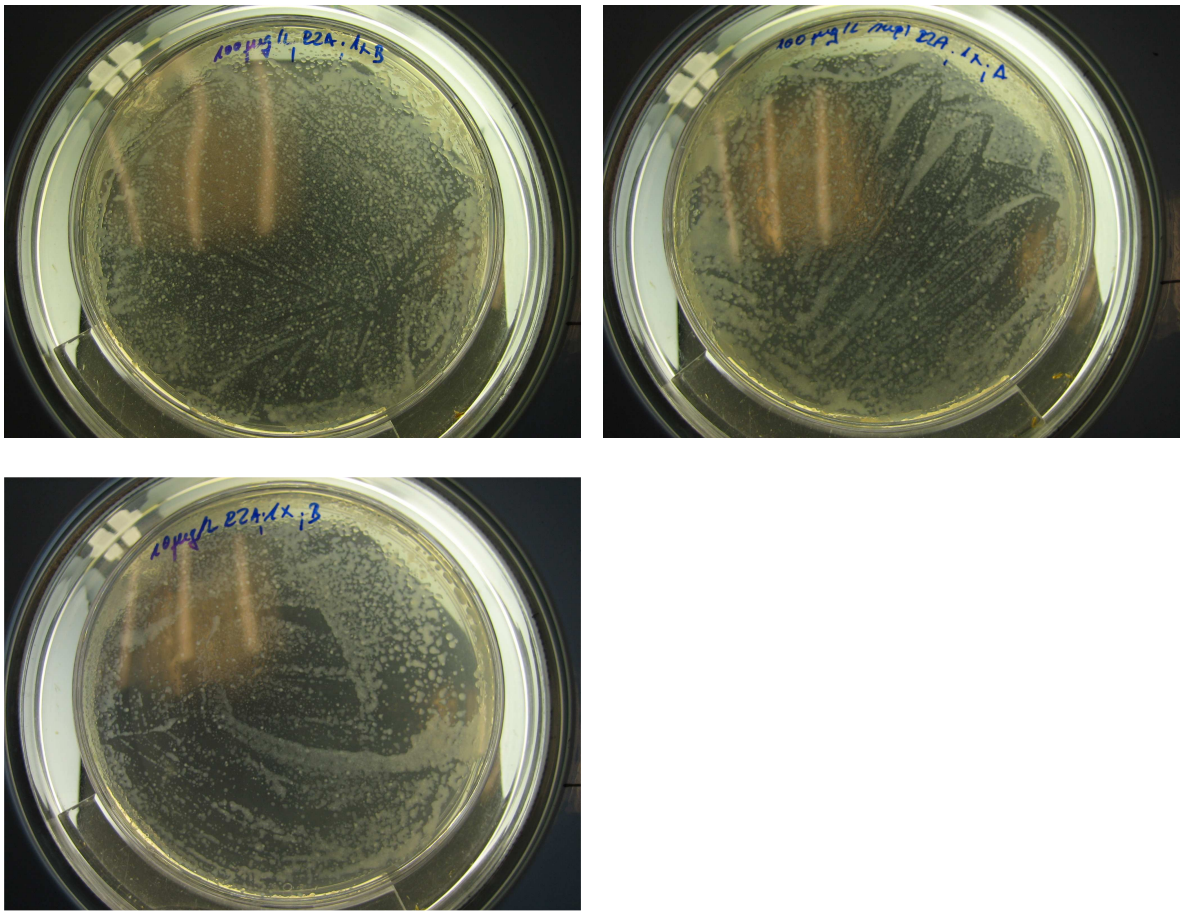


Figure 16 Documented growth on the plates of the 1st series isolation

The plates are taken out from the temperature of 10 °C to the laboratory after 7 days of incubation and the colonies are documented. Some of the colonies, those which can be isolated from other colonies, are transferred into following mineralization batches.

During this time, at a temperature of approximately 20 °C, a new kind of colonies growth is shown. Two new types of colonies are shown. The first one has a yellow color (Figure 17). On one of the other plate, a colony with stick shape has grown (Figure 18).

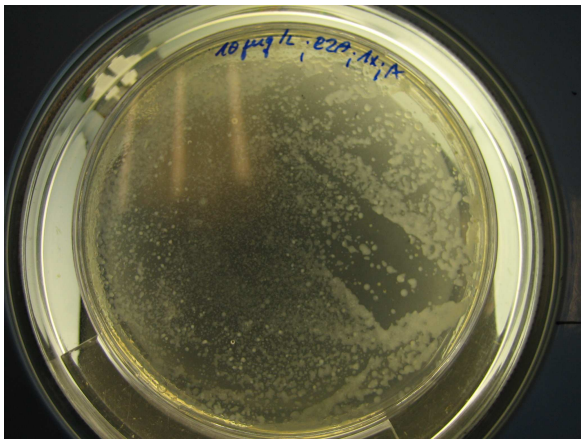


Figure 17 The reported yellow colonies

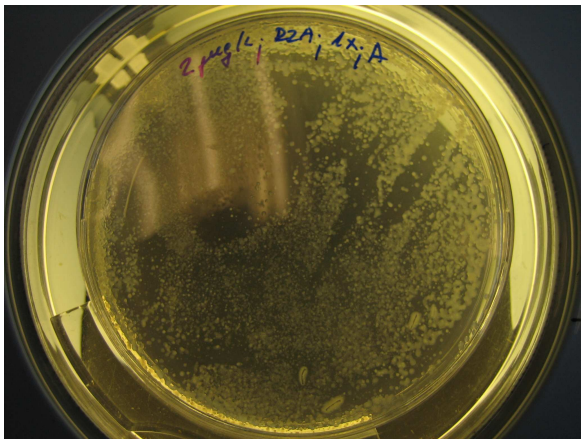


Figure 18 The reported stick shape colonies

4.3.2 Incubation mineralization of batches with R2A grown colonies I

After 15 days of incubation, no significant change in the ^{14}C-mecoprop concentration or $^{14}CO_2$ production is shown. The 13 % decrease is reported in the most active batches within this time.

After 28 days of incubation, a significant decrease in the ^{14}C-mecoprop concentration and $^{14}CO_2$ production is shown in five of the batches (Figure 19). In those batches the ^{14}C activity is measured again on day 33 and the mineralization activity of the bacteria present in those batches is confirmed. The decrease in ^{14}C-mecoprop concentration in the active batches is reported about 37 – 45 % with $^{14}CO_2$ production 21 - 29 % within 33 days incubation. The half life time of mecoprop is reported about 123-288 days.

The following R2A plate isolation is done on the 35th day of incubation, since the systems are reported currently active. The counting of ^{14}C-mecoprop concentration and $^{14}CO_2$ production of the day 33 proves the similarity of the mineralization progress with the active batches of the 2nd transfer. According to this similarity, a decrease in the mineralization activity is expected. This decreasing trend is already confirmed by the ^{14}C values of the day 33 and it is shown in Figure 19. The complete data are shown in the Appendix VIII.

According to the result of the mineralization following the R2A plate's isolation, no significant influence of the three shades of white colonies color on the mineralization progress is reported. Thus it is assumed that the difference in white shade is due to the amount of bacteria in the colony.

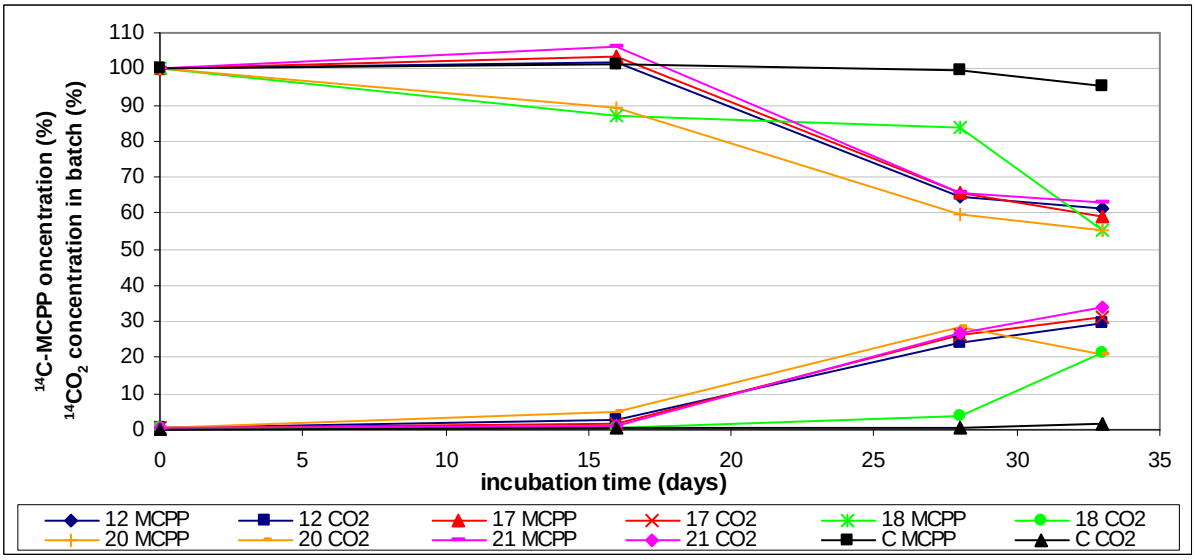


Figure 19 The progress of the ^{14}C-mecoprop concentration and $^{14}CO_2$ production during the incubation of the 1st series of batches containing colony growth on pesticide polluted R2A plate

4.3.3 Bacterial growth on mecoprop polluted R2A plates II

A significant difference is shown for plated 100x and 1000x dilution. The amount of colonies grown on the top of the plates with plated dilution 100x is in all the cases > 6 000 000 CFU.mL^{-1}. In the case of 1000x dilution of the mother suspension, a growth of 760 000 CFU.ml^{-1} – 3 000 000 CFU.mL^{-1} is reported. Only in the case of two plates with plated dilution 1000x the same amount of CFU.mL^{-1} as in the case of 100xs dilution is grown.

The colonies appear to have the same characteristics than the colonies in the first isolation. Two kinds of colonies have grown on the plates, the white one and the yellow one. The yellow colonies growth is documented also in the room with a temperature of 10±0.1 °C and the growth of those colonies is more frequent than on the first isolation plates (Figure 20).

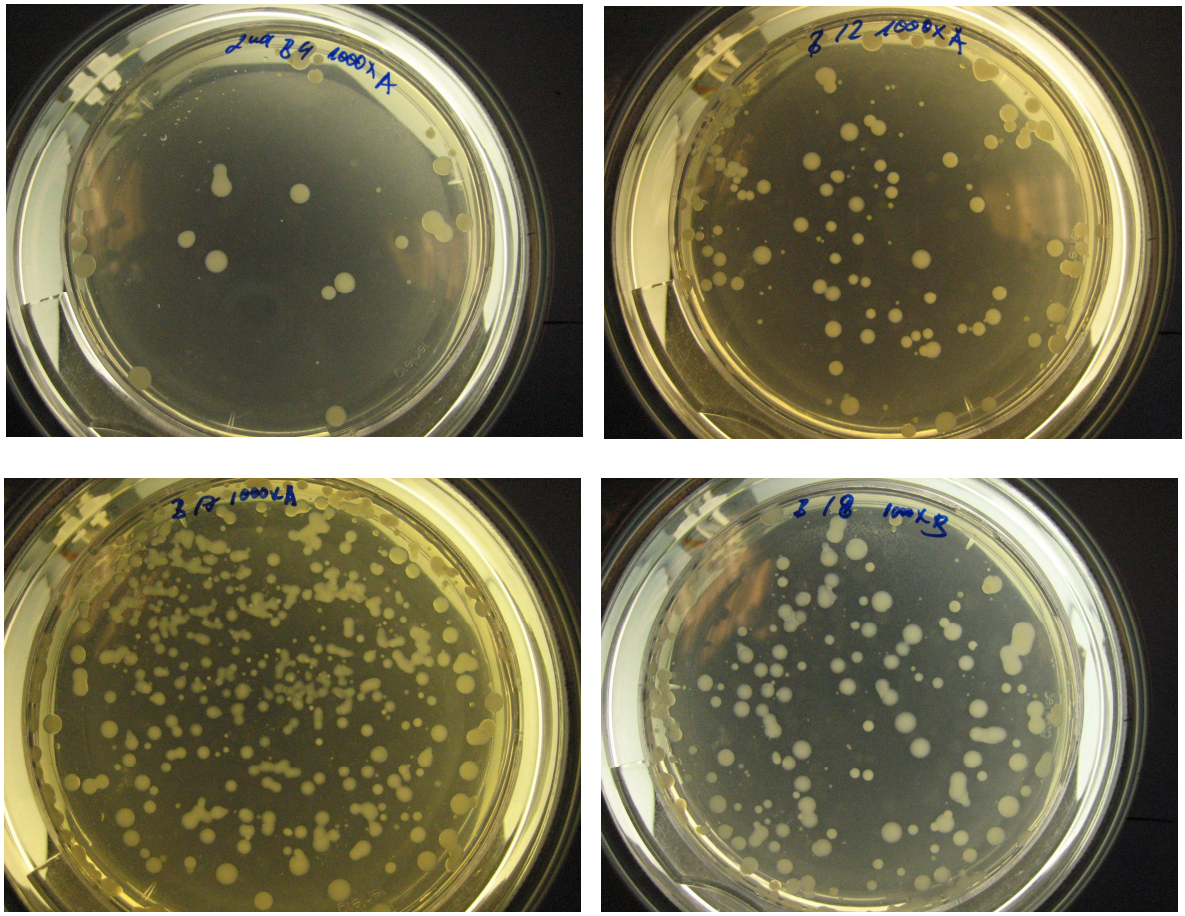


Figure 20 Documented growth on the plates of the 2nd series isolation

4.3.4 Incubation mineralization of batches with R2A grown colonies II

No change in the ^{14}C-mecoprop concentration and $^{14}CO_2$ production is reported within 42 days of incubation as shown in Figure 21. This can be due to the high dilution used for the plating of culture. That increases the possibility that pure cultures are present in the colonies grown on the R2A plates, and thus the risk that colonies which don't contain any culture mineralizing mecoprop are transported into the mineralization batches is higher. In that case no mineralization activity can be reported. Detailed data are shown in Appendix IX.

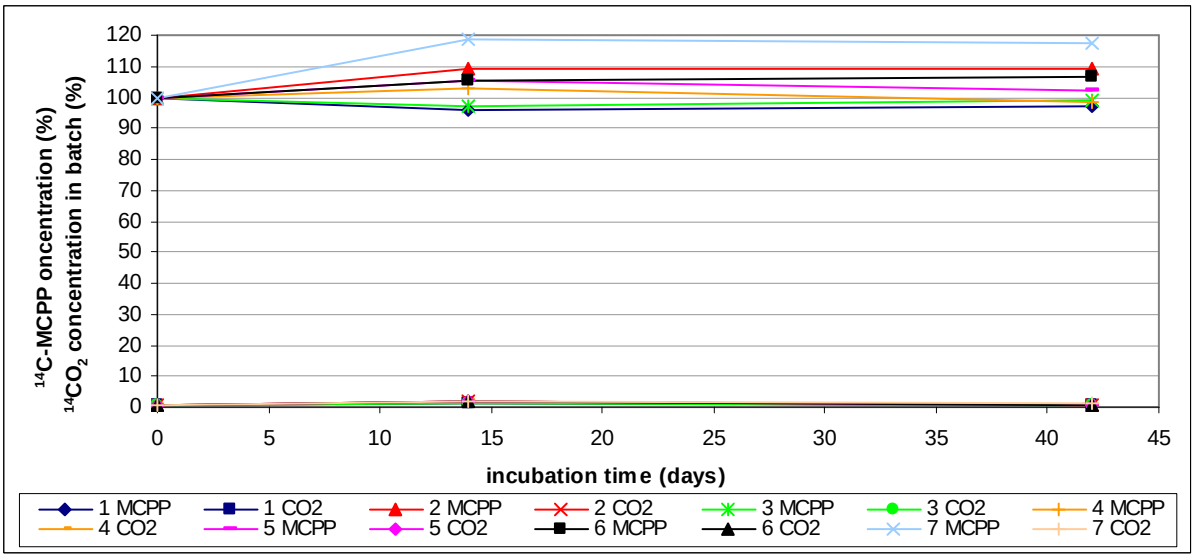


Figure 21 The progress of the ^{14}C-mecoprop concentration and $^{14}CO_2$ production during the incubation of the 2nd series of batches containing colony growth on pesticide polluted R2A plate

4.3.5 Stabilization of the active bacterial culture for following experiments

Due to the lack of any mineralization activity of the twice isolated colonies, no clear strain conservation is done. The suspensions from the five batches of the first series of batches containing cultures grown on the first series of mecoprop polluted R2A plates are chosen to be kept. Also suspensions from their transfer and the most active batch from the 2nd transfer are kept.

4.4 Effects possibly influencing the rate of mineralization studied batches containing culture grown on the mecoprop polluted R2A plates

4.4.1 The suspension transfers effect – R2A

The data of this experiment focused on the transfer of suspension from the batches containing a colony grown on the R2A plates, are compared after 48 days of incubation with the data of the original batches containing a colony grown on the R2A plates. In the batches containing transferred microorganisms, a shorter (or no) lag phase before the rapid biodegradation is shown, while a significant lag phase of approximately 15 – 20 days is shown in the original batches, Figure 22.

The $T_{1/2}$ in the original batches is reported between 123 and 188 days with a lag phase of approximately 15 - 20 days, the $T_{1/2}$ in batches with transferred suspension is reported between 85 and 106 days with no lag phase.

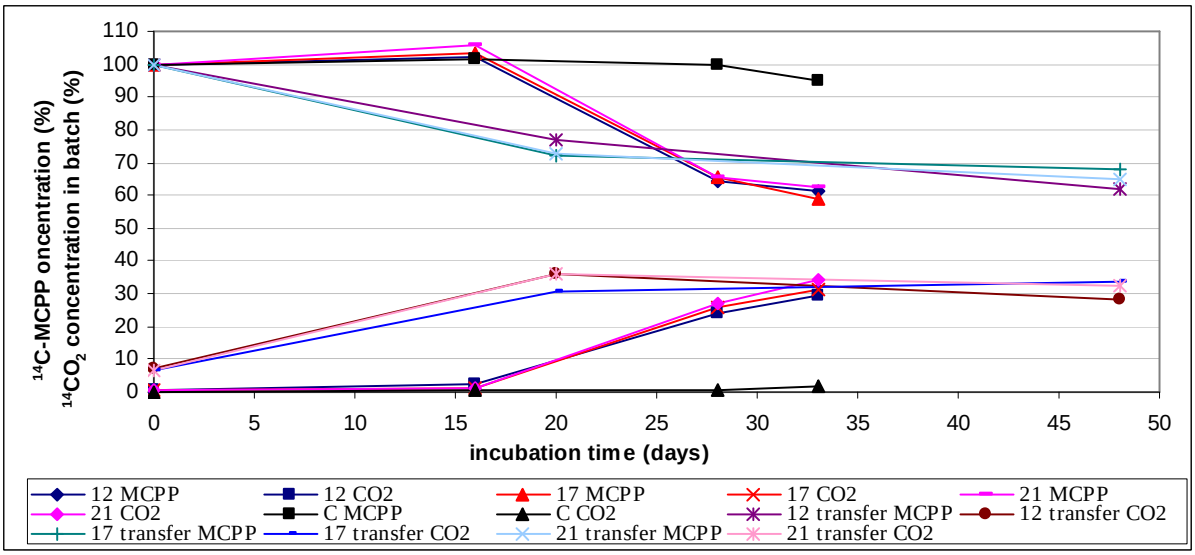


Figure 22 The suspension transfer effect: progress of ^{14}Cmecoprop concentration and $^{14}CO_2$ production in the original 1st series of batches contain colony grown on R2A plate and in the transfer made from those batches

4.4.2 Effect of using microorganisms pre-exposed to pesticide, repeated pesticide pollution

The comparison of mineralization rates in the original batches containing a colony grown on the R2A plate and the same batches with repeated mecoprop pollution is shown in Figure 23 and 24. In the Figure 23, the progress of the ^{14}C-mecoprop concentration and $^{14}CO_2$ production in the original batches is shown with a significant lag phase of approximately 15-20 days and mecoprop $T_{1/2}$ of 123-288 days. In the Figure 24 the progress of the ^{14}C-mecoprop concentration and $^{14}CO_2$ production in the same set of batch after repeated mecoprop pollution is shown. No lag phase is reported in this case and the mecoprop $T_{1/2}$ decreases to 113-187 days. Detail data of progresses are shown in the Appendix VII.

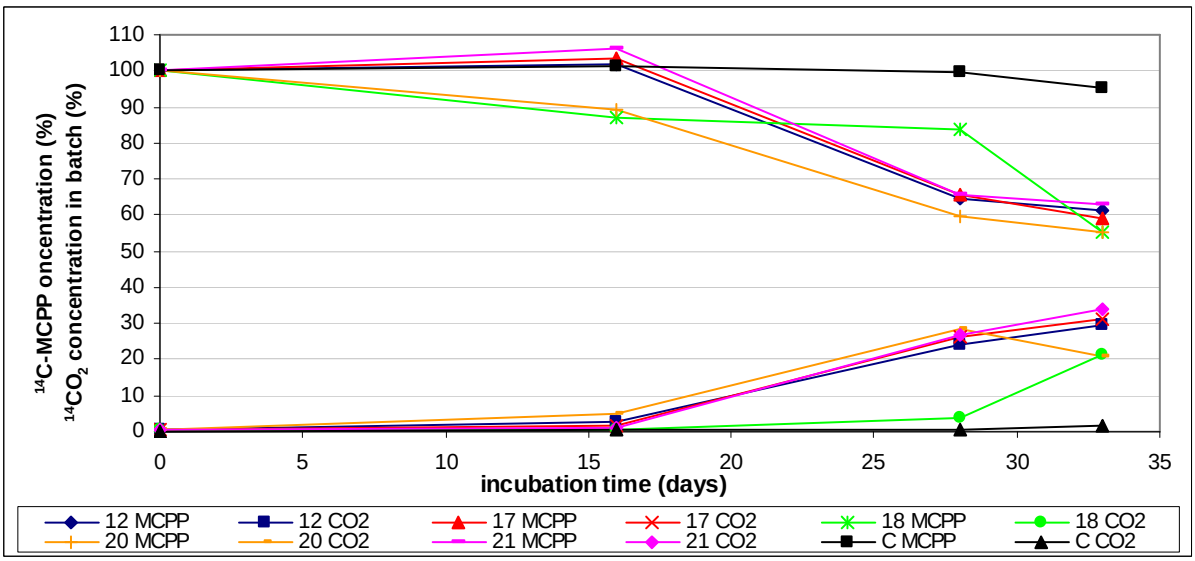


Figure 23 Effect of repeated pesticide pollution: progress of ^{14}C-mecorpop concentration and the $^{14}CO_2$ production in the original 1st series of batches containing a colony grown on R2A plate.

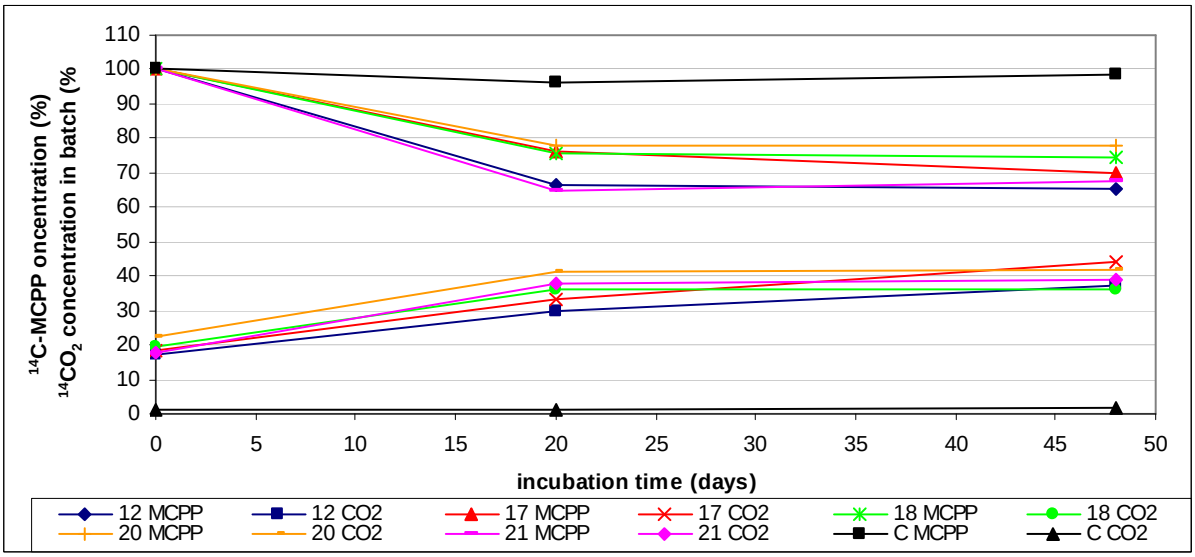


Figure 24… and the same batch, when the mecoprop pollution is repeated in the non-active phase

The repeated mecoprop pollution of the same sediment or bacterial culture leads to a rapid elimination of the lag phase before its own mineralization. The shape of the ^{14}C-mecoprop degradation curve as well as the curve of the $^{14}CO_2$ production matches in both cases. The final concentration of ^{14}C-mecoprop is in both cases around 35 % of the initial concentration lower, than the concentration of ^{14}C-mecoprop at the point where a rapid mineralization is starting. The final concentration of $^{14}CO_2$ produced is in both cases close to 40 % of the initial concentration with respect to the same rule as a ^{14}C-mecoprop concentration.

4.5 BAM mineralization

A decrease of the ^{14}C-BAM concentration of 11-17 % and a $^{14}CO_2$ production of 1-26 % is shown in this experiment within 68 days of incubation. In the control batch 12 % decrease in ^{14}C-BAM concentration and 1-13 % production of $^{14}CO_2$ is shown. Thus no significant mineralization activity is reported in this experiment.

In the second mineralization batches, which are done as the result of bacterial isolation, no mineralization activity is shown within 28 days of incubation.

Detail data of both incubations are shown in Appendix XI.

5. CONCLUSION

So far, the effect of dilution of the sediment containing active culture able to degrade pesticides had not been studied. The main goal of this project was to investigate if the sediments used in previous experiments and containing cultures with biodegradation potential were still active, possible to be used in a diluted microcosm, able to degrade mecoprop repeatedly and isolate this active culture. Based on the data acquired from experiments and presented previously, the general conclusions are:

- One of the Bréville site sediments contains cultures able to aerobically mineralize mecoprop, in its different dilutions and repeatedly. This culture is localized in core Pz17c in depth 4.50 – 4.65 mbs with crumbly limestone structure of subsurface. This culture is able to aerobically degrade mecoprop in low concentrations with 40 % efficiency within 50 days.

- The dilution of active mecoprop biodegradating cultures into the new microcosm results in a decrease of the biodegradation activity, meaning that the time needed for the acclimation is longer and that the final amount of mineralized mecoprop is lower.

- A repeated exposition of the same microcosm system to mecoprop minimizes the acclimation period, keeping the same mineralization capacity. The culture is able to degrade around 40 % of mecoprop in low concentration.

- The isolated active culture is able to degrade mecoprop in an environment of sterile sand instead of its natural sediment.

- The culture present in the sediment and grown on mecoprop polluted R2A plates is a group of microorganisms, where just some of them are able to degrade mecoprop.

- No monoculture is isolated and identified in this project.

- No biodegradation activity is reported for BAM biodegradating cultures.

The results obtained in this project are giving some future perspectives in research of used sediments containing cultures able to degrade mecoprop:

- The need of sediment in the system needs to be studied further. Can the culture degrade mecoprop without sticking to the sediment or sand, or is the sediment one of the mandatory parts of the system?

- The second isolation to obtain purer cultures needs to be repeated and should result in setting a bigger amount of mineralization microcosms. Then clear culture able to degrade mecoprop can be isolated and identified.

The ability of the Hvidovre sediments to degrade BAM repeatedly needs to be confirmed or excluded by another investigation.

This project shows that the biodegradation on the waterworks sand filters is a possible way of pesticide removal and in future can be considered as an alternative technique to the current pesticide treatment technique, the sorption to the activated carbon filter. However a long research way still needs to be achieved before this technique can take place in drinking water pre-treatment practice.

6. BIBLIOGRAPHY

[1] Albrechtsen, H.-J., Arvin E., Degradation I and Degradation II, Lecture note for 12330 Soil and Groundwater Pollution – Processes and Remediation, E&R DTU, Lyngby 2002

[2] Albrechtsen, H.-J., Mills, S.M., Aamand, J. & Bjerg, P.L.:. Degradation of herbicides in shallow Danish aquifers: an integrated laboratory and field study, *Pest Management Science*, 2001, vol. **57**, no. 4 pp. 341-350. ISSN 1526498x

[3] Bækgaard, A.: Introduction to Water Supply system in Denmark, *Water Supply* 1997, vol. 08, pp. 270-273, ISSN 1606-9749

[4] Bitton, G., Gerba, C.P.: Groundwater Pollution Microbiology, 1st ed. John Wiley & Sons Inc., 1984. 377 p. ISBN 0471096563

[5] Beyon, K.I., Wright, A.N.:, The fates of the herbicides chlorthiamid and dichlobenil in relation to residues in crops, soils and animal, *Residue Rev.*,1972, vol. 10 no. 43, pp. 23-53. ISSN 0080181X

[6] Buss, S.R, Trasher, J., Morgan, P., Smith & J.W.N. 2006. A review of mecoprop attenuation in the subsurface, *Quarterly Journal of Engineering Geology and Hydrogeology*, 2006, vol. 39, no.3, pp. 283-292. ISSN 14709236

[7] Cheng, H.H.: Pesticide in the Soil Environment, Processes, Impacts, and Modeling, 1st ed.. Soil Science Society of America, Inc., 1990, 554 p. ISBN *0-89118-791-X*

[8] Clausen, L., Arildskov, N.P, Larsen, F., Aamand, J. & Albrechtsen, H.-J. : Degradation of the herbicide dichlobenil and its metabolite BAM in soils and subsurface sediments, *Journal of Contaminant Hydrology*, 2007, vol. 89, no. 3-4, pp. 157-173. ISSN 01697722

[9] Clausen, L., Larsen F. & Albrechtsen, H.-J.: Sorption of the herbicide dichlobenil and the metabolite 2,6-dichlorobenzamide (BAM) on soil and aquifer sediments. *Environ. Sci. Technol.* 2004, vol. 38, no. 17, pp. 4510-4518. ISSN 0013936x

[10] Cox, C.: Mecoprop (MCPP). *Journal of Pesticide Reform, 2004, vol.* 24 no. 1 , pp. 10-14. ISSN 0893-357X

[11] CHMU, e-mail communication with Mr. Jindřich Freisleben, February 2008

[12] European Commission Drinking Water Directive 98/83/EC: http://ec.europa.eu/environment/water/water-drink/index_en.html - last. check 20.4.2008

[13] Fletcher, C.A., Bubb, J.M. & Lester, J.N.: Agricultural inputs of mecoprop to salt marsh system: its fate and distribution within the sediment profile, *Marine Pollution Bulletin*,1995, vol. 30, no. 12, pp. 803-811. ISSN 0025-326X

[14] GEUS, Groundwater monitoring: http://www.geus.dk/publications/grundvandsovervaagning/grundvandsovervaagning-uk.htm, last check: January 3rd, 2008

[15] Helweg, A.: Degradation and adsorption of ^{14}C mecoprop (MCPP) in surface soil and in subsoil. Influence of temperature, moisture content, sterilization and concentration on degradation, *Science of the Total Environment*, vol. 132, no 2-3, pp. 229-241. ISSN 00489697

[16] Holtze, M.S.. Degradation of the Herbicide Dichlobenil and its Metabolite 2,6-Dichlorobenzamide (BAM) in Soil and Bacterial Cultures, PhD thesis, KVL, GEUS, Copenhagen 2005

[17] Holtze, M.S, Hansen, H.C.B., Juhler, R.K., Sørensen, J. & Aamand, J.: Microbial degradation pathway of the herbicide dichlobenil in soils with different history of dichlobenil-exposure, *Environmental Pollution*, 2007, vol. 148, no.1, pp. 343-351, ISSN 0269-7491

[18] Holtze, M.S, Hansen, H.C.B., Juhler, R.K., Sørensen, J. & Aamand, J.: Degradation pathways of the herbicide 2,6-dichlorobenzonitrile and its metabolites 2,6-dichlorobenzamide (BAM) and dichlorobenzoic acid in soil in Degradation of the Herbicide Dichlobenil and its Metabolite 2,6-Dichlorobenzamide (BAM) in Soil and Bacterial Cultures, PhD thesis, KVL, GEUS, Copenhagen 2005

[19] Holtze, M.S, Hansen, H.C.B., Juhler, R.K., Sørensen, J. & Aamand, J.: Enrichment and characterization of 2,6-dichlorobenzamide-mineralising soil bacterial communities from dichlobenil-exposed soil, Degradation of the Herbicide Dichlobenil and its Metabolite 2,6-Dichlorobenzamide (BAM) in Soil and Bacterial Cultures, PhD thesis, KVL, GEUS, Copenhagen 2005

[20] Johnson, A.C., White, C., LaL Bhardwaj, C.& Dixon, A. 2003. Potential for isoproturon, atrazine and mecoprop to be degraded within a chalk aquifer system, *Journal of Contaminant Geology*, 2000, vol 44, no.1, pp. 1291-1302, ISSN 0169-7722

[21] Kemibrug, www.kemibrug.dk, last check 30.5.2008

[22] Lindberg, E. 2008.: Detection and quantification of subsurface pesticide degrading microbial populations, PhD thesis, E&R DTU, Lyngby, 2008

[23] Lyngkilde, J. & Christensen, T.H.: Fate of organic contaminants on the redox zones of a landfill leachate pollution plume (Vejen, Denmark), *Journal of Contaminant Hydrology*, 1992, vol.10, no. 4, pp. 291-307, ISSN 0169-7722

[24] MZP CR - Ministry of the Environment of the Czech Republic, database EKODISK, information about usage of herbicide dichlobenil, product Casoron G: http://www.env.cz/ris/ais-ris-info-copy.nsf/aa943fb38bfdd406c12568e70070205e/205b8c1632b6b7f280256833003f4946?OpenDocument , last check: 22.5.2008

[25] NIOSH - National Institute for Occupational Safety and Health, *Registry of Toxic Effects of Chemical Substances*, 1993.7-9: http://www.cdc.gov/niosh/rtecs/ last check: 15.5. 2008

[26] Nilsson, B., Dahl, M., Juhler, R.K., Hoffmann, C.C. & Engesgaard, P.: Fate of Two Pesticides in a Freshwater Wetland. Comparison of Field Injection and Laboratory Results. In: [27] Bjerg, P.L., Engesgaard, P. & Krom, T.D. (eds): Groundwater 2000. Proceedings of the International Conference on Groundwater Research, Copenhagen, Denmark, June 6-8, 2000. Rotterdam: Balkema, pp 275-276

[28] Council Directive 98/83/EC of 3 Nov. 1998 on the quality of water intended for human consumption. Official Journal of the European Communities: L 330/32–54. 5.12.1998.

[29] Pitter P., Hydrochemistry "UK", Hydrochemie (Czech original), 3rd ed., Vydavatelství VŠCHT Praha, 1999, 568 p. ISBN 80-7080-340-1

[30] SCR Phys-Prop Database, http://www.syrres.com/esc/datalog.htm last check: 30.6.2008

[31] Simonsen, A., Holtze, M.S., Sørensen, S.R., Sørensen, J.S. & Aamand, J.: Mineralisation of 2,6-dichlorobenzamide (BAM) in dichlobenil-exposed soils and isolation of BAM-mineralising *Aminobacter* sp., in Degradation of the Herbicide Dichlobenil and its Metabolite 2,6-Dichlorobenzamide (BAM) in Soil and Bacterial Cultures, PhD thesis, KVL, GEUS, Copenhagen 2005

[32] Smith, A.E.: Degradation, fate and persistence of the phenoxyalkanoic acid herbicides in soil, *Review of Weed Science*,1989, vol. 4, 1-24

[33] S.R. Buss, J. Thrasher, P. Morgan and Smith J.W.N..: A review of mecoprop attenuation in the subsurface, *Quarterly Journal of Engineering Geology and Hydrogeology*, 2006, vol. 39, no.3, pp. 283 – 292. ISSN 14709236

[34] Torang, L., Nyholm,N. & Albrechtsen, H.-J.: Shifts in biodegradation kinetics of the herbicides MCPP and 2,4-D at low concentration in aerobic aquifer materials, *Environmental Science and Technology*, 2003, vol. 37, no. 14, pp 3095-3103. ISSN 0013-936X

[35] Tuxen, N.: Oxygen-Enhanced Biodegradation of Phenoxy Acids in Ground Water at Contaminated sites, *Ground water*, 2006, vol. 44, No. 2, pp. 256-265, ISSN: 0017467x

[36] Tuxen, N., De Lipthay, J.R., Albrechtsen, H.-J., Reitzel, L.A., Pedersen, J.K. & Bjerg, P.L.: Effect of exposure history on microbial herbicide degradation in an aerobic aquifer affected by appoint source, *Environmental Science and Technology*, 2002, vol. 36, No.10, pp. 2205-2212, ISSN 0013936x

[37] US EPA, 1998. Registration Eligibility Decision (RED). Dichlobenil, EPA-738-F-98-005

[38] US EPA, 1988. Office of Pesticides and Toxic Substances. 1988. Fact Sheet No. 192: 2-(2-Methyl-4-chlorophenoxy) propionic acid and its salts and esters. U.S.EPA. Washington, DC

[39] Williams, G.M., Harrison, I., Carlick, C.A & Crowley, O.: Changes in enantiomeric fraction as evidence of natural attenuation of mecoprop in limestone aquifer, *Journal of Contaminant Hydrology*, *2003*, *vol.*64, no. 3-4, pp. 253-267, ISSN 0169-7722

[40] Heron, G., Christensen, T.H.: Degradation of the herbicide mecoprop in an aerobic aquifer determined by laboratory batch studies, *Chemosphere*, 1992, vol. 24, no. 5, pp. 547-557. ISSN 0045-6535

[41] Janniche, G.S.: non published materials from previous research.

7. LIST OF USED ABBREVIATIONS

BAM	2,6-Dichlorobenzamide
DWD	Drinking Water Directive
EC	European Commission
EU	European Union
GEUS	Nationale Geologiske Undersøgelser for Danmark og Grønland (Geological Survey of Denmark and Greenland)
CHMU	Český Hydrometeorologický Ústav (Czech Hydrometeorological Institute)
K_{ow}	Water/ octanol partition coefficient
LD50	Dose at which 50% of subjects will die
MAC	Maximum Allowed Concentration
mbs	meters bellow surface
MCPP	Mecoprop
MZP CZ	Ministerstvo Životního Prostředí České Republiky (Ministry of the Environment of the Czech Republic)
pK_a	$-\log_{10} K_a$, where Ka is acid dissociation constant
US EPA	United States Environmental Protection Agency
WHO	World Health Organization

8. LIST OF APPENDIXES

9. APPENDIXES

APPENDIX I – Overview of the biodegradation activities in the 1st transfer sediments

1st transfer of sediments potentially contains microorganisms biodegradating mecoprop, pesticide concentration 100 µg.l^{-1}

1st transfer of sediments - Gry 2007							
day 0 - 26.1.2007		day 32 - 27.2.2007		day 77 - 13.4.2007		day 123 - 29.6.2007	
% MCPP	%CO_2	% MCPP	%CO_2	% MCPP	%CO_2	% MCPP	%CO_2
100	0	47	29	43	29	46	26
100	0	79	2	75	1	66	3
100	0	40	19	33	18	20	22
100	0	30	29	9	19	4	42
100	0	76	3	68	4	56	5
100	0	95	0	95	0	83	0

1st transfer of sediments potentially contains microorganisms biodegradating BAM, pesticide concentration 700 µg.l^{-1}

	% BAM				
Time	25.X.06	29.XI.06	31.I.07	20.IV.07	29.VI.07
(day)	0	35	98	177	247
Blind	100	84	80	77	26
HV-C	100	79	81	74	1
HV-D	100	66	75	53	4

	% CO_2				
Time	25.X.06	29.XI.06	31.I.07	20.IV.07	29.VI.07
(day)	0	35	98	177	247
Blind	0	2	2	4	0
HV-C	0	2	2	6	16
HV-D	0	2	6	13	10

APPENDIX II - **The influence of sample filtration to the measured ^{14}C-activities**

batch	non - filtered		filtered	
	DPM/ml	% MCPP	DPM/ml	% MCPP
5	52.442	108	49.169	102
1/100	55.197	100	51.813	94
2/100	54.918	102	54.479	102
3/100	58.088	107	54.426	100
4/100	35.123	110	29.015	91
5/100	59.961	118	49.542	98

batch	non - filtered		filtered	
	DPM/ml	% CO_2	DPM/ml	% CO_2
5	2.226	5	2.429	5
1/100	1.429	3	1.698	3
2/100	1.716	3	1.820	3
3/100	2.568	5	2.655	5
4/100	14.779	46	14.346	45
5/100	3.191	6	2.478	5

APPENDIX III - Provenance of the transferred colonies into the batches contain colony grown on R2A plate I and II

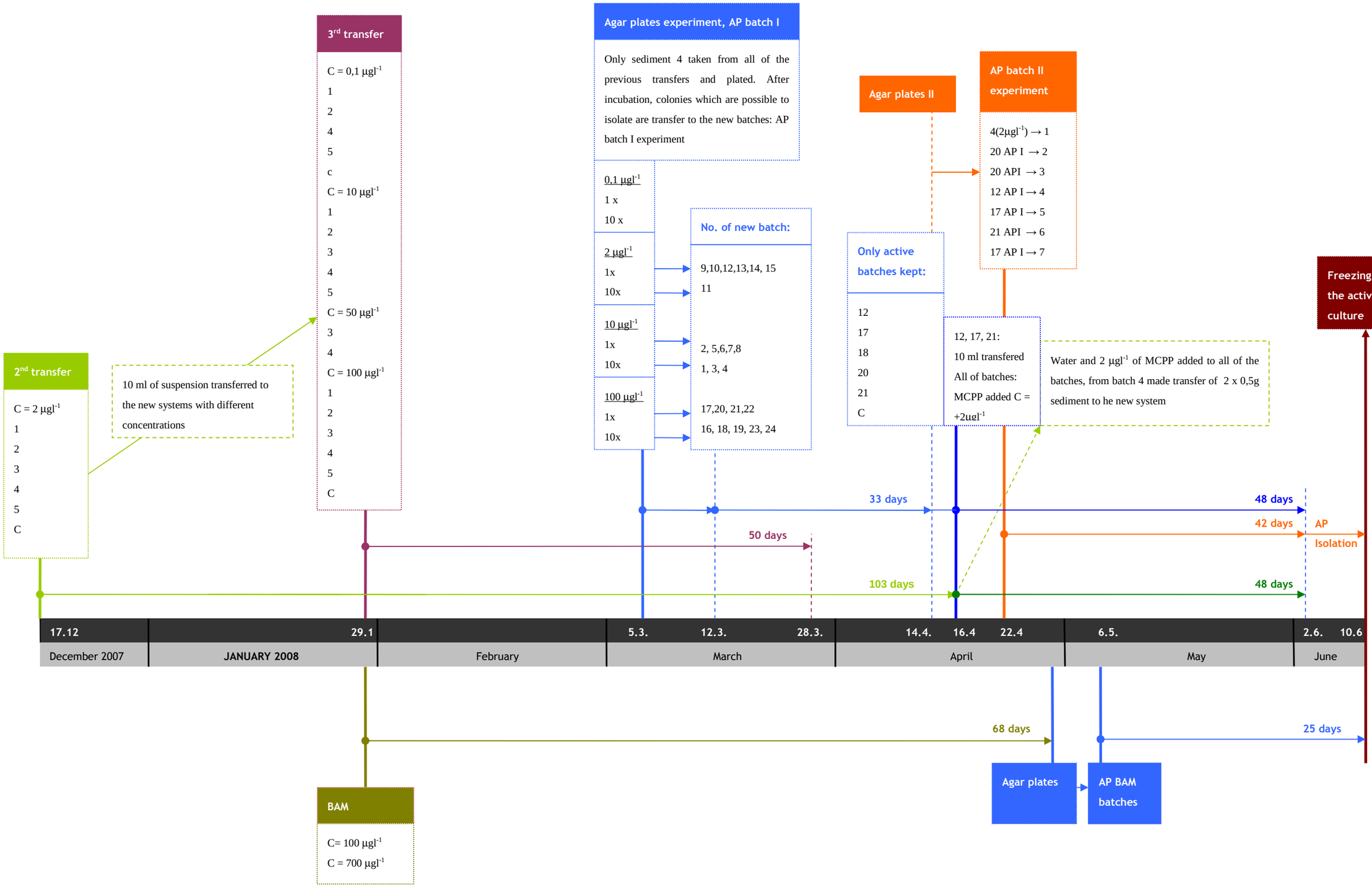
A) Batches contain colony grown on R2A plate I

No of batch	Colony added (mother batch, R2A+plated MCPP/supplemented R2A, plated dilution - kind of transferred colony)
1	10µg/L, R2A 10x – light white colony
2	10 µg/L, supl. R2A, 1xA – light white colony
3	10 µg/L, supl. R2A, 10xB – light white colony
4	10 µg/L, supl. R2A, 10x A – light white colony
5	10 µg/L, R2A, 1x A – yellow colony
6	10 µg/L, R2A, 1x A – milk white colony
7	10 µg/L R2A, 1x B – intensive white colony
8	10 µg/L R2A, 1xB - light white colony
9	2 µg/L, supl R2A 1x B – yellow colony
10	2 µg/L, supl R2A 1x B – light white colony
11	2 µg/L, R2A 10x A – light white colony
12	2 µg/L, R2A 1x A – light white colony
13	2 µg/L, R2A 1x B - light white colony
14	2 µg/L, supl R2A 1x A – light white colony
15	2 µg/L, R2A 1x A – intensive white colony
16	100 µg/L, R2A 10 x A – intensive white colony
17	100 µg/L, supl R2A 1 x A – intensive white colony
18	100 µg/L, supl R2A 10 x A – light white colony
19	100 µg/L, supl R2A 10 x A – milk white colony
20	100 µg/L, R2A 1 x B – intensive white colony
21	100 µg/L, R2A 1 x A – light white colony
22	100 µg/L, R2A 1 x C – intensive white colony
23	100 µg/L, supl R2A 10 x B – yellow colony
24	100 µg/L, R2A 10 x B – light white colony
control	No colony

Note 1: The pink one are batches where mineralization activity is reported

B) Batches contain colony grown on R2A plate I

Batch No.	Mothers batch	Plate - dillution	Kind of colony
1	2nd transfer batch 4	1000x	White small
2	20	1000x	White small
3	20	1000x	White big
4	12	1000x	White small
5	17	1000x	White small
6	21	1000x	White big
7	17	100x	White small


2nd transfer
C = 2 µgl-1
1
2
3
4
5
C
10 ml of suspension transferred to the new systems with different concentrations
3rd transfer
C = 0,1 µgl-1
1
2
4
5
c
C = 10 µgl-1
1
2
3
4
5
C = 50 µgl-1
3
4
C = 100 µgl-1
1
2
3
4
5
C
Agar plates experiment, AP batch I
Only sediment 4 taken from all of the previous transfers and plated. After incubation, colonies which are possible to isolate are transfer to the new batches: AP batch I experiment
0,1 µgl-1
1 x
10 x
2 µgl-1
1x
10x
10 µgl-1
1x
10x
100 µgl-1
1x
10x
No. of new batch:
9,10,12,13,14, 15
11
2, 5,6,7,8
1, 3, 4
17,20, 21,22
16, 18, 19, 23, 24
Agar plates II
AP batch II experiment
4(2µgl-1) → 1
20 AP I → 2
20 API → 3
12 AP I → 4
17 AP I → 5
21 API → 6
17 AP I → 7
Only active batches kept:
12
17
18
20
21
C
12, 17, 21:
10 ml transfered
All of batches:
MCPP added C = +2µgl-1
Water and 2 µgl-1 of MCPP added to all of the batches, from batch 4 made transfer of 2 x 0,5g sediment to he new system
Freezing of the active culture
33 days
48 days
42 days
AP Isolation
50 days
103 days
48 days
17.12
29.1
5.3.
12.3.
28.3.
14.4.
16.4
22.4
6.5.
2.6.
10.6
December 2007
JANUARY 2008
February
March
April
May
June
68 days
25 days
Agar plates
AP BAM batches
BAM
C= 100 µgl-1
C = 700 µgl-1

APPENDIX IV – Laboratory experiment overview

APPENDIX V – Overview of the biodegradation activities in the 2nd transfer sediments potentially contains microorganisms biodegradating mecoprop

2nd transfer 2µg/L								
Concentration of pesticide: approximate 2**µg.L^{-1}**:1ml of 100µg.L^{-1} in 100ml batch (30g sand, 60ml water, 10 ml subsample).								
	point 0- 17.12.07		**day 29 - 15.1.08**		**day 39 - 25.1.08**		**Day 50 - 5.2.2008**	
batch No.	**DPM/ml**	**% MCPP**	**DPM/ml**	**% MCPP**	**DPM/ml**	**% MCPP**	**DPM/ml**	**% MCPP**
1	464.080	100	394.470	85	380.738	82	388.809	83,8
2	484.774	100	449.821	93	436.571	90	421.157	86,9
3	448.746	100	413.454	92	422.416	94	414.390	92,3
4	466.633	100	388.519	83	258.207	55	221.921	47,6
5	477.020	100	382.999	80	377.694	79	373.364	78,3
control	522.302	100	491.473	94	498.681	95	486.395	93,1

	day 57 - 12.2.08		**day 64 - 22.2.08**		**day 64 - 22.2.08**		**day 103 - 29.3.08**	
batch No.	**DPM/ml**	**% MCPP**	**DPM/ml**	**% MCPP**	**DPM/ml**	**% MCPP**	**DPM/ml**	**% MCPP**
1	393.370	85	371.976	80	391.863	84	380.185	82
2	401.002	83	370.520	76	388.466	80	403.540	83
3	402.595	90	383.126	85	427.242	95	390.299	87
4	214.849	46	203.752	43	192.800	41	215.065	46
5	355.569	75	354.818	74	347.692	73	345.557	72
control	478.383	92	449.165	86	472.853	91	477.146	91

	point 0- 17.12.07		**day 29 - 15.1.08**		**day 39 - 25.1.08**		**Day 50 - 5.2.2008**	
batch No.	**DPM/ml**	**% CO2**	**DPM/ml**	**% CO2**	**DPM/ml**	**% CO2**	**DPM/ml**	**% CO2**
1	524	0	6.998	2	8.235	2	9.248	2
2	532	0	7.325	2	9.346	2	10.917	2
3	597	0	12.877	3	15.612	4	9.975	2
4	598	0	27.344	6	129.477	28	135.293	29
5	475	0	15.786	3	16.472	4	18.450	4
control	423	0	952	0	1.379	0	2.009	0

	day 57 - 12.2.08		**day 64 - 22.2.08**		**day 64 - 22.2.08**		**day 103 - 29.3.08**	
batch No.	**DPM/ml**	**% CO2**	**DPM/ml**	**% CO2**	**DPM/ml**	**% CO2**	**DPM/ml**	**% CO2**
1	9.890	2	11.192	2	11.385	3	13.267	3
2	10.690	2	11.144	2	13.605	3	15.834	3
3	17.644	4	17.297	4	18.948	4	20.638	5
4	146.511	31	130.436	28	157.790	34	166.826	36
5	18.072	4	19.378	4	19.959	4	23.006	5
control	2.084	0	2.651	1	2.758	1	3.738	0

APPENDIX VI - Overview of the biodegradation activities in the 3rd transfer sediments potentially contains microorganisms biodegradating mecoprop

3rd transfer: 0,1µg/L, 10µg/L, 100 µg/L and 50 µg/L

MCPP 0,1 µg/L

		day 0 - 29.1.08		day 7 - 5.2.08		day 14 - 12.2.08		day 24 - 22.2.08		day 36 - 5.3.08		day 50 - 29.3.08	
2nd transfer batch	batch No.	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP
1	1/0,1	51.786	100	50.886	98	51.390	99	51.208	98	49.493	96	48.975	95
2	2/0,1	56.136	100	55.116	98	55.893	99	57.277	102	55.111	98	53.942	96
3	xx												
4	4/0,1	33.331	100	33.698	101	31.038	93	30.140	90	29.834	89	27.909	84
5	5/0,1	48.376	100	51.379	106	48.435	100	49.169	102	47.567	98	47.747	88
C	c/0,1	76.562	100	65.963	86	71.763	93	71.519	93	72.126	94	68.170	89

CO_2 0,1 mg/L

		day 0 - 29.1.08		day 7 - 5.2.08		day 14 - 12.2.08		day 24 - 22.2.08		day 36 - 5.3.08		day 50 - 29.3.08	
2nd transfer batch	batch No.	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2
1	1/0,1	1.357	3	1.098	2	1.192	2	1.365	3	1.764	3	2.363	5
2	2/0,1	1.649	3	1.354	2	1.452	3	1.545	3	2.898	5	2.633	5
3	xx					0							
4	4/0,1	11.945	36	17.742	53	18.907	57	19.334	58	20.328	61	20.107	60
5	5/0,1	2.336	5	2.112	4	2.312	5	2.429	5	422	1	2.712	6
C	c/0,1	536	1	393	1	323	1	313	4	418	1	683	1

MCPP 10,0 mg/L

		day 0 - 29.1.08		day 7 - 5.2.08		day 14 - 12.2.08		day 24 - 22.2.08		day 36 - 5.3.08		day 50 - 29.3.08	
2nd transfer batch	batch No.	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP
1	1/10	39.897	100	40.929	103	39.992	100	40.243	100	39.863	100	38.890	98
2	2/10	53.067	100	56.938	107	53.863	101	52.422	99	51.930	98	52.029	98
3	3/10	57.344	100	55.483	96	54.605	95	55.352	96	55.950	98	53.681	94
4	4/10	32.714	100	32.295	98	31.188	95	30.201	92	29.981	92	27.088	83
5	5/10	51.554	100	53.582	104	51.803	100	51.172	99	51.312	99	49.432	96
C	xx												

APPENDIX VII - Using of microorganisms pre-exposed to pesticide, repeated pesticide pollution

A) 2nd transfer batches

2nd transfer batches - substrate added 16.4.08						
	day 0 - 16.4.08		day 20 - 6.5.08		day 47 - 2.6.08	
batch No.	DMP/ml	% MCPP	DMP/ml	% MCPP	DMP/ml	% MCPP
1	301.905	100	301.809	100	281.334	93
2	267.054	100	260.853	98	268.239	100
3	308.393	100	308.613	100	309.704	100
4	273.342	100	168.874	62	162.186	59
5	298.248	100	296.667	99	280.752	94
control	223.405	100	218.238	98	230.312	103

	day 0 - 16.4.08		day 20 - 6.5.08		day 47 - 2.6.08	
batch	DMP/ml	$\%CO_2$	DMP/ml	$\%CO_2$	DMP/ml	$\%CO_2$
1	6.300	2	8.939	3	10.386	3
2	7.271	3	5.725	2	10.417	4
3	8.546	3	13.850	4	14.328	5
4	32.763	12	106.594	39	114.405	42
5	13.428	5	15.834	5	18.450	6
control	3.290	1	2.045	1	2.085	1

B) Batches contain colony from R2A plates

1st R2A Batches - substrate added 16.4.2008						
	day 0 - 16.4.08		day 20 - 6.5.08		day 47 - 2.6.08	
	DMP/ml	% MCPP	DMP/ml	% MCPP	DMP/ml	% MCPP
12	116.438	100	77.337	66	75.873	65
17	108.155	100	82.613	76	75.454	70
18	110.130	100	83.083	75	82.228	75
20	93.865	100	73.256	78	72.974	78
21	118.023	100	76.712	65	79.924	68
cap	138.685	100	133.379	96	136.304	98

	day 0 - 16.4.08		day 20 - 6.5.08		day 47 - 2.6.08	
	DMP/ml	$\%CO_2$	DMP/ml	$\%CO_2$	DMP/ml	$\%CO_2$
12	20.204	17	34.661	30	43.150	37
17	19.631	18	35.829	33	47.450	44
18	21.765	20	39.681	36	39.827	36
20	21.049	22	38.777	41	39.031	42
21	21.210	18	44.773	38	46.231	39
cap	1.920	1	1.367	1	2.512	2

APPENDIX VIII - 3 layers experiment results

			mixture		water		bacteria layer		sediment	
	day 0 - 29.1.08		day 36 - 5.3.08		day 36 - 5.3.2008		day 36 - 5.3.2008		day 36 - 5.3.2008	
batch No.	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP	DPM/ml	% MCPP
1/100	55.013	100	49.657	90	52.624	96	51.109	93	52.604	96
2/100	53.673	100	53.229	99	53.937	100	47.446	88	51.525	96
3/100	54.524	100	58.465	107	54.031	99	53.087	97	55.780	102
4/100	31.955	100	28.320	89	28.016	88	29.372	92	28.320	89
5/100	50.741	100	51.238	101	47.221	93	50.145	99	51.171	101
c/100	70.772	100	67.118	95	61.412	87	62.319	88	61.050	86

	day 0 - 29.1.08		day 36 - 5.3.08		day 36 - 5.3.2008		day 36 - 5.3.2008		day 36 - 5.3.2008	
batch No.	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2	DPM/ml	% CO_2
1/100	1.150	2	1.734	3	1.924	3	1.733	3	1.898	3
2/100	1.616	3	1.788	3	2.043	4	1.674	3	1.967	4
3/100	2.299	4	2.752	5	2.581	5	2.471	5	2.468	5
4/100	16.221	51	20.363	64	20.654	65	19.763	62	20.052	63
5/100	2.326	5	2.558	5	2.823	6	2.817	6	2.900	6
c/100	569	1	456	1	528	1	546	1	549	1
			the lowest value				the highest value			

APPENDIX IX - 1st series of batches contain colonies grown on mecoprop polluted R2A plates

	day 0 - 12.3.08		day 16 - 28.3.08		day 28 - 9.4.08		day 33 - 14.4.08	
batch	**DMP/ml**	**%CO_2**	**DMP/ml**	**%CO_2**	**DMP/ml**	**%CO_2**	**DMP/ml**	**%CO_2**
1	158	0	358	1	620	1		
2	87	0	389	1	946	2		
3	162	0	212	0	877	1		
4	101	0	305	0	531	1		
5	149	0	259	0	518	1		
6	180	0	323	0	781	1		
7	445	1	668	1	513	1		
8	368	1	230	1	480	1		
9	330	1	218	0	389	1		
10	786	1	724	1	660	1	540	1
11	268	0	334	1	400	1		
12	256	0	1.615	3	14.748	24	17.984	29
13	248	0	507	1	776	1		
14	282	0	553	1	647	1		
15	245	0	298	0	610	1		
16	280	0	325	0	541	1		
17	212	0	994	1	17.690	26	21.078	31
18	256	0	567	1	3.410	4	19.767	21
19	202	0	303	1	392	1		
20	281	0	3.506	5	19.963	28	14.474	21
21	240	0	845	1	18.032	27	22.774	34
22	163	0	462	1	1.049	2		
23	173	0	281	0	796	1		
24	180	0	274	0	768	1		
control	170	0	258	0	577	1	1.324	2

APPENDIX X - 2nd series of batches contain colonies grown on mecoprop polluted R2A plates

2nd R2A plates batches (isolation of bacteria from 1st AP experiment)						
	day 0 - 22.4.08		day 14 - 6.5.08		day 41 - 2.6.08	
batch	DMP/ml	% MCPP	DMP/ml	% MCPP	DMP/ml	% MCPP
1	77.091	100	73.805	96	75.056	97
2	75.062	100	81.975	109	81.795	109
3	74.359	100	72.193	97	73.494	99
4	66.699	100	68.441	103	65.841	99
5	75.315	100	79.205	105	76.783	102
6	60.162	100	63.432	105	64.314	107
7	59.890	100	71.172	119	70.218	117

	day 0 - 22.4.08		day 14 - 6.5.08		day 41 - 2.6.08	
batch	DMP/ml	%CO_2	DMP/ml	%CO_2	DMP/ml	%CO_2
1	477	1	1.072	**1**	514	1
2	500	1	1.347	**2**	619	1
3	487	1	979	**1**	692	1
4	411	1	1.166	**2**	517	1
5	458	1	1.391	**2**	493	1
6	384	1	994	**2**	430	1
7	333	1	1.035	**2**	758	1

APPENDIX XI - Overview of the biodegradation activities in the 3rd transfer sediments potentially contains microorganisms biodegradating BAM

	day 0 - 29.1.08		day 14 - 12.2.2008		day 36 - 5.3.2008		day 50 - 29.3.2008		day 68 - 15.4.2008	
batch No.	**DPM/ml**	**% BAM**	**DPM/ml**	**% BAM**	**DPM/ml**	**% BAM**	**DPM/ml**	**% BAM**	**DPM/ml**	**% BAM**
BAM 1 100	842	100	756	90	810	96	770	91	748	89
BAM 2 100	971	100	829	85	861	89	817	84	843	87
BAM C 100	535	100	518	97	527	98	583	109	634	119
BAM 1 700	34.958	100	32.553	93	31.496	90	33.030	94	29.163	83
BAM 2 700	28.819	100	29.561	103	29.265	102	29.476	102	28.669	99
BAM C 700	33.991	100	33.216	98	33.610	99	32.510	96	30.003	88

	day 0 - 29.1.08		day 14 - 12.2.2008		day 36 - 5.3.2008		day 50 - 29.3.2008		day 68 - 15.4.2008	
batch No.	**DPM/ml**	**% CO_2**	**DPM/ml**	**% CO_2**	**DPM/ml**	**% CO_2**	**DPM/ml**	**% CO_2**	**DPM/ml**	**% CO_2**
BAM 1 100	189	22	132	16	157	19	295	35	407	48
BAM 2 100	220	23	146	15	196	20	231	24	404	42
BAM C 100	180	34	41	8	53	10	136	25	250	47
BAM 1 700	258	1	158	0	165	0	302	1	700	2
BAM 2 700	233	1	163	1	149	1	280	1	233	1
BAM C 700	120	0	36	0	50	0	136	0	452	1