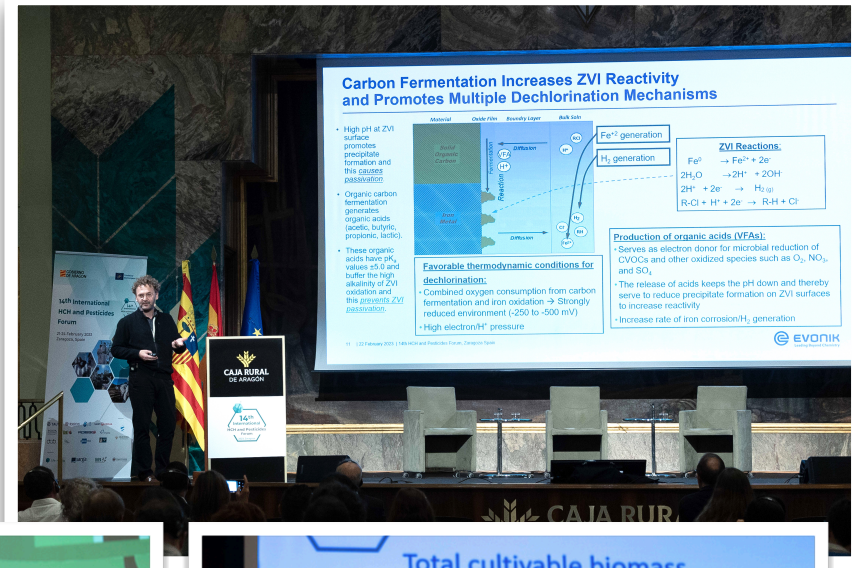
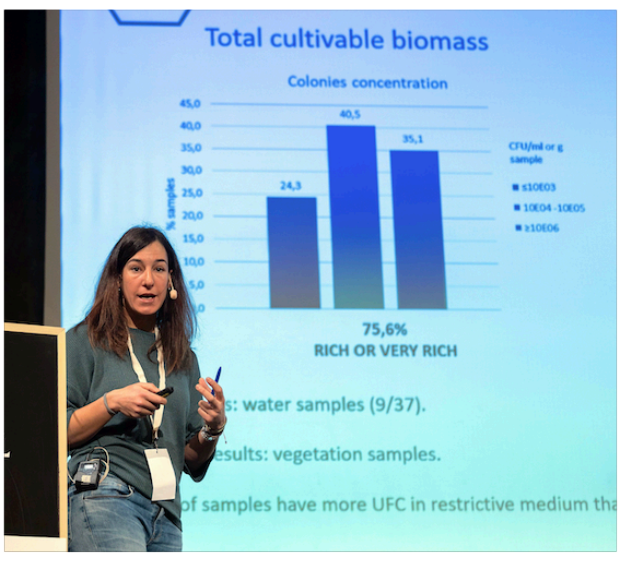
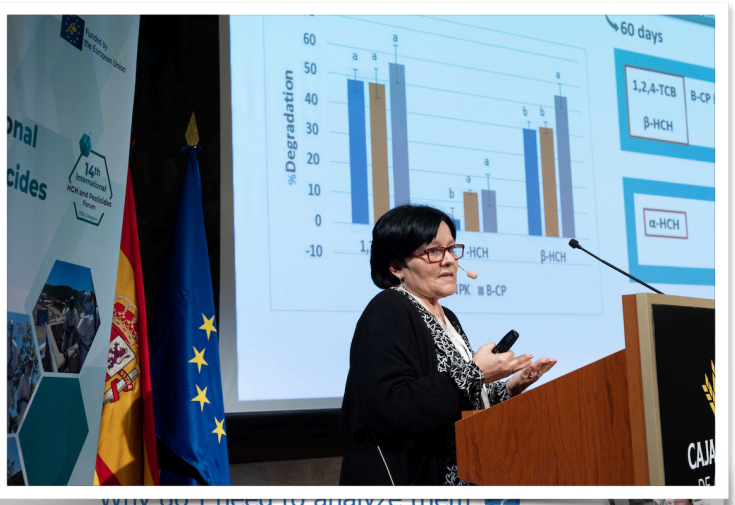
Block 5

BIOREMEDIATION

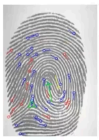






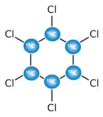
Isotope signature of the primary compound

$\delta^{13}C$ = -26 ‰ VPDB
$\delta^{37}Cl$ = +4 ‰ SMOC
$\delta\ ^{2}H$ = -50 ‰ SMOW
$\delta^{18}O$ = +2 ‰ SMOW
= +7 ‰ AIR

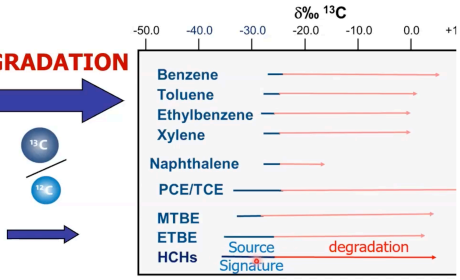


FINGERPRINT – Source Identification

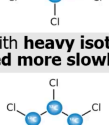
^{12}C-HCH



DEGRADATION



nt molecules with **heavy isotopes** (^{13}C) are **degraded more slowly**.



^{13}C-HCH

www.isodetect

4

MICROBIOME BASED REMEDIATION AND OTHER NATURE BASED TECHNIQUES

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Summary

There is an immense task on soil & groundwater remediation of contaminated sites in the EU, amongst which HCH-sites. This paper gives a compact overview of the current research and development progress regarding potential nature based solutions (NBS) for HCH in the EU. These NBS are focused on the use and support of natural processes, like the natural flowrate of (ground)water, sorption to green adsorbents, degradation by bacteria, atmospheric oxygen/UV and phytoremediation. The search for HCH-specific microbiomes to subsequently upscale them for depollution purpose at contaminated sites has just started within the framework of EU-Horizon project MIBIREM. The quality improvement of surface water impacted by contaminated groundwater has just been shown by the use of a reactive mat filled with green adsorbents in another EU-project: RESANAT. For HCH-contaminated sites, which are frequently large and sometimes remote, NBS have a high potential and fit into the current philosophy that the environmental, social and economic value of a remediation work should be optimized.

Keywords

HCH, EU, sustainable remediation, nature based solutions, microbiome, MIBIREM, Natural Catch^{TAUW}

Introduction, scope and main objectives

The EC-document on the Roadmap regarding the 'New Soil Strategy - Healthy soil for a healthy life' states: "Local pollution is also present in all EU-countries and 14% of an estimated total of 2.8 million potentially polluted sites from industrial activities are expected to require remediation, that is 390,000 sites. By 2018, only some 65,500 of these sites were remediated".

The 'Inventory of sites potentially impacted by HCH in EU Member States' points out that there are about 300 sites in 22 EU countries where HCH was handled, of which a large part still needs to be remediated.

This gives an impression of the immense remediation task in EU-countries, with regard to the number of sites as well as with regard to complexity (technical, financial and organizational). Traditional intensive and high-tech techniques, like excavation and is situ chemical oxidation, will only solve a part of the problem. This is because of the high cost, the scale of sites, accessibility to contamination (depth, hindrance due to present buildings and infrastructure) and the impact on sustainability indicators like landscape disturbance, nuisance and biodiversity. The environmental, social and economic value of the remediation work should be optimized . Nature based solutions (NBS) will often be the key for a sustainable approach to sites decontamination. In general the NBS-approach is cheaper, less disturbing, emits less CO2 and operational maintenance is less extensive which is a pro for remote and large sites with diffuse contamination. Several current EU funded innovation projects within the framework of Horizon, Life and Interreg are focusing on this approach to remove soil & groundwater contaminants.

These NBS are focused on the use and support of natural processes, like the natural flowrate of (ground)water, sorption to green adsorbents, degradation by bacteria and fungi, atmospheric oxygen and UV, alkaline gravel beds and groundwater extraction by trees.

In this paper we list and touch on some NBS projects funded by the EU relevant for persistent contaminants like the HCH-isomers and pesticides. We zoom in to two of them:

- The set-up and expectations of the freshly started project MIBIREM.
- The set-up and results of the Natural CatchTAUW as one of the pilots of nearly finalized project RESANAT.

Nature based solutions (NBS) and current EU-projects

Currently there are several EU-projects that are relevant for HCH-isomers and pesticides and focused on NBS. To have an overview of these innovative initiatives, which are all integral desk-, lab- and field studies, some of them are listed below, together with their focus, partners and a link to the specific website for more info:

- H2020 GREENER (2019-2023). This project integrates several bioremediation strategies with innovative bio-electrochemical technologies for PH, **pesticides**, PAH and heavy metals. Cooperation of 20 partners: universities, knowledge institutes, consultancies and project management parties

- Interreg RESANAT (2019-2023). In this project nature-based remediation techniques (reactive mat, phytoremediation, injection electron acceptors) for residual contaminants PAH, PH and BTEX are design and tested the field. Cooperation of 9 partners: national authority, knowledge institute, consultancies and contractors
- Life POPWAT (2020-2023). This project promotes innovative technology based on constructed wetlands for treatment of **pesticide/ HCH** contaminated waters. Cooperation of 7 partners: local authority, universities, knowledge institutes and consultancies
- H2020 RECYCLE (2020-2025). Removal and Mitigation of Pollution from the Use of **Pesticides**: Prevention, Recycling and Resource Management. Cooperation of 12 partners/ participants: universities, knowledge institutes and consultancies
- H2020 REMEDI (2021-2025). Trapping and Removal of X-ray Contrast Medium agents from water resource and stream Sediments - New Concepts in Trapping, Recycling and Management. Cooperation of 5 partners: regional engineering department, university, consultancies
- Horizon Europe MIBIREM (2022-2027). Toolbox for Microbiome based remediation of sites contaminated with **HCH**, PH and cyanide. Cooperation of 11 partners: universities, knowledge institutes, consultancies/contractors and project management party.

Though some of these projects are focused on other contaminants, the NBS-mechanisms for removal like adsorption, aerobic/anaerobic biodegradation and chemical reductive dechlorination and phytocontainment are promising for HCH-isomers and/or pesticides as well. At the same time, these projects may complement each other and have interesting mutual benefits. This is the case for the projects POPWAT and MIBIREM for example with regard to microbial site characterization. In the end, a lot of practical information will be generated, which will lead to an increase of successful use of NBS. The silver bullet for sites will mostly be in the combination of several NBS-techniques, if necessary supplemented by a high-tech step when time and/or space are limited; to speed up processes and/or to minimize the occupation of land. One of the examples is the combination of a preliminary high-tech dechlorination step by using zero valent iron (ZVI) and a successive bio-adsorption and aerobic degradation by a constructed wetland.

Set up of MIBIREM

The EU-funded MIBIREM project was launched in October 2022. MIBIREM consists of a consortium of 11 partners from several EU-countries: AIT, RTDS, TAUW, DND Biotech, ALTAR, Sensatec, CNRS and Universities of Ghent, Hasselt, Pisa and Utrecht. The project will take 4.5 years.

The project will use the potential of microbiomes for soil and groundwater bioremediation: it will create and apply a unique toolbox to identify, analyze, isolate, cultivate and upscale microbiomes for implementation at and depollution of contaminated sites. With its toolbox, the project will provide innovative methods to improve the functions of microbiomes in degrading contaminants in soil and groundwater. By testing the improved microbiomes under real field conditions in selected sites, MIBIREM aims to pave the way for long-term upscaling of microbiome-based bioremediation.

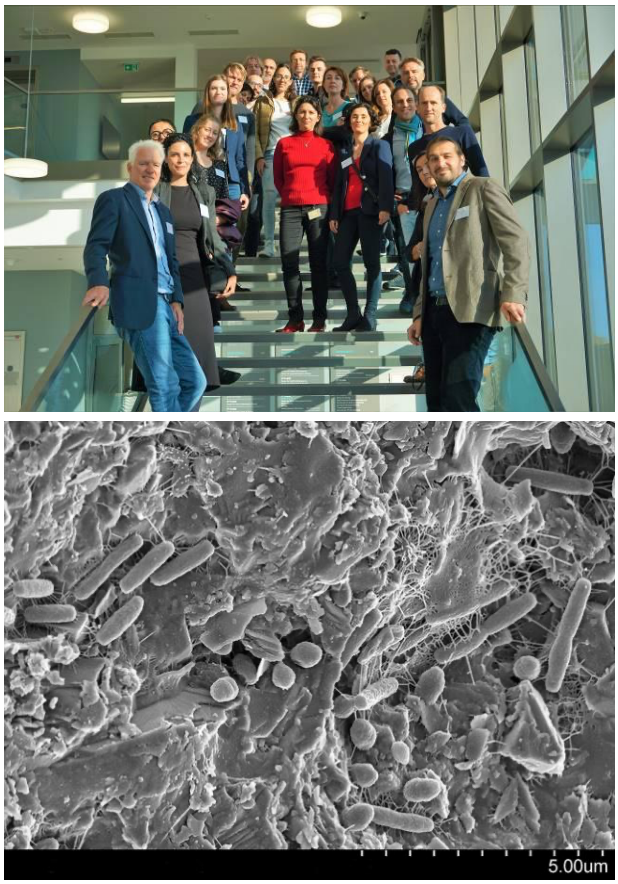

FIGURE 1. (A) THE MIBIREM-CONSORTIUM AND (B) A COMMUNITY OF SOIL BACTERIA (LEWIS LAB AT NORTHEASTERN UNIVERSITY)

The project is focusses on the more persistent contaminants petroleum hydrocarbons, cyanide and HCH. For each of those contaminants 5 sites will be sampled for identification of environmental conditions, composition of contamination and isolation of microbiomes, strains and individual species relevant for biodegradation of the contaminants. One of each 5 sites will be selected for piloting.

The following disciplines from all the project partners will come into place in a unique melt: molecular biology, bioinformatics, mathematical modelling, microbiology, laboratory evolution, environmental designing and engineering, chemistry and environmental science. Safety,

regulation and policy will be part of the project as well, which can be different in each country.
The activity is divided in several working packages, ranging from sampling to microbiome analysis, cultivation and evolution to collection of degrading microbiomes and strains, implementation of prediction tools to field pilot testing.
At this moment the quest for sites to sample has almost completed. For all three contaminants sites have been pre-selected. With regard to HCH sites we have now a pre-selection of sites in Germany, France, Italy, Spain and the Czech Republic, of which the latter is a site which is part of the LIFE project POPWAT. We prioritize the sites to their potential, contact the site owners for their permission to take samples and start field work (measurements and sampling) in Q1 and Q2 of 2023. After that, the microbiome analyses, functionality and identification of bacteria will start.
One of the preliminary studies that will be used as input for MIBIREM was carried out by DND Biotech and the University of Pisa. In this study, a microbiota capable of transforming different isomers of HCH was isolated and grown from a soil contaminated by halogenated pesticides. The microbiota has good capability to deplete the technical lindane (γ-HCH) comprising the β isomer in liquid culture. The characterization of the microbiota is still ongoing and will be implemented in bioaugmentation approaches in the RoboNova®, a terrestrial drone capable of testing and validating the efficacy of biodegradation mechanisms with identified and specifically isolated bacterial strains.

NBS-field experience: Natural Catch

In Europe, there is a large number of locations where a long term inflow of contaminated groundwater from industrial sites, brownfields or chemical landfills negatively influences the quality of draining surface waters. The situation at this field study is an example of such a location.
This case study was funded by the EU, the Dutch Ministry of Economic Affairs and the project partners OVAM, TAUW, Envisan, iFlux, TTE and Witteveen+Bos. TAUW headed the project.
Canal the Lieve in Ghent (B) has been impacted by an adjacent historically contaminated site of a former industrial production of tar and carbon black. This has led to soil and groundwater contamination by aliphatic, monocyclic and polycyclic aromatic hydrocarbons (PAH). Secifically the PAH have a lot in common with (halogenated) pesticides when it comes to adsorption characteristics.
Contaminated groundwater from this site drains into the Lieve, which leads to concentrations in the surface water exceeding the environmental quality standard (70-300 times for several PAH-components). Because of significant residual soil and groundwater contamination and improvement of the drainage capacity of the canal, this problem will last for decades.
TAUW came up with the concept of using natural materials as an adsorbent in a permeable barrier in a mat structure to intercept the pollutants in contaminated groundwater draining into surface water. TAUW have called this technology Natural Catch^{TAUW}, which received the 2013 NICOLE Technology Award. The mat structure is placed on the bed of a surface water body, like a brook or canal. The functioning of this Natural Catch^{TAUW} exploits three nature-based processes:
1. The natural drainage capacity of the canal as propelling force; no pump is needed;
2. The use of a naturally occurring renewable adsorbent in the mat that is inert and has a high adsorption capacity;
3. A biologically active interface at the mat surface that provides aerobic biodegradation.
In one of the segments the influx of contamination is about 100 mg/m2/day total hydrocarbons, of which about 10% benzene, 30% naphthalene, 18% phenanthrene and 7% acenaphthene. See Figure 2.
In September 2020 the geotextile mat elements were filled on site with ballast material (gravel) and adsorption material: 16 upstream mat elements with biochar and 11 downstream mat elements with peat. The mat elements were then hoisted into the canal and fixed to the banks (Figure 3). The biochar mats are used for the segments 1 and 2 with the highest influx with a length of 65 meters, the peat mats are used for segment 3 with the lowest influx with a length of 45 meters.
During the first 16 months a high efficiency on the reduction of contaminant concentrations in the surface water was observed: 80-99% for PAH, BTEX and C6-C10. Exception is benzene with 70-80% because of its lower adsorption characteristics (high mobility). The water flux through the mat was shown to be upward and only a slight mass flux of benzene through the mat could be measured above detection limit. From this it is concluded that the use of a reactive mat filled with green adsorbents like biochar and peat can significantly improve the quality of a waterbody (canal, brook) that receives groundwater contaminated with PAH, BTEX and C6-C10.
In the period May to July 2022 the concentrations in surface water increased in segment 2. It was shown by flux measurements in July 2022 that the biochar was far from saturation with contaminants, so this was not the cause. The primary cause turned out to be a combination of (a) the presence of free product in the waterbed directly below the reactive mat and (b) the loss of mutual connection between some mat elements. Consequence is a preferential flow path of free product to the surface water and impact on the surface water quality. Before implementing a reactive mat, any free product in the waterbed should be removed from the upper

part. Furthermore, the use of two water-tight foil flap over the joints (connection between two mat elements), one at the bottom and one at the top, reduces this risk of preferential flow paths. In Ghent only the flap on top was designed and constructed.

	Segment 1	Segment 2	Segment 3
Benzene	0.00	11.8	0.00
Xylenes (sum)	0.17	20.2	0.04
Naphthalene	2.36	28.4	0.35
Phenanthrene	7.93	17.8	5.17
Pyrene	2.21	4.59	4.83
Acenaphthene	4.98	7.24	2.24
C6-C10	0.00	3.02	0.00

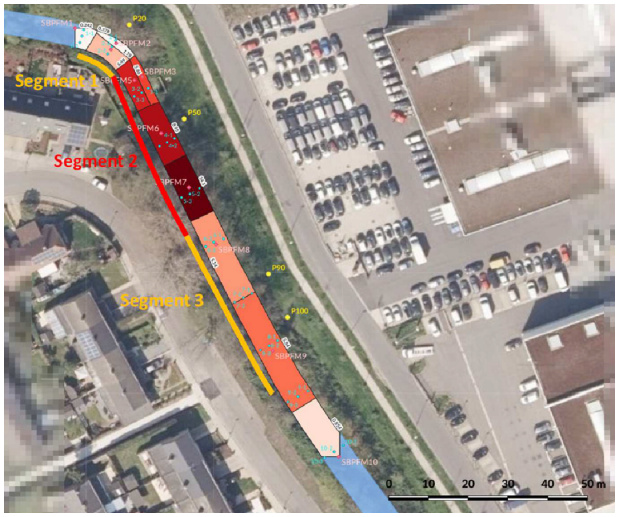

FIGURE 2. INFLUX OF CONTAMINANTS INTO SURFACE WATER FROM GROUNDWATER PER SEGMENT IN MG/M²/DAY)

Aerobic biodegradation is a key part of the Natural Catch^{TAUW} concept and is being tracked in the Lieve Canal project. For this, at three moments in time the water at the mat surface was analysed by a qPCR-test to identify the presence of specific micro-organisms. In addition to anaerobic biodegraders, aerobic biodegraders of BTEX, PAH and alkanes were continuously (BTEX) or temporally (PAH and alkanes) present at the interface in low to moderate numbers (10^1 – 10^4cells/ml), which means aerobic biological degradation is taking place of residual contaminants that pass the adsorbent in the reactive mat in small amounts.

A sustainability assessment on 22 indicators on People, Planet and Prosperity dimensions showed the Natural Catch^{TAUW} is a more sustainable alternative than excavation or smart pump&treat (flow interception), amongst others because of a low carbon footprint, a minimum of nuisance and health & safety issues, low cost and a low impact on air quality and biodiversity.

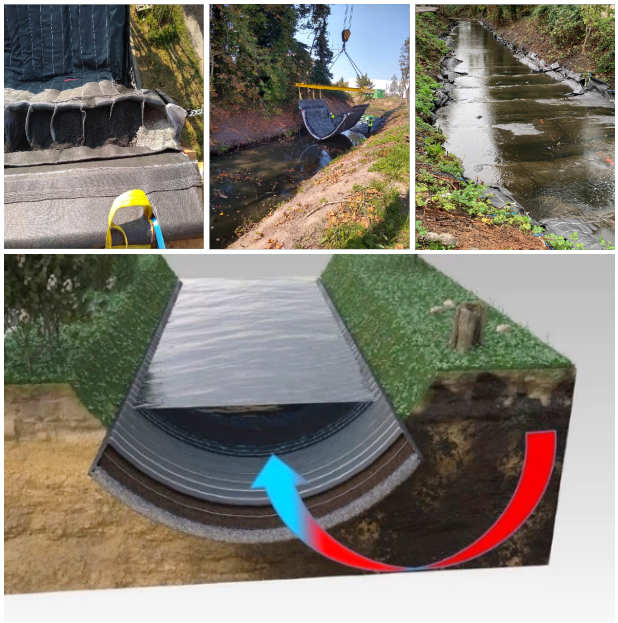

FIGURE 3. PICTURES OF CONSTRUCTION OF REACTIVE MAT IN THE LIEVE AND ARTIST IMPRESSION

For similar situations with draining surface waters threatened by contaminated groundwater with pesticides (e.g. lindane), isomers (e.g. ß-HCH) and other by-products (e.g. chlorobenzenes) and intermediates (e.g. MCB) the Natural Catch^{TAUW} construction can be a sustainable solution. Depending on the site specific circumstances like dimensions of the surface water and specific contaminants, the construction can be adapted.

Conclusions

Nature based solutions are key to control or degrade soil and groundwater contamination, taking into account their sustainable approach (incl. cost-efficiency and low footprint) and the high number of contaminated sites in Europe (> 300,000, of which 300 HCH-sites) that still need to be tackled. A lot of EU-projects on this subject have been initiated the last couple of years, the first results have been booked (like RESANAT) and promising new NBS-approaches are expected within 3-5 years (like MIBIREM).

Acknowledgements

We would like to thank the EU for their funding programs for research and information on sustainable development goals. In addition, we would like to thank our organizations TAUW and DND Biotech for their faith in putting effort and financial means into nature based innovations. We thank our project-partners for doing the same and for the pleasant and constructive cooperation.

INTEGRATED SUSTAINABLE APPROACH TO LINDANE BIODEGRADATION

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Summary

Background/Objectives. Lindane was used in the second half of the 20th century as pesticide, until in 2000 it was banned in the EU. Its production was inefficient as 8 to 12 tons of waste isomers were produced per ton of lindane. These wastes were dumped near the production sites, usually creating uncontrolled landfills. Lindane and its isomers are POPs difficult to biodegrade. The project presented here aims at identifying and studying microbial population and species that can degrade effectively lindane and its isomers as well as testing their efficiency in laboratory and pilot scale trials in order to find a sustainable and cost-effective approach for widespread diffused contamination.

Approach/Activities. A model site in Italy, Colleferro industrial area and Valle del Sacco, has been studied and characterized. Microorganisms have been isolated based on their capacity to degrade lindane and HCH isomers. Both single strains and mixed culture are being tested for in-situ and ex-situ remediation treatments. Microbial community analysis will be performed by a molecular approach, to identify the bacterial and fungal communities and to predict their functional behavior. At a later stage, the ex-situ on-site testing will be conducted using the proprietary pilot testing plant RoboNova®.

Results/Lessons learned. Based on the results of the site characterization and on the aim of the project to define a strategy for bioremediation of lindane and its isomers, it was decided to proceed with isolation of microbiota and strains to be later tested at lab scale and pilot scale using RoboNova®. Three fungal and five bacterial strains have been isolated from cultures grown on medium with HCH as sole carbon source. One microbial community has also been established and is currently being tested at lab scale for its abilities to degrade HCH.

Keywords

Lindane; HCH; isomers; sediments; groundwater; contamination; bioremediation; pilot testing

Introduction

Lindane (γ HCH) was produced and used as a broad-spectrum insecticide and treatment against ectoparasites between 1945 and 2000. Production was inefficient because each ton of Lindane resulted in the production of 8 to 12 tons of waste isomers. These waste isomers were dumped at production facilities and often led to huge uncontrolled landfills. More than 4.8 million tons of HCH-waste were and vastly still are present worldwide. Lindane and the other HCH isomers barely degrade in the environment, bio-accumulate through the food chain, and present a risk to human health and the environment. Lindane and HCH isomers were banned in the EU in 2000 and placed under the Stockholm Convention on POPs in 2009.

A site of interest in Italy, the industrial area of Colleferro and the surrounding Valle del Sacco, has been selected as a case study for the project (Figure 1). At this site, lindane was produced from mid '40s to the late '70s and two disposal waste areas in disuse reported high concentrations of HCH isomers (α, β, γ). In 2005 high concentrations of the beta isomer were detected in cow milk and dairy products from farms nearby. The state of environmental crisis was declared by the Italian government for the area and epidemiological surveillance was implemented. The two disposal areas were studied and characterized; containment plans were put in place to permanently separate the contaminated soil. However, the sediments of the river, as well as agricultural soil, are believed to have absorbed the contaminant, releasing it during flooding events.

The project described here aims at studying the possibilities for the remediation of lindane and its isomers by means of bio-based techniques. For this, both lab and pilot scale tests have been put in place.

FIGURE 1. CASE STUDY SITE OF VALLE DEL SACCO COLLEFERRO

Activities & Results

The two disposal areas have been studied in order to characterize the contamination and its proportions in the soil. In area 1, a "white substance" has been found while excavating the

soil, resulting in about 2.500 m^3 of "white soil" in which the waste isomers accounted for about 50% of its weight, and 1.000 m^3 of contaminated soil, in which HCH accounted for about 10% of the weight (Figure 2). The contaminated soil was moved to a containment facility for permanent storage. In area 2, the contaminated material and underlying soil (70.000 m^3) is currently being moved to a permanent confinement storage as well (Figure 3).

FIGURE 2. EXCAVATION DURING SITE CHARACTERIZATION OF AREA 1

The surrounding agricultural areas are under characterization, as well as surface and groundwater. The sediments of the river should also be studied, as it is believed that they carry attached particles of the contaminants and during floodings they get released in the water and spread.

Soil, water and sediments samples have been taken from this site and used for microbial analysis. From the samples, strains and microbiota have been cultured and studied for their ability to grow on and degrade HCH (α-, β-, and γ-HCH) through their enzymatic activities. Three fungal and five bacterial strains have been isolated from cultures grown on medium with HCH as sole carbon source. One microbial community has also been established and is being tested at lab scale for its abilities to degrade HCH.

Metagenomic analysis will be performed on the isolated microorganisms to identify them and infer their enzymatic abilities. Additionally, the degradation pathways of these strains will be investigated by means of mass spectrometry analysis on the metabolic intermediates and final products obtained during the degradation process.

Next steps

The selected microbes will be tested in mesocosm and pilot scale, to better understand their abilities to decontaminate soil from HCH and define the best treatment protocols. The tests consist of different combinations of amendments (bulking agents, nutrients), inoculation of bacterial and/or fungal biomass, process conditions (water content, pH, temperature, tilling/aeration). Parameters such as contaminants depletion, nutrient consumption, bacterial growth and physical parameters will be measured regularly to compare the performance of the different treatment protocols.

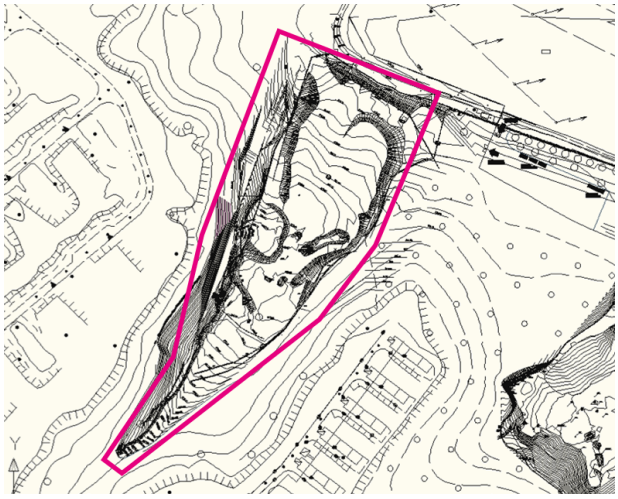

FIGURE 3. PLAN FOR THE PERMANENT STORAGE OF CONTAMINATED SOIL FROM AREA 2

The pilot scale testing will be performed with the best performing protocols from mesocosm scale, using the pilot plant RoboNova® (Figure 4). This innovative system makes it possible to model the interventions on site, working on the specific matrix and field conditions; to perform the monitoring of processes in terms of kinetics of degradation and toxicity of matrices; to measure the environmental impacts (consumption, emissions, waste products) of the technology. Metagenomics will be performed throughout the treatment trials as well, to follow the evolution of the microbial communities while the degradation process advances.

FIGURE 4. ROBONOVA® PILOT PLANT

BIODEGRADATION OF THE LINDAN THROUGH THE USE OF THE DAB TECHNOLOGY

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Summary

The gamma isomer of the hexaclohexane (γ-HCH) or lindane, is an organochlorine compound included in the list of persistent organic pollutants (POP) of the Stockholm Convention. Lindane was produced in Spain in four factories, one of them being INQUINOSA factory in Huesca. Lindane production wastes were dumped, solid and liquid, in an almost non-controlled fashion in the area.

The key to biodegradation of lindane is the removal of the halogen (Cl) atom. During this step, the halogen atoms, which are usually responsible for the toxic and xenobiotic character of the compound is most commonly replaced by hydrogen or a hydroxyl group. Gram negative bacteria (such as our Rhodopseudomonas and nitrifying bacteria) and gram positive bacteria (as our three Bacillus species) will be responsible of carrying out the degradation in this assay. The method is via Preparation tanks (aerated and with controlled temperature) being used to acclimatize the product to lindane and generate enzymes, favoring bacterial growth in a hostile environment through an adaptation mechanism.

Three efficiency tests were carried out at the landfill facilities during our collaboration with the UELA.

In the first one, a stable bacterial population was generated, achieving a bioaugmented high-yield product with evolved transfer over several generations. Adaptive and degradative efficacy of lindane was verified.

During the second test, trials were extended to 16 individualized tests, generating different scenarios with varying conditions, bacteria mix (using Blue Planet bacteria), different substrates, and inoculums.

Finally, the third test consisted of a Biopiles system to create aerobic, anaerobic, and mixed environments, using different substrates, seeking effective methods of decontamination of soils.

All the tests carried out, both aerobic and anaerobic, presented a significant reduction in total HCH using Blue Planet technology.

Keywords

Hexacolohexane; lindane; biodegradation; bacteria; decontamination; soils; biopiles.

Introduction

Lindane is classified as “Moderately Hazadous” by the World Health Organization, and its international trade is restricted and regulated under the Rotterdam Convention on Prior Informed Consent. In 2009, the production and agricultural use of lindane was banned under the Stockholm Convention on persistent organic pollutants. There is documented work showing biodegradation of lindane by a variety of bacteria, including *Pseudomonas putida* (GC-MS Study on Microbial Degradation of Lindane, International Journal of Applied Chemistry ISSN 0973-1792 Volume 6 Number 3 (2010) pp. 363–366). The key to the biodegradation of lindane is removal of the halogen (Cl) atom. During this step, the halogen atoms, which are usually responsible for the toxic and xenobiotic character of the compound is most commonly replaced by hydrogen or a hydroxyl group. Halogen removal reduces both recalcitrance to biodegradation and the risk of forming toxic intermediates during subsequent metabolic steps. A long list of gram negative (such as our *Rhodopseudomonas* and nitrifying bacteria) and gram positive (our three *Bacillus* species) bacteria exist that degrade HCH. Different aerobic and anaerobic bacteria have been found to be capable of using halogenated compounds as a growth substrate. These include:

- *Bacillus sp*. and *Pseudomonas sp.* (Tu C.M. Utilization and degradation of lindane by soil microorganisms. Arch. Microbiol. 1976;108:259–263)
- *Bacillus circulans*, *Bacillus brevis* (Metabolism of &-hexachlorocyclohexane by Arthrobactercitreus strain BI-100: Identification of metabolites. J. Gen. App. Microbiol. 2000;46:59–67)
- *Pseudomonas aeruginosa* and *Pseudomonas sp.* (Determination of organochlorine pesticides in agricultural soil with special reference to γ-HCH degradation by Pseudomonas strains. Bioresour. Technol. 2000;46:59–67)
- *Nitrosomonas europaea* (Oxidation of monohalogenatedethanes and n-chlorinated alkanes by whole cells of Nitrosomonas europaea, Journal of Bacteriology)

While BluePlanet has not specifically tested ACF for its lindane digestion capability, the pathways for degradation for some of the individual species are noted in the literature. This gives us considerable confidence in the ability of ACF to successfully bioremediate lindane. The key is using tanks to

acclimate ACF to lindane. This will be a first application with the objective of evaluating the material needs and means necessary for the development, proving its effectiveness with different load.

Materials and methods

1. Study area

Lindane was produced in Spain in four factories: two of them located in the Basque Country, a third one in Galicia, and a fourth one in Huesca province, the INQUINOSA factory. Inquinosa factory synthesised lindane from 1975 to 1988, ceasing its commercial activity permanently in 1992. Throughout that period, it produced an estimated 150.000 tonnes of waste with high content in HCH and other organochlorine compounds. Lindane production wastes were dumped, solid and liquid, in two sites in an almost non-controlled way: Sardas dumping site and Bailín dumping site. From 1984 till 1989, Inquinosa used Bailin dumping site for the disposal of its wastes and from 1978 till 1983, it used Sardas dumping. The latter will be the study area of this essay.

2. Description of treatment system

Test 1

Two bioreactors (CUL 1 and CUL 2) were available for the generation of a stable bacterial population in the presence of small concentrations of HCH. These had aeration, recirculation and a temperature of 27 ºC. CUL 1 was used to condition the bacterial population and was made up of 420 l of water, 20 l of leachate contaminated with lindane, 3 kg of clogged activated carbon and doped perlite (as a contamination support), 4 gallons of ACF-32 and 4 pounds AD Activator4. CUL2 was used with the same additions of support substrates and leaching, and bacteria adapted from CUL 1 were incorporated into it. 4 cycles of approximately 1 week each were carried out. In each of the cycles samples were taken from the top and the bottom of the liquid at the beginning and the end of the cycle.

A complementary test was performed with the liquid resulting from the previous test. To do this, 400 l were poured into a storage pond in three cycles one week apart and samples were taken for analysis: an initial one, after a week had passed in cycle 2, and another week in cycle 3.

Test 2:

In test 2, 4 bioreactors (CUL 1, CUL 2 and CUL 3) were used, which were the basis for the subsequent treatment in bottles. The bioreactors were designed as follows:

CUL1 and CUL 2 were the same as those of test 1. CUL 3 was an anaerobic bioreactor with a 200 l capacity and a temperature of 27 ºC to which 1 Gal ACF-32 + 1 Lb AD Activator was added.

After the stage of growth and adaptation of the bacterial consortia, a new stage of trials began. A series of laboratory tests were started with a small volume format to control the degradative activity and tolerance in different oxidation conditions and without the addition of nutrients as shown in the following:

- Bottle 1: 1 l of CUL1, 1 l of a-HCH leachate, 1 l of clean water. With air and agitation at 200 rpm.
- Bottle 2: 1 l of CUL1, 1 l of g-HCH leachate, 1 l of clean water. With air and agitation at 200 rpm.
- Bottle 3: 1 l of CUL1, 1 l of Romanian HCH leachate, 1 l of clean water. With air and agitation at 200 rpm.
- Bottle 4: 1 l of CUL1, 1 l of BT1 leachate, 1 l of clean water. With air and agitation at 200 rpm.
- Bottle 5: 1 l of CUL1, 1 l of a-HCH leachate, 1 l of clean water. Without air and without agitation.
- Bottle 6: 1 l of CUL1, 1 l of g-HCH leachate, 1 l of clean water. Without air and without agitation.
- Bottle 7: 1 l of CUL1, 1 l of Romanian HCH leachate, 1 l of clean water. Without air and without agitation.
- Bottle 8: 1 l of CUL1, 1 l of BT1 leachate, 1 l of clean water. Without air and without agitation.
- Bottle 9: 1 l of CUL3, 1 l of a-HCH leachate, 1 l of clean water. Without air and without agitation.
- Bottle 10: 1 l of CUL3, 1 l of g-HCH leachate, 1 l of clean water. Without air and without agitation.
- Bottle 11: 1 l of CUL1, 3 l of Romanian HCH leachate, 1 l of clean water. Without air and without agitation.
- Bottle 12: 1 l of CUL3, 1 l of BT1 leachate, 1 l of clean water. Without air and without agitation.
- Bottle 13: 1 l of a-HCH leachate, 2 l of clean water. Without air and without agitation.
- Bottle 14: 1 l of g-HCH leachate, 2 l of clean water. Without air and without agitation.
- Bottle 15: 1 l of Romanian HCH leachate, 2 l of clean water. Without air and without agitation.
- Bottle 16: 1 l of BT1 leachate, 2 l of clean water. Without air and without agitation.

Samples of all of them were taken after finishing the treatment.

Test 3

For the work with the Biopiles, different environments were built in to separately assess the effectiveness with Aerobic techniques, Anaerobic techniques and even an intermediate term to test with Mixed Environment Biopiles (aerobic-anaerobic). In total five (5) Aerobic cells, seven (7) Anaerobic cells and two (2) Mixed cells. (14 units total).

The backfill of each biopila is made up of a mixture of materials from different sources: sediments from

the dismantled HCH cell and from cleaning ditches and sand traps (VV Sediments), construction and demolition waste (RCD's), sewage sludge (WWTP sludge), sludge from the Sardas pond (Sardas sludge) and organic amendments (rice husks and sludge), whose purpose is to assess the feasibility of degradation with different polluting loads and different amendments and microorganisms.
The conditions of the biopiles are described in Table 1 and Table 2, respectively:

TABLE 1. COMPOSITION OF AEROBICAL, ANAEROBIAL

Condition	Biopiles	Operative
Aerobical	AER-1, AER-3, AER-5, AER-7, AER-9	In Concrete Structure, with Aeration
Anaerobical	RED-2, RED-4, RED-6, RED-8, RED-10, RED-11, RED-12	In an IBC (No Aeration, No Turning, No Sun)
Mixed	MIX-13, MIX-14	In Concrete Structure, Periodical Aeration

TABLE 2. COMPOSITION OF AEROBICAL, ANAEROBIAL AND MIXED BIOPILES

Composition (kg)	AER-1	AER-3	AER-5	AER-7	AER-9
Sediment VV	360	360	360	450	450
RCD Inquinosa	840	840	630	630	700
Manure	---	---	40	40	---
WWTP Sludge	60	60	100	70	70
Sardas Pond Sludge	---	---	60	60	60
Rice Husk	Yes	Yes	Yes	Yes	Yes
Leachate					
CUL-1	Yes	Yes	---	Yes	Yes
CUL-3	---	---	---	---	---
Nutrients	Yes	Yes	Yes	Yes	Yes
Inorganic	Yes	---	---	---	Yes

Composition (kg)	RED-2	RED-4	RED-6	RED-8	RED-10	RED-11	RED-12
Sediment VV	720	720	630	720	720	720	720
RCD Inquinosa	420	420	420	420	420	420	420
Manure	---	---	40	40	40	80	---
WWTP Sludge	90	90	100	70	70	---	100
Sardas Pond Sludge	---	---	60	60	60	60	60
Rice Husk	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Leachate							
CUL-1	---	---	---	---	Yes	---	---
CUL-3	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Nutrients	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Inorganic	Yes	---	---	---	---	---	---

Composition (kg)	Mix-13	Mix-14
Sediment VV	540	540
RCD Inquinosa	560	560
Manure	40	40
WWTP Sludge	70	50
Sardas Pond Sludge	60	60
Rice Husk	Yes	Yes
Leachate		
CUL-1	Yes	Yes
CUL-3	Yes	Yes
Nutrients	Yes	Yes
Inorganic	Yes	No

3. Analytical methods

- Determination of semi-volatile organic compounds (VOC) in soils samples by capillary gas chromatography with mass detector

The foundation of Gas-Mass Chromatography consists in the separation, by the retention at different times in the phase of the column, of each one of the Composed of complex mixtures. Being analyzed, with great precision, in the detector the characteristic ions obtained by the rupture of the molecules on contact with the power source (filament), depending on the signal emitted by the ratio mass-charge (m/z). Allowing their qualification and quantification in relation to calibration standards. The semi-volatile organic compounds, a previous extraction of the compounds with organic solvents in a SOXTEC. The extract is pre-concentrated before being injected into the chromatograph. The injection systems are coupled to the gas chromatograph with columns capillaries and a mass detector. For the identification of the compounds, we use the retention time in patterns of pure compounds as well as the spectra of corresponding masses. For quantification, pure standards of known concentration were used.

The presence of the following VOC was determined:

- α (a)-HCH
- β (b)-HCH
- γ (g)-HCH
- δ (g)-HCH
- ε (e)-HCH

Results and discussion

Test 1

Table 3 shows the HCH results after each contribution of contaminated substrate in CUL 2.

TABLE 3. REDUCTION (%) OF HCH IN CUL 2

Cicle	1	2	3	4
a-HCH	65,0	97,1	93,9	99,9
b-HCH	47,6	100,0	99,7	100,0
g-HCH	58,5	98,7	99,9	100,0
d-HCH	74,5	95,7	81,7	100,0
e-HCH	65,9	90,1	76,7	99,2
TOTAL HCH	61,6	97,8	91,4	99,9

In relation to the reduction of HCH in CUL 1, as shown in Table 4, the yields are lower than CUL 2, except in cycle 4, which were maintained for a longer time. There are even differences in the type of isomers, with increases with respect to day zero, although they are not with respect to some intermediate sampling.

TABLE 4. REDUCTION (%) OF HCH IN CUL 1

Cicle	At the top 1	At the bottom 1	At the top 2	At the bottom 2	At the top 3	At the bottom 3	4
a-HCH	55,4	56,1	53,7	87, 2	65,2	67,7	98,0
b-HCH	-163,4	-84,0	98,6	99,4	97,4	97,6	81,3
g-HCH	51,7	50,2	84,8	83, 2	94,1	92,9	98,5
d-HCH	41,8	42,2	-31,2	66, 6	-102,2	-92,6	98,5
e-HCH	28,0	33,3	-96,5	80,3	-87,2	-74,5	84,0
TOTAL HCH	15,1	27,0	41,5	85,0	27,1	30,6	97,7

In the complementary test (Table 5) the degradation of all the components can be observed with an overall performance of around 90%.

TABLE 5. CONCENTRATION (MG/L) OF HCH ISOMERS IN COMPLEMENTARY TEST

	Cicle 1	Cicle 2	Cicle 3
a-HCH	82,5	0,9	5,3
b-HCH	203,8	25,8	19,4
g-HCH	93,3	0,2	3,5
d-HCH	364,4	257,0	2,1
e-HCH	71,3	126,7	63,8
TOTAL HCH	815,5	410,7	94,2

Test 2

Figure 1, Figure 2 and Figure 3 shown the results of reduction of HCH isomers in bottles with different conditions.

The best results were obtained from the aerobic by-product generated in CUL1 when aeration and agitation were applied, reaching almost complete degradation of the HCH of the different types used for the tests (Bottle 1, Bottle 2, Bottle 3 and Bottle 4). Furthermore, in those with the contribution of CUL1, under anoxic conditions and without agitation (Bottle 5, Bottle 6, Bottle 7 and Bottle 8), the results are repeated with very high efficiency.

In tests conducted without providing Blue Planet bacteria (Bottle 3, Bottle 14, Bottle 15, and Bottle 16), the reductions were irrelevant, and the results would be conditioned by the activator as a nutrient supply, regardless of incorporating freeze-dried bacteria.

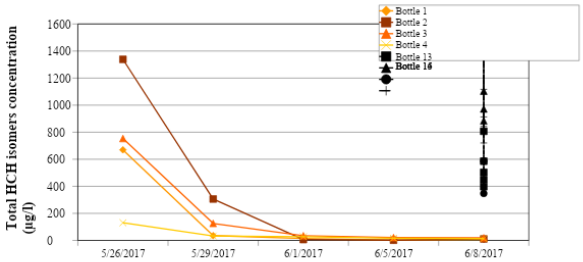

FIGURE 1. REDUCTION OF TOTAL HCH ISOMERS (AEROBIC CUL 1)

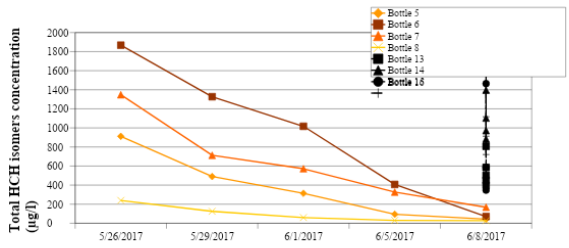

FIGURE 2. REDUCTION OF TOTAL HCH ISOMERS (ANOXIC CUL 1)

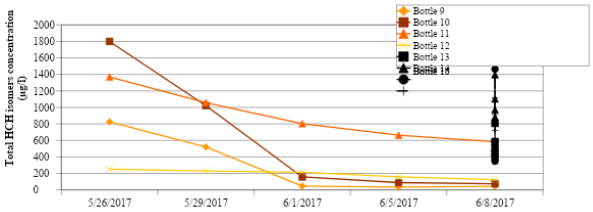

FIGURE 3. REDUCTION OF TOTAL HCH ISOMERS (CUL 3)

Test 3

Table 6 shows the total concentration of HCH in the aerobic biopiles for approximately two months. As can be seen, the highest concentration is found in the AER-9 biopile on 10/16/17, reaching 3 mg/kg after two months. Similarly, in the rest of aerobic biopiles, the final concentration is around 2-5 mg/kg of total HCH.

TABLE 6. TOTAL HCH CONCENTRATION (MG/KG) IN AEROBIAL BIOPILES WITH THE TIME.

	16/10/17	25/10/17	8/11/17	22/11/17	5/12/17	21/12/17	Reduction
AER-1	59,86	37,15	16,00	18,22	30,44	2,76	95%
AER-3	59,86	20,69	6,47	19,03	11,10	5,80	90%
AER-5	45,05	11,89	4,56	6,97	5,51	2,18	95%
AER-7	45,27	23,51	3,47	6,95	4,94	2,54	94%
AER-9	165,38	45,21	33,70	12,18	11,33	3,25	98%

Table 7 shows the total concentration of HCH in the anaerobic biopiles. In the RED-10 biopile, the initial concentration is the highest, however, the lowest final concentration is also found in this biopile, with a 99% reduction. The percentage reduction for the rest of the anaerobic biopiles is between 74-94%, except for the RED-6 biopile, which is 16%.

The reductions in the mixed biopiles decrease progressively with a reduction percentage of 98 and 97% for MIX-13 and MIX-14, respectively.

TABLE 7. TOTAL HCH CONCENTRATION (MG/KG) IN ANAEROBIAL BIOPILES WITH THE TIME

	09/10/17	13/10/17	20/10/17	03/11/17	17/11/17	30/11/17	15/12/17	Reduction
RED-2	94,08	113,67	29,34	103,98	60,66	30,63	11,50	88%
RED-4	94,08	23,41	35,08	64,51	63,49	19,44	11,14	88%
RED-6	107,28	86,79	70,52	41,30	14,00	41,84	89,98	16%
RED-8	169,13	75,29	37,98	58,55	91,80	45,41	44,09	74%
RED-10	1094,37	151,90	91,27	86,39	165,71	51,43	8,43	99%
RED-11	406,15	160,29	22,55	106,39	49,26	42,86	23,05	94%
RED-12	178,86	104,46	29,20	47,17	68,33	38,74	32,48	82%

TABLE 8. TOTAL HCH CONCENTRATION (MG/KG) IN MIXED BIOPILES WITH THE TIME.

	17/10/17	25/10/17	8/11/17	22/11/17	5/12/17	21/12/17	Reduction
MIX-13	267,45	116,76	32,15	27,10	7,02	4,57	98%
MIX-14	75,98	15,69	22,92	8,12	3,14	2,57	97%

Conclusions

The aerobic results present a reduction of over 90% of the total HCH in all the tests and with the contribution of Blue Planet products adapted in the CUL, as well as with the contribution of sludge and cow manure.

It is perhaps in the anaerobic part, where it is seen as if by itself the inoculums of mud and manure get the worst results (RED-6, RED-8 and RED-12), being the best results in which Blue Planet bacteria are applied in conjunction with the rest of the products (RED-10, MIX-13 and MIX-14).

Finally, it can be affirmed with the results obtained the effectiveness and efficiency of the treatment through the adaptive method of the Blue Planet for the degradation of lindane in polluted waters, both in aerobic conditions and with energy expenditure and by anaerobic conditions with the presence of adapted bacteria.

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LAB STUDIES LEADING TO DECISION-MAKING FOR IN SITU BIOREMEDIATION OF ORGANOHALIDES

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Summary

Bioremediation is an economical and environmentally friendly technology that can degrade a wide variety of persistent pollutants, such as pesticides, hexachlorocyclohexanes (HCHs), and chlorinated benzenes, with high specificity and efficiency. In this lab study, we evaluated the feasibility of applying bioremediation to the polluted groundwater of Sardas (Spain) and tested different conditions to select the best treatment to apply at field scale. Microcosms experiments were set up in different conditions and satisfactory bioremediation results were achieved. Aerobic treatment biostimulated with phosphate presented degradation of less chlorinated compounds (monochlorobenzene, dichlorobenzene and trichlorobenzene) in short period of time (3 d) and the degradation of the most chlorinated pollutants (tetrachlorobenzenes, pentachlorobenzenes, hexachlorobenzenes and hexachlorocyclohexanes) were almost completely achieved after 15 d. In addition, the anaerobic treatment biostimulated with phosphate led to the degradation of the most chlorinated compounds including all hexachlorocyclohexanes isomers, but no dechlorination of the less chlorinated pollutants was obtained. The presence of aerobic and facultative anaerobic bacteria known to dechlorinate these pollutants were identified in the different stages of the lab studies. Our findings pointed out that oxygen and phosphate integrate a suitable biostimulation alternative to be carried out in the alluvial groundwater.

Keywords

Aerobic, anaerobic, biostimulation, microcosms, microorganisms, lindane, treatment train.

Introduction

For the decontamination of aquifers, bioremediation processes, that is the use of the capacity of microorganisms to degrade pollutants, have emerged as an alternative given their ability to degrade them below the established legal limits. Depending on the bioremediation treatment to be performed, these contaminants can be used by microorganisms as a carbon source or energy source, or they can be degraded co-metabolically, without the microorganisms obtaining any metabolic benefit.

Halogenated pollutants generally have a low solubility and are denser than water, so that when they reach the aquifer they accumulate in the form of a micelle and slowly dissolve forming pollution plumes that can reach hundreds of meters. For the bioremediation of these compounds in anoxic aquifers, the key microorganisms are organohalide respiring bacteria (OHRB). These bacteria use these toxic compounds to breathe, generating less chlorinated compounds than the originals, and thus transforming the toxicological and physicochemical properties of the parent compound. By this reaction the chlorinated compound is transformed into smaller forms, making it susceptible to be oxidized by other microorganisms aerobically as previously reported (Trueba-Santiso et al., 2022) or reduced again by other OHRB organisms under anaerobic conditions.

To study and propose a feasible bioremediation strategy to be implemented at the alluvial groundwater of Sardas, lab studies were performed to conduct biodegradation strategies within microcosms under aerobic, anaerobic, and sequential treatment conditions. The purpose of this is to determine the degrading biological activity of the aquifer, to identify the microbial population present and to evaluate the effects of the biostimulation with nutrients in the different bioremediation strategies experimented to finally decrease the impact of chlorinated pollutants in the environment.

Methodology

After analysing the hydrogeological parameters of the polluted site, four wells were chosen to perform the microcosms, they were in the intermediate zone of the plume. All microcosms were prepared in sterile glass bottles with a volume of 100 mL of groundwater. In the first microcosms experiments, three wells were studied, PS16E, PS16D and PS16G at 13ºC. PS16E well was also studied at 25ºC. The treatments studied were (i) natural attenuation (named ATN): contained only well water; (ii) anaerobic biostimulation (named BS-AN): contained well water plus 3 mM lactate; (iii) aerobic biostimulation (named BS-OX): contained well water plus oxygen to saturation; (iv) killed control (named control): contained water from the well plus 1 mL of 4 M HNO_3, obtaining a final pH <2, to inactivate the microorganisms it may

contain. Samples were taken for organochlorine analysis at: the initial time, 15 d, 30 d, 55 d and 85 d. After 85 d, the anaerobically biostimulated microcosms were being aerobically stimulated with the addition of oxygen (+1.4 bar) to study a possible treatment train (BS-AN-OX sequential treatment), and samples were taken at 11 d, 25 d and 53 d.

In the second microcosms experiment set up PS16I and PS16E were the selected wells to be studied. All microcosms were incubated at 13ºC and the anaerobic and aerobic biostimulation treatments were performed with three alternative of nutrients amendment each: (i) 20 mg/L of NaH_2PO_4; (ii) 500 mg/L of $(NH_4)_2CO_3$; and (iii) 20 mg/L of NaH_2PO_4 plus 500 mg/L of $(NH_4)_2CO_3$. Samples were taken for organochlorine analysis at: the initial time, 3 d, 7 d and 15 d. After 15 d, extra microcosms anaerobically biostimulated were aerobically stimulated with the addition of oxygen to study a possible treatment train (BS-AN-OX sequential treatment), and samples were taken again at 3 d, 7 d and 15 d. Microcosms were also prepared in parallel for molecular biology analysis of each condition at the end of each treatment.

Results and discussion

The results obtained in the three wells are very similar. Natural attenuation microcosms presented degradation but with a very low degradation rate compared to the biostimulated treatments. The microcosms treated at two different temperatures did not present significant differences between 13°C and 25°C, which indicates that the degrading microorganisms are adapted to 13°C, the average temperature of the aquifer.

Aerobic treatment (BS-OX) showed better results at lower times, for less chlorinated organic compounds compared to anaerobic treatments at the same time. The decrease in concentrations of the isomers of dichlorobenzene (DCB) is presented in Figure 1A. In addition to these results at short period of time, long term aerobic treatment can lead to an almost complete degradation of HCH (Figure 1B).

Anaerobic treatment (BS-AN) shows the best results for bioremediation of the most chlorinated organic compounds such as the HCH family, in a short period of time. We can observe that the degradation of the γ-HCH isomer is completed after 15 days (Figure 1B). The ε-HCH isomer was detected in very low concentrations and follows the same degradation trend. Treatment train showed that it is possible to increase the elimination of all compounds. These results are especially relevant to eliminate less chlorinated compounds (benzene, MCB, DCB and TCB) after the first anaerobic treatment, where no elimination was detected (Figure 1).

The second microcosms set up experiments were performed with aerobic and anaerobic biostimulation treatments adding phosphate (P), nitrate (N), and phosphate plus nitrate (P+N) showed the same results in both wells studied. To simplify, only well PS16E is displayed (Figure 2). Aerobic biostimulation (BS-OX) showed the same tendency among all the less chlorinated compounds, is faster in biostimulated microcosms with P, and with the mixture of P+N treatments. In general, 100% elimination is achieved within 3-7 days. Moreover, the addition of nutrients as P and P+N improved the elimination of HCH family although it was not complete in 15 days. Anaerobic biostimulation (BS-AN) shows that for the most chlorinated organochlorine compounds, HCH family and the sum-HCH a faster degradation is achieved than with aerobic treatments and when P or P+N is added the degradation is even faster. This increment in the degradation rate is not observed when only N is added to any of the treatments

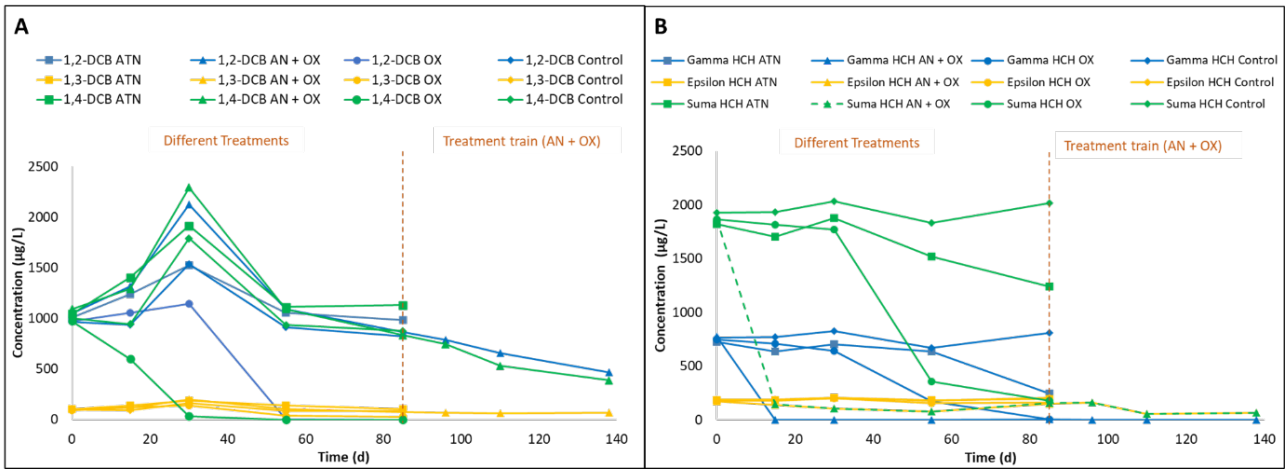

FIGURE 1. THE RESULTS OF MICROCOSMS STUDIES WITH DIFFERENT TREATMENT STRATEGIES APPLIED ON PS16E WELL GROUNDWATER AT 13ºC IS PRESENTED IN THE PANEL: (A) EVOLUTION OF THE CONCENTRATIONS OF DICHLOROBENZENE ISOMERS AND (B) THE EVOLUTION OF Γ-HCH, E-HCH ISOMER CONCENTRATIONS AND SUM-HCH.

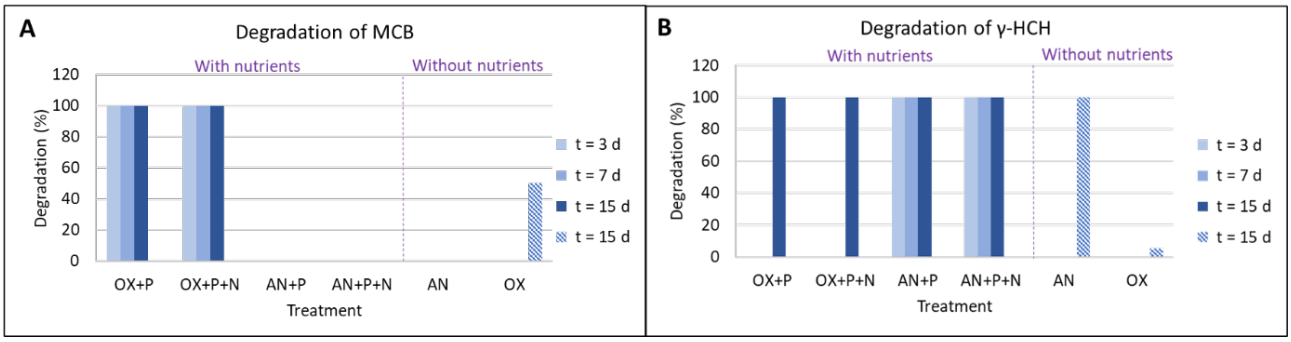

FIGURE 2. MICROCOSMS RESULTS WHEN NUTRIENTS ARE ADDED TO THE DIFFERENT BIOSTIMULATION TREATMENTS ON PS16E WELL GROUNDWATER AT 13ºC COMPARED TO PREVIOUS MICROCOSMS WITHOUT NUTRIENT ADDITION. (A) PERCENTAGE OF DEGRADATION OF MCB, (B) PERCENTAGE OF DEGRADATION OF Γ-HCH.

When we combine both lab studies, we can clearly see how the addition of phosphate to the aerobic treatment improves the percentage of degradation and achieves a degradation rate of 100%, in 3 days, for the less chlorinated. Additionally, the same was observed in the HCH family, for the anaerobic treatment with P. In addition, in the aerobic treatment after 15 days with nutrients, elimination yields over 60% of the sum-HCH are reached, including the degradation of ε-HCH, nevertheless, the anaerobic biostimulation without nutrients is greater than 90% for the HCH family.

A great bacterial diversity is detected in all samples from the wells, both from the initial samples and from the samples at the end of the biostimulation treatments. In aerobic biostimulation treatments, *Proteobacteria* was mainly identified, the genera that have been detected in greater relative abundance are *Pseudomonas* and *Acidovorax* (Field & Sierra-Alvarez, 2008). These microorganisms have been identified as responsible for the biodegradation of the less chlorinated chlorobenzenes. Under anaerobic conditions, the relative abundance of the phyla *Firmicutes* and *Chloroflexi* increases. The bacteria of these phyla are very slow growing, so their relative abundance with respect to aerobic bacteria is lower, but the genera *Dehalococcoides* and *Dehalogenimonas* are detected, which have been described in the literature as degraders of chlorobenzenes. There are no significant differences in the distribution of populations between nutrient and non-nutrient studies. The presence of nutrients affects the rate of degradation but did not present a shift in the population present in this study.

Conclusions

Bioremediation proved to be a suitable strategy to treat the alluvial groundwater of Sardas with the presence of autochthonous degrading microbes. The microcosms set up results performed showed that there is biological degrading activity in all the different treatments applied (natural attenuation, aerobic, anaerobic, treatment train and addition of nutrients) and this capability increases with the appropriate combination of oxygen, phosphate and lactate depending on the bioremediation strategy. Therefore, we conclude that in this case, aerobic biostimulation with phosphate as nutrient is the best strategy to applying in real scale for a more integrated degradation treatment.

Acknowledgements

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SOURCE ALLOCATION AND DEGRADATION EVALUATION OF HCHS WITHIN A CONTAMINATED AQUIFER USING COMPOUND-SPECIFIC STABLE CARBON ISOTOPE ANALYSIS (CSIA)

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Summary

Hexachlorocyclohexane (HCH) isomers are pollutants of particular concern because of their widespread distribution in the environment, toxicity and persistence. Especially at sites of pesticide production, formulation and dumping, significant soil and groundwater pollutions of HCHs have been detected. For cost-efficient and highly productive remediation strategies of contaminated sites, it is necessary to investigate pollutant sources and sinks. In recent years, compound-specific stable isotope analysis (CSIA) has gained more and more attention as a tool for characterizing and assessing contaminant sources and in situ degradation of organic pollutants, respectively.

The applicability of compound-specific stable carbon isotope analysis (CSIA) for assessing degradation of hexachlorocyclohexane (HCH) isomers was investigated in a contaminated aquifer at a former pesticide processing facility. A CSIA method was developed and tested for efficacy in determining carbon isotope ratios ($^{13}C/^{12}C$) of HCH isomers in groundwater samples using gas chromatography - isotope ratio mass spectrometry (GC-IRMS).

The carbon isotope ratios of HCHs confirmed contaminant source zones at former processing facilities, a storage depot and a waste dumpsite. This finding was confirmed by the concentration patterns of the contaminants and historical information.

The ^{13}C-enrichment in HCHs provided evidence for degradation of HCHs especially downstream of the contaminant source zones. CSIA from monitoring campaigns in several years revealed temporal trends in HCH degradation. Thus, the impact and progress of natural attenuation processes could be evaluated within the investigated aquifer. Conservative calculations based on the Rayleigh equation approach yielded extends of HCH degradation ranging from 30 to 86%. Moreover, time- and distance-dependent in situ first-order degradation rate constants were estimated.

In summary, our study highlights the applicability of CSIA for evaluating sources and sinks of HCHs within contaminated aquifers located at sites of pesticide production, formulation and dumping.

Keywords

Isotope, CSIA, HCH, source allocation, degradation evaluation

Introduction

One of the most promising tools for monitoring *in situ* degradation of organic contaminants in aquifers is compound-specific stable carbon isotope analysis (CSIA) (US-EPA, 2008, Thullner *et al.*, 2012, Fischer *et al.*, 2016). Molecules with light carbon isotopes (^{12}C) in the reactive position require less energy for bond cleavage and, thus, tend to be degraded faster than molecules containing a heavy carbon isotope (^{13}C), resulting in an ^{13}C-enrichment in the remaining stock of the pollutant. This process is called stable isotope fractionation and can be detected via changes in carbon isotope ratios ($^{13}C/^{12}C$, most commonly given as $\delta^{13}C$) of a pollutant towards more positive $\delta^{13}C$-values. Therefore, CSIA allows for the assessment of pollutant degradation based on the degree of carbon isotope fractionation observed at a contaminated field site.

In laboratory studies, significant changes in carbon isotope ratios have been observed for HCH biodegradation under both oxic and anoxic conditions. Anaerobic degradation exhibited higher carbon isotope fractionation of HCHs than aerobic degradation (Badea *et al.*, 2011; Badea *et al.*, 2009; Bashir *et al.*, 2013). However, limited knowledge exists on the applicability of CSIA for the evaluation of *in situ* degradation at HCH-contaminated field sites.

In our study, CSIA was applied for assessing biodegradation of HCHs in a contaminated aquifer. Besides hydrogeochemical parameters and pollutant concentrations, carbon isotope ratios ($^{13}C/^{12}C$) of HCHs were measured for three monitoring campaigns, in order to determine the progress and sustainability of HCH biodegradation. A comprehensive description of our study is given in (Bashir *et al.*, 2015).

Materials and Methods

The field site is located in the area of a former pesticide formulating plant that included both a formulation site and a packaging facility. As known from historical information, HCH was not produced

on-site, but technical HCH was purchased from suppliers and γ-HCH was purified for use in pesticide formulation. HCH contamination of soil and groundwater were mainly caused by losses of HCH-containing raw materials and products during purification, pesticide formulation and storage as well as irrigation and dumping of production-related wastes.

For the CSIA monitoring, 13 groundwater wells were sampled in first monitoring campaign and 15 wells in second and third monitoring campaign, respectively (Figure 1). Wells 1 and 3 were established before the second monitoring campaign, in order to monitor the pollutant distribution in more detail within the groundwater upstream flow and the western fringe of the contaminant plume.

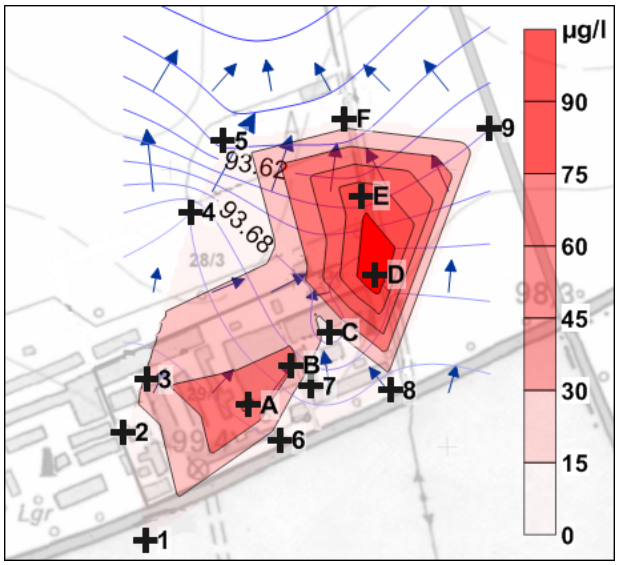

FIG. 1:DISTRIBUTION OF HCHS (SUM OF CONCENTRATIONS OF HCH ISOMERS [MG/L] IN THIRD MONITORING CAMPAIGN) AND GROUNDWATER FLOW DIRECTION
(blue arrows) within the upper aquifer of the investigated field site. The main parts of the contaminant plume are: upstream flow (well 1), central flow (wells A to F), western fringe (wells 2-5), eastern fringe (wells 6-9).

For CSIA of HCHs, two 1L groundwater samples of each well were extracted three times with 30 mL dichloromethane in a separating funnel. The DCM extracts obtained from the two groundwater samples were combined and dried with anhydrous sodium sulfate. The combined DCM extracts were reduced to approximately 1 mL using a rotary evaporator. The extraction procedure did not result in significant changes in carbon isotope ratios of HCHs. CSIA of HCHs were performed by gas chromatography - isotope ratio mass spectrometry (GC-IRMS), using a system described elsewhere (Ivdra *et al.*, 2014). The carbon isotope ratios ($^{13}C/^{12}C$) of HCHs were reported in the delta notation ($\delta^{13}C$) relative to the international standard Vienna Pee Dee Belemnite (VPDB) according to Eq. 1.

$$\delta^{13}C_{sample} = \frac{R_{sample}}{R_{standard}} - 1 \quad (1)$$

R_{sample} and $R_{standard}$ are the $^{13}C/^{12}C$ ratios of the sample and VPDB, respectively. The $\delta^{13}C$-values were reported in per mil (‰).

Results

Since δ-HCH was the predominant HCH-isomer, most conclusions on sources and biodegradation of HCHs could be derived from $\delta^{13}C$-values of δ-HCH in conjunction with its concentrations (Figure 2).

The $\delta^{13}C$-values of HCHs confirmed three distinct contaminant source zones at the field site, which were indicated by concentration data. Based on historical information, the source at well A can be considered to result from contamination at former processing facilities and the source at wells D and E from contamination at the former dump site of HCH wastes (Figure 3). Moreover, a distinct γ-HCH source at well 3 seemed to originate from contamination at a former storage depot (Figure 3). Since γ-HCH was the predominant HCH isomer, it can be concluded that it was stored in the vicinity of well 3 after purification of technical HCH.

The $\delta^{13}C$-values of HCHs provided evidence of HCH biodegradation downstream of the HCH source zones at the former processing facilities and dump site, revealing that biodegradation contributed to the natural attenuation of HCHs within the investigated aquifer (Figure 3). However, in some cases the decrease in concentration of HCHs was caused by physical processes. Since sorption and evaporation of HCHs can be neglected due to both the low organic matter content in the aquifer's matrix (0.014%) and the low tendency of volatilization of HCHs from water (Sahsuvar et al., 2003), dispersion, dilution and HCH recharge from the unsaturated zone into the groundwater are likely the most relevant physical processes influencing the concentration of HCHs within the aquifer.

HCHs showed a decrease in concentrations over time concomitant with more positive $\delta^{13}C$-values of HCHs, indicating that the contribution of biodegradation to natural attenuation of HCHs increased over time. At few wells, HCHs displayed an inconsistent relationship between changes in $\delta^{13}C$-values of HCHs and changes in concentrations. In those cases, trends of concentrations of HCHs could provide information on the overall natural attenuation or recharge of HCHs but only limited indications for biodegradation. In addition, CSIA could more precisely reveal whether biodegradation contributed to natural attenuation of HCHs and, therefore, provided considerable information on the fate of pollutants at the field site.

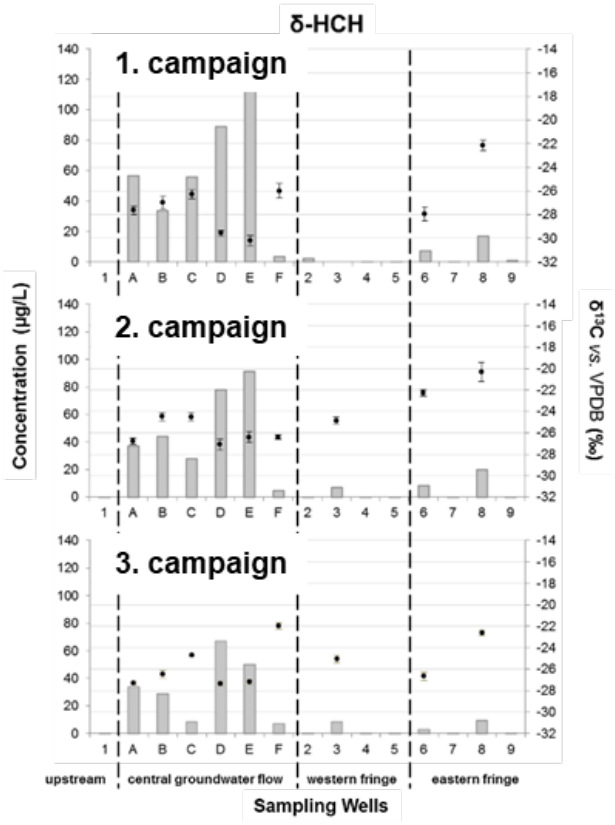

FIG. 2:CONCENTRATIONS (BARS) AND Δ^{13}C-VALUES (DOTS) OF Δ-HCH FOR THE FIRST, SECOND AND THIRD MONITORING CAMPAIGN.
Wells 1 and 3 were established in before the second monitoring campaign, thus, concentration and isotope data are not available for the first monitoring campaign. Uncertainty of concentration analysis is < 10% in all cases. Errors of δ^{13}C-values are indicated as error bars.

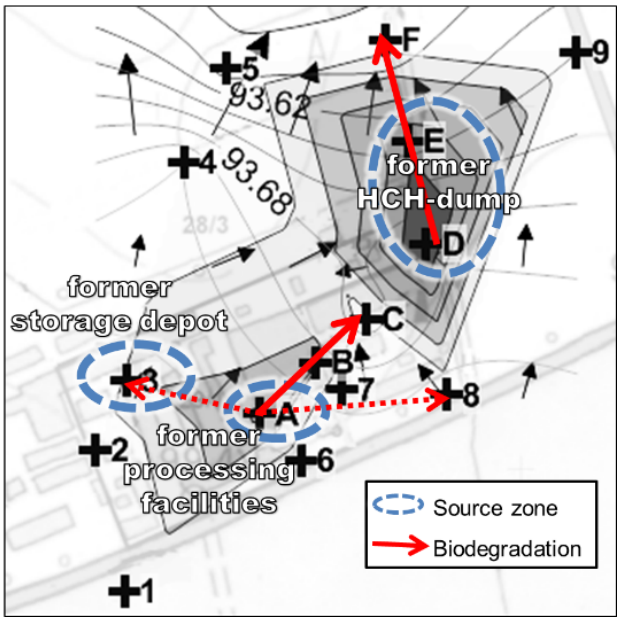

FIG. 3:CONCEPTUAL SITE MODEL FOR SOURCES AND SINKS OF HCHS IMPROVED BY THE CSIA STUDY.
Dashed ellipses show pollutant source zones. Solid red arrows illustrate HCH biodegradation, whereas dotted red arrows show expected HCH biodegradation because direct flow paths between wells are ambiguous.

Using the Rayleigh-equation approach (Thullner *et al.*, 2012), calculations of the percentage of biodegradation (B [%]), distance- and time-dependent *in situ* first-order biodegradation rate constants (λ_s [1/m], λ_t [1/d]) for HCHs were carried out for flow paths within the main groundwater flow direction. Calculations yielded levels of HCH biodegradation ranging from 30 to 86%. Moreover, time- and distance-dependent *in situ* first-order biodegradation rate constants were estimated with maximal values of 3×10^{-3} d^{-1} and 10×10^{-3} m^{-1} for α-HCH, 11×10^{-3} d^{-1} and 37×10^{-3} m^{-1} for β-HCH, and 6×10^{-3} d^{-1} and 19×10^{-3} m^{-1} for δ-HCH, respectively.

Discussion

Based on our study, we could highlight:

- the applicability of CSIA for the assessment of biodegradation and the source identification of HCHs within contaminated aquifers,
- the potential of CSIA for the quantification of HCH biodegradation,
- that time-resolved CSIA can reveal temporal variations in HCH biodegradation and provide information on the influences of various processes on natural attenuation.

Due to the intensive production of HCHs and their worldwide usage, there are a huge number of HCH-contaminated production, formulation and dump sites (Vijgen *et al.*, 2011). At these sites, time-resolved CSIA could be applied to identify trends in attenuation of HCH isomers and help to predict the evolution of contaminant plumes, as exemplified in our study. *In situ* biodegradation rate constants could be integral in modeling the current status and future development of contaminant plumes.

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ANALYSIS OF MICROBIAL COMMUNITIES FOR THE IDENTIFICATION OF INOCULANTS FOR AN *IN-SITU* BIOREACTOR FOR TREATING HCH CONTAMINATION IN GROUNDWATER

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Summary

In situ bioremediation using microorganisms is a growing and developing discipline for clean-up sites contaminated by pollutants of different nature. Several bioremediation technologies can be applied at pilot and field scale levels based on different approaches as natural attenuation, biostimulation or bioaugmentation. However, when applied to the field, the previous techniques do not always offer an optimal yield due to slow degradation rates, logistic limitations or difficulties to control field parameters that relate with microbial degradation of target pollutants. In those cases, bioreactors can be a more controlled, robust and effective approach that can provide more accurate and optimum conditions for microbial growth for its use in bioremediation processes.

This study evaluates the feasibility of implementation of an *in-situ* bioreactor for the groundwater bioremediation of a site impacted by a HCH spill at a former dumpsite. Comprehensive characterization of indigenous samples of different nature and collected at the site was carried out in laboratory scale experiments to identify matrices to be used as bacterial starters or inoculums in the following steps of bioreactor scale-up. For that purpose, different laboratory experiments were carried out to preliminary investigate the microbial communities in order to identify those samples with better characteristics and potentially containing HCH-degrading bacteria.

In total, 13 samples collected from locations historically impacted by HCH were characterized in four laboratory experiments, including the study of (1) viable microbial population growth in generic medium, (2) viable population of indigenous microorganisms capable of growing in minimal medium containing HCH, (3) diversity community composition and (4) tolerance to different HCH concentrations. The analysis of the samples revealed microbial populations adapted to site conditions with rather similar populations growing in the generic and minimal mediums with values of $10^4/10^5$ CFU for all the samples. Functional diversity studies showed diverse and metabolically active communities capable of growing in plate conditions which, at the same time, revealed to also be tolerant to HCH concentrations among 1 to 35 ppm.

Keywords

Bioremediation; HCH; bioreactor; microbial communities; laboratory

Introduction

Bailín's landfill, located in the north of Spain, is a former unlined landfill placed over a complex fractured bedrock media. At this location industrial waste generated during the manufacture of lindane was disposed for several years which eventually formed a multi-component DNAPL (Dense Non Aqueous Phase Liquid) that has spread through the formation according to the geological layers and the fracture network.

Over the years, several remediation techniques have been successfully applied to remove the DNAPL and control and reduce the contamination in groundwater. However, the applicability of some of these techniques may be limited over time (e. g. pump and treat) and would require for alternatives to pursue remediation of the site. Among these alternatives bioremediation techniques based on microorganisms might be an interesting option as they are usually more sustainable and cost-effective techniques when properly applied.

The microorganisms used in bioremediation systems can be endogenous, present in the medium itself, or added from external sources. In both cases, however, the viability of bioremediation will depend on the extent and duration of the activity and growth of the microbial communities over time. One way to sustain the microbial activity is the use of bioreactor systems that can be installed in a piezometer and promote in situ biodegradation processes within the aquifer.

However, the application of this type of systems to the field with the minimum cost and high performance (productivity) is a very complex process that involves several optimization and scaling-up stages starting from the laboratory. In the first laboratory stages, one of the fundamental aspects is the selection of the starting sample, called inoculum or bacterial starter, from which the

microbial community of the biological system will proliferate.

The aim of this work is to carry out the characterization of samples of the different nature (i.e., water, soil, sediment and sludge) to identify matrices with interesting microbial characteristics to be used as inoculum or bacterial starters.

Materials and methods

Samples

Up to 13 indigenous samples of different nature (i. e., water, soil, sediment and sludge) have been evaluated to identify matrices with interesting microbial characteristics to be used as inoculum. All samples were taken from areas of the Bailin landfill affected by historical episodes of HCH contamination. Table 1 shows the samples selected for the study, including matrix type and a brief description of their main characteristics.

All samples were collected in sterile containers with appropriate aseptic procedures according to matrix type and specific characteristics of each sample and location (e. g., samples located in the different ponds were taken with different sampling methods due to accessibility considerations). In all cases samples may be minimally disturbed in order to preserve their integrity and representativeness, as well as to avoid cross-contamination from external sources. Once collected, they were taken to the laboratory, prepared and analyzed on the same day.

TABLE 1. SAMPLES SELECTED IN THE CURRENT STUDY

No.	Matrix	Type	Description
M1	Soil	Soil on the way to the new landfill	Soils impacted during the dismantling of the former landfill and transportation of residues to the new landfill.
M2	Sludge	Downstream upwelling	An upwelling located downstream and in the lower part of the old landfill basin. It receives leachates from the former landfill surface and contributions from contaminated upwellings. Possibility to collect water and sludge at the same location
M3	Water	Downstream upwelling	
M4	Soil	Soil upstream the former landfill	Soils upstream of the former landfill. Impacted first during the exploitation of the old landfill and, later, while dismantling and transferring residues to the new landfill.
M5	Sludge	Eastern shotcrete pond	Shotcrete pond located at the former landfill. Accumulates sediments dragged from the surface of the landfill, basically carbonate silts and some clays, with the presence of particulate HCH. Pond practically clogged and with some vegetation growing.
M6	Sludge	Western shotcrete pond	Shotcrete pond located at the former landfill. Accumulates sediments dragged from the surface of the landfill basin, basically carbonate silts and some clays, with the presence of particulate HCH. The pond floods in storms and has not developed vegetation. Sampling at two levels to evaluate aerobic and anaerobic zones.
M7	Sludge	Western shotcrete pond	
M8	Groundwater	Piezometer	Piezometer with low concentrations of contaminants of concern. Mainly drilled in limestone. Low fracture density and limited water renewal.
M9	Groundwater	Piezometer	Piezometer with high concentrations of contaminants of concern. Mainly drilled in sandstone and located just downstream the former landfill
M10	Sediment	BT2 storm pond	Storm pond that accumulates sediments dragged from the former landfill and residues from other locations and current activities carried out at the site. It historically received a high pollutant load. It accumulates water and also has an area with solid and consolidated material.
M11	Soil	BT2 storm pond	
M12	Sediment	BT4 storm pond	Storm pond that accumulates sediments dragged from the former landfill. It has not received high contaminant loads over time
M13	Sediment	BV1 discharge pond	Discharge pond at the outlet of the water treatment plant. Low HCH concentrations.

On one hand, collected matrices were analyzed to determine pollutant burden, nutrient content (TOC, N, P, K, etc.) and physico-chemical parameters (pH, ORP and conductivity). On the other hand, microbial experiments were carried out to study different microbial attributes for the comprehensive characterization of microbial communities of each sample.

Analytical characterization

The specific protocols and methods used for the analytical characterization depend on the type and nature of the analyzed samples.

The analysis of the pollutant burden of each of the selected samples has been carried out in the laboratory of the Government of Aragon (Sabiñánigo, Huesca) analyzing 34 common pollutants routinely monitored at the site. Among them, the main pollutants benzene, monochlorobenzene and total HCH (which account for more than 90% of the total pollutant mass) has been analyzed. Other organic and chlorinated compounds present in lower concentrations such as chlorobenzenes and chlorophenols, among others, are also included in the analysis. Those analysis have been carried out by gas chromatography-mass spectrometry (GC-MS). Nutrient (N, K, P and TOC) and physicochemical parameters analyses were externalized to a routine laboratory and analyzed using specific protocols and methods depending on the type and nature of the analyzed samples.

Microbiological experiments

The microbial characterization comprises a series of experiments for the assessment of significant

attributes that may allow a comprehensive study of microbial communities present in each kind of samples. Thus, the proposed approach included the development of four experiments using Petri dishes and microplates with different culture media:

1. Plate count of viable and culturable bacteria growing in generic media.
2. Plate count of viable and culturable bacteria growing in a minimal medium composed by HCH as carbon source.
3. Metabolic activity and diversity of microbial communities using Biolog EcoPlates™.
4. Metabolic activity and tolerance of microbial communities to different HCH concentrations (i. e., 1 to 35 ppm) using Biolog MT2 microplates.

As mentioned above, the proposed approach required samples to be prepared on the same day to avoid variability. Therefore, after sample collection, different extracts and dilutions were prepared to carry out sample inoculation for each experiment. Finally, microbial growth was monitored in each case for an average period of three weeks when microbial activity in the microplates may stabilize. Figure 1 depicts a simplified scheme of the workflow followed in the current study.

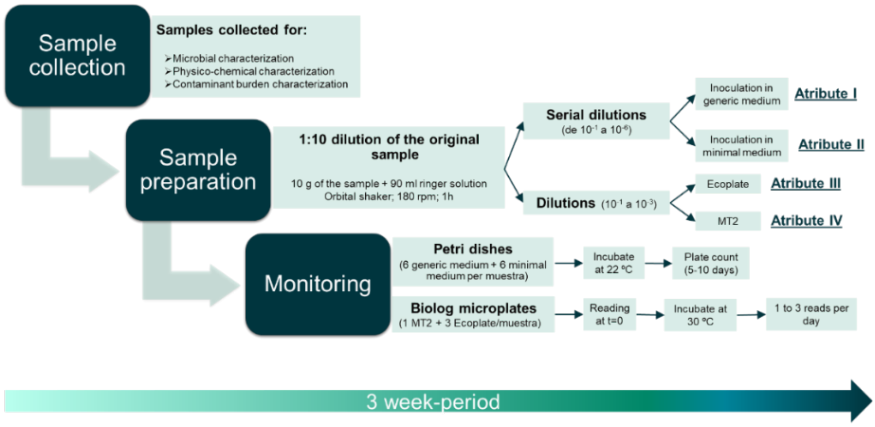

FIGURE 1. DESIGN WORKFLOW FOR THE CHARACTERIZATION OF SAMPLES INCLUDED IN THE STUDY

Petri dishes were incubated at 22ºC and microbial growth was assessed by counting the colonies (CFU) observable on each dilution. On the other hand, microplates were incubated at 30ºC according to existing literature. Microbial growth of microplates was measured using a UV-microplate reading at 595 nm, since Biolog technology is based on Tetrazolium dye reaction due to the presence of metabolic activity.

Results and discussion

The analytical characterization of the selected samples showed variable HCH concentrations in the range of 0,1-529 mg/kg for solid samples and 0,2-4,4 mg/l for water samples. The highest concentrations of HCH in the solid samples were obtained for those collected in some of the ponds, both in the shotcrete ponds (M4, M5 and M6) and in the BT2 storm pond (M10, M11). On the other hand, the effect seemed significantly lower in the collected soils from the upwelling downstream the landfill and in BV1 and BT4 ponds. These ponds are currently in use and the renewal of their composition could be more dynamic.

In general, the analytical results for nutrients and physico-chemical parameters (results not shown) would be compatible with the existence and proliferation of microbial populations in the different matrices evaluated. In some cases (e. g., M8) low nutrient concentrations were observed. This result would show possible lines of improvement to enhance microbial growth by adding amendments to adjust the nutrient content. Further investigation and optimization would be carried out in successive phases with those samples that present the best results for the studied microbiological attributes.

For the microbial characterization, Figure 2 shows the results for the 13 samples included in the study for each of the microbial attributes described above. The upper panels show the CFU population obtained when samples were inoculated in generic (left) and minimal media (right), respectively. In both cases, CFU population where among 10^3 and 10^5 g or ml/sample. These values, in general, would be suitable for the potential application of biostimulation treatments that enhance microbial activity.

On one hand, the generic medium will approach the total viable and cultivable microbes capable of growing in laboratory conditions. While, on the other hand, the minimal medium represents a selective medium, where there will be a preferential growth of those microorganisms capable of degrading the substrates that composed the medium. As can be seen, in most of the Bailin's samples the number of specialized bacteria is close to the total population of bacteria. This could be

indicative of the existence of a high rate of specialization and adaptation in the Bailin landfill ecosystem, in which almost all existing microorganisms are capable of metabolizing the contaminants of concern. As can be seen, the samples that presented the highest CFU populations were those belonging to BT2 storm pond (M10 and M11) with values higher than 10^5 CFU/g.

Biolog microplates (used for the study of attributes III and IV) are 96-well microplates based containing a tetrazolium salt that proportionally dyes (color development) in response to microbial activity. Sequential readings of the microplates using a UV-spectrophotometer may allow to study the growth of the microbial communities over time and, thus, obtain what is known as a bacterial growth curve. The lower panels in Figure 2 show the microbial growth curves obtained for Biolog Eco-plate (left) and MT2 (right). For most of the samples both microplates showed apparent color development and a curve that may fit a typical microbial growth curve. That is, when microorganisms are placed in a new media require a time of adaptation to the new conditions (known as the lag or latency phase). After that, the microorganisms already adapted to their new environment grow exponentially, consuming substrates and nutrients (log growth phase), until they reach the stationary phase in which the number of cells that grow and those that die are in equilibrium. Finally, if the systems stops working there will be a final stage were cells would die and the microbial activity decreases. The behavior of the microbial communities during their growth cycle will be to some extent related to the viability and robustness of the system (e. g., bioreactor), being desirable to have robust bacterial communities or consortia that last over time.

In general, microbial activity was higher in Biolog Ecoplate than in MT2 which is consistent with the greater degradability of the substrates comprise in this type of microplates. In general, Ecoplate curves reveal the presence of diverse microbial communities with high diversity indexes in all samples (e. g., above 3 for the Shannon index). In addition, MT2 microplates revealed that the microbial communities of the analyzed samples were able to tolerate different concentrations of HCH ranging from 1 to 35 ppm.

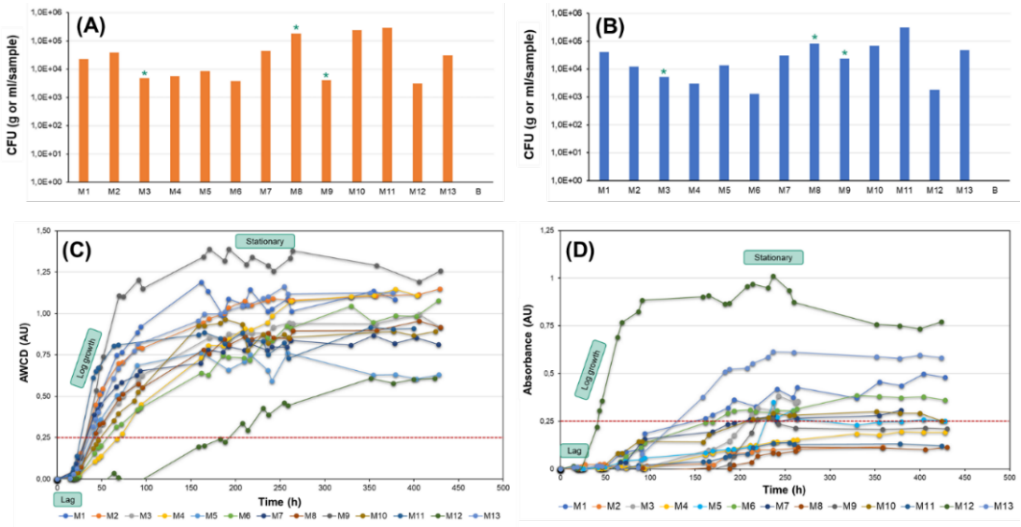

FIGURE 2. RESULTS OBTAINED FOR THE MICROBIAL ATTRIBUTES EVALUATED IN THE STUDIED SAMPLES: (A) CFU POPULATION IN GENERIC MEDIUM, (B) CFU POPULATION IN MINIMAL MEDIUM, (C) MICROBIAL GROWTH CURVES IN BIOLOG ECOPLATES AND (D) MICROBIAL GROWTH CURVES IN MT2 MICROPLATES

Conclusions

Up to 13 samples of different nature collected at the site and impacted by HCH have been evaluated. Six potential candidates (M1, M3, M8, M10, M11 and M13) to be used as microbial starters have been identified in the preliminary screening based on their CFU population (>10^5 CFU), microbial community diversity (Shannon indices above 3) and tolerance to concentrations of HCH between 1 to 35 ppm. The most promising samples corresponded to matrices from areas of the site historically affected by HCH and with relatively homogeneous conditions over time. Future experiments will be carried out to study if co-inoculation of different samples enhance microbial activity or to approach which environmental conditions result in a better performance of the system.

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CHARACTERIZATION OF MICROBIAL POPULATION NATURALLY PRESENT AT SARDAS' LANDFILL AND INQUINOSA FACTORY IN SABIÑANIGO, HUESCA

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Summary

Microorganism bioremediation is a technique that is increasing its importance in the last years. The microorganisms naturally present in the soil are capable of using polluting compounds as a carbon source, contributing to their degradation and therefore, to the environment decontamination. In this essay, we have evaluated the diversity of microbial colonies naturally present at strategic points in the emplacements of Sardas' landfill and Inquinosa factory, both located in Sabiñánigo, Huesca, and their degradation capacity of chlorinated organic compounds (COCs). For it, 8 samples from Inquinosa area and 29 from Sardas site, including soil, mud, water and plant material were studied. A microbiological characterization was carried out for each sample. Within the microbiological analyses, the total and special bacterial biomass was determined; also, the diversity of the population through analysis with Biolog EcoPlate™ microplates and "Shannon" and "Simpson" diversity indexes. In addition, toxicity tests were carried out using Biolog MT2™ microplates, with the objective to evaluate the growth capacity of microbial populations using the organic carbon of the contaminants. From the Inquinosa area, rich population densities were obtained in 3 samples (10^4-10^5 CFU) and very rich in other 3 ($\geq 10^6$ CFU). On the other hand, 7 of 8 samples obtained high values of diversity indexes. Regarding the Sardas samples, 26 of 29 presented high diversity indexes and in terms of population density, 10 samples can be considered rich and 12 very rich. All the samples that have shown biological activity in the toxicity tests, two samples, one from each site, showed more promising results in the future.

Keywords

COCs, Biolog EcoPlate ™, Biolog MT2 ™, bioremediation, Lindane, CFU (colony forming unit), microbial diversity.

Introduction

Microorganisms present in the soil act as indicators of its environmental state. Soils with a high functional diversity are considered to be more efficient in terms of productivity, resistance and resilience [1]. Likewise, microbiological parameters are very effective indicators of changes that may occur in soil conditions, even more than some physicochemical parameters [2], allowing early detection and monitoring of environmental problems. On the other hand, certain microorganisms naturally present in the environment are able to use polluting compounds as a carbon source and, therefore, contribute to the bioremediation of degraded spaces [3]. One of the methods that exist to evaluate the microbiological diversity of a soil is the use of microplates *Biolog Ecoplate*, which allow evaluating the metabolic activity of the community, establishing a characteristic physiological profile of the same [4] and also determining ecological parameters such as diversity or species richness, both indicators of the state of the community. Included in the control and decontamination of lindane and its derivative compounds carried out by the Government of Aragon, through the Department of Agriculture, Livestock and Environment, through the Contaminated Soils service, bioremediation is an important bet as a technique for the decontamination of the environment.

With the aim of using this technique in areas contaminated by HCH and compounds derived from its production, a microbiological characterization of different points within the Sardas landfill and the Inquinosa factory has been carried out, to see the state of the microbial communities and carry out an inventory of possible inoculums for future use, both in bioaugmentation as biostimulation techniques.

Material and methods

For this work, a total of 37 samples were taken; 8 from Inquinosa (4 water samples and 4 soil samples) and 29 from Sardas (10 water samples, 8 soil, 7 mud from the ponds and 4 vegetation). See Annex 1. The microbiological characterization carried out for each sample can be summarized in the following attributes:

1. *Total biomass:* Sowing of samples in generic medium plates (plate count agar) to evaluate the amount of microorganisms present.
2. *Specialized biomass:* Sowing in a restrictive medium, in which the only available carbon

source are the compounds present in a mixture of DNAPL and water. The presence of microorganisms specialized in degrading COC's is evaluated. Considering the concentrations of microorganisms calculated, such as CFU/ml or CFU/g of sample, those with values equal to or less than 10E+03 are considered to be poor; those between 10E+04 and 10E+05 are rich and those with results higher than 10E+06 are very rich samples.

3. *Population parameters:* with the Biolog EcoPlate™ plates, the latency time, metabolic activity, and ecological indices (Shannon's diversity index, Shannon's evenness index, and Simpson's index) are calculated for each sample, by absorbance data, based on the colour variation of the microplate wells. The Shannon diversity index allows evaluating the functional diversity of the microbial community based on two factors, the number of species present or richness and their relative abundance. The values of maximum diversity are reached when all species are equally present [5]. Shannon index values between 0.1 and 1.5 are considered low diversity, between 1.6 and 3.0 medium diversity and between 3.1 and 5.0 high diversity. Regarding the Simpson and Shannon equity indices, the values oscillate between 0 and 1, with the most diverse samples being those close to unity.
4. *Toxicity or tolerance to COCs:* this test studies the resistance of bacteria to contamination, using MT2 microplates, to which a dilution of DNAPL in distilled water is added, as a carbon source, in different concentrations.

Results

Total and specialized biomass

Below, for each sample and type of medium, the concentration of microorganisms is presented at the Table 1, expressed as Colony Forming Units (CFU) per mL or gr of sample, calculated for two different culture times: 3 and 10 days.

TABLE 1: CONCENTRATION OF MICROORGANISMS IN GENERIC MEDIUM AND MINIMAL MEDIUM. THOSE SAMPLES IN WHICH THE CONCENTRATION IN THE MINIMAL MEDIUM IS HIGHER THAN THAT IN THE GENERIC MEDIUM ARE MARKED WITH *.

Sample	Matrix	Incubation time 3 days		Incubation time 10 days	
		Generic Medium (CFU/ml o CFU/g)	Minimal Medium (CFU/ml o CFU/g)	Generic Medium (CFU/ml o CFU/g)	Minimal Medium (CFU/ml o CFU/g)
M15*	Soil	9,6E+03	3,2E+03	1,2E+04	2,4E+04
M16	Soil	4,7E+03	0 CFU	>300CFU D1	1,4E+04
M17*	Soil	2,5E+06	9,6E+05	4,9E+06	8,7E+06
M18*	Soil	3,2E+04	0 CFU	5,8E+04	1,1E+05
M19	Liquid	1,3E+04	0 CFU	1,4E+04	5,7E+03
M20	Liquid	<30CFU	<30CFU	3,2E+03	<30CFU
M21*	Liquid	7,0E+04	0 CFU	1,3E+05	3,5E+05
M22*	Liquid	2,7E+03	7,9E+03	3,6E+03	7,4E+04
M 25*	Liquid	<30CFU	0 CFU	<30CFU	1,6E+04
M 26	Mud	5,3E+04	0 CFU	7,6E+04	8,2E+04
M 27	Mud	<30CFU	<30CFU	2,0E+05	4,4E+04
M 28*	Liquid	4,7E+03	0 CFU	8,7E+03	1,8E+04
M29	Liquid	8,7E+03	0 CFU	1,8E+04	2,6E+04
M 30*	Mud	1,0E+04	<30CFU	1,9E+05	1,7E+06
M 31	Mud	3,2E+04	0 CFU	3,0E+05	1,7E+05
M 32	Mud	1,5E+06	<30CFU	2,9E+06	1,2E+05
M 33*	Vegetation	7,0E+06	>300CFU D2	2,1E+07	1,8E+08
M 34	Vegetation	3,0E+05	>300CFU D1	1,5E+07	6,8E+06
M 35	Mud	5,1E+05	>300CFU D1	2,4E+06	1,3E+06
M 36*	Vegetation	2,9E+06	>300CFU D1	1,3E+07	1,1E+08
M 37*	Mud	4,3E+04	6,4E+04	4,8E+04	2,8E+05
M 38*	Vegetation	3,3E+07	>300CFU D3	8,9E+07	6,2E+07
M 39*	Soil	2,1E+05	1,8E+05	2,5E+05	3,2E+06
M 40	Soil	1,7E+05	<30CFU	1,8E+05	1,2E+05
M 41	Soil	6,2E+05	7,3E+04	2,0E+06	1,1E+06
M 42	Soil	1,1E+06	<30CFU	2,9E+06	2,5E+05
M 43	Soil	8,3E+05	<30CFU	2,5E+06	1,6E+05
M 44	Soil	3,3E+06	8,4E+04	6,2E+06	1,2E+06

Sample	Matrix	Incubation time 3 days		Incubation time 10 days	
		Generic Medium (CFU/ml o CFU/g)	Minimal Medium (CFU/ml o CFU/g)	Generic Medium (CFU/ml o CFU/g)	Minimal Medium (CFU/ml o CFU/g)
M 45	Soil	1,7E+06	1,3E+05	3,4E+06	2,3E+05
M 46	Soil	1,19E+06	7,0E+04	1,53E+06	1,57E+05
M 47	Liquid	<30CFU	<30CFU	<30CFU	<30CFU
M 48	Liquid	0 CFU	0 CFU	0 CFU	0 CFU
M 49*	Liquid	6,40E+04	9,20E+03	1,38E+05	1,57E+06
M 50*	Liquid	1,16E+05	<30CFU	1,41E+04	1,36E+05
M 51	Liquid	<30CFU	0 CFU	<30CFU	1,48E+04
M 52	Liquid	<30CFU	0 CFU	<30CFU	0 CFU
M 53	Liquid	<30CFU	<30CFU	3,50E+03	4,70E+03

Based on the counts in generic medium plates, 76% of the samples are rich or very rich in terms of microorganism density and only 9 present very low values, all of them being water samples. The highest concentration of microorganisms has been recorded, in the case of Inquinosa in the soil sample from the upper terrace (M17) and in the case of Sardas, in the vegetation samples (M33, M34, M36 and M38).

Finally, in 38% of the samples, despite the fact that the colonies are more tenuous, there are higher concentrations of CFU in the minimal medium cultures.

Population parameters

The indices calculated from the analysis of the Biolog Ecoplate plates are shown below (Table 2), for time 1/2 and as well as the maximum absorbance recorded in the MT2 microplates.

TABLE 2: DATA AND INDICES OBTAINED FROM MICROPLATES BIOLOG ECOPLATE AND BIOLOG MT2.

Sample	t1/2 (h)	Phase lag(h)	AWCD max.	AWCD (t1/2)	NUS (t1/2)(n)	Shannon (t1/2)	Simpson (t1/2)	S.Eveness (t1/2)	AWCD max. MT2
M15	57	17	1,360	0,680	22	4,310	0,94	0,96	0,297
M16	65	17	1,030	0,570	20	4,124	0,94	0,96	0,081
M17	68	20	1,460	0,633	22	4,140	0,95	0,97	0,218
M18	64	17	1,340	0,577	21	4,215	0,94	0,97	0,361
M19	75	25	1,320	0,507	14	3,612	0,91	0,96	1,679
M20	363	-	<0,25	-	-	-	-	-	0,517
M21	193	41	0,510	0,484	20	4,171	0,94	0,96	0,480
M22	33	12	1,100	0,394	16	3,733	0,92	0,96	0,540
M25	55	18	1,045	0,447	16	3,911	0,93	0,97	0,087
M26	39	18	0,894	0,377	15	3,565	0,81	0,98	0,031
M27	102	21	0,720	0,480	14	3,668	0,92	0,98	0,759
M28	64	21	0,986	0,438	16	3,847	0,92	0,95	0,100
M29	79	21	0,578	0,409	14	3,652	0,91	0,96	0,069
M30	122	21	1,036	0,584	16	3,690	0,91	0,94	0,078
M31	60	21	0,951	0,462	15	3,724	0,92	0,97	0,044
M32	40	21	0,975	0,377	15	3,773	0,92	0,97	0,061
M33	50	15	0,805	0,590	23	4,383	0,95	0,97	0,942
M34	36	15	0,62	0,438	20	3,984	0,92	0,97	0,330
M35	39	17	1,198	0,360	13	3,236	0,79	0,95	0,084
M36	44	17	0,484	0,404	21	4,238	0,94	0,97	0,198
M37	34	19	1,202	0,297	11	2,765	0,75	0,96	0,067
M38	37	<12	0,907	0,776	24	4,538	0,96	0,99	0,118
M39	45	19	0,82	0,481	19	3,969	0,90	0,96	0,227
M40	157	41	0,901	0,635	19	4,066	0,93	0,96	0,211
M41	44	20	1,332	0,404	14	3,563	0,90	0,95	0,118
M42	> 450	-	0,940	-	-	-	-	-	0,247
M43	94	32	1,01	0,472	15	3,566	0,90	0,93	0,107

Sample	t1/2 (h)	Phase lag(h)	AWCD max.	AWCD (t1/2)	NUS (t1/2)(n)	Shannon (t1/2)	Simpson (t1/2)	S.Eveness (t1/2)	AWCD max. MT2
M44	47	20	1,448	0,417	17	3,860	0,92	0,95	0,188
M45	25	19	1,443	0,208	8	2,786	0,84	0,97	0,151
M46	29	<12	1,164	0,460	17	3,904	0,93	0,98	0,201
M47	257	45	0,58	0,498	11	3,178	0,88	0,94	0,133
M48	-	-	<0,25	-	-	-	-	-	0,112
M49	48	21	1,101	0,466	17	3,873	0,93	0,95	0,136
M50	153	21	1,045	0,851	24	4,397	0,95	0,96	0,241
M51	101	23	0,745	0,400	13	3,501	0,90	0,94	0,034
M52	170	46	0,457	0,312	8	2,845	0,86	0,98	0,455
M53	47	17	0,88	0,466	17	3,932	0,93	0,97	0,062

Based on the results obtained in the Shannon and Simpson index, 84% of the samples have a high ecological diversity. In addition, the values close to the unit of the Shannon evenness index indicate that there is a good degree of homogeneity in terms of the relative abundance of the species composition of practically all the inoculums.

Samples with a Shannon index of less than 3.1, and therefore with less ecological diversity, would be sample M37 (mud from the new pond), M45 (soils from the leachate area near the road) and M52 (groundwater with a high pollution); all of them samples of the Sardas site. On the other hand, in the case of samples M20, M42 and M48, the indices could not be calculated due to absorbances lower than 0,25 or very long 1/2 times, which indicates little metabolic activity.

COCs Tolerance Test

All the samples have an increase in the absorbance with time, but only those in which the registered absorbance is higher than 0,25 have been taken into account, value from which it is considered that the oxidation of substrate occurs by microorganisms. In the case of Inquinosa, 6 of 8 samples show degradation of the substrate, while, at the Sardas site, this only happens in 4 samples. Of the samples analyzed, the ones that have presented the best results in the toxicity test have been the M19 inoculum from Inquinosa, corresponding to water from the S-21 piezometer, with a high contaminant load, and the M33 inoculum, scraping from the roots of Phragmytes sp. from the Sardas old pond (Figure 1).

In both samples, the maximum absorbances recorded in the MT2 and Ecoplate microplates are similar (Table 2). In the case of sample M19, the highest degrading activity occurs in the 1:50 dilution, reaching an approximate AWCD of 1.68.

In case of sample M33, the maximum degrading activity occurs in the most concentrated dilution, reaching values of absorbance of 0,94 and decreasing as the concentration of pollutants decreases. Comparing the absorbances obtained in these two samples, we could say that the M19 sample is the one with the highest metabolic activity and is very effective in degrading the compounds from the 1:50 dilution, while the M33 inoculum has better resistance to highly toxic conditions, maintaining its maximum activity in the most concentrated dilution.

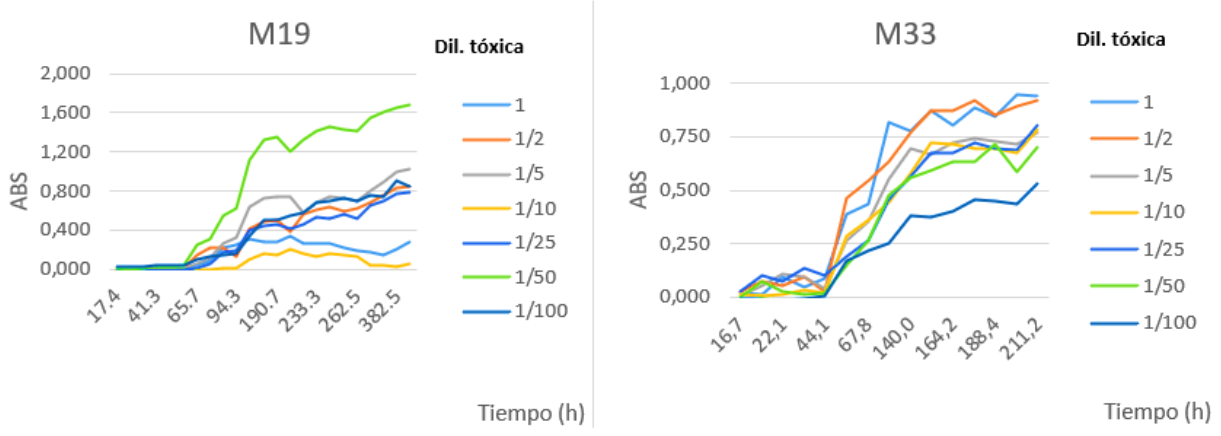

FIGURE 1: EVOLUTION OF ABSORBANCES IN THE MT2 ASSAY OF SAMPLES M19 AND M33

Discussion

- It is considered, in general, that the places studied have a good diversity of microorganisms and they are well adapted to the environment. For this, high diversity indices have been obtained, as well as, the results of sowings in generic and selective medium. In some samples, higher densities of microorganisms are reached in selective medium opposite generic medium. It could indicate that the specialized microorganisms use organic compounds as a carbon source and, in the absence of other dominant and faster growing species, colonize the environment without problem.
- Calcium concentrations above 100 ppm cause interference between the tetrazolium and the substrate, giving rise to false positives in the Biolog system [6]. This could explain the high diversity values that have been obtained, through this system, in some samples with very low colony counts, such as samples M47 and M51, since both present concentrations of this cation above the mentioned value.
- As expected, the metabolic activity of microorganisms decreased on MT2 plates. Despite this, in some samples, the absorbances in these plates are slightly higher than those obtained in the Ecoplate, and this could again be related to the lack of competition. M19 from Inquinosa and M33 from Sardas samples are featured, in which the maximum absorbances recorded on both plates (MT2 and Ecoplate) are very similar, indicating that these inoculums maintain their metabolic activity, even though they only have contaminating compounds as a source carbon.
- Based on the results obtained, it is considered that the M19 and M33 inoculum samples could be used in bioaugmentation techniques, given their degrading activity. On the other hand, taking into account the presence of microorganisms adapted to environmental contamination conditions, we consider it interesting to apply biostimulation methods to increase their degrading activity.

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ANNEX 1 - SAMPLE DESCRIPTION

Sample	Matrix	Site	Description	Sample	Matrix	Site	Description
M15	Solid	Lower terrace	Soil (0-15cm) next to platform	M36	Solid	Old Pond	Vegetation of vegetation zone
M16	Solid	Lower terrace	Soil (>50cm) next to platform	M37	Solid	New Pond	Muds (0-20 cm)
M17	Solid	Upper terrace	Soil (0-15cm) next to the blue tank	M38	Solid	New Pond	Roots vegetation
M18	Solid	Upper terrace	Soil (>50cm) next to the blue tank	M39	Solid	Treatment Plant	Soil at the discharge point of the treatment plant
M19	Liquid	Lower terrace	Groundwater of borehole S-21 (high pollution)	M40	Solid	Biopiles Soils	Biopiles soils. Point 1, (0-5 cm)
M20	Liquid	Lower terrace	Groundwater of borehole S-17 (low pollution)	M41	Solid	Biopiles Soils	Biopiles soils. Point 1, (>20 cm)
M21	Liquid	Upper terrace	Groundwater of borehole S-22 (high pollution)	M42	Solid	Biopiles Soils	Biopiles soils. Point 2, (0-5 cm)
M22	Liquid	Upper terrace	Groundwater of borehole S-25 (low pollution)	M43	Solid	Biopiles Soils	Biopiles soils. Point 2, (>20 cm)
M25	Liquid	Old Pond	Water of un vegetation zone	M44	Solid	Ditch	DNAPL outcrop
M26	Solid	Old Pond	Mud (0-5 cm) of un vegetation zone	M45	Solid	Ditch	Urban waste leachates
M27	Solid	Old Pond	Mud (>20 cm) of un vegetation zone	M46	Solid	Ditch	Alkaline zone
M28	Liquid	Old Pond	Water of ecotone zone	M47	Liquid	Landfill inside	Groundwater inside the landfill of borehole S-37 (low pollution)
M29	Liquid	Old Pond	Water of vegetation zone, away from polluted effluent	M48	Liquid	Landfill inside	Groundwater inside the landfill of borehole S-39F (high pollution)
M30	Solid	Old Pond	Mud (>20 cm) of ecotone, *Typha sp.* area	M49	Liquid	Silt	Silt groundwater of borehole PS5E (high pollution)
M31	Solid	Old Pond	Mud (0-5 cm) of ecotone, *Phragmytes sp.* area	M50	Liquid	Silt	Silt groundwater of borehole PS20B (low pollution)
M32	Solid	Old Pond	Mud (>20 cm) of ecotone *Phragmytes sp.* area	M51	Liquid	Alluvial	Alluvial groundwater of borehole ST2 (low pollution)
M33	Solid	Old Pond	Phragmytes sp. roots	M52	Liquid	Alluvial	Alluvial groundwater of borehole PS14E (high pollution)
M34	Solid	Old Pond	Thypa sp. roots	M53	Liquid	New Pond	Water
M35	Solid	Old Pond	Mud (>20 cm) of vegetation zone				

APPLICATION OF MOLECULAR BIOLOGICAL TOOLS AND ISOTOPIC ANALYSIS FOR BIOGEOCHEMICAL CHARACTERIZATION OF FRACTURED BEDROCK AQUIFER IMPACTED BY DNAPL

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Summary

Biological degradation processes of target compounds are often desired in contaminated aquifers for the successful application of bioremediation strategies. However, the understanding and characterization of the occurring biological processes is still limited, since many projects rely on classical approaches just considering geochemical and hydrogeological data to support the existence of biodegradation. Those approaches would provide valuable information on reductions in concentrations, changes in inorganic species, generation of secondary products or variations in physicochemical parameters, although insights on microbial data are neglected.

In that sense, Molecular Biological Tools (MBTs) and isotopic analysis comprise a group of non-culture dependent laboratory techniques used to fill these gaps and estimate biodegradation processes in contaminated aquifers. With its application it is possible to identify key microorganism, microbial communities and microbiological processes involved in the degradation of the contaminants of concern. It is also possible to obtain probing data that contaminant biodegradation is actually occurring and/or information about the parameters that affect microbial activity.

This work would provide a general overview on the application of commercially available MBTs at fractured bedrock aquifer impacted by DNAPL mainly composed by benzene, chlorobenzenes and HCH isomers. The combination of various MBTs and its integration with hydrogeochemical data, allowed the collection of multiple lines of evidence to assess the biodegradation potential or degradation mechanisms existing under different site conditions. Comprehensive site characterization would facilitate understanding of microbial processes and time/spatial variations thereof for the potential selection and design of future remediation strategies and monitoring campaigns.

Keywords

Bioremediation; Molecular Biological Tools; biodegradation; HCH

Introduction

Lindane (gamma-hexachlorociclohexane) is a chlorinated pesticide widely produced in many European countries but banned by the Stockholm Convention since 2009 due to its severe environmental impact [1]. It was produced through a non-selective process where huge amounts of wastes were generated per each ton of the active product [2]. The large-scale production of lindane occasionally led in many cases to an uncontrolled management of the production wastes often dumped in unlined landfills which, over the years, derived in a serious environmental problem due to natural lixiviation and infiltration processes.

A lindane production factory was located in the town of Sabiñánigo (Huesca, Spain), where the former company INQUINOSA produced lindane between 1975 and 1994 [3]. The wastes of the production process were dumped during several years in various landfills close to the site.

One of these dumping spots was the Bailín landfill, which was placed over a complex fractured bedrock media and used for almost 10 years to dispose wastes of the manufacturing process [4]. Leachate from the waste deposited on the ground generated a multi-component DNAPL (Dense Non Aqueous Phase Liquid) composed by more than 30 organic compounds where the main contaminants are benzene, chlorobenzenes and different HCH-isomers. Over time the DNAPL has infiltrated and spread through the subsurface in response to the geological layers and the fracture network of the formation creating a 1.5 km plume in a complex geological system.

In previous years, several remediation techniques have been successfully applied to remove the DNAPL, as well as control and reduce the contamination in groundwater. However, the applicability of some of these techniques may be limited over time (e. g. pump and treat) and would require for alternatives to pursue remediation of the site. The application of bioremediation techniques has been considered lately due to the presence in groundwater of biologically reactive compounds to provide alternatives when the application of active remediation techniques no longer makes sense.

Bioremediation approaches rely on the metabolic activity of microbial communities in order to

achieve contaminant degradation in contaminated aquifers. In that process, the understanding of biological processes may be important in order to properly select and optimize potential treatments that enhance biodegradation through engineer processes.

In that sense, it is important to obtain site-specific and quality data that allow the characterization of microbial processes occurring in this kind of environments and may complement the information provided by classical chemistry and geochemistry analyses. Molecular Biological Tools (MBTs) are a group of molecular genetic analyses that can be used at field-scale to characterize and evaluate microorganisms degrading-capabilities and their related activity [5]. Those techniques in conjunction with isotopic analysis comprised a suite of effective techniques that may be useful to properly implement bioremediation approaches [6].

During the last decades there has been an increase development on these kinds of microbial and molecular techniques applied the remediation field to investigate microbially-related processes related with common contaminants present in contaminated sites [7]. Indeed, nowadays, there is a wide range of commercially available MBTs and isotopic analyses that can be used for site managers to obtain multiples lines of evidence related with contaminant degradation (Figure 1) [8].

This paper addresses the use of the most common commercially available MBTs at fractured bedrock aquifer impacted by DNAPL. The use of these techniques would provide information on microbially mediated degradation processes in contaminated aquifers and would be useful to complete the conceptual site model (CSM) in order to approach new remediation strategies based on biodegradation processes.

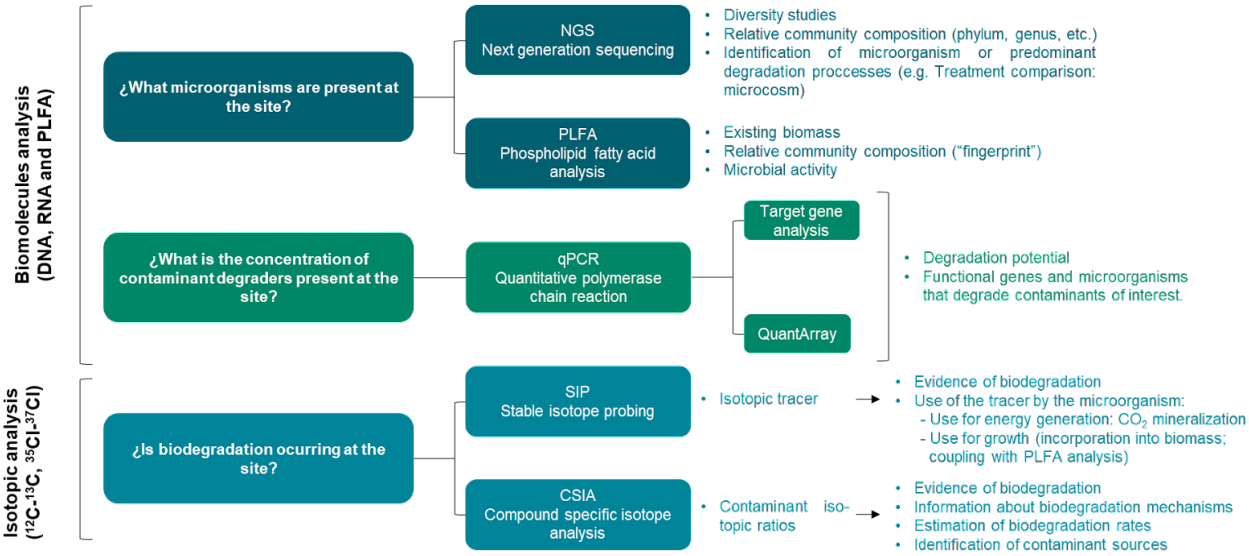

FIGURE 1. LINES OF EVIDENCE PROVIDED BY MBTS AND ISOTOPIC ANALYSIS WHEN APPLIED TO THE REMEDIATION FIELD (ADAPTED FROM [9])

Materials and methods

Sampling methods

Different approaches and microbial samplers can be used to obtain a representative sample of the site depending on the analysis and scope of the investigation. For example, passive samplers are usually selected to obtain an integrated sample that allows collection of microbes over time for better understanding of biodegradation potential [10, 11]. Those samplers are usually cylindrical devices composed by a polymeric material that provide a suitable surface and medium for microbial colonization. The devices are usually installed in existing wells to the depth of interest and for an estimated exposure time. After a sufficient incubation period (commonly between 30 and 60 days) in the zone of interest that ensures microbial proliferation, the samplers are retrieve and sent for laboratory analyses of the accumulated biomass and preferred MBTs. One of the advantages of the passive samplers is that the microorganisms that colonize the cells are living microorganisms, and therefore, the analysis does not include genetic material of dead microorganisms, which cannot be differentiated with other sampling methods.

Another possibility comprises filtering groundwater using filter cartridges [5]. These filters are connected to a pump (e.g., peristaltic or air bladder) and a certain volume of groundwater from the borehole is passed through the filter. The microorganisms get attached to the surface of the filter, which is cooled and sent for analysis. Filtering procedures must ensure that enough volume pass through in order to have sufficient biomass for laboratory analysis. This method is a rapid sampling procedure that allows obtaining a

discrete sample that, for example, can be useful for the assessment of changes in the microbial community in response to specific treatments. It is also the recommended procedure for DNA/RNA analysis, where an individual filters are collected for DNA and RNA analysis, respectively. In this case, for RNA sampling, a preservative should be added to the sampling filter to minimize degradation of these molecules. Other alternative procedures are possible for collection of samples of different nature.

MBTs and isotopic analyses

As depicted in Figure 1 different commercially available techniques can be selected for microbial characterization to obtain multiple lines of evidence of occurring microbial processes and existing degradation of contaminants of concern.

In the Bailín aquifer, several microbial and analytical techniques have been consistently applied since 2017 (Figure 3). The use of field-scale MBT data and combination of various techniques over the years, allowed completion of the CSM and the analysis of time/spatial variations to assess the potential selection and design of future remediation strategies and monitoring campaigns.

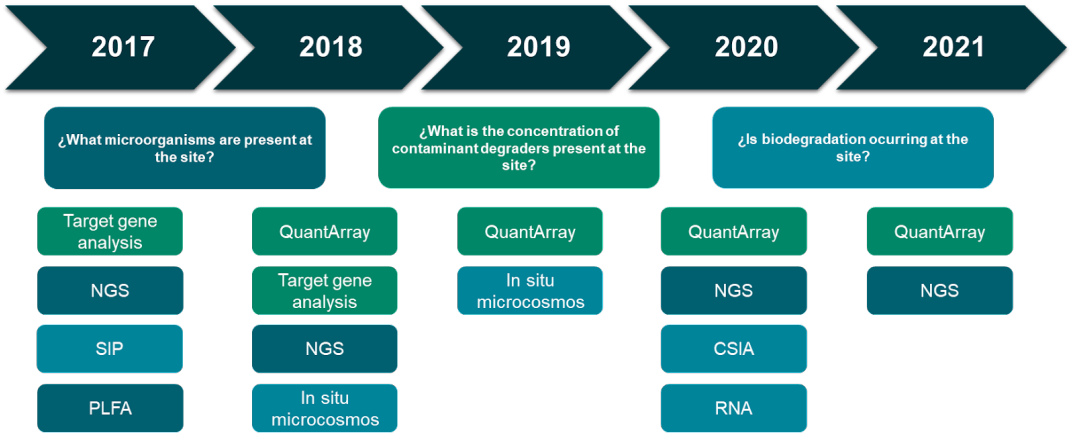

FIGURE 3. MBTS AND ISOTOPIC ANALYSES APPLIED IN THE BAILÍN AQUIFER

Case studies

The application and added value of the use of MBTs is shown below for different case studies carried out in the Bailín aquifer.

Case study 1 – Microbial community changes based on variable contaminant concentrations

Case study #1 assesses the use of technique Next Generation Sequencing (NGS) for the characterization of the microbial community in groundwater samples. This technique provides accurate identification of microorganisms present in the sample down to the genus level allowing the characterization of the community composition. Overall, the application of this technique could give some insight about the existence of degradation potential for a given compound and possible degradation mechanisms based on detected microorganisms.

In this case, up to 22 passive samplers were installed in boreholes from different areas of the site with different hydrogeological characteristics. Contaminant of concern (COCs) concentrations differ by several orders of magnitude between points located in different areas.

NGS analysis was performed for the characterization of the microbial communities of each location and, if possible, the identification of key microorganisms and degradation mechanisms related with the COCs. Besides, a comparison of microbial classes between all samples involved in the study have been carried out.

The analysis revealed substantial variations in the microbial communities from different areas of the site. In summary, the boreholes that are generally less affected had microbial profiles much similar than boreholes that historically had higher concentrations of contaminants in groundwater (Figure 4).

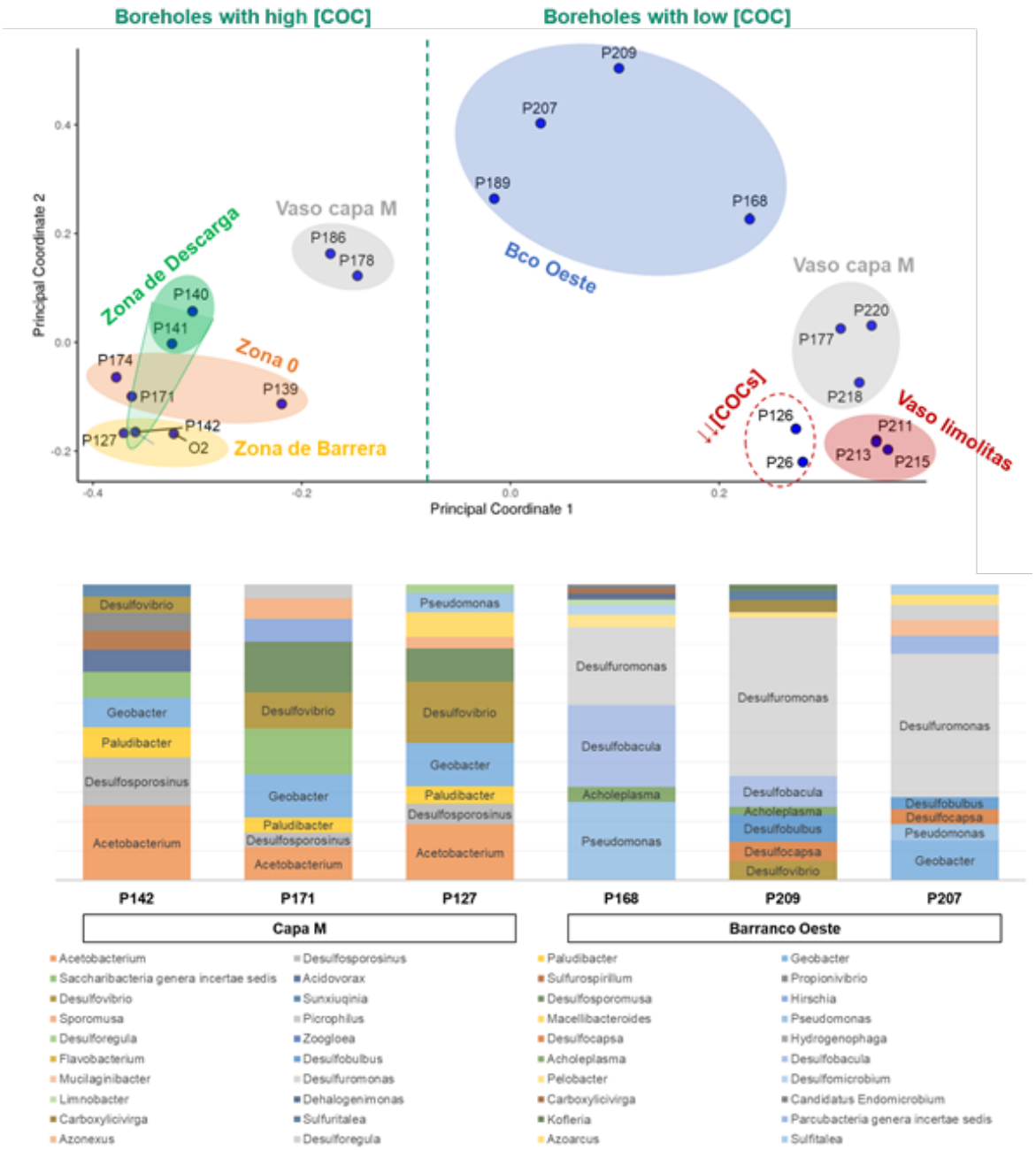

FIGURE 4. PRINCIPAL COMPONENT ANALYSIS RESULTS AND MICROBIAL COMPOSITION OBTAINED FROM NGS ANALYSES OF 22 BOREHOLES INCLUDED IN CASE STUDY #1

As an example, among the less impacted boreholes, microorganisms of the genus *Pseudomonas* can be identified as one of the predominant microbes. Bacteria belonging to this genus are usually quite versatile in terms of the type of substrates and terminal electron acceptors they use for their growth. However, among them, different strains of this genus have been defined that are capable of degrading chlorobenzenes and even HCH under aerobic conditions (e.g., *Pseudomonas putida* and *Pseudomonas aeruginosa*) [12]. On the other hand, among the boreholes that showed higher concentrations in groundwater, one of the most abundant genera is that of microorganisms belonging to the *Geobacter* genus, which are characterized by being iron-reducing bacteria, but of which some species have been related to the anaerobic degradation of hydrocarbons such as benzene (e.g., *Geobacter metallireducens*) [13].

Case study 2 – qPCR for biodegradation potential assessment

Case study #2 addresses the application of qPCR arrays for the study of the biodegradation potential existing in different areas of the site. qPCR analysis quantified target genes and microorganisms related with aerobic and anaerobic degradation pathways of benzene, chlorobenzenes, chlorophenols and HCH isomers.

Figure 5 shows concentrations of biomarkers obtained in three different boreholes located within the dissolve plume but with rather different impact (P127 and O2 are usually more impacted than P26

well). Concentrations of genes responsible for COC biodegration in impacted wells (P127 and O2) indicated high concentrations of anaerobic degraders under existing site conditions. The concentration of the aerobic functional genes was much more similar in all cases.

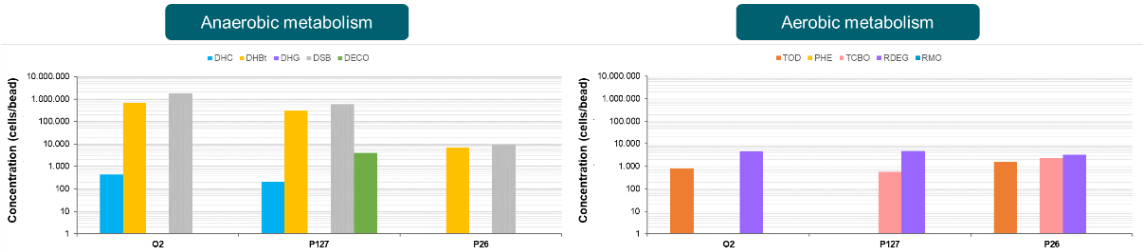

FIGURE 5. CONCENTRATION OF BIOMARKERS RELATED WITH THE DEGRADATION OF BENZENE, CHLOROBENZENES, CHLOROPHENOLS AND HCH ISOMERS BY AEROBIC AND ANAEROBIC PATHWAYS ANALYZED USING QPCR ARRAYS

Overall, the highest concentrations (>10^5 cells/bead) were detected for microorganisms related with the biodegradation of chlorinated compounds, particularly bacteria belonging to *Dehalobacter spp.* (DHBt), related with the degradation of monochlorobenzene, dichlorobenzenes and HCHs and *Desulfitobacterium spp.* (DSB) whose presence may be indicative of pentachlorophenol, trichlorophenols and dichlorophenols biodegradation.

This result provides a direct line of evidence for the ocurrence of degradation potentials of the COC existing in the Bailin aquifer.

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PRELIMINARY STUDIES TO IMPLEMENT A PILOT REACTOR FOR THE BIOLOGICAL REMOVAL OF PESTICIDES FROM AGRICULTURAL WASHING WASTEWATER

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Summary

Agricultural washing wastewater (AWW) is an important source of pesticides that has a high potential to be treated by fungal bioremediation using white rot fungi. In the present study, two AWW treatment strategies were compared: a fluidized-bed bioreactor (FBB) with *T. versicolor* pellets and a rotating drum bioreactor (RDB) with *T. versicolor* immobilized on wood. The RDB effluent showed better results in all studied parameters compared to those of the FBB, including pesticide removal (85%), toxicity, laccase activity, COD, absorbance and microbial communities. Additionally, the fungal assemblage showed that *T. versicolor* was successfully immobilized in the RDB, which triggered a major shift in the initial community. Afterwards, solid by-products were treated in a fungal biopile-like system reaching high biodegradation rates. Therefore, this study validates the fungal RDB as a viable alternative for AWW treatment, opening up the possibility of a further in-situ and full-scale application.

Keywords

Fungal bioremediation, Pesticides, Agricultural wastewater, Fluidized-bed bioreactor, Rotating-drum bioreactor

1. Introduction

Pesticides are essential to ensure pest control and food production worldwide. Nevertheless, the growing occurrence of pesticides in water resources has become a worldwide concern, as these compounds are considered persistent, bioaccumulative and toxic to human beings and the environment, even at low concentrations. In this respect, particular emphasis should be placed on agricultural washing wastewater (AWW), also known as rinsate, which is generated on farms when washing agricultural machinery and equipment (European Commission, 2009).

Physical-chemical treatments are effective processes for removing organic pollutants, but these methods are limited by their high costs and the generation of transformation products. In contrast, bioremediation is gaining increasing attention as an environmentally friendly, efficient and low-cost approach (Marican and Duran-Lara, 2018). In particular, bioremediation using white rot fungi (WRF) seems to be a very promising technology for AWW treatment. The powerful enzyme system of WRF allows the removal of a wide range of organic compounds, including pesticides, considered recalcitrant in other treatment systems (MirTutusaus et al., 2018).

Nevertheless, one of the main drawbacks of fungal treatments is bacterial competition, hence different strategies are frequently used to enhance fungal dominance, such as biomass immobilization. There are two main types of biomass immobilization: in pellet form (also known as autoimmobilization) or on lignocellulosic materials (such as wood). In this regard, a comparative study between two types of reactors, a fluidized-bed bioreactor (FBB) with *T. versicolor* pellets and a rotating drum bioreactor (RDB) with *T. versicolor* immobilized on wood, has been carried out to elucidate which approach is more viable for scaling up the treatment of AWW from rinsing pesticide application equipment.

2. Materials and methods

2.1. Rotating drum bioreactor

The RDB was constructed with a methacrylate tube supported on a polyvinylchloride gutter. The wastewater was contained within the channel while the colonized wood chips were placed inside the tube. Approximately 30% of the biomass was submerged in the liquid phase, while the rest was in direct contact with air. The inner tube was connected to an electric motor that rotates (1.5 turns every 24 h) to alternate the submerged biomass fraction. The RDB (2.3 L) was fed in sequential batches for two cycles of 17 days (RDB-1 and RDB-2 for the first and second cycle, respectively) under non-sterile conditions. The pH was automatically controlled at 4.5 by adding either 1 M HCl or NaOH. The inner tube contained 1100 g DW of colonized wood (2.2 g DW·L^{-1} of fungal biomass).

2.2. Fluidized-bed bioreactor

A total of 2.2 g·L^{-1} DW pellets were transferred to an FBB (1.5 L). The AWW was previously

autoclaved at 121ºC for the experiment under sterile conditions. The reactor was operated in batch mode for 17 days, both under sterile (FBB-S) and non-sterile (FBB) conditions, and at 25 ºC. The pH was controlled at a constant value of 4.5 by adding 1 M NaOH or 1 M HCl. Fluidized conditions were achieved by introducing a 1 s air pulse every 4 s through a solenoid valve placed at the bottom part of the reactor. The aeration rate was set at 0.8 $L \cdot min^{-1}$. Glucose and NH_4Cl were fed for *T. versicolor* maintenance at a molar C/N ratio of 7.5.

2.3. Solid-phase treatment in a biopile-like system

Solid by-products produced in the RDB were piled inside Scott glass bottles (Duran, Inc; 250 mL, 95 × 105 mm) equipped with an open-port screw cap opened with a passive air inlet through a 0.45 µm filter. A total of 30 g DW of by-products (in triplicate) obtained at the end of the second batch in the RDB were treated under non-sterile conditions and 25 ºC for 27 days.

3. Results and discussion

3.1. Pesticide removal performance

Pesticide removal profiles in the FBB and RDB treatments are shown in Fig. 1. The FBB-S was more efficient than the FBB concerning total pesticide removal, reaching 88 and 51%, respectively, which indicates that *T. versicolor* was involved in pesticide degradation, and that other microorganisms, such as bacteria, competed for substrate and reduced fungal metabolic activity over time. Under non-sterile conditions, total elimination in RDB (85%) was higher than in FBB (51%). In fact, equilibrium was not reached in the FBB during the 17 days of treatment, while the RDB showed maximum removals after the first 5 days. This result is particularly interesting for future full-scale reactor applications, as operating periods, and thus reactor volumes, could be considerably reduced. However, a significant amount of pesticides could be sorbed on the wood in the RDB.

3.2. Toxicity

The initial toxicity (13.6 TU) was reduced to 8.6 and 2.2 TU in the FBB and RDB, respectively. As occurred in the case of the detected pesticides, sterile conditions favored the reduction of toxicity in the FBB, denoting a better degradation activity of the fungal consortium in the absence of competing microorganisms. However, even under non-sterile conditions, the removal capacity of the RDB prevailed over that of the FBB. In any case, toxicity values were lower than the wastewater discharge limit (25 TU) established in Catalonia (DOGC, 2003). Similar results were reflected in the phytotoxicity test. The treated water obtained from the RDB presented better quality than that from the FBB in terms of relative root elongation (93.7% versus 82.9%, respectively) and relative seed germination (111.8% versus 76.5%, respectively).

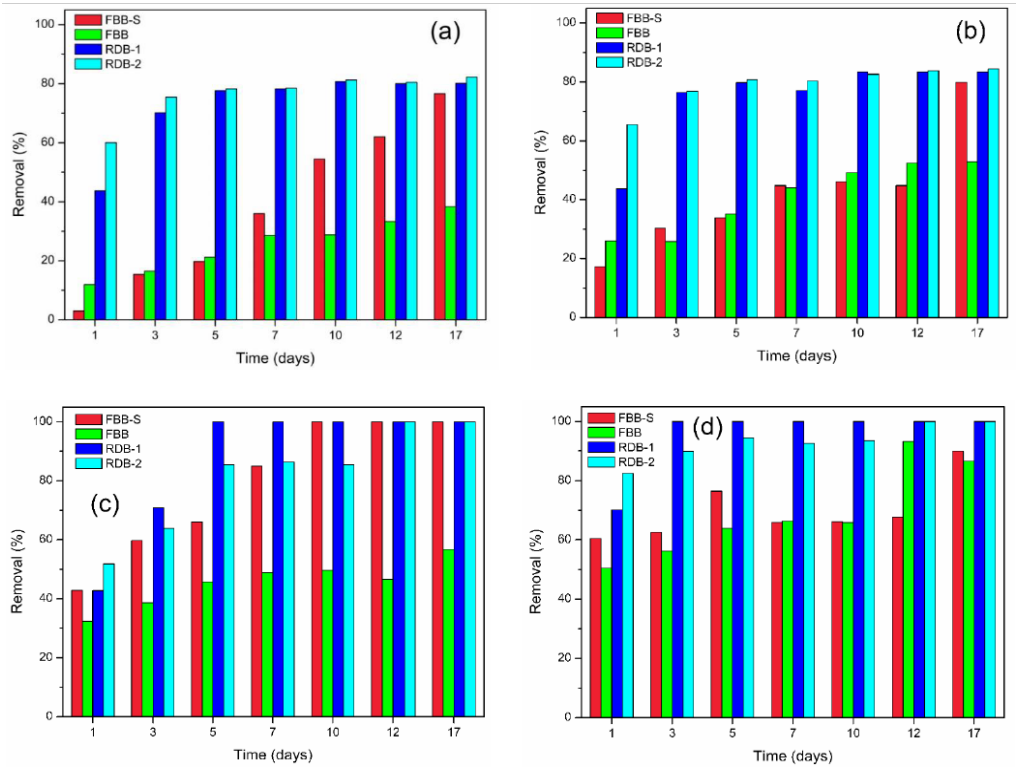

FIGURE 1. PESTICIDE REMOVALS ACHIEVED FOR THIA (A), CHLOR (B), AZO (C) AND TEBU (D) IN EACH EXPERIMENT

3.3. Fungal community assemblage

Samples corresponding to AWW displayed high fungal diversity, with 3 prominent genera representing more than half of the fungal community: Penicillium sp., Phanaerochaete sp. and Meyerozima sp. Trametes sp. was not identified in any of the three replicates, indicating its absence from the initial AWW. After the treatment, Trametes sp. was detected in the liquid phase, but its presence in the RDB effluent was actually minor, whereas in the final sample of the FBB effluent Trametes sp. represents almost 50% of the fungal diversity. This result suggests that the strategy of immobilization on wood used in the RDB was more effective in retaining biomass than the auto-immobilization (pellets) approach implemented in the FBB.

3.4. Solid-phase study and treatment

Solid by-products resulting from the RDB were subsequently treated in a biopile-like reactor during 27 days to evaluate the ability of the remaining fungus to biodegrade sorbed pesticides, achieving notable biodegradation rate of total pesticides ($3.25 \cdot 10^{-4}$ mg·g wood $DW^{-1} \cdot day^{-1}$).

Conclusions

AWW with an inherently high pesticide content was treated in two different fungal reactors. The RDB proved to be a better candidate than the FBB according to all studied parameters, including 87 % versus 51% in pesticide removal, respectively. Fungal community study showed that *T. versicolor* was especially dispersed in the FBB, while this fungus was successfully immobilized in the RDB. In addition, solid by-products were treated with *T. versicolor* in a biopile system achieving remarkable biodegradation rates of pesticides of 3.25 $mg \cdot \cdot g^{-1} \cdot day^{-1}$. These results suggest that the RDB is a promising approach for further AWW full-scale treatments.

Acknowledgements

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DESIGN, DEVELOPMENT AND SCALE-UP OF AN AEROBIC *IN-SITU* BIOREACTOR FOR REMOVAL OF HCH IN GROUNDWATER

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Summary

Lindane and other hexachlorocyclohexane isomers (HCH) are complex target pollutants for the implementation of bioremediation strategies of contaminated sites due to its persistency and low solubility. Nonetheless, microbial degradation of HCH is known to be possible through different degradation mechanisms under either aerobic or anaerobic conditions, although the implementation of these biological processes as remediation strategy is challenging.

In that sense, different bioremediation technologies are possible but its successful implementation for field-scale treatment should address for multiple considerations and design parameters. One of these growing alternatives is the implementation of bioreactor systems which can be an efficient, robust and viable way to approach HCH removal from contaminated environments.

This work involves a scaling strategy for the development of an aerobic bioreactor from laboratory experiments until its implementation in a pilot field test for groundwater treatment. The first steps of the study involve the initial screening of samples of different nature using a multi-criteria approach that may allow the selection of the best candidates to be used as inoculum in the successive phases (e. g., microbial populations, diversity or tolerance to contaminant concentrations). At this stage co-inoculation of different samples to enhance microbial activity will also be addressed. Secondly, experiments in flasks will be carried out to for the assessment of degradation efficiency and basic design conditions and parameters such as optimal pH, nutrients, minerals or contaminant bioavailability. At the final stages, a laboratory scale bioreactor would be carried out to optimize and adjust operating conditions that could be implemented at field scale and that support biomass growth during project lifecycle.

Keywords

Bioremediation; HCH; bioreactor; microbial communities; laboratory; pilot test

Background / site characteristics

Bailin's landfill is a former unlined landfill where industrial waste generated during the manufacture of lindane was disposed for more than 10 years. Leachate from the waste deposited on the ground generated over time a multi-component DNAPL (Dense Non Aqueous Phase Liquid) that has infiltrated and created a 1.5 km plume in a complex geological system.

The site is composed by a fractured bedrock, consisting of a series of interbedded vertical sandstone and limestone layers with rather different hydrogeochemical characteristics that influence contaminant distribution. For example, limestone layers have a much lower fracture density and hydraulic conductivity that consequently involves a lower advection flow through the fractured network. Besides, some of the sandstone layers have a greater continuity downstream and present a greater number of fractures at depth. Interlayer connection is limited. Figure 1 shows the disposition of the geological layers in the landfill and depicts a diagram detailing the different fracture density that could be found in both lithologies.

Since the formation is basically rock, contamination mainly affects the aquifer beneath the surface which is confined through the fractured network until finally discharges downstream in the Gallego river.

Aforementioned characteristics imply that, outside the source, groundwater impact is usually greater in the sandstone layers than in limestone. Such conditions present two different scenarios in terms of the way and priority of treatment of the different areas on the site.

Since 2004 several active remediation techniques such as pump and treat, chemical oxidation or AS-SVE have been successfully applied at the site. In most cases, when working outside the source, these remedial treatments have been widely applied in sandstone layers, while works in limestone have been scarce. However, limestone areas may be a good starting point to study the application of other techniques that cannot currently be applied in the sandstone layers, like for example bioremediation, since they usually have much lower concentrations in groundwater that could be affordable using this type of techniques.

This work describes the study and development, using a scaling strategy, of an *in-situ* aerobic bioreactor from laboratory experiments, until its intended implementation in a pilot field test for groundwater treatment of limestone areas.

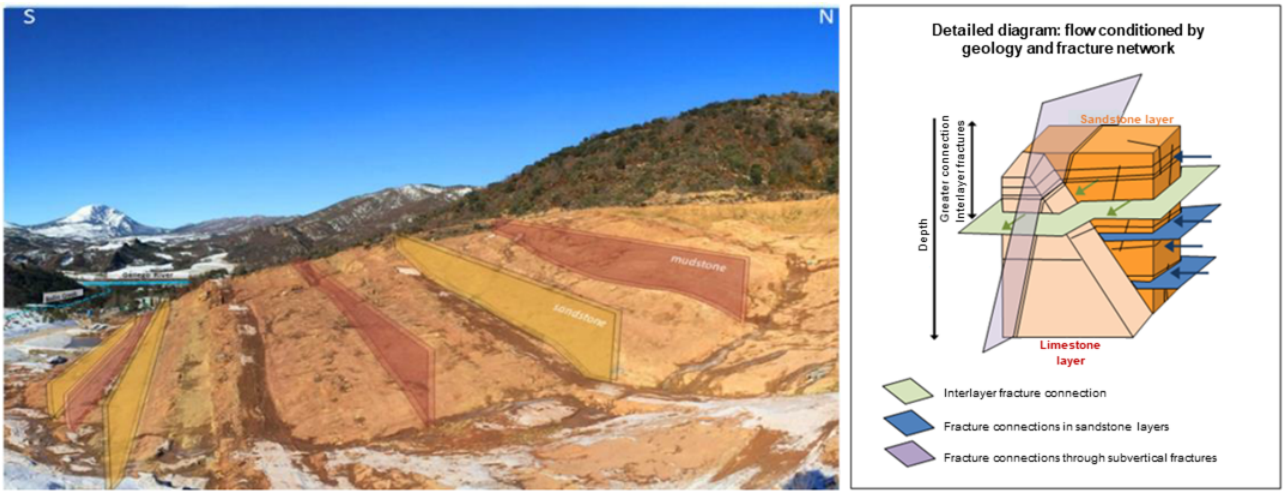

FIGURE 1. GEOLOGICAL CHARACTERISTICS OF THE SITE

Bioreactors as an in-situ bioremediation system

Bioremediation can be understood as a group of techniques based on the use of biological systems, such as plants, fungi or especially microorganisms, for the remediation of contaminated sites. In the latter case, strategies based on the use of microorganisms rely on the availability in the environment of microorganisms capable of degrading the contaminants existing on the site. These microorganisms can be endogenous, present in the medium itself, or added from external sources. In both cases, however, the viability of the remediation will depend on the sustained activity and growth of the microbial communities over time.

However, the design, control and optimization of a bioremediation process is complex since it involves multiple factors often interrelated among them. For example, the rate of biodegradation of contaminants may depend on the presence of contaminant-degrading microbial communities, the type of matrix (soil, water, etc.), the bioavailability of the contaminants, the conditions of the medium (pH, temperature, etc.) and the presence of electron acceptors and nutrients, among others. Figure 2 summarizes some of the key factors that may play an important role in biodegradation processes.

Bearing in mind the number of factors involved in this type of process the suitability of the bioremediation strategy may be often site-specific so, therefore, the availability of a solid conceptual model gains importance.

Nowadays there are different treatment options to carry out a potential bioremediation using naturally occurring microorganisms to degrade contaminants. One possibility is the addition of microbial communities to the medium through the use of bioreactors.

A bioreactor can be defined as a system capable of producing a controlled and isolated environment that guarantees and maximizes the growth of a culture of microorganisms. Its application to environmental remediation may be configured as an in-well system where an exogenous microbial community is carried to the contaminated site which, after a period of adaptation, will proliferate and degrade *in-situ* the contaminants of interest.

Nonetheless, the application of this type of systems to the field with the minimum cost and high performance (productivity) is a very complex process, that involves several optimization and scaling-up stages starting from the laboratory. Throughout these stages, different aspects must be evaluated, such as the most appropriate starting samples, nutrients or amendments. Previous investigations are intended to ensure an optimal and robust performance of the system over time and could also include the evaluation of economic feasibility of its application on a large scale.

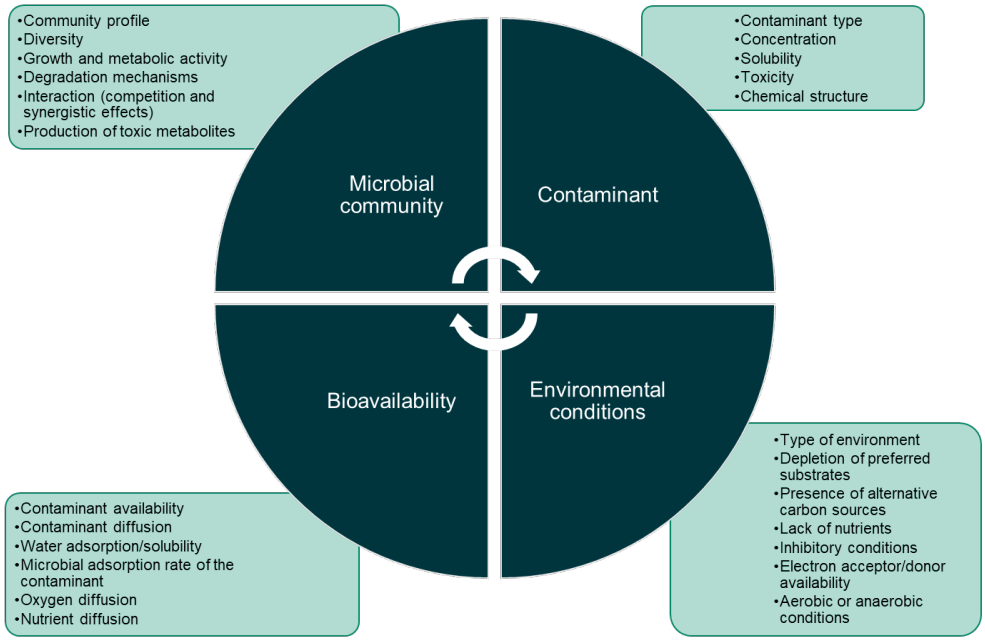

FIGURE 2. FACTORS INVOLVED IN A BIOREMEDIATION PROCESS

Conceptual design and treatability test development

This work proposes the design of a workflow to study the feasibility and potential application of an *in situ* bioreactor as a pilot system in the Bailín aquifer. The bioreactor will provide a pool of HCH-degrading bacteria and pollutants of interest capable of carrying out a sustained remediation of the aquifer over time.

As previously discussed, in general, limestone areas have lower impact and much lower hydraulic conductivity than sandstone layers. Those conditions imply a more stable environment and concentrations of contaminants that may be suitable for the implementation of bioremediation treatments. The upper areas of the former landfill have hydrogeochemical characteristics fitting the previous statement and, hence, would constitute a good location for the potential application of this technology.

In the current work, a multi-step process as shown in the workflow depicted in Figure 3 have been design for the development of a robust bioreactor as remediation strategy. The more thorough the entire design process is, the more likely it is that the system will be viable and that the microorganisms will proliferate and show activity when applied to real field conditions.

First steps involve laboratory experiments aimed to find a bacterial consortium with a high HCH-degrading capacity. Those experiments include the characterization of samples of the different nature (i.e., water, soil, sediment and sludge) to identify matrices with interesting microbial characteristics to be used as inoculum or bacterial starters. The inoculum is the basis on which the biological system will be developed. The selection of a proper inoculum is crucial to obtain consortia of adapted microorganisms that can acclimatize more easily to the conditions of the environment, that are viable, versatile and with a high capacity to degrade the pollutants of interest.

Samples for that purpose would be collected at the site because it is more likely that microbial communities are adapted to the complex mixture of contaminants existing in the Bailin aquifer. Several microbial attributes can be selected and study in this preliminary screening of the samples to obtain information about their microbial potential. For example, plate count experiments to assess CFU population in generic and HCH-selective media may be, respectively, indicative of total viable and cultivable microbial populations, as well as specialized microbial populations. This information can be complemented with microplate experiments that provide information about metabolic activity, microbial community diversities and their tolerance to contaminants of concern. In general, these classical microbiological techniques have shown to be an excellent, cost-effective and time saving methods to carry out a preliminary screening.

Depending on the results of the previous stage, it may be necessary to study new candidates or to further investigate the samples that yielded the best results. Among the possibilities to carry out the additional characterization, for example, it is possible to test mixtures of various samples to evaluate co-inoculation as an effective strategy to enhance microbial activity of individual samples.

Further in the scaling process, flask experiments would be implemented to study different treatments and environmental conditions to the most promising samples. At this stage the selected consortium would be incubated for a long period of

time, during which the degradation of the pollutants of interest, the physicochemical parameters of the sample and the bacterial populations can be evaluated.

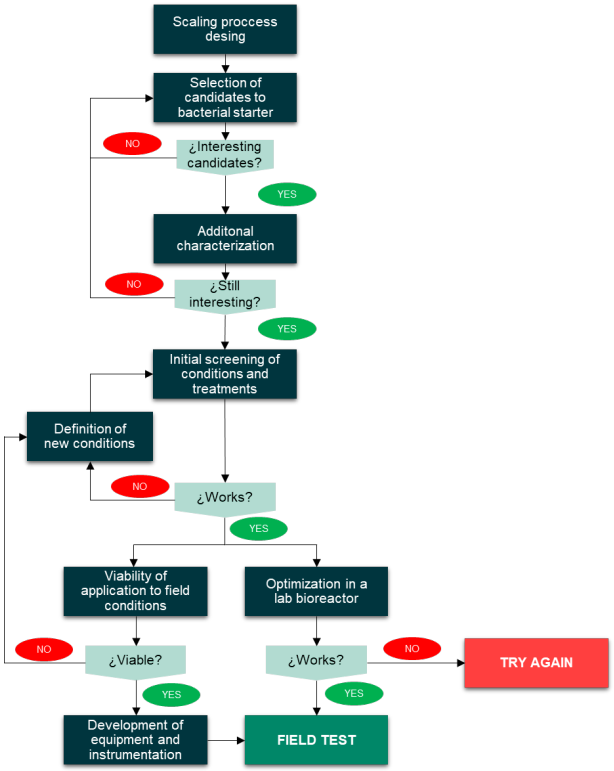

FIGURE 3. WORKFLOW FOR THE DEVELOPMENT OF AN *IN SITU* BIOREACTOR AS A PILOT BIOREMEDIATION SYSTEM

Depending on the observed behavior, it will be possible to decide whether the evaluated conditions are interesting or whether it is necessary to evaluate new ones.

Later on, the laboratory characterization will conclude with the incubation of the bacterial consortium in a laboratory bioreactor where the conditions selected in the previous step can be more accurately studied and controlled. This step follows to optimize and acclimatized the consortium in order to enhance the degrading capacity prior to its application to field conditions.

At the same time, it would be necessary to study methods for transferring the bacterial consortium from the laboratory to the aquifer and the feasibility of effectively applying in the field those treatments that sustain the microbial activity and the performance of the system.

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APPLICATION OF THE METHOD OF PHYTOREMEDIATION OF PESTICIDE CONTAMINATED SOILS IN A FIELD EXPERIMENTAL PLOT IN CHIM-KORGON VILLAGE

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Summary

The widespread use of pesticides in agricultural practices has led to the fact that all countries of the world, in one way or another, face the problem of pesticide waste. The main problem is the elimination of unused and obsolete pesticide stockpiles, as well as the reclamation of adjacent areas. The sources of pesticides entering the ecosystem are old pesticide storage facilities, landfills, and agricultural aviation airfields. Many storage facilities are either completely or partially destroyed or converted to other needs. For the Kyrgyz Republic, the problem of disposal of obsolete pesticides is relevant, since there are more than 200 storage sites in the republic, and more than half of them are in an unusable condition, with contaminated degraded soil. In this study, an experimental site was selected - the former airfield in Chym-Korgon village (N 42049'23.9" and E 75031'49.8"); since 2021, aerobic biodegradation technology by soil bacteria has been carried out at this site. Chromatographic analysis in the spring of 2022 showed the presence of residual amounts of organochlorine compounds. Therefore, the phytoremediation method was applied at this site, which is an *in-situ* strategy, cheap and sustainable, suitable to remove this type of pollutants, for self-remediation of soil processes. To evaluate the potential of phytoremediation of soils, we chose the seeds of agricultural plants, since the seeds of agricultural crops are easily available, and has a short growing season. It was found that in the soil-plant system, the efficiency of the process of phytoextraction of pesticides increases.

Keywords

Obsolete pesticides, contaminated soil, phytoremediation, seeds of agricultural plants, types of pesticides.

Introduction

Organochlorine types of pesticides are very stable in the environment, they are very difficult to decompose and tend to migrate in the ecosystem. These types of pesticides are classified as persistent organic pollutants (POPs) [1-3, 6-8]. These types of pesticides have been banned for more than 50 years, and yet they pose a threat to the ecological stability of the area where they are located. With the support of the international organization IHPA (they have been partially or completely eliminated [2]. Even when the pesticides have been disposed of, the soils around the storage facilities are heavily contaminated with pesticides and their persistent metabolites. Studies show that concentrations of banned or obsolete pesticides in the soil around inactive storage facilities exceed sanitary and hygienic standards by hundreds of times [4]. These soils are highly degraded, these areas are completely devoid of vegetation, and at a distance of 700-1000 meters, there is a strong smell of pesticide residual compounds. Cleaning soils from pesticides the most difficult process, because of the peculiarities and diversity of both soil types, soils, topography, etc. Among remediation strategies, phytoremediation is a relatively inexpensive, effective method of cleaning soils from contaminants, which contributes to the creation of safe agricultural soils [9].

The purpose of this study for the selections of plants that accumulate pesticide residues in their tissues without the plants themselves losing their vitality.

Material and methods

From 2021-2022, the technology of bioremediation of contaminated soils with obsolete pesticides is being carried out at the experimental site, is the former airfield in Chym-Korgon village (JPS data: N 42049'23.9" and E 75031'49.8", and elevation 974 m above sea level). This site, was used during agricultural operations to refuel aircraft and spray pesticides from the air, and use for pesticide storage. Aerobic bioremediation technology was applied with a biopreparation created on the basis of soil bacteria, which were isolated from pesticide dumps, from the Suzak district, and one strain was isolated from contaminated soils of storage facilities. A number of agro-technological measures (application of clean mountain soil, constant loosening, constant soil moistening) were also included. Soil samples were taken from a depth of 25-30 cm. Chromatographic analysis (Master GS, standard NEN-6980 of the Netherlands Institute of Standardization) [11] for the quantitative composition of organochlorine compounds was performed in the spring and autumn period. The area of the plot is 250 m^2. The phytoremediation potential of the contaminated soils was tested for

the seeds of agricultural plants. In the plot was sown with seeds of maize (*Zea mays*), wheat (*Triticum aestivum*) and beet (*Beta vulgaris*). Plant samples were taken according to the experimental variants at sprouting phase, tillering phase, and ripening phase. The above-ground and underground parts of plants were dried separately. Mean values and standard deviations of three replications were calculated using data analysis tools IBM SPSS Statistics.

Results and discussion

It was found that seeds soaked in a biological preparation have high germination rate and they have a positive effect on growth and development of plants. Therefore, seeds were pre-treated with biopreparation from our laboratory collection, based on Streptomyces genus [5]. The effectiveness of phytoremediation of soils depends on the productivity of plants, and the greater the biomass of plants, the more pollutants it can accumulate in their tissues. A number of studies [9] have shown a direct dependence of plant productivity on their photosynthetic activity, which is one of the most important indicators determining the efficiency. Site area is 250 m^2. Potential of phytoremediation of contaminated soils was tested on seeds of agricultural plants. The plot was sown with seeds of maize (Zea mays), wheat (Triticum aestivum) and beet (Beta vulgaris).

TABLE 1. PESTICIDE CONTENT IN UNDERGROUND AND ABOVEGROUND PLANT PARTS

№	Pesticide	Concentration in control soil (fertile soil + no bioproduct) (mg/kg ± SD)		Concentration in experimental soil (fertile soil + bioproduct) (mg/kg ± SD)		Concentration in control soil (fertile soil + no bioproduct) (mg/kg ± SD)		Concentration in experimental soil (fertile soil + bioproduct) (mg/kg ± SD)		Concentration in control soil (fertile soil + no bioproduct) (mg/kg ± SD)		Concentration in experimental soil (fertile soil + bioproduct) (mg/kg ± SD)	
		Zea mays				Triticum aestivum				Beta vulgaris			
		underground part	above ground part	underground part	above-ground part	underground part	above-ground part	underground part	above-ground part	under-ground part	above ground part	under-ground part	above-ground part
1	A-BHC	0.022± 0,04	0.004 ± 0,09	0.034 ± 0,17	0.004± 0,13	0.023± 0,05	0.024± 0,07	0.053± 0,05	0.049± 0,03	0.010± 0,07	0.022± 0,07	0.026± 0,17	0.013 ± 0,08
2	G -BHC	0.014± 0,08	-	0.074 ± 0,23	0.004± 0,01	0.022± 0,01	0.037± 0,06	0.053± 0,06	0.041± 0,08	0.070± 0,16	0.010± 0,27	0.044± 0,17	0.026 ± 0,08
3	B -BHC	0.092± 0,05	0.024 ± 0,02	0.272 ± 0,06	0.021± 0,13	0.109± 0,04	0.036± 0,05	0.110± 0,08	0.112± 0,07	0.133± 0,07	0.129± 0,07	0.167± 0,13	0.071 ± 0,12
4	D-BHC	0.036± 0,12	0.003 ± 0,07	-	0.059± 0,10	0.046± 0,03	0.010± 0,06	0.007± 0,02	0.061± 0,06	0.037± 0,21	0.083± 0,03	0.015± 0,07	0.028 ± 0,11
5	Heptachlor	0.029± 0,07	0.004 ± 0,01	0.056 ± 0,11	0.018± 0,09	0.009± 0,01	0.015± 0,07	0,073± 0,09	0.043± 0,06	0.061± 0,04	0.045± 0,04	0.034± 0,11	0.018 ± 0,17
6	Aldrin	0.096± 0,08	0.005 ± 0,02	0.131 ± 0,05	0.017± 0,11	0.029± 0,02	0.016± 0,04	0.337± 0,08	0.112± 0,05	0.293± 0,04	0.069± 0,05	0.086± 0,03	0.024 ± 0,16
7	Heptachlor-epox	0.085± 0,08	0.011 ± 0,02	0.120 ± 0,02	0.009± 0,01	0.034± 0,03	0.004± 0,05	0.283± 0,04	0.384± 0,03	0.152± 0,03	0.056± 0,06	0.006± 0,02	0.086 ± 0,17
8	Trans-Chlordane	0.008± 0,05	0.006 ± 0,18	0.013 ± 0,06	0.013± 0,06	0.003± 0,06	0.003± 0,04	0.121± 0,05	0.097± 0,05	0.090± 0,04	0.065± 0,04	0.021± 0,07	0.018 ± 0,14
9	G-Chlordane Endosul-1-A-Chlordane	0.052± 0,02	0.010 ± 0,05	0.128 ± 0,08	0.011± 0,02	0.056± 0,04	0.018± 0,04	0.045± 0,06	0.092± 0,04	0.104± 0,04	0.036± 0,05	0.143± 0,11	0.056 ± 0,11
10	4,4 DDE	0.086± 0,17	-	0.172 ± 0,05	0.013± 0,08	0.021± 0,05		0.033± 0,02	0.065± 0,05	0.055± 0,05	0.025± 0,03	0.033± 0,12	0.022 ± 0,08
11	Dieldrine	0.285± 0,02	0.016 ± 0,08	0.107 ± 0,06	0.067± 0,15	0.045± 0,07	0.045± 0,11	0.537± 0,06	0.761± 0,04	0.807± 0,09	0.099± 0,03	0.695± 0,14	0.021 ± 0,09
12	Endrine	0.012± 0,19	0.002 ± 0,11	0.024 ± 0,07	0.003± 0,06	0.019± 0,04	0.009± 0,06	0.053± 0,04	0.128± 0,05	0.041± 0,02	0.023± 0,02	0.052± 0,14	0.024 ± 0,12
13	4,4 DDD	0.018± 0,13	0.004 ± 0,04	0.185 ± 0,05	0.003± 0,15	0.100± 0,05	0.004± 0,05	0.190± 0,06	0.373± 0,01	0.205± 0,07	0.079± 0,14	0.159± 0,15	0.077 ± 0,12
14	Endosulfane-2	0.202± 0,07	0.007 ± 0,07	0.208 ± 0,06	0.032± 0,16	0.150± 0,06	0.026± 0,04	0.183± 0,05	0.368± 0,03	0.271± 0,09	0.107± 0,14	0.211± 0,11	0.093 ± 0,02
15	Endrine-Aldehid	0.133± 0,23	0.003 ± 0,03	0.106 ± 0,05	0.013± 0,03	0.092± 0,09	0.019± 0,05	0.235± 0,03	0.219± 0,06	0.209± 0,02	0.049± 0,11	0.241± 0,11	0.062 ± 0,11
16	4,4 DDT	-	-	0.107 ± 0,02	0.026± 0,04	0.094± 0,12	0.030± 0,06	0.123± 0,01	0.206± 0,04	0.261± 0,02	0.026± 0,15	0.097± 0,13	0.020 ± 0,04
17	Endosulfan-sulfat	0.021± 0,14	0.019 ± 0,02	0.047 ± 0,03	0.024± 0,13	0.015± 0,04	0.015± 0,05	0.057± 0,09	0.079± 0,03	0.056± 0,03	0.018± 0,17	0.062± 0,13	0.012 ± 0,03
18	Metoxichlor	0.007± 0,28	0.015 ± 0,08	0.035 ± 0,08	0.014± 0,18	0.011± 0,03		0.214± 0,01	0.056± 0,02	0.065± 0,05	0.006± 0,02	0.050± 0,11	0.007 ± 0,07
19	Endrine-ketone	0.024± 0,06	0.012 ± 0,11	0.019 ± 0,18	0.010± 0,14	0.023± 0,7	0.011± 0,07	0.195± 0,06	0.154± 0,07	0.161± 0,02	0.027± 0,02	0.153± 0,12	0.012 ± 0,07

Chromatographic analysis in the spring of 2022 showed the presence of residual amounts of organochlorine compounds. Therefore, the phytoremediation method was applied at this site, which is an in situ strategy, cheap and sustainable, suitable to remove this type of pollutants, for self-remediation of soil processes. The area of the site is 250 m^2. To assess the potential of phytoremediation of soils, we chose the seeds of agricultural plants, because the seeds of agricultural crops are easily available, and has a short growing season.

In our studies, it was confirmed that plants are able to accumulate more metabolite of obsolete pesticides in their vegetative organs. It has been observed that in the presence of growth-promoting substances, the process of metabolite migration from the soil to the root system is enhanced. Table 1 shows, the root system of plants actively accumulates 4,4 DDT, in maize roots it was contained to – 0.107± 0.02 mg/kg soil, in wheat roots – 0.123± 0.01mg/kg; and in beet roots, 4,4 DDT content was - 0.261± 0.02 mg/kg.

Conclusions

The efficiency of the phytoremediation process depends on the plant species, in beet roots their amount was higher than in other plants. Microbiological activity was also higher in these soils, which indicates the gradual removal of pesticides and has a positive effect on the fertility of agricultural soils.

Acknowledgments

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ASSISTED-BIOREMEDIATION FOR THE DEGRADATION OF ORGANOCHLORINE COMPOUNDS

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Summary

For several decades, the organochlorine pesticide lindane (γ-hexachlorocyclohexane, γ-HCH) has been used in agriculture and medicine due to its wide field of application. Its high toxicity and low degradation have caused negative effects on the environment and the human health, resulting in the presence of contaminated sites that must be remediated. The object of this study was to evaluate the bioremediation assisted with compost in a soil contaminated with organochlorine compounds. The soil comes from a site located in Sabiñánigo (Huesca). The initial analysis showed concentrations of chlorinated compounds above the legislative limits, being the majority compounds 1,2,4-trichlorobenzene and the α-HCH and β-HCH isomers. The experiment was carried out in mesocosms (500 g soil) for 60 days. The effect of natural attenuation (NA) was compared with the application of a compost from sewage sludge with pruning waste (B-CP), and a mineral treatment from a NPK fertilizer (B-NPK). The NA achieved around 45% degradation, not observing a positive effect when providing soluble nutrients (B-NPK). The application of compost (B-CP) induced an increase in degradation to values around 57% in the 60 days of incubation probably due to the double effect of biostimulation by the contribution of nutrients and organic matter and a potential bioaugmentation due to the incorporation of exogenous microorganisms. The application of compost showed a positive effect in the recovery of soil functionality, observing increases in biological activity of the soil as well as the decrease of its phytotoxicity.

Keywords

Polluted soil, lindane, HCH, biostimulation, natural attenuation, biological activity

Introduction

Soil contamination alters soil biodiversity and reduces the organic matter it contains and its ability to act as a filter (FAO, 2022).

Lindane was used for decades as an insecticide, and is currently withdrawn from the market. However, its continued use gave rise to contaminated soils that must be recovered, considering the environmental and human health risks due to the stability and hydrophobicity of this compound and other HCH isomers (Rigas et al., 2009). Bioremediation uses the degradative capacity of soil microorganisms to eliminate a wide variety of contaminants, which can be transformed into innocuous products, at a low cost and without environmental risks. The bioremediation process is highly influenced by environmental conditions, the functional capacity of the soil microbiota, and the properties of the pollutant and the soil. Different studies have evaluated the ability of specific microorganisms to degrade lindane and other HCH isomers (Asemoloye et al., 2017, Usmani et al., 2021). In general, the assays have been carried out using soils enriched with lindane, therefore the results obtained cannot be extrapolated to real conditions. The use of compost from waste, to facilitate bioremediation, supposes the supply of nutrients and organic matter, and the improvement of the physical characteristics of the soil that stimulate the activity of native soil microorganisms. On the other hand, given the origin of the compost, its application incorporates exogenous microorganisms with a very diverse metabolic potential into the soil that can contribute to the degradation of organochlorine compounds (Kästner and Miltner, 2016).

The aim of the study is to evaluate the efficiency of bioremediation assisted with compost in a soil polluted with organochlorine compounds.

Methodology

The soil used (S) comes from a site located in Sabiñánigo (Huesca). The initial analysis showed concentrations of chlorinated compounds above the legislative limits (67 mg/kg HCH), with 1,2,4-TCB compounds and the α-HCH and β-HCH isomers being the majority.

To evaluate its degradation, an incubation test was carried out under controlled conditions of temperature (26ºC) and humidity (60% of field capacity) for 60 days. A treatment with compost (CP, 10% w/w) from sewage sludge and pruning remains (B-CP) was used to favour biodegradation and its effect was compared with a natural attenuation treatment (NA) supplying water and aeration to the soil and a biostimulation treatment providing nutrients from an NPK fertilizer (15:15:15) at a dose of 47 kgN/ha (B-NPK). The characteristics of the soil and the compost are shown in Table 1.

After 60 days of incubation, 1,2,4-TCB was determined by HS-GC/MS according to the UNE-EN ISO 22155:2016 / EPA 8260b method. For the determination of the α-HCH and β-HCH isomers, GC-MS was used according to the NEN 6980:2006 method. The biological activity of the soil after the treatments was evaluated through the measurement of the enzymatic activities β-galactosidase (GAL), β-glucosidase (GLU), urease (URE), acid phosphatase (FAC), alkaline phosphatase (FAL) and arylsulfatase (ARIL), related to the C, N, P and S cycles. The colorimetric substrates were measured in 96-well plates applying the ISO 20130, 2018 standard. The different soil samples were divided into three subsamples and the analysis was performed in three repetitions for each subsample. After the corresponding incubation times, the absorbances were measured in a microplate photometer (Multiskan, FC). A germination test was carried out to evaluate the potential loss of phytotoxicity of the soil after incubation according to the methodology described by Zucconi et al. (1985). An analysis of variance ($p<0.05$) was carried out and Duncan's test was applied to evaluate the differences between the different treatments.

TABLE 1. SOIL AND COMPOST (CP) PROPERTIES

	pH	E.C.	O.M.	N	P	Ca	Mg	Na	K	Pb	Cd	Cu	Ni	Zn	Cr
		dS/m	%		mg/kg										
S	8.2	0.4	1.09	0.09	8	3737	146	30	187	25	0.4	14	20	154	25
CP	6.9	18.2	33.9	3.32	1067	13267	1494	765	1714	35	1.39	111	17	322	44

Results and Discussion

The results of the degradation of the organochlorine compounds after the incubation assay are observed in Figure 1. The natural attenuation process (NA) and the biostimulation with mineral nutrients (B-NPK) showed similar results for the degradation of 1,2,4-TCB (49%) and β-HCH (40%). The use of compost (B-CP) achieved the highest values, obtaining degradation percentages between 55-60% for both compounds. In the case of α-HCH, biodegradation was low, observing increases with respect to NA when the process is biostimulated with NPK (B-NPK) or compost (B-CP).

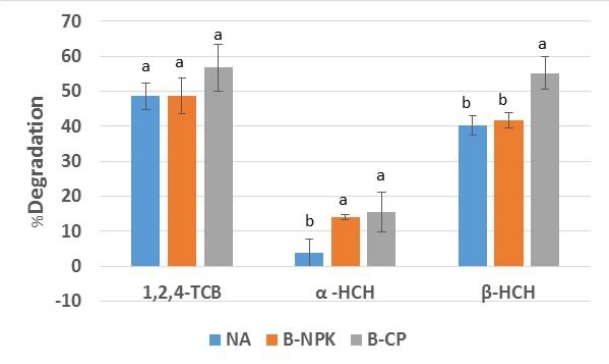

FIGURE 1. DEGRADATION (%) OF ORGANOCHLORINE COMPOUNDS AFTER TREATMENTS. BARS WITH THE SAME LETTER DO NOT DIFFER SIGNIFICANTLY (P < 0.05)

The analysis of the enzymatic activities at 60 days shows that the application of compost caused an increase in microbial activity, affecting all the enzymatic activities (Figure 2).With respect to the natural attenuation (NA) treatment, significant increases of the order of 250% in GLU, FAL and ARIL, 150% in FAC, 90% in URE and greater than 700% in GAL were observed in the biostimulation treatments with compost. However, no significant differences were observed in the NPK biostimulation treatments, with the exception of FAL and ARIL activities that showed a significant decrease in enzymatic activity of around 25%.

These results shows that the application of the organic amendment favours the biostimulation of the soil microbiota due to the effect of organic matter on the activity of autochthonous microorganisms. Nevertheless, the incorporation of exogenous microorganisms from the compost that can promote the degradation of HCH isomers together with autochthonous microorganisms should also be considered (Kästner and Miltner, 2016).

Another possible cause of the increase in microbial activity would be related to the decrease in the bioavailability of pollutants and their effect on the populations of microorganisms. Due to its physicochemical characteristics, HCH isomers have a tendency to be adsorbed to soil organic matter, which decreases its bioavailability against degradation, reducing stress for microorganisms (Kumar and Pannu, 2018).

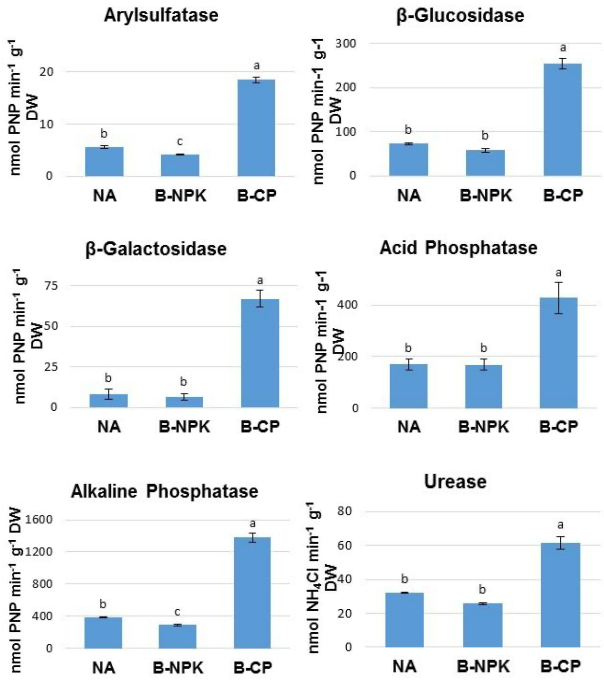

FIGURE 2. ENZYMATIC ACTIVITIES IN THE SOIL AFTER TREATMENTS. BARS WITH THE SAME LETTER DO NOT DIFFER SIGNIFICANTLY (P < 0.05)

The results of the toxicity test (Figure 3) show that after 60 days the natural attenuation treatment (NA) and biostimulation with nutrients (B-NPK) present germination index values <50%, which implies the persistence of the high toxicity of the samples. The

application of compost increases the germination rate to 67%, showing moderate toxicity.

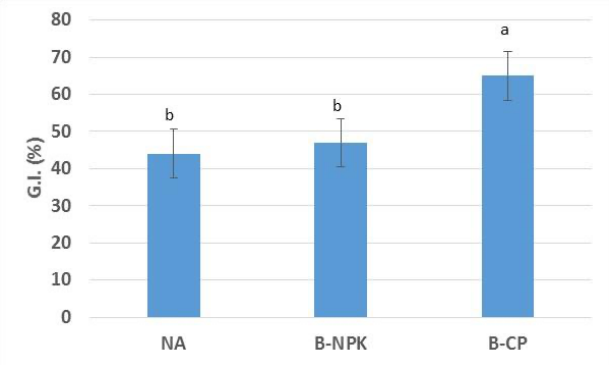

FIGURE 3. GERMINATION INDEX (GI) IN SOIL AFTER TREATMENTS. BARS WITH THE SAME LETTER DO NOT DIFFER SIGNIFICANTLY (P < 0.05)

Conclusions

The organochlorine compounds detected in the soil undergo a degradation process under the evaluated conditions. The natural attenuation (NA) achieves degradations of around 45%, not observing a positive effect when providing soluble nutrients (B-NPK). However, the application of compost induces an increase in degradation to values around 57% in the 60 days of incubation, probably due to the double effect of biostimulation due to the contribution of nutrients and organic matter and a potential bioaugmentation due to the incorporation of exogenous microorganisms. The application of the compost shows a positive effect in the recovery of the functionality of the soil, observing increases in the biological activity of the soil as well as the decrease in its phytotoxicity.

Acknowledgments

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ENHANCED REMEDIATION OF SOIL CONTAMINATED WITH LINDANE, OTHER CHLORINATED PESTICIDES, AND ORGANIC EXPLOSIVES USING ZVI/ORGANIC CARBON REAGENTS

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Summary

Industrial production, agricultural and horticultural uses of organochlorine pesticides (OCPs) has resulted in contamination of soil in many countries. Lindane is the gamma isomer of hexachlorocyclohexane (γ-HCH), and it was extensively used worldwide as an insecticide for almost five decades, from the early 1950s through the late 1990s. Due to its toxicity and environmental persistence, Lindane as well as the alpha and beta isomers of HCH, are recognized as Persistent Organic Pollutants (POPs) in many countries, and the use of Lindane has been banned in more than 50 countries. HCH compounds and several other chlorinated pesticides have proven resistant to traditional soil bioremediation methods. This prompted research on alternate remediation methods and led to the development of a novel biochemical treatment using soil amendments formulated using microscale reduced iron powder, processed organic carbon, and an emulsifying agent. A key finding from the research was that greater degradation of OCPs could be achieved when the soil was treated with ZVI plus organic carbon, than when ZVI alone or organic carbon alone was used. Soils contaminated with Lindane, DDT, Toxaphene, Chlordane, and Dieldrin have been successfully remediated at many industrial and agricultural sites using this soil amendment approach. The ZVI/organic carbon soil amendment has also proven very effective for remediation of soil contaminated with organic explosive compounds, including TNT, DNT, RDX, HMX, and Tetryl. Full-scale soil remediation projects using the iron/carbon soil amendments have been successfully completed in Brazil, Canada, China, Colombia, El Salvador, and the United States. Treatment has been conducted *in situ* on surface soil (i.e., without excavation) as well as on excavated soil with attainment of common remedial goals for industrial and residential land use. Findings from bench-scale research and full-scale projects will be presented and discussed from the perspectives of performance and cost. The ZVI/organic carbon soil amendment approach provides a reliable, economical, and an environmentally sustainable alternative to excavation and off-site soil disposal, soil washing, or thermal treatment.

Introduction

HCH compounds including Lindane, as well as a wide variety of other chlorinated organic pesticides and herbicides, have been used in agricultural, commercial, industrial, and military applications in both developed and developing countries. During their manufacture, formulation, storage, and use these compounds have been deposited to soil. After deposition to soil, they are bound to varying degrees, can be carried to surface water bodies with runoff, or migrate through the soil profile to groundwater. Most chlorinated pesticides are acutely and chronically toxic to terrestrial and aquatic life and persist for extended periods in soil or groundwater; hence, they are classified as POPs and highly hazardous pesticides (HHPs). In addition, some are recognized by the US EPA as probable human carcinogens (i.e., Toxaphene, Chlordane) or tumor promoters (i.e., Lindane, DDT) and many can bioaccumulate and move upward through the food chain (Dich et al., 1997). Microbial biodegradation was identified as a major pathway for the removal of pesticide residues in soil more than 50 years ago (Guenzi and Beard, 1968; Plimmer et al., 1968). Later, a positive relationship between the presence of an active iron redox system in soils with higher organic matter content and shorter DDT half-life was identified (Glass, 1972).

Research and technology development

Between 1992 and 1994, research conducted in Canada revealed that amending soil with a combination of elemental iron powder and certain types of nutrient-rich organic carbon (i.e., processed plant fiber) significantly increased degradation of chlorinated pesticides (Seech et al., 1995). The iron powder/organic carbon soil amendment approach was commercialized by Adventus Remediation Technologies beginning in the early 2000s (US EPA, 2006) and has since been successfully applied to soil contaminated with pesticides, herbicides, and organic explosive compounds in Brazil, Canada, China, Colombia, El Salvador, and the United States. The iron powder/organic carbon soil amendments are now offered under the tradename Daramend® by Evonik Corporation (hereinafter identified as Daramend® reagents).

Treatment mechanisms and advantages of Daramend® reagent

Remediation of pesticide-contaminated soil using the Daramend® reagent is a form of bioremediation known as *In Situ* Chemical Reduction (ISCR). Application of ISCR enhances pesticide degradation rates by promoting both chemical and microbiological processes that remove dissolved oxygen, nitrate, and sulfate from the soil's aqueous phase, and thereby create highly reduced conditions under which the thermodynamic conditions for reductive dechlorination are more favorable (Dolfing, 2003) including dechlorination reactions proceeding more rapidly (Dolfing et al., 2008). Under strongly reduced conditions the release of ferrous iron from ZVI particles is increased; thereby ensuring a good supply of an effective reducing agent capable of participating in pesticide dechlorination reactions. ISCR using the Daramend reagent can be applied to soil *in situ* using a variety of soil mixing equipment, or *ex situ* to soil in bio-piles or treatment cells. Remediation of pesticide-contaminated soil using this approach offers several significant advantages over other approaches, as follows:

Elimination of Soil Bulking: The Daramend® soil amendment is a fine-grained (<500 µm) flowable powder typically applied at low dosages between 1% and 5% by weight of soil undergoing treatment. As a result, the post treatment soil volume is not substantially increased, and geotechnical soil characteristics are not significantly altered. In contrast, traditional composting requires blending of the contaminated soil with large volumes of compost materials (e.g., food waste, manure, wood chips) and results in >30% increased soil volume.

Complete Degradation of Chlorinated Pesticides: Radioisotope studies conducted with ^{14}C-Lindane and ^{14}C-DDT indicated that during treatment of soil with Daramend® reagent, both are dechlorinated with ring cleavage and ultimate mineralization of breakdown products to $^{14}C\text{-}CO_2$. These observations indicate that the reductive dechlorination process stimulated in soil treated with the Daramend reagent results in complete degradation of Lindane and DDT, rather than only partial dechlorination with accumulation of breakdown products. Given that full biodegradation of Lindane and DDT to carbon dioxide has been proven when soil is treated with the Daramend® reagent, it is reasonable to assume that the fate of other pesticides such as Chlordane and Toxaphene during Daramend® treatment is full biodegradation to CO_2.

Cost Effectiveness and Sustainability: Use of the Daramend® reagent to remediate soil *in situ* (without excavation) or on-site (without transportation to an off-site location) enables a more sustainable approach to soil remediation and accrues substantial cost savings versus other approaches such as landfilling, thermal treatment, or soil washing. Elimination of soil excavation, handling, and transportation means that the Daramend® reagent approach is more sustainable.

Results from Laboratory Treatability Testing

Soil contaminated with Lindane and other HCH compounds from an industrial site used to manufacture Lindane was treated with the Daramend® reagent. The objectives of the laboratory testing were to determine the degree to which soil Lindane concentration could be reduced and to identify the fate of Lindane during treatment. The results (Table 1) indicated that substantial degradation of Lindane and the other HCH compounds was achieved in response to treatment of the soil with the Daramend® amendment. The observed sharp reduction in Lindane concentration can be attributed to biodegradation based on radioisotope studies which revealed that almost 50% of the ^{14}C-Lindane (γ-HCH) added to the soil was recovered as $^{14}C\text{-}CO_2$ over a period of 220 days. It is reasonable to believe the observed reductions in other HCH compounds were also a result of biodegradation.

TABLE 1. INFLUENCE OF BENCH-SCALE DARAMEND® TREATMENT ON CONCENTRATIONS OF LINDANE AND OTHER HCH COMPOUNDS IN SOIL.

Condition	α-HCH		β-HCH		γ-HCH (Lindane)		δ-HCH		Total HCH	
	(mg/kg)									
	Initial	Final[1]	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Control (no treatment)	2,710	2,572	826	389	491	77	95	160	4,122	3,197
Daramend®	2,710	197	826	156	491	3	95	5	4,122	361

[1]Final soil HCH concentrations were determined after 250 days of treatment.

Case Studies

Large-scale remediation of soil and sediments contaminated with chlorinated pesticides including Lindane, DDT, Dieldrin, Chlordane, Toxaphene, and major organic explosive compounds has been successfully conducted at many sites worldwide using the Daramend® reagent. Brief case studies from several completed remediation projects are provided.

Case Study 1: Soil at an industrial site was contaminated with Lindane and other HCH compounds during preparation of agricultural insecticide formulations. *In situ* treatment of soil with Daramend reagent was conducted by soil mixing to a depth of 0.6 m and irrigation. The influence of treatment on concentrations of Lindane and other HCH compounds is presented in Table 2.

TABLE 2. INFLUENCE OF FIELD SCALE IN-SITU DARAMEND® TREATMENT ON CONCENTRATIONS OF LINDANE AND OTHER HCH COMPOUNDS IN SOIL (AGRICULTURAL SITE, UNITED STATES).

Condition	α-HCH		β-HCH		γ-HCH (Lindane)		δ-HCH		Total HCH	
	(mg/kg)									
	Initial	Final[1,2]	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Daramend® Treatment	17	1.1	13	1.0	14	1.1	3.5	1.0	47.5	4.2

[1]Final soil HCH concentrations were determined after 192 days of treatment.
[2]Laboratory reporting limits were between 1.0 and 1.1 mg/kg for individual HCH compounds

Case Study 2: Soil at an industrial pesticide site was contaminated with Lindane. *In situ* treatment of soil to a depth of 0.6 m with Daramend® reagent was conducted by soil mixing and irrigation. The influence of treatment on Lindane concentration is presented in Table 3.

TABLE 3. INFLUENCE OF FIELD-SCALE IN-SITU DARAMEND® TREATMENT ON CONCENTRATION OF LINDANE IN SOIL (INDUSTRIAL SITE, UNITED STATES).

Condition	Initial	154 days	371 days	Reduction (%)
	Lindane (mg/kg)			
Control (no treatment)	266	289	481	n/a
Daramend® Treatment	1,610	471	133	91.7

Case Study 3: A 14-hectare agricultural site in Canada which was used for fruit production during a period of more than 75 years was being converted to residential land use. Soil at the site was contaminated with DDE, a recognized breakdown product of the insecticide DDT which had been used as an insecticide. *In situ* bioremediation to a depth of 60 cm using the Daramend® soil amendment was selected based on bench-scale testing results, substantial cost savings, and greater sustainability versus excavation of the soil and off-site disposal at a landfill. The remedial goal for DDE was 260 µg/kg and representative results of treatment are presented in Table 4. The cost of *in situ* Daramend® treatment was less than the cost calculated for excavation, transportation, and disposal at a landfill.

TABLE 4. INFLUENCE OF FIELD-SCALE IN-SITU DARAMEND® TREATMENT ON DDE CONCENTRATION IN SOIL (AGRICULTURAL SITE, CANADA).

Sampling Area	Initial	42 days	90 days	151 days
	DDE[1] Concentration (µg/kg)			
Zone 1	990	429	344	190
Zone 2	390	355	65	ns[1]
Zone 3	930	180	160	ns
Zone 4	840	373	288	170
Zone 5	610	540	450	180

[1]"ns" indicates not sampled

Case Study 4: Ex-Situ Daramend® Bioremediation of Soil Contaminated with Organic Explosives

Following successful application to a wide range of chlorinated pesticides and herbicides, a series of laboratory tests were conducted on soil from US Navy, US Army, and US Marine Corps sites to determine the effectiveness of the Daramend reagent on soil contaminated with major organic explosive compounds including 2,4,6-trinitrotoluene (TNT), hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). The results indicated that concentrations of these organic explosive compounds were rapidly reduced when soil was treated with the Daramend reagent. Following completion of the laboratory tests several large scale-applications were completed at US military sites. The results from Daramend treatment of four 1,200-ton batches of soil contaminated with TNT and RDX at the US Navy facility in Yorktown, Virginia USA are presented in Table 5.

TABLE 5. INFLUENCE OF EX-SITU DARAMEND® TREATMENT ON CONCENTRATIONS OF TNT AND RDX IN SEDIMENT (MILITARY SITE, UNITED STATES)

Organic Explosive Compound	Batch 1		Batch 2		Batch 3		Batch 4	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
	(mg/kg)							
TNT	10,151	6.2	9,906	4.5	15,359	5.3	8,119	3.6
RDX	209	3.0	255	0.6	1,090	2.0	44	0.7

Discussion and conclusions

Over the past two decades, use of the Daramend reagent for enhancing degradation of Lindane, other HCH compounds, chlorinated pesticides, and organic explosive compounds has proven effective, and economical during numerous large-scale applications and under a wide variety of conditions in Canada, the United States, South America, and China. Despite the prevalent usage of OCPs, HHPs and POPs across Europe during past decades, it is apparent that widespread adoption of such well-proven ISCR technologies has been relatively slow. Despite a handful of small ISCR soil treatment projects in countries such as Moldova, Poland, Romania and Sweden, much of the wider EU, EFTA and UK geographies have been dormant. There are likely a variety of reasons for this, including limited public knowledge of where such environmental impacts may exist, lack of awareness or technical sophistication within national regulatory bodies, institutional resistance on the part of established contracting firms (e.g., highly profitable excavation and incineration contractors), other physical removal practitioners (e.g., dig and dump operations), et cetera.

From a more positive perspective, there have recently been a myriad of European Union and NGO funded research and site characterization initiatives for such sites. These efforts are bearing fruit, in the form of wider private and public sector awareness, greater information sharing across the spectrum of site remediation specialists, and an increased prioritization of Lindane impacted sites (in particular) for treatment. On a practical level, dramatically increased tipping fees for disposal of excavated materials into increasingly scarce hazardous waste landfills has catalyzed a search for more environmentally responsible and cost-efficient destruction technologies.

Concerning the increasingly dominant theme of Sustainability, the Daramend technology has a particularly compelling value proposition for decision makers to consider. There is a growing consensus amongst contaminated land stakeholders that sustainability should become a main driver in selecting the appropriate remediation solution. Scoring options across Social, Environmental, and Economic metrics is key, and there are many methods that can be applied. The Daramend reagent can be used to provide a sustainable approach to remediation of soils contaminated with Lindane, other HCH compounds, chlorinated pesticides, and organic explosive compounds.

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PRELIMINARY STUDY OF THE BIOREMEDIATION CAPACITY OF HORSE AMENDMENT IN SOILS CONTAMINATED WITH HCHS

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Summary

One of the objectives of the REMSURFOX research project is the evaluation of bioremediation in soils contaminated by hexachlorocyclohexanes (HCHs) as the final part of a treatment train after the use of chemical oxidation. In this work, an initial evaluation of the bioremediation capacity of organic horse amendment on soils with different HCH concentrations is carried out.

Four treatments were established: 1) soil with High Concentrations (HC) of HCH (130.27 mg/kg), 2) soil with Low Concentrations (LC) of HCH (0.97 mg/kg), 3) soil HC together with Horse Amendment (HC-HA) at 5% dry weight and 4) LC soil with Horse Amendment (LC-HA) at 5% dry weight. After an initial physical-chemical characterization of the soils, a study of the temporal evolution of the biological activity of the soils over 55 days was carried out, by determining enzymatic activities and HCH concentrations.

The application of horse amendment generated an improvement in the physical-chemical properties of the soil by reducing its pH and by increasing the content of organic matter and nutrients, which generated an increase in the biological activity of the soil. The dehydrogenase enzymatic activities (related to the quantity of microorganisms in the soil), phenol-oxidase (related to the degradation of complex organic compounds) and the phosphatase and urease activities (related to P and N, both macronutrients that are usually limiting in soils) were the most benefited from the application of the amendment. The temporal evolution of the enzymatic activities indicates a decrease in them, possibly due to a decrease in the availability of nutrients throughout the experiment.

The use of organic amendment produced a strong decrease in the concentrations of alpha, gamma, and delta HCH isomers during the first week of the experiment, reaching decrease percentages of around 90%. Regarding the beta and epsilon isomers, the horse amendment used reduced their concentrations by 50% at the end of the experiment.

The results obtained in this preliminary study point to the possibility of using the organic horse amendment studied within the treatment of soils contaminated by HCH, especially in the final phases within a treatment train.

Keywords

Lindane, bioremediation, organic amendment, biological activity, enzymatic activities.

1. Introduction

The use of pesticides in agriculture led to an increase in crop yields from the second half of the 20th century. One of the most widely used pesticides after World War II was technical hexachlorocyclohexane (HCH), made up of variable amounts of the 8 HCH isomers. From the 1950s, the γ isomer, commercially known as lindane, began to be used individually since it is the only HCH isomer with insecticidal properties. In 2001, the α-HCH, β-HCH and γ-HCH isomers were classified as Persistent Organic Pollutants (POPs) and their use was banned by the Stockholm Convention due to their harmful effects on human health and the environment.

In addition to the problems derived from the use of lindane in crop fields, it is necessary to take into account that for each ton of lindane, between 8 and 12 tons of other unwanted products are produced, mainly α and β isomers (Vijgen et al., 2011). The incorrect management of this waste caused in some cases its uncontrolled dumping in the surroundings of the lindane factories themselves. This is the problem that occurs in the town of Sabiñánigo (Huesca, Spain), in which, between 1975 and 1992, the Inquinosa company dumped between 115,000 and 160,000 tons of HCH waste in the Sardas and Bailín landfills, which caused the affectation of the Gállego river and the surrounding soils (Dominguez et al., 2018).

The recovery of soils contaminated by HCHs is an urgent matter, not only for the recovery of the functionality of these soils, but also to prevent the possible entry of this contaminant into the water or air. Among the possible decontamination techniques, bioremediation through the degradation of HCHs by soil microorganisms is the most effective method in economic terms and the most

eco-friendly (Zhang et al., 2020). In this work, an initial evaluation of the bioremediation capacity of organic horse amendment on soils with different HCH concentrations is carried out, the working hypothesis is that the organic horse amendment will provide nutrients to the microorganisms present in the soil capable of degrading the HCHs.

2 Materials and methods

2.1 Study zone

The study area is located in the north of the Iberian Peninsula in the municipality of Sabiñánigo (Huesca, Spain). The climate would be classified by Cfb, typical oceanic, according to the Köppen climate classification, with an average annual rainfall of 964 mm and average annual temperatures of 10.1 ºC (**Climate-data, 2022**). Geologically, the study area is located on the northern flank of the Guarga Syncline, characterized by alternating layers of red shale and subvertical Tertiary sandstone, hydrogeologically connected to the Gállego River (Montes **Santiago, 2012)**. The predominant soils in the area according to the WRB (2007) classification are Cambisols, Leptosols, Calcisols and Regosols (**Badía et al., 2009**).

2.2 Experimental design and analytical methods

In the experiment carried out, four treatments were prepared: i) soil with high contamination, ii) soil with low contamination, iii) soil with high contamination and organic amendment, and iv) soil with low contamination and organic amendment. In this study, commercial horse amendment was used at 5% dry weight. The samples were incubated at 60% of their field capacity humidity and at a temperature of 25 ºC, controlling these conditions three times a week. With the objective of being able to identify the temporal evolution of the different HCH isomers and of the microbial activity, samples were taken on days 0, 1, 7 and 55 of the experiment.

From each of the soil samples taken, one subsample was air-dried, sieved at < 2 mm, and stored until soil the physicochemical analyses. pH was determined by ISRIC method (**ISRIC, 2002**) with a soil:water ratio of 1:2.5 (w/v) measured with a HANNA EDGE pH-meter. Organic carbon (OC) was determined by the **Walkley and Black (1934)** method using a Metrohm 665 DOSIMAT automatic titrator. **Olsen and Sommers (1982)** was followed to obtain available phosphorus (Pav) which was measured in a UV-visible spectrophotometer with a TECAN NANOQUANT INFINITE M200 PRO multi-well plate reader. The available ammonium (NH_4^+) was extracted by the method of **Keeney and Nelson (1982)**, being determined by colorimetry in a M200 PRO-TECAN spectrophotometer.

To study soil microbiological activity six enzyme activities were analyzed in the samples. The enzymes related to C cycling were: β-glucosidase and phenoloxidase; with N cycling: arylamidase, and urease and with P cycling: phosphatase. Dehydrogenase activity was analyzed due to its relationship with the total amount of living microorganisms. Phenoloxidase activity was obtained following the **DeForest (2009)**, dehydrogenase was obtained following **Schaefer (1963)** and the remaining enzymatic activities following the ISO 20130 methods (**ISO, 2018**). All activity measurements were made on a UV–vis spectrophotometer TECAN NANOQUANT INFINITE M200 PRO.

3 Results and discussion

3.1 Physical-chemical characteristics of soils

The physical-chemical characteristics of the soil samples are presented in Table 1. The samples without amendments (HC and LC) showed low concentrations of total organic carbon, available phosphorus and NH_4^+. The use of organic horse amendment generated that the soils have greater amounts of these nutrients, while the pH value decreased by 0.3 units in the samples of the two levels of contamination studied.

TABLE 1: SOIL PARAMETERS

Soil parameters	HC	HC-HA	LC	LC-HA
pH	8,29	8	8,3	8,04
Total Organic Carbon (%)	0,928	2,236	0,487	3,322
Available P (mg/kg)	39,52	141,93	24,08	142,07
$NH4_+$(mg/kg)	16,21	41,17	13,78	58,19

HC=high concentrations of HCHs, HC-HA= high concentrations of HCHs with horse amendment, LC=low concentrations of HCHs, LC-HA= low concentrations of HCHs with horse amendment

3.2 Soil biological activity

Figure 1 shows the temporal evolution of the enzyme activities analyzed for the different treatments. The enzymatic activities in the low contamination soils were higher than the activities in the highly contaminated soils, this would imply the ability of some microbial species to exist in soils with low levels of HCHs, on the contrary, the high concentrations of HCHs inhibited the microbial activity revealing the toxicity of this type of contaminant. The application of organic horse amendment achieves an increase in enzymatic activity in all cases, even in highly contaminated soils. This increase may be due to the own microorganisms of the amendment or to the beneficial action of the nutrients provided by the amendment that generates the proliferation of those already existing and adapted to the concentrations of HCHs in the soil. In all cases, the low

contamination sample with organic amendment had the highest enzymatic activities; however, the high concentration sample with horse amendment presented high levels of phenol oxidase activity, this enzyme is responsible for the degradation of lignin in soil and influences the degradation of HCH (Kaur et al., 2016). These results would indicate the biostimulation effects of the organic horse amendment on the microorganisms present in the samples, although it is not possible to rule out a possible bioaugmentation exerted by itself.

3.2 Temporal evolution of HCHs

The low contamination (LC) and low contamination with amendment (LC-HA) sample had a significant change in the concentrations of each HCH isomer from the first day of the temporal study (Figure 2). This seems to indicate that the microbial populations present in soils with a low level of contamination are capable of efficiently degrading low concentrations of HCHs, such as those presented in this study, and independently of the addition of amendment. The β-HCH isomer makes an exception, which became the majority, maintaining its levels throughout the incubation period. This isomer is the most hydrophobic and the most difficult to degrade because it has the chlorines of the molecule in an equatorial position, which gives it greater physical and metabolic stability (Willett et al., 1998), and with a greater affinity for organic matter, highlighting manifested its greater persistence in the environment (Kumar et al., 2006).

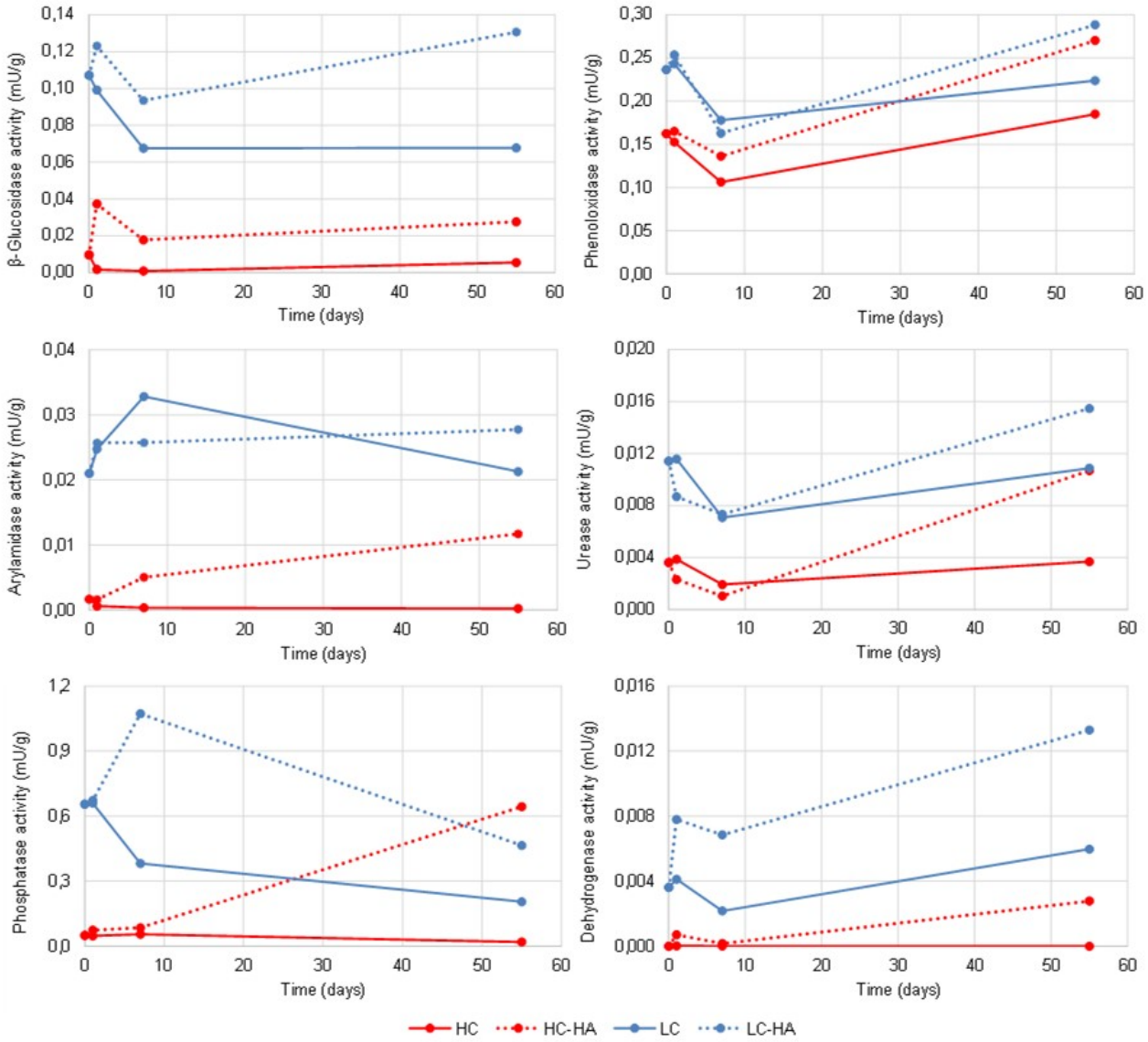

FIGURE 1: TEMPORAL EVOLUTION OF THE ENZYMATIC ACTIVITIES THROUGHOUT THE EXPERIMENT, HC=HIGH CONCENTRATIONS OF HCHS, HC-HA= HIGH CONCENTRATIONS OF HCHS WITH HORSE AMENDMENT, LC=LOW CONCENTRATIONS OF HCHS, LC-HA= LOW CONCENTRATIONS OF HCHS WITH HORSE AMENDMENT

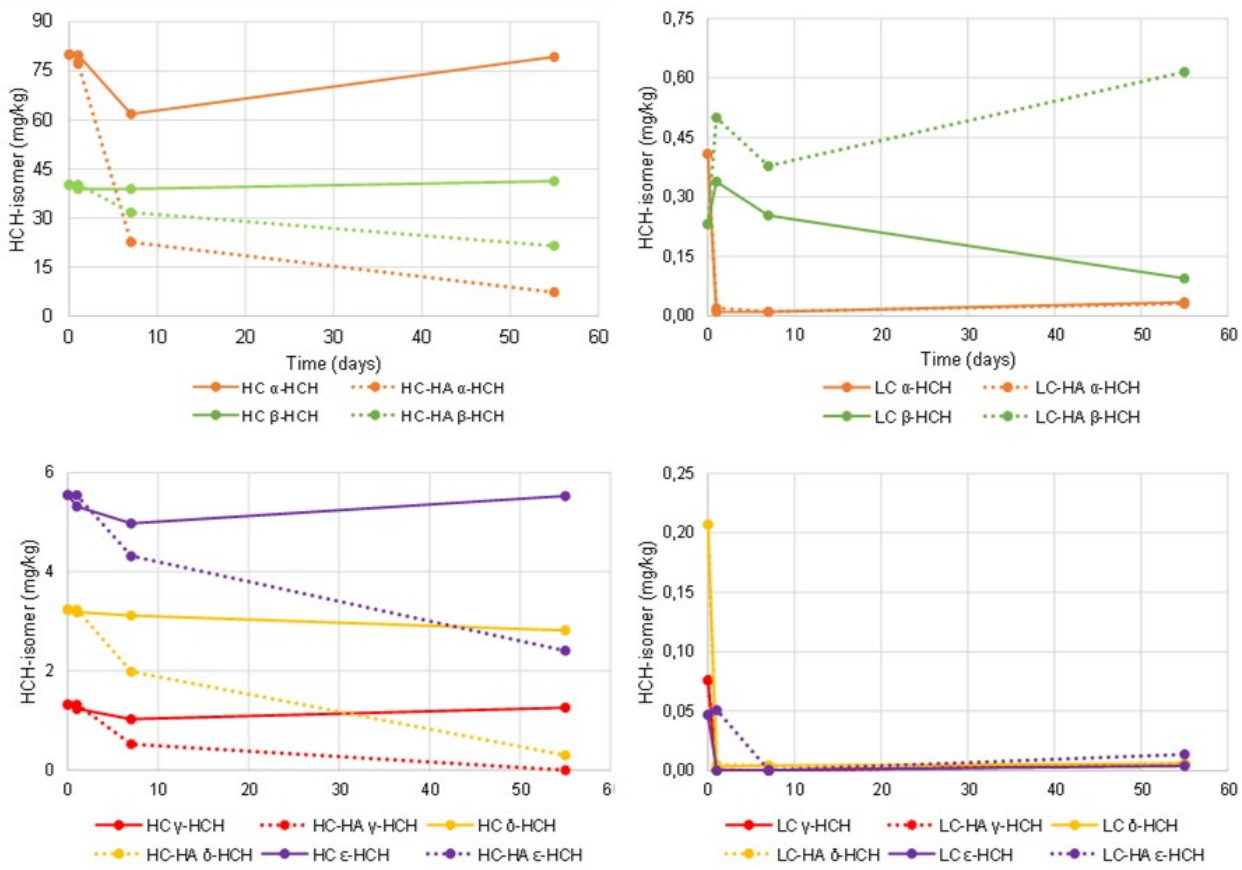

FIGURE 2: CONCENTRATIONS OF THE DIFFERENT ISOMERS OF HCH THROUGHOUT THE EXPERIMENT. HC=HIGH CONCENTRATIONS OF HCHS, HC-HA= HIGH CONCENTRATIONS OF HCHS WITH HORSE AMENDMENT, LC=LOW CONCENTRATIONS OF HCHS, LC-HA= LOW CONCENTRATIONS OF HCHS WITH HORSE AMENDMENT

In the high concentration treatment, the soil without amendment (HC-HA) maintained the concentrations of HCHs without variation until the end of incubation (Figure 2), with α-HCH being the main isomer, which would indicate that this concentration of HCHs inhibited microbial activity, as previously discussed. The addition of the organic amendment in highly contaminated soils (HC-HA) produced a decrease in all HCH isomers: α-HCH, γ-HCH and δ-HCH isomers with a strong decrease a week after the start of incubation and decreasing their values until the end of the experiment, reducing α-HCH and δ-HCH concentrations by more than 90% after 2 months of incubation and causing the disappearance of γ-HCH. The concentrations of β-HCH and ε-HCH isomers decreased by 50% throughout the incubation, demonstrating the effectiveness of the organic amendment to reduce the concentrations of these isomers that are more persistent in the medium (**Lal et al., 2010**). The temporal evolution of the HCH isomers did not reach the asymptote after two months of the study, so a longer incubation period could be effective to observe when a new amendment application would be necessary and to determine the maximum reductions in the HCH concentrations that can be achieved with this technique.

Conclusions

The use of organic horse amendment in the bioremediation of soils contaminated by HCHs was evaluated through an incubation study. The use of this amendment was shown to be effective in the degradation of the different HCH isomers. The reduction of HCHs concentrations did not reach the asymptote at the end of the incubation experiment, so it is possible that in longer times the reduction was greater. The use of organic horse amendment stimulated the enzymatic activity of the samples analyzed, due to the improvement in the nutrient content, which would be used by the microorganisms adapted to the exposure of HCHs.

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