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Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous HCH tolerant vegetation

--Manuscript Draft--

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	Peter Schroeder, Prof. Dr.
Abstract:	Given the toxicity, volatility and persistence of hexachlorocyclohexane (HCH), reclamation of contaminated areas is priority for the health and welfare of neighboring human communities. Microbial diversity and functions at field scale, in relation to spontaneous vegetation in post-excavation situations, are essential indicators for development of bioaugmentation or microbe-assisted phytoremediation strategies. This study aimed to evaluate effects of long-term HCH contamination on soil and plant-associated microbial communities, and whether HCH contaminated soil has potential to act as bacterial inoculum in post-excavation bioaugmentation and microbe-assisted phytoremediation strategies. In addition, the potential nitrogen fixation of free-living and symbiotic diazotrophs of Lotus tenuis was assessed as a measure of nutrient cycling functions under contamination. Potential nitrogen fixation was not affected by HCH, except a temporary lower nifH gene count in contaminated rhizospheres which is most probably a short-term HCH effect on early bacterial succession in this compartment. HCH was the main shaping factor of microbial communities in long-term contaminated bulk soil, where we identified possible HCH tolerating genera like Sphingomonas and Altererythrobacter. In Lotus tenuis rhizosphere, microbial community composition was influenced by HCH contamination and plant growth stage. Sphingobium and Massilia were genera characteristic for HCH contaminated rhizospheres. Lotus tenuis growth and development was negatively affected by long-term HCH contamination. The root-associated bacterial community composition however, was driven solely by plant age, whereas HCH effects were negligible. Our results showed that in contamination conditions, L. tenuis acquired possible HCH tolerant bacteria which could at the same time offer plant growth promoting (PGP) benefits for the host, such as the Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium clade, Sphingomonas, Massilia or Pantoea. Finally, we identified an inoculum with possibly HCH tolerant, PGP bacteria transferred from contaminated bulk soil to L. tenuis roots through the rhizosphere compartment, consisting of Mesorhizobium loti, Neorhizobium galegae, Novosphingobium lindaniclasticum, Pantoea agglomerans and Lysobacter bugurensis.
Response to Reviewers:	Response to reviewers Helga paper Reviewer 1 R: This paper concerns about the post-reclamation microbial diversity and functions in

	hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous vegetation. The topic is highly interesting and relevant for many areas contaminated with organic persistent pollutants. The manuscript structure and experimental approach is appropriate. The comprehensive approach including the analysis of bulk soil, rhizosphere and roots is very good. The manuscript is suitable for STOT.. Overall, it is a very interesting and good manuscript but a potential to improve, suggest major/minor revision (not sure if major is needed). A: the authors are grateful to the reviewer for his constructive comments. R: Graphical abstracts: I don't find the PCAs so useful but not any harm. Normally, I would day a map of the country is not interesting, but in this case, it is a point to show Romania. (somewhere between score 1 and 2. A: we have included a map of Romania with the site indicated in the supplementary section where it can be presented in higher resolution. R: Title: Suggest to include a hint about vegetation which can improve HCH-degradation. It would make it more interesting for a wider publicum. A: DONE - the title has been expanded to "Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous HCH tolerant vegetation". R: The highlights: should include one point related to the potential of bioaugmentation. A: DONE R: Keywords: again, suggest to focus on degradation and in-situ natural attenuation, and maybe bioaugumentation or something related to plants (maybe phytoremediation?) A: DONE - "bioremediation" has been added as keyword R: Abstract: Introduction: suggest shortening the focus at HCH and introduce some of the interesting parts from the result section about potential with use of plants, e.g. Lotus tenuis, nitrogenase and nifH gene, and bioaugmentation. A: DONE - Lotus tenuis is quoted in line 42, and the nif-gene is quoted in lines 44-46, bioaugmentation or microbe-assisted phytoremediation strategies are mentioned in L. 37/38 R: Materials and methods: Good and thoroughly description. The term old and new is not the best, maybe in-situ-Poll (polluted), in-situ-Ref (low pollution), ex-situ (polluted) and ex-situ-Ref (low pollution) is better? A: DONE - changed with "excavPoll" and "excavRef" (instead of "new HCH" and "new clean", respectively) and with "undistPoll" and "undistRef" instead of "old HCH" and "old clean", respectively R: Results: There are some choices and statements which should be given some explanation; e.g. line 352; HCH significantly affected the pH and line 354, significant positive relationship between HCH conc and OM, DOC and DON. Since HCH is hydrophobic the correlation with organic matter is excepted, but such information could be included. A: DONE the pH values are now given in lines 356-357, and the OM/DOC and DON values are available in Supplement 1 R: Since I don't have much experience with PCA data, I asked a colleagea to look into this part. His comments is: "Data analysis and conclusions indicate a very weak impact of HCH presence and e.g., bacterial richness. Figure 1 indicates almost no impact of HCH presence on bacterial richness in root while the response in bulk and rhizosphere on HCH presence is very weak and might me statistically unimportant including the precision level of applied measurement methods. Following the Figure 2 being a different way of describing a data from Figure 1 the same problem is visible. The obtained relationships in Figure 2 are very week (see sum of CAP1+CAP2 < c. 19%). Due to a way how, the obtained data was treated any analyzed the obtained conclusions are very weak. Although, I truly believe that the obtained raw data carries an interesting and good value but the significant improvement in data analysis and data interpretation is required". A: These valuable comments can be answered together in one: It is true that the level of HCH contamination did not have a significant effect on bacterial α-diversity in roots. However, this was to be expected, since HCH is a hydrophobic substance that is not taken up by plants. In excavated bulk soil and undisturbed rhizosphere, we found a significant impact of HCH on the α-diversity with a coefficient of determination (R^2) of 0.6 each. While these numbers cannot be judged by themselves without comparisons, we consider these relationships relevant, since results from field studies are always also influence by a variety of uncontrollable environmental conditions. We consider precision levels of our measurement methods sufficient for the presented results, since

	the HCH levels varied over three orders of magnitude (cf. the new table in supplementary materials), which should be detectable by any decent measurement method. The precision level for the number of ASVs is well established for Illumina amplicon sequencing and was also ensured by rarefaction curves. For the ordination analysis presented in Fig. 2 we would like to stress the point that “weak” or “strong” relationships cannot be assigned by the numbers of statistical measures alone. It would rather be necessary to cross-compare with results from other studies in the area to judge about the effect size in our conditions. Unfortunately, there is very little results available from field studies investigating the effects of HCH pollution on the bacterial soil community. As such, the size of the HCH pollution effect has so far been unknown under field conditions, which is why we consider our results all the more relevant to the scientific audience. Lastly, we would like to clarify that figure 2 represents the changes in community composition of samples of the same soil compartments, i.e. of bulk, rhizosphere and root compartments alone. In other studies, these compartments are often analyzed together, leading to much greater numbers of “explained variance” because of the natural difference in community composition between these compartments (e.g. Feng et al. 2019), while hiding to some extent the changes in the community caused by the pollution. To make the results of the statistical analyses more clear to the reader, we revised the figure captions of figures 1 and 2 and the relevant sections of the results in the main text. R: I find the manuscript very interesting and fit well in STOTEN. My recommendations/suggestions might improve the relevance in addition, get more readers interested. A: authors did everything to improve the relevance of the manuscript according to the reviewer´s recommendations and hope it is now acceptable for publication. Reviewer 2 R: This article represented the results of a field study on a long-term HCH contamination site and reported the effects on microbial diversity and functions by several factors. Although the topic referring to the soil bacterial community and the result of HCH levels is interesting, the authors could not adequately document the relationship among the HCH contamination in soil, the microbial diversity and the spontaneous vegetation. A: Done. By now, these relationships have been clearly shown in the correlation analyses… In addition we have added the HCH concentrations as a table in the supplements R: In terms of the writing of this manuscript, the author should reduce the length to meet the requirement on the word limit of the journal. A: Done: the manuscript has been edited and shortened significantly, now meeting the word limit of the journal. R: This study was relatively comprehensive like a simplified degree dissertation, and its main topic was not clear and should be rephrased to be more specific. A: the main topic of the paper is to show the importance of the microbial community and its shaping by plants for the remediation of HCH. This is now strongly pointed out in the abstract and conclusion. R: The HCH contamination, the rhizosphere microbial communities, the plant growth and the nitrogen fixation potential were analyzed, but the levels of HCH in soil and plants were not reported in the manuscript or the supplementary material. A: Done. Please see the first answer. We have added the HCH concentrations as a table in the supplements R: In general, I would not suggest the editor accepting this manuscript for publication in

	Science of the Total Environment. A: the authors are confident that the manuscript will be acceptable for publication in the STOTEN after the significant improvements made with view to the reviewer´s comments and to the constructive remarks of the second reviewer.

Cover Letter

Helmholtz Zentrum München · P.O. Box 11 29 · 85758 Neuherberg

Prof. Dr. Damià Barceló
Co-Editor
Science of the Total Environment

Prof. Dr. Dr. Peter Schröder

Research Unit Comparative Microbiome Analysis - COMI

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04/10/20

Re: Evaluation of ms STOTEN-D-20-18896

Dear Prof. Barceló, Dear Damià,

please find enclosed our revision to the manuscript: "Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous vegetation".
We thoroughly went through all reviewer comments and accepted all proposed changes.
While reviewer 1 was very detailed and on the point in his very constructive instructions, we had to interpret the comments of reviewer 2 a bit more, but we are confident that we met all critical points mentioned in the review and hence were able to improve the manuscript considerably.
All changes have now been listed in the response to reviewers section, and marked in color in the main manuscript. The text has also been streamlined, and checked for language and style by experienced researchers.
We hope that our manuscript can by now be considered for publication.

Kind regards, also on behalf of the co-authors.

Yours sincerely,

Peter
(Prof. Dr. Peter Schröder)

Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous HCH tolerant vegetation

Helga E. Balázs[a,b], Christoph A. O. Schmid[a], Catarina Cruzeiro[a], Dorina Podar[c], Paul-Marian Szatmari[b,d], Franz Buegger[e], Gudrun Hufnagel[a], Viviane Radl[a], Peter Schröder[a]*

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Given the toxicity, volatility and persistence of the organochlorine pesticide hexachlorocyclohexane (HCH), reclamation of contaminated areas is a priority for the health and welfare of neighboring human communities. Microbial diversity and functions at field scale, in relation to spontaneous vegetation in post-excavation situations, are essential indicators to consider when bioaugmentation or microbe-assisted phytoremediation strategies are developed. Thus, the present study aimed to evaluate the effects of long-term HCH contamination on soil and plant-associated microbial communities, and whether HCH contaminated soil has the potential to act as a bacterial inoculum in post-excavation bioaugmentation and microbe-assisted phytoremediation strategies. To scrutinize the role of vegetation, the potential nitrogen fixation of free-living and symbiotic diazotrophs of the legume *Lotus tenuis* was assessed as a measure of nutrient cycling functions in soil under HCH contamination. Potential nitrogen fixation was generally not affected by HCH contamination. The single exception was a temporary lower nifH gene count in the excavated contaminated rhizosphere which is most probably a short-term HCH effect on early bacterial succession in this compartment. HCH was the main shaping factor of the microbial communities in long-term contaminated bulk soil, where we identified possible HCH tolerating genera such as *Sphingomonas* and *Altererythrobacter*. In *L. tenuis* rhizosphere, microbial community composition was influenced by both HCH contamination and plant growth stage. *Sphingobium* and *Massilia* were the bacterial genera characteristic for HCH contaminated rhizospheres. *L. tenuis* growth and development was negatively affected by long-term HCH contamination. The root-associated bacterial community composition however was driven solely by plant age, whereas the HCH effect was negligible. In contamination conditions, *L. tenuis* seems to acquire potentially HCH tolerant bacteria which could at the same time offer plant growth promoting (PGP) benefits for the host, such as the *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* clade, *Sphingomonas, Massilia* or *Pantoea*. Finally, we identified an inoculum with possibly HCH tolerant PGP bacteria transferred from the contaminated bulk soil to *L. tenuis* roots through the rhizosphere compartment, consisting of *Mesorhizobium loti, Neorhizobium galegae, Novosphingobium lindaniclasticum, Pantoea agglomerans* and *Lysobacter bugurensis*.

Response to reviewers Helga paper

Reviewer 1

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A: the authors are grateful to the reviewer for his constructive comments.

R: Graphical abstracts: I don't find the PCAs so useful but not any harm. Normally, I would day a map of the country is not interesting, but in this case, it is a point to show Romania. (somewhere between score 1 and 2.

A: we have included a map of Romania with the site indicated in the supplementary section where it can be presented in higher resolution.

R: Title: Suggest to include a hint about vegetation which can improve HCH-degradation. It would make it more interesting for a wider publicum.

A: DONE - the title has been expanded to "Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous HCH tolerant vegetation".

R: The highlights: should include one point related to the potential of bioaugmentation.

A: DONE

R: Keywords: again, suggest to focus on degradation and in-situ natural attenuation, and maybe bioaugumentation or something related to plants (maybe phytoremediation?)

A: DONE - "bioremediation" has been added as keyword

R: Abstract: Introduction: suggest shortening the focus at HCH and introduce some of the interesting parts from the result section about potential with use of plants, e.g. Lotus tenuis, nitrogenase and nifH gene, and bioaugmentation.

A: DONE - Lotus tenuis is quoted in line 42, and the nif-gene is quoted in lines 44-46, bioaugmentation or microbe-assisted phytoremediation strategies are mentioned in L. 37/38

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A: DONE - changed with "excavPoll" and "excavRef" (instead of "new HCH" and "new clean", respectively) and with "undistPoll" and "undistRef" instead of "old HCH" and "old clean", respectively

R: Results: There are some choices and statements which should be given some explanation; e.g. line 352; HCH significantly affected the pH and line 354, significant positive relationship between HCH conc and OM, DOC and DON. Since HCH is hydrophobic the correlation with organic matter is excepted, but such information could be included.

A: DONE the pH values are now given in lines 356-357, and the OM/DOC and DON values are available in Supplement 1

R: Since I don't have much experience with PCA data, I asked a colleagea to look into this part. His comments is: "Data analysis and conclusions indicate a very weak impact of HCH presence and e.g., bacterial richness. Figure 1 indicates almost no impact of HCH presence on bacterial richness in root while the response in bulk and rhizosphere on HCH presence is very weak and might me statistically unimportant including the precision level of applied measurement methods. Following the Figure 2 being a different way of describing a data from Figure 1 the same problem is visible. The obtained relationships in Figure 2 are very week (see sum of CAP1+CAP2 < c. 19%). Due to a way how, the obtained data was treated any analyzed the obtained conclusions are very weak. Although, I truly believe that the obtained raw data carries an interesting and good value but the significant improvement in data analysis and data interpretation is required".

A: These valuable comments can be answered together in one: It is true that the level of HCH contamination did not have a significant effect on bacterial α-diversity in roots. However, this was to be expected, since HCH is a hydrophobic substance that is not taken up by plants. In excavated bulk soil and undisturbed rhizosphere, we found a significant impact of HCH on the α-diversity with a coefficient of determination (R^2) of 0.6 each. While these numbers cannot be judged by themselves without comparisons, we consider these relationships relevant, since results from field studies are always also influence by a variety of uncontrollable environmental conditions. We consider precision levels of our measurement methods sufficient for the presented results, since the HCH levels varied over three orders of magnitude (cf. the new table in supplementary materials), which should be detectable by any decent measurement method. The precision level for the number of ASVs is well established for Illumina amplicon sequencing and was also ensured by rarefaction curves.

For the ordination analysis presented in Fig. 2 we would like to stress the point that "weak" or "strong" relationships cannot be assigned by the numbers of statistical measures alone. It would rather be necessary to cross-compare with results from other studies in the area to judge about the effect size in our conditions. Unfortunately, there is very little results available from field studies investigating the effects of HCH pollution on the bacterial soil community. As such, the size of the HCH pollution effect has so far been unknown under field conditions, which is why we consider our results all the more relevant to the scientific audience. Lastly, we would like to clarify that figure 2 represents the changes in community composition of samples of the same soil compartments, i.e. of bulk, rhizosphere and root compartments alone. In other studies, these compartments are often analyzed together, leading to much greater numbers of "explained variance" because of the natural difference in community composition between these compartments (e.g. Feng et al. 2019), while hiding to some extent the changes in the community caused by the pollution.

To make the results of the statistical analyses more clear to the reader, we revised the figure captions of figures 1 and 2 and the relevant sections of the results in the main text.

R: I find the manuscript very interesting and fit well in STOTEN. My recommendations/suggestions might improve the relevance in addition, get more readers interested.

A: authors did everything to improve the relevance of the manuscript according to the reviewer´s recommendations and hope it is now acceptable for publication.

Reviewer 2

R: This article represented the results of a field study on a long-term HCH contamination site and reported the effects on microbial diversity and functions by several factors.
Although the topic referring to the soil bacterial community and the result of HCH levels is interesting, the authors could not adequately document the relationship among the HCH contamination in soil, the microbial diversity and the spontaneous vegetation.

A: Done. By now, these relationships have been clearly shown in the correlation analyses…
In addition we have added the HCH concentrations as a table in the supplements

R: In terms of the writing of this manuscript, the author should reduce the length to meet the requirement on the word limit of the journal.

A: Done: the manuscript has been edited and shortened significantly, now meeting the word limit of the journal.

R: This study was relatively comprehensive like a simplified degree dissertation, and its main topic was not clear and should be rephrased to be more specific.

A: the main topic of the paper is to show the importance of the microbial community and its shaping by plants for the remediation of HCH. This is now strongly pointed out in the abstract and conclusion.

R: The HCH contamination, the rhizosphere microbial communities, the plant growth and the nitrogen fixation potential were analyzed, but the levels of HCH in soil and plants were not reported in the manuscript or the supplementary material.

A: Done. Please see the first answer. We have added the HCH concentrations as a table in the supplements

R: In general, I would not suggest the editor accepting this manuscript for publication in Science of the Total Environment.
A: the authors are confident that the manuscript will be acceptable for publication in the STOTEN after the significant improvements made with view to the reviewer´s comments and to the constructive remarks of the second reviewer.

Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous HCH tolerant vegetation

Helga E. Balázs[a,b], Christoph A. O. Schmid[a], Catarina Cruzeiro[a], Dorina Podar[c], Paul-Marian Szatmari[b,d], Franz Buegger[e], Gudrun Hufnagel[a], Viviane Radl[a], Peter Schröder[a]*

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Abstract

Given the toxicity, volatility and persistence of the organochlorine pesticide hexachlorocyclohexane (HCH), reclamation of contaminated areas is a priority for the health and welfare of neighboring human communities. Microbial diversity and functions at field scale, in relation to spontaneous vegetation in post-excavation situations, are essential indicators to consider when bioaugmentation or microbe-assisted phytoremediation strategies are developed. Thus, the present study aimed to evaluate the effects of long-term HCH contamination on soil and plant-associated microbial communities, and whether HCH contaminated soil has the potential to act as a bacterial inoculum in post-excavation bioaugmentation and microbe-assisted phytoremediation strategies. To scrutinize the role of vegetation, the potential nitrogen fixation of free-living and symbiotic diazotrophs of the legume *Lotus tenuis* was assessed as a measure of nutrient cycling functions in soil under HCH contamination. Potential nitrogen fixation was generally not affected by HCH contamination. The single exception was a temporary lower nifH gene count in the excavated contaminated rhizosphere which is most probably a short-term HCH effect on early bacterial succession in this compartment. HCH was the main shaping factor of the microbial communities in long-term contaminated bulk soil, where we identified possible HCH tolerating genera such as *Sphingomonas* and *Altererythrobacter*. In *L. tenuis* rhizosphere, microbial community composition was influenced by both HCH contamination and plant growth stage. *Sphingobium* and *Massilia* were the bacterial genera characteristic for HCH contaminated rhizospheres. *L. tenuis* growth and development was negatively affected by long-term HCH contamination. The root-associated bacterial community composition however was driven solely by plant age, whereas the HCH effect was negligible. In contamination conditions, *L. tenuis* seems to acquire potentially HCH tolerant bacteria which could at the same time offer plant growth promoting (PGP) benefits for the host, such as the *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* clade, *Sphingomonas*, *Massilia* or *Pantoea*. Finally, we identified an inoculum with possibly HCH tolerant PGP bacteria transferred from the contaminated bulk soil to *L. tenuis* roots through the rhizosphere compartment, consisting of *Mesorhizobium loti, Neorhizobium galegae, Novosphingobium lindaniclasticum, Pantoea agglomerans* and *Lysobacter bugurensis*.

Keywords:

Hexachlorocyclohexane; Soil clean-up; Soil functions; Bacterial community; Bioremediation

1. Introduction

Hexachlorocyclohexane (HCH) was one of the most widely used organochlorine pesticides in agriculture between the 1950s and 1980s where a total of nearly 8 million tons of toxic and persistent waste was deposited worldwide at former production sites (Willett et al., 1998). Due to inadequate disposal techniques, HCH has been flagged for regulatory intervention and elimination to limit its dispersion via air or water and avoid accumulation into plant and animal food resources (Vijgen et al., 2011; Ogbeide et al., 2016). Since many countries lack funding for proper clean-up and restoration, excavation remains the cheapest conventional clean-up approach for HCH contaminated sites (Caliman et al., 2011; Morillo and Villaverde, 2017). However, there are often no follow-up interventions (*e.g.* landfilling or phytoremediation approaches) to prevent further dispersion of contaminant left-overs via air transport to neighboring residential areas.

Microbial diversity and functions at field scale, in relation to spontaneous vegetation in post-remediation situations, are essential criteria for the development of customized strategies to enhance and speed up the reclamation of brownfields representing a potential danger to local human communities. There are, however, very few studies of microbial diversity and functions as ecological indicators in HCH polluted soil (Dadhwal et al., 2009; Bala et al., 2010), and only one which tackles these aspects in post-reclamation situations (Balázs et al., 2020). Nonetheless, the latter study handles only lindane as a contaminant, and it was limited to controlled greenhouse conditions.

The partial excavation of the HCH waste deposits from Turda (Romania, Suppl. Mat. Fig. 1) offered an opportunity to study how soil and plant associated bacterial communities adapt to long-term pollution and remediation attempts. As HCH tolerant bacterial consortia proved to decrease HCH levels in soil at both pot and field scale (Garg et al., 2016), we hypothesized that post- excavation traces of contaminated soil might serve as an inoculum with native, HCH- tolerant bacteria for subsequent bioaugmentation strategies. Such inocula could prove more efficient than artificial consortia, given that native bacterial communities are already adapted to contamination and to the local physico-chemical conditions.

In addition, microbe-assisted phytoremediation studies showed that soil inoculation with HCH tolerant/degrading strains and plant growth promoting rhizobacteria could enhance HCH dissipation in rhizospheres and increase plant performance and tolerance to toxicity (Becerra-Castro et al., 2013a,b; Alvarez et al., 2015). Plants grown in contaminated soils become an important sink for organochlorine compounds via soil-plant and or air-plant route, as they are capable of accumulating high amounts of HCH (Pereira et al., 2006). Given that *L. tenuis* is growing spontaneously at the Turda site, we hypothesized that contaminated soil may potentially serve as an inoculum with HCH tolerant bacterial

strains for the plant, and that these strains might support *L. tenuis* growth and performance as first colonizer, as well as its function in nitrogen fixation.

Finally, recovery of microbial functions after excavation is a critical step in achieving soil restoration and a functional ecosystem. Since the excavated soil had already been colonized by the legume *Lotus tenuis*, potential nitrogen fixation was one of the most important bacterial functions at this stage of ecological succession. Hence, our third objective was to assess effects of HCH on potential nitrogen fixation in bulk and rhizosphere soil, as well as at root level of the early colonizer *Lotus tenuis*, as a measure of post-reclamation ecosystem functionality.

To summarize, the present field study aimed to address the following hypotheses: (H1) HCH contaminated soil acts a bacterial inoculum in post-reclamation bioaugmentation strategies; (H2) such an inoculum can improve plant performance and tolerance to HCH in early vegetation succession stages; and (H3) HCH affects potential nitrogen fixation of free-living and symbiotic diazotrophs. For this purpose, we measured soil physico-chemical parameters, plant fitness, and characterized soil and root-associated bacterial communities by means of 16S rRNA gene amplicon sequencing in long-term HCH- contaminated soil and their subsequent response to excavation procedures.

2. Materials and methods

2.1 Site description

The former chemical plant (46°55´N, 23°78´E) is located within the precincts of Turda city, Romania, near the river Arieș (Suppl. mat. 1). Between 1954 and 1983, approximately 15,000 tons of waste HCH were inadequately deposited within that enclosure (Prodan et al., 2011). The chemical plant shut down in 1998, but the close proximity to residential areas and easy access of unauthorized persons on the grounds represent a continuous pollution source and health concern for the local community. The factory area includes two HCH hotspots: one is a waste heap on the right bank of the river Arieș, and the other corresponds to the former lindane production department (Balázs et al., 2018). In spring 2018, an area including the former production department HCH hotspot and several neighboring low-contamination plots were excavated and leveled out, as a measure of soil reclamation (Suppl. mat. 1). The sampling campaign took place two months after the excavation operation, at the end of May 2018.

2.2 Sampling design and procedures

To assess the presence of a possible bacterial inoculum and the effect of post-reclamation HCH levels on nitrogen fixation and plant performance, we compared the previously identified HCH hotspots with two neighbouring low contaminated plots (Balázs et al., 2018). One of the HCH hotspots and one low contaminated plots were freshly excavated, while the other two were undisturbed and covered with spontaneous vegetation (Suppl. mat. 1). The undisturbed plots are further named "undistPoll" (high contamination), "undistRef" (low contamination) while the freshly excavated and leveled plots were named "excavPoll" (high contamination) and "excavRef" (low contamination). All plots were colonized by *Lotus tenuis* WALDST. & KIT. ex WILLD., but with different coverage. *Lotus tenuis* was chosen for this field study as it already grew spontaneously on the premises in HCH hotspots (Balázs et al., 2018). As an early perennial colonizer and member of the Fabaceae family, *L. tenuis* has an important role in creating optimal conditions for the further development of a spontaneous vegetation cover. Furthermore, it provides important ecosystem services such as nitrogen fixation, erosion control, build-up of soil organic matter or development of a diverse microbial community in the soil.

For optimal coverage, a five sampling point pattern corresponding to five biological replicates was randomly set within the limits of each of the four plots. Each of the equally spaced five sampling points consisted of a circular area with one meter radius. Five bulk soil sub-samples were collected at random from within the limits of each sampling point and mixed into one composite sample, resulting in five composite biological replicates for each of the four plots: "undistPoll", "undistRef", "excavPoll" and "excavRef". Rhizosphere and plant samples were subsequently collected from within the circular sampling points established for bulk soil.

Bulk soil was collected from the upper 10 cm, mixed into composite samples for each sampling point, sieved through 2 mm mesh, filled into sterile tubes and temporarily stored on ice. Samples meant for DNA extraction were stored at -80°C and samples for physico-chemical measurements were stored at 4°C. Samples meant for HCH quantification were freeze-dried and subsequently stored at 4°C.

To collect rhizospheres, soil was loosened around the plant roots which were then carefully pulled out with adherent rhizosphere soil. Plants collected from one sampling point were pulled together into a composite sample, were shaken on top of a sieve equipped with a tray and brushed to retrieve as much rhizosphere material as possible. The soil was then sieved and stored in the same manner as the bulk soil. The plant material was washed with tap water, followed by autoclaved distilled water, separated into roots and shoots and stored at -80°C until further use. Before the plant material was separated, several plants from each sample were weighed to estimate mean fresh biomass.

2.3 Soil physico-chemical parameters, microbial biomass carbon and plant C/N ratio

Microbial biomass carbon (C_{mic}) was determined according to DIN ISO 14240-2:2011-09 after chloroform fumigation and subsequent extraction with 0.01M $CaCl_2$ solution (1:4 (w/v)). Dissolved organic carbon and nitrogen (DOC, DON) as well as ammonium, nitrate and nitrite concentrations were extracted in the same manner, excluding the fumigation step. All extractions were done in triplicates and stored at -20°C for later measurements. Organic C and N were quantified on a Total Carbon Analyzer (Shimadzu TOC 5050, Tokyo, Japan). C_{mic} was calculated as the difference of total C between fumigated and non-fumigated samples, using a fraction of 0.45 as extractable part of microbial biomass carbon (Joergensen, 1996). Total nitrogen and ammonium were determined using a continuous-flow photometric analyser (CFA-SAN Plus; Skalar Analytik, Germany). Soil pH was measured according to the ISO 10390:2005-02 method, in 0.01M $CaCl_2$ (soil: solution ratio of 1:5 (w/v)) after a 2 h incubation time. Soil organic matter content (OM) was determined after drying the soil for 24 hours at 65° C followed by heating at 450°C for 5 hours in a muffle oven. OM content was determined by weighing before and after heating samples to 450°C, the difference in weight corresponding to the incinerated soil OM.

Plant material (roots and leaves) was dried at 65° C for 48 h and ground to a fine powder with a Tissue LyserII (Qiagen GmbH, Germany). Approximately 1.5 mg of the powder was weighted into 3.5 mm × 5 mm tin capsules (HEKAtech GmbH, Wegberg, Germany). Total carbon and nitrogen contents in *L. tenuis* roots and leaves were determined using an Elemental-Analysator 'Euro-EA' (Eurovector, Milano, Italy).

2.4 HCH extraction and analysis from bulk soil

The extraction of HCH isomers from soil followed the QuEChERS method described by Fernandes et al. (2013). HCH was extracted from 5 g of dried sieved bulk soil hydrated with 3 mL MiliQ water after one hour incubation. 10 mL of acetonitrile were added to the solution, followed by vigorous vortexing and shaking. A powder mixture of 4 g anhydrous $MgSO_4$, 1 g NaCl, 1 g Na_3citrate dihydrate and 0.5 g Na_2citrate sesquihydrate was added to the acetonitrile solution, subsequently vigorously shaken and vortexed, sonicated for 5 minutes, ending with a 5 minute centrifugation at 3000 rpm (Avanti J-25, Beckman Coulter, USA). 1.5 mL of supernatant was cleaned-up with 150 mg $MgSO_4$, 50 mg C18 and 50 mg PSA. The mixture was vortexed, shaken vigorously, and centrifuged for 5 minutes at 4000 x g. 1mL of supernatant was spiked with hexachlorobenzene (HCB) as internal standard (10 ppm end concentration in the solution), and subsequently evaporated to dryness at 40° C under nitrogen gas flow and reconstituted with 1 mL n-hexane. The identification and quantification of α-, β- and γ-HCH isomers was done using an Agilent 6890N gas chromatograph equipped with a 7686B series injector,

both from Agilent Technologies (CA, USA) and coupled with a IRMS detector (Delta plus Advantage, Thermo Finnigan, Waltham, Massachusetts). Isomers were separated on a DB-5 column (30m x 0.25mm x 0.25µm, P/N 122-5032, J&W Scientific from Agilent Technologies) with helium (99.999 % purity) used as carrier gas, at a flow of 1mL/min. The column oven temperature was programmed to 80°C for 10 min, increased to 175° C at 20° C/min, followed by a 1° C/min increase until 185°, until it reached 300° at a rate of 35° C/min and then held for 2 min. A volume of 5 µL was injected in splitless mode with a 40-mm length needle (P/N 5181-1267, Agilent Technologies) into the injection port at 250°C. The HCH isomer concentrations were calculated based on a linear five-point calibration curve, ranging from 5 ppm to 20 ppm. Integrations were done using the Isodat-Gas Isotope ration MS software (version 3.0) from Thermo Scientific. HCH values are synthesized in Suppl. mat 2.

2.5 16SrRNA gene library preparation

Total DNA was extracted from 0.5g of soil and 0.3g of ground root material (both fresh weight), using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. Empty sterile extraction tubes were prepared for each soil compartment and root DNA extraction, to serve as negative extraction controls. The concentration of DNA extracts was quantified with a 5200 Fragment Analyzer System (Agilent, US-CA), using the Genomic DNA 50Kb Analysis Kit. Ten ng of the DNA extracts were used to amplify the V3-V4 region of the 16S rRNA gene, using the primer pair 338F (5'- GCTGCCTCCCGTAGGAGT- 3') /789R (5' -GGAATCCTCTCTCACCACATTGCCCAGG CAGACC- 3') with Illumina adapter sequences, which was reported to exclude chloroplast amplification (Dorn-In et al., 2015). PCR reactions were carried out in three technical replicates using the NEBNext High-Fidelity 2× PCR Master Mix (New England Biolabs, Ipswich, US-MA) with the addition of tetramethyl ammonium chloride at 36mM final concentration in the PCR reaction. Non target control (NTC) and positive controls containing the target gene were also performed in triplicates. PCR conditions were: initial denaturation step, 30 s at 98 °C; 25 cycles (10 s denaturation at 98 °C; 30 s annealing at 60 °C; and 30 s elongation at 72 °C); and the final elongation step at 72 °C, 5 min. The technical replicates of the PCR products were checked on 1% agarose gel, and afterwards pooled for purification using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), according to the manufacturer´s protocol. The purified product underwent quality check and quantification with the 5200 Fragment Analyzer System (Agilent, US-CA), using the NGS Fragment Kit (1-6000bp). 10 ng of the purified 16S rRNA gene amplicon were indexed using the Nextera XT Index Kit v2 (Illumina Inc., San Diego, US-CA) for multiplexed short-read sequencing. PCR conditions were: initial denaturation step, 30 s at 98 °C; 8 cycles (10 s denaturation at 98 °C; 30 s annealing at 55 °C; and 30 s elongation at

72 °C); and the final elongation step at 72 °C, 5 min. The indexed product was checked on 1% agarose gel and purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Quality and concentration of the indexing product were assessed using the above mentioned kit from Agilent. The samples were diluted to 4nM and pooled equimolarly. 10 pmol DNA were sequenced with a MiSeq System (Illumina, San Diego, US-CA) using the MiSeq Reagent Kit v3 (600 cycle) for paired end sequencing with PhiX (Illumina, San Diego, US-CA) as a spike-in positive control.

2.6 Quantification of nitrogen fixation potential

DNA extracts used for 16S rRNA gene library preparation were also used to quantify the *nifH* gene (coding for a nitrogenase) copy numbers by means of real time quantitative PCR (qPCR). The *nifH* gene abundance was quantified using the primer pair *nifH*-f (5'-AAA GGY GGW ATC GGY AAR TCC ACC AC-3') and *nifH*-r (5'-TTG TTS GCS GCR TAC ATS GCC ATC AT-3') (Rösch et al., 2002). The source organism for the nitrogenase reductase standard was *Sinorhizobium meliloti* 30136, and the mean PCR reaction efficiency ± SD was 2.04 ± 0.1 for bulk soil, 2.04 ± 0.09 for rhizosphere and 1.88 ± 0.1 for plant samples. Serial dilutions of 1:8, 1:16, 1:32 and 1:64 of the DNA extracts were tested for DNA polymerase inhibitors. The dilution showing the best PCR efficiency (1:64 v/v) was used for quantification, using the PowerSYBR® Green PCR Master Mix (Life Technologies, Warrington, UK) according to the manufacturer's instructions. Bovine serum albumin (BSA) was added to all reactions at 0.06% final concentration to reduce inhibitory effects of polyphenolic compounds co-extracted from soil. To maintain similar PCR conditions throughout sample types, BSA was added in the same concentration to root samples as well.

Samples were measured in three technical replicates on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, US-CA). The PCR program consisted of the following steps: initial denaturation 95°C, 10 min; amplification 40 cycles (denaturation 45s at 95°C, annealing 45s at 55°C, elongation 45s at 72°C); melting curve (15s at 95°C, 30s at 60°C and 15s at 95°C). The specificity of the PCR reactions was checked by melting curve analysis. Initial fluorescence values obtained for each sample were converted to gene copy numbers per gram of dry soil / dry root biomass, using standard curves created with 10-fold dilutions of the above mentioned standard with known copy number. The baseline was subtracted and normalized, followed by data analysis using the package qpcR (Spiess, 2018) applying the mechanistic cm3 model. This model is insensitive to low PCR efficiencies and minimizes effects of PCR inhibitors (Carr and Moore, 2012). Kinetic outliers were discarded automatically.

2.7 Data analysis

2.7.1 Bioinformatics analysis

The raw sequencing data were demultiplexed on the MiSeq system. Subsequently, primer and adapter sequences were removed using AdapterRemoval v. 2.1.7 (Lindgreen, 2012). 6.52 million raw reads were generated in total, with 97,242 ± 36,417 (mean ± SD) reads per sample. Rarefaction plots were constructed to assess sequencing coverage. There was saturation of the sequence coverage in all samples (data not shown). Sequencing run quality and the amount of trimming necessary for further processing of the sequences was assessed using quality plots using the R package DADA2 v. 1.10.1 (Callahan et al., 2016). All reads were trimmed by 10 bp at the beginning, while forward reads were trimmed after 290 bp and reverse reads after 210 bp. After removing remaining PhiX sequences, quality filtering and trimming, 99.3% of the initial reads remained for analysis after this step. The forward and reverse reads were denoised separately (91.3% from the forward reads and 93.2% of the reverse reads remaining) and merged (66.6% of the total reads remaining) using the R package DADA2. Amplicon Sequence Variants (ASVs) were generated, followed by removal of chimeric sequences (54.5% of reads remaining, i.e. 3,548,214 reads or 24,482 ASVs). DADA2 infers sample sequences which are resolved at differences of one nucleotide and generates ASVs as opposed to operational taxonomic units. The ASVs were taxonomically annotated using the SILVA database v. 128 (Quast et al., 2012). This data set was then imported in R and further filtered using the R package phyloseq v.1.26.1 (McMurdie and Holmes, 2013). ASVs assigned to kingdoms other than Bacteria, to chloroplasts or mitochondria were removed. Contaminant ASVs were identified and removed using the R package decontam (Davis et al., 2017), accounting for a total of 0.29% of the initial number of reads. Bacterial richness was estimated at this step from the remaining 24,407 ASVs as the number of observed ASVs per sample using the R function *estimate_richness()* from the phyloseq package.
For further analysis of the data set, a prevalence filter was applied to sort out the ASVs that were only present in a single sample. This abundance cut-off attempts to reduce the high number of ASVs that account for only a few reads, thus minimizing the influence of rare ASVs on the statistical models. The final data set contained 39.33% of the initial raw reads (3,010,093 reads, accounting for 8,334 ASVs). The nucleotide sequence data reported are available in the SRA database under the BioProject ID PRJNA648690.

2.7.2 Statistical analysis and identification of potential bacterial inocula

Constrained analysis of principal coordinates (CAP) on generalized UniFrac distances was conducted to assess effects of HCH contamination and excavation on the microbial community composition in

bulk soil, rhizosphere and root samples. Explanatory variables of the model were soil physico-chemical parameters or plant C/N ratio. Environmental parameters significantly influencing β-diversity were highlighted by means of an automatic model selection procedure, using the ordistep function of the R package vegan v. 2.5.5 (Oksanen et al., 2019), based on permutation p-values. The initial model for bulk and rhizosphere samples was based on the following variables: total HCH, disturbance status (excavated or undisturbed), pH, OM, gravimetric water content, ammonium, total inorganic N, DOC and DON. For root samples, in addition to the above mentioned rhizosphere soil parameters, we considered root C/N ratios as explanatory variables. The significance of the final CAP model, of the axes and of the selected variables was tested using the R function anova.cca() with 5,000 permutations. ASVs were subsetted according to Schmid et al. (2020) to reveal those ASVs present exclusively in a single of the four conditions (undistPoll, undistRef, excavPoll, excavRef). The "undist" plots were undisturbed, while the freshly excavated plots were called "excav". Subsetting aimed to identify bacterial taxa, specific for HCH contamination that could serve as an inoculum with HCH tolerant strains in soil clean-up procedures. Comparing the undistRef and the undistPoll plots allowed us to identify those bacterial taxa which are characteristic to long-term HCH contaminated bulk and rhizosphere soil, and to the root-associated bacterial communities of *L. tenuis*. Plants growing on these plots were of similar age. By comparing the undistPoll plot against the excavPoll plot, we assessed which HCH tolerant taxa initially adapt to soil disturbance and which ones settle only later on. We additionally identified HCH tolerant bacterial taxa that are first acquired by young *L. tenuis* plants after excavation, and those acquired in later growth stages. Finally, we compared excavPoll and excavRef rhizosphere and root-associated bacterial communities to evaluate which factor has greater influence on the development of these communities: the age of plants or HCH contamination. The five most frequently occurring ASVs (addressed as families and genera) in each of these comparisons were further analyzed as those taxa which reacted strongest to HCH contamination. Unclassified taxa and those which appeared in only one condition were not considered for further analysis. To assess whether the main responding families were also the most abundant ones throughout the samples, we used the simple ranking of mean relative abundance per family, per sample in bulk, rhizosphere and roots. The subsetting method was also used to identify possible bacterial inoculants to serve as HCH tolerant bacteria with beneficial properties for the plant, acquired by the roots of *Lotus tenuis* from the surrounding soil. The inoculation path considered was undistPoll bulk soil via excavPoll bulk, excavPoll rhizosphere, with the final destination, the roots of the young plants growing on the freshly excavated HCH contaminated plot (excavPoll). The bacterial taxa were identified at species level using BLASTN (Zhang et al., 2000).

Robust two-way analysis of variance (ANOVA) was used to test the effect of HCH and excavation on the bacterial taxa identified by means of subsetting as main responders to HCH contamination. The *t2way()* function was used to assess overall statistical significance (Wilcox, 2017). Differences in mean relative abundance of the specific comparisons mentioned above (undistPoll vs. undistRef, undistPoll vs. excavPoll and excavPoll vs. excavRef) were tested through pairwise comparisons using the function *yuenv2*. The p-values of multiple comparisons were corrected using the Benjamini-Hochberg method. Ordinary linear regressions in semi-log space were conducted to test effects of HCH on soil pH, OM, DOC, DON, soil ammonium concentration, bacterial richness, C_{mic}, plant C/N ratio and nitrogen fixation potential (*nifH* gene copy numbers) in soil and plant samples. Statistical data analysis was performed in R v.3.5.2 (R Core Team, 2018). All plots were generated using the package ggplot2 v.3.2.0 (Wickham, 2016).

3. Results

3.1 Plant performance and soil physico-chemical parameters and potential nitrogen fixation

The fresh biomass of plants collected from excavRef, excavPoll and undistPoll plots had similar average values (1.2g, 0.9g and 0.8g respectively), while that of plants collected from the undistRef plot was 27g. Regardless of the fresh biomass estimates, the C/N ratio of *L. tenuis* roots and leaves remained unaffected by HCH (data not shown). HCH concentrations detected in bulk soil ranged between 392 and 22068 ppm in the highly contaminated plots and between 3.1 and 403 ppm in the low contaminated plots (Suppl. Mat. 2). HCH significantly affected the pH of undisturbed bulk soil ($p < 0.05$; $R^2 = 0.6$) and excavated rhizosphere ($p < 0.001$; $R^2=0.9$) which in both cases declined from 7.7 to 7.4 with increasing HCH concentration (data not shown). There was a general significant positive relationship between HCH concentration and OM, DOC and DON content in bulk and rhizosphere soils (Suppl. mat. 3). Ammonium values increased significantly with HCH concentration in undisturbed ($p < 0.05$; $R^2 = 0.5$) and excavated ($p < 0.05$; $R^2 = 0.4$) rhizosphere soils (not shown).

We observed a general positive relationship between *nifH* gene abundance and HCH concentration in the undisturbed bulk and rhizosphere soil, as well as in the roots of mature *L. tenuis* plants (from undisturbed plots). Nevertheless, this effect was only marginal, as potential nitrogen fixation was not significantly affected by HCH contamination. HCH had a significant negative effect on *nifH* gene abundance only in rhizospheres of young *L. tenuis* plants ($p < 0.05$; $R^2 = 0.4$) (data not shown).

360 3.2 Microbial biomass carbon and bacterial diversity

361 Microbial biomass carbon (C_{mic}) increased significantly in undistPoll bulk soil (Suppl. mat. 5), while in
362 excavated bulk soil and undisturbed rhizosphere, HCH had a similar but marginal effect. α- diversity
363 (*i.e.* species richness) decreased significantly with increasing HCH concentration in excavated bulk soil
364 and in undisturbed rhizosphere of *L. tenuis* (Fig. 1). The trend was similar but not significant at 5%
365 level, in the case of root-associated bacterial communities of young plants growing on excavated plots.

366

367 3.3 Main responders to HCH contamination

368 HCH was the main shaping factor for bacterial community composition in bulk soil. HCH
369 concentration was strongly correlated with OM content as co-explanatory variable ($p < 0.001$; Pseudo-
370 $F_{1,18} = 1.39$) (Fig. 2a). ASV distribution in the rhizosphere was shaped mostly by the growth stage of
371 plants ($p < 0.05$, Pseudo-$F_{1,15} = 1.35$), while HCH had a smaller effect, just above the 5% significance
372 level ($p = 0.064$, Pseudo-$F_{1,15} = 1.24$). HCH was positively correlated with ammonium ($p < 0.05$,
373 Pseudo-$F_{1,15} = 1.31$) and total N content ($p < 0.01$, Pseudo-$F_{1,15} = 1.64$) (Fig. 2b). The composition of
374 the root-associated bacterial communities was determined solely by the age of plants ($p < 0.01$, Pseudo-
375 $F_{1,16} = 1.30$), with no effect of HCH contamination detected at this level (Fig. 2c).

376 In bulk soil, main responders to HCH contamination were ASVs belonging to the Burkholderiaceae,
377 Nitrosomonadaceae and Xanthomonadaceae bacterial families, and to the genera *Sphingomonas* and
378 *Altererythrobacter* (both part of the Sphingomonadaceae family), and *Lysobacter* (fam.
379 Xanthomonadaceae) (Suppl. mat. 4). HCH had a significant effect on the mean relative abundance of
380 Burkholderiaceae ($p < 0.01$; Suppl. mat. 6). Further pairwise comparisons showed significantly higher
381 abundance of this family in excavPoll soil as compared to undistPoll ($p < 0.05$) and in undistRef soil as
382 compared to undistPoll ($p < 0.01$) (Suppl. mat. 6 and 7). Nitrosomonadaceae were negative responders
383 to HCH, pairwise comparisons between undistPoll and undistRef plots showing a significantly lower
384 abundance in the first condition ($p < 0.05$) (Suppl. mat. 6 and 7). Xanthomonadaceae were more
385 abundant in excavPoll bulk soil, as compared to undistPoll soil ($p < 0.05$; Suppl. mat. 7).

386 Genus *Lysobacter* was significantly affected by HCH contamination in bulk soil, as it was more
387 abundant in excavPoll bulk soil as compared to undistPoll soil ($p < 0.01$), but higher in undistPoll bulk
388 soil when compared to undistRef soil ($p < 0.05$) (Fig. 3; Suppl. mat. 7). Genus *Altererythrobacter* was a
389 positive responder to HCH contamination ($p < 0.05$), with slightly higher abundance in the undistPoll
390 plot than in all other conditions. However, this effect was not further detected in pairwise comparisons
391 (Fig. 3; Suppl. mat. 7).

In the rhizosphere, the most abundant responders at family level were Burkholderiaceae, Nitrosomonadaceae and Sphingomonadaceae. At genus level, the most common differential ASVs corresponded to the genera *Massilia* (Burkholderiaceae), *Sphingobium* and *Sphingomonas* (both Sphingomonadaceae) (Suppl. mat. 4). Similar to bulk soil, Nitrosomonadaceae were negative responders to HCH contamination, pairwise comparisons between undistPoll and undistRef plots showing a significantly lower abundance of this family in the first condition ($p < 0.001$) (Suppl. mat. 6 and 7). As second main responder to contamination, there was a significantly higher abundance of Burkholderiaceae in excavPoll rhizosphere as compared to undistPoll ($p < 0.05$) and in undistRef rhizosphere as compared to undistPoll soil ($p < 0.01$) (Suppl. mat. 6 and 7). HCH ($p < 0.01$) and the excavation process ($p < 0.05$) had a significant effect on the mean relative abundance of Sphingomonadaceae in rhizosphere soil (Suppl. mat. 6 and 7). However, the HCH effect was only marginal, as it was not further detected in pairwise comparisons.

Genus *Sphingobium* was a significant responder to HCH contamination in the rhizosphere. Pairwise comparisons showed a significantly higher abundance of the genus *Sphingobium* in the undistPoll rhizosphere than in undistRef one ($p < 0.01$). Similarly, *Sphingobium* was more abundant in excavPoll rhizosphere than in undistPoll rhizosphere ($p < 0.05$) or in excavRef rhizosphere ($p < 0.01$) (Fig. 3; Suppl. mat. 7). Plant age had a notable effect on the abundance of *Sphingobium*, which was significantly higher in excavated rhizosphere plots, regardless of contamination ($p = 0.001$). Genus *Massilia*, was significantly affected by both HCH and excavation in rhizospheres (Fig. 3; Suppl. mat. 7). Pairwise comparisons revealed a significantly higher abundance of this genus in excavPoll soil when compared to undistPoll rhizosphere ($p < 0.05$) as well as in comparison to the excavRef soil ($p < 0.05$) (Fig. 3; Suppl. mat. 7).

In roots of *L. tenuis*, the most frequently occurring differential ASVs belonged to Burkholderiaceae, Rhizobiaceae, and Sphingomonadaceae (Suppl. mat. 4). At genus level, the most frequent responders were *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (Rhizobiaceae) and *Sphingomonas* (Sphingomonadaceae). Burkholderiaceae from the root-associated microbiome were significantly affected by HCH. Pairwise comparisons showed higher abundance of this taxon in the undistPoll as compared to excavPoll roots ($p < 0.05$) and in undistRef roots as compared to undistPoll roots ($p < 0.05$)(Suppl. mat. 7). Additionally, Burkholderiaceae were marginally more abundant in the excavPoll roots than in the excavRef roots ($p = 0.06$). Rhizobiaceae were identified as negative responders to HCH in the root-associated bacterial communities ($p < 0.01$) (Suppl. mat. 6 and 7). This effect was however not strong enough to be further detected in the pairwise comparisons. Plant age had a clear effect on the abundance of Sphingomonadaceae in root-associated bacterial communities ($p < 0.05$).

They were more abundant in the old roots, regardless of the contamination level (Suppl. mat. 6 and 7). Furthermore, this family was positively affected by HCH, as they were significantly more abundant in undistPoll roots ($p < 0.01$), and nearly significant when compared to undistRef roots ($p > 0.05$). The *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* clade was a strong positive responder to HCH in the root compartment of *L. tenuis*, pairwise comparisons showing higher abundance of this clade in undistPoll roots, as compared to excavPoll roots ($p < 0.01$). Genus *Sphingomonas* had significantly higher abundance in undistPoll roots when compared to excavPoll roots ($p = 0.001$) (Fig. 3; Suppl. mat. 7).

All identified main responders were also among the most abundant taxa at family level in our bulk, rhizosphere and root samples (Suppl. mat. 8). Further, we identified those HCH tolerant bacteria which were common to undistPoll bulk soil, excavPoll bulk soil, excavPoll rhizosphere and to roots of young plants germinating on excavPoll plot (Suppl. mat. 9). Bacteria transferred from bulk soil to the root-associated microbiome were identified as *Mesorhizobium loti* (100% similarity), *Neorhizobium galegae* (100% similarity), *Novosphingobium lindaniclasticum* (similarity 99%), *Pantoea agglomerans* (prev. *Enterobacter agglomerans*) (similarity 100%), *Lysobacter sp.* clone T28265 and *Lysobacter bugurensis* (99% similarity) (Suppl. mat. 9).

4. Discussion

4.1. Bulk soil bacterial communities adaptation to HCH contamination and excavation procedures

Our results showed that HCH and OM alone were the most significant environmental descriptors for the differentiation of the bulk soil microbial community. Because bacterial α-diversity (richness) was lower in the excavPoll plot than in all other three conditions, soil disturbance combined with HCH contamination may have led to a slower recovery of species richness in freshly excavated bulk soil. On the other hand, microbial growth (expressed here as C_{mic}) appeared to be sustained in both excavated and undisturbed highly contaminated bulk samples by higher DOC and OM contents. Similar to previous findings of Kalbitz and Popp (1999) and Balázs et al. (2020), high soil organic matter content was positively correlated with HCH concentrations present in both excavated and undisturbed plots. Kalbitz and Popp (1999) attributed higher OM and DOC concentration to either drought in summer or litter accumulation in autumn. Since our sampling campaign took place at the beginning of June when local temperatures were relatively high, this might be a plausible explanation for the OM peak in bulk soil, triggering the adsorption of HCH to soil particles rich in OM, resulting in mobilization of the

contaminant, followed by HCH uptake in plants (Wahid and Sethunathan, 1979; Kalbitz and Popp, 1999).

Finding ASVs from the Sphingomonadaceae and Burkholderiaceae families as main responders to long term HCH contamination is not surprising at all, as they all include versatile taxa found in a wide range of habitats. As sphingomonads are well known to degrade recalcitrant xenobiotics and polyaromatic compounds of both anthropogenic and natural origin (Glaeser and Kämpfer, 2014), we expected to find taxa from this bacterial group that are specific for HCH contamination at the Turda site. However, possibly because of their ubiquitousness, which allows these chemoorganotrophs to inhabit various ecosystems (Amils, 2014; Glaeser and Kämpfer, 2014), HCH did not affect mean relative abundances of Sphingomonadaceae in bulk soil. Burkholderiaceae are a very versatile group as well, and well known hydrocarbon degraders (Castorena et al., 2006). Nevertheless, they appear to thrive on easy degradable hydrocarbons rather than more recalcitrant compounds such as diesel oil (Bell et al., 2013) or HCH in our case, which could explain the higher occurrence of this group in clean bulk soil. Although ammonia-oxidizing bacteria have been proposed for the remediation of chlorobenzene contaminated soil (Sayavedra-Soto et al., 2010), the Nitrosomonadaceae in our study were in general negative responders to HCH. This supports results of Ibiene and Okpokwasili (2011), which showed that the type genus of the family, *Nitrosomonas* sp., is sensitive to lindane contamination. Excavated HCH bulk soil samples were much richer in Bulkhorderiaceae, Xanthomonadaceae and Sphingomonadaceae ASVs than undistPoll bulk soil. Xanthomonadaceae are known hydrocarbon degraders with many applications in bioremediation of oil / petroleum or trifluralin (herbicide) contaminated soil (Popp et al., 2006; Chang and Zylstra, 2010; Du et al., 2018). The fact that they were generally more abundant in the excavated conditions regardless if clean or contaminated, suggests that these taxa are fast-growing generalists and early colonizers. Thus, despite their metabolic capabilities which allowed them to tolerate and degrade HCH, they merely take advantage of free niches left available by previously established bacterial communities.

At genus level we found two taxa to be considered as HCH tolerant in long term contamination: *Sphingomonas* and *Altererythrobacter*, which is consistent with our previous findings in lindane contaminated soil (Balázs et al., 2020). There are several examples of *Sphingomonas* strains isolated from HCH contaminated soils found capable to tolerate and mineralize HCH isomers (Pal et al., 2005; Singh and Lal, 2009; Teramoto et al., 2010; Tonon et al., 2014). Our results show that HCH did not have any notable effect on the general abundance of this genus, highlighting the ability of *Sphingomonas* to tolerate HCH and the possibility of employing them in *in situ* bioremediation projects. *Sphingomonas* and *Lysobacter* were antagonistic responders to the excavation of HCH

contaminated soil. *Sphingomonas* was negatively affected by the excavation process, consistent with observations of Dong et al. (2017) and Shi et al. (2017), who reported lower abundance of this genus in tilled agricultural soil or land subsidence. Nevertheless, *Sphingomonas* was replaced by another known HCH degrader in excavPoll bulk soil, the xanthomonad *Lysobacter* (Rani et al., 2016; Margesin et al., 2018), suggesting quick adaptation of bacterial communities to HCH contamination after excavation procedures.

4.2. Rhizosphere soil bacterial communities adaptation to HCH contamination and the interaction with plant developmental stage

Rhizosphere is a transition compartment between bulk soil and the root environment. Accordingly, we expected the bacterial communities in this zone to reflect both soil HCH contamination and age of *L. tenuis*, as plants can select different subsets of microbes at different stages to fulfill age-specific functions (Chaparro et al., 2013). In line with this, the age of the rhizosphere had a significant influence on bacterial community composition, followed by HCH contamination. As such, HCH shaped the microbial community in the undisturbed rhizosphere, while excavated rhizosphere samples showed a high degree of similarity regardless of contamination, thus highlighting the importance of plant growth stage in acquiring a specific rhizosphere microbiome. Ammonium and total N content were the most important environmental descriptors in rhizosphere soil, and positively correlated with HCH concentration. This is consistent with results of Blondel et al. (2017) in lindane and chlordecone contaminated maize rhizospheres where the plants enabled efficient C- and N- turnover and maintained a normal ammonification process as opposed to lindane contaminated soil without plants. OM, DOC and DON concentrations in rhizosphere followed the same pattern as in bulk soil, where they were positively correlated with HCH concentration in undisturbed plots and possibly triggered an increased microbial carbon biomass. Higher C_{mic} content and at the same time a lower bacterial richness in the undistPoll rhizosphere suggests that contamination might offer suitable growth conditions for only few HCH tolerant taxa, which are able to thrive under these conditions.

The Sphingomonadaceae were positive responders to HCH in the rhizosphere compartment. This might have occurred either due to root exudates which could promote their growth (el Zahar Haichar et al., 2008), or by following a selection process of the rhizosphere microbiome by plants as stress response to contamination. Chapelle et al. (2016) showed that sphingomonads were enriched in the rhizosphere of sugar beet as a stress response upon fungal infection, while Tsavkelova et al. (2007) suggested that these bacteria are selected by orchid plant roots as they produce the growth hormone indole-3-acetic acid. The strongest positive responder to HCH contamination in rhizosphere from this bacterial family

was *Sphingobium*, well known for its HCH degrading capabilities (Lal et al., 2008; Sangwan et al., 2014). Burkholderiaceae are degraders of a vast range of aromatic compounds (Goldfarb et al., 2011), and the presence of the copiotrophic genus *Massilia* in the early stages of succession of the rhizosphere and root microbiome when supply of labile organic carbon in soil is high, is not surprising (Shrestha et al., 2007; Li et al., 2014). Being copiotrophic organisms, *Massilia* are good abiotic stress tolerants (*e.g.* to high HCH concentration in the excavated rhizosphere) but not particularly tolerant to biotic stress (Shrestha et al., 2007; Abou-Shanab et al., 2010). Considering that lower species richness (α- diversity) in the excavPoll rhizosphere also means lower competition between species, this low biotic stress is probably the reason for the dominance of *Massilia* in this condition, as opposed to the excavRef compartment.

In undistPoll rhizosphere, sphingomonads *Altererythrobacter* and *Sphingobium*, were the most abundant differential genera, all of which have known HCH tolerant representatives (Pal et al., 2005; Singh and Lal, 2009; Teramoto et al., 2010). The excavPoll rhizosphere however, showed higher diversity of bacterial families, dominated by *Massilia* (Burkholderiaceae), *Sphingomonas* (Sphingomonadaceae) and *Lysobacter* (Xanthomonadaceae). This configuration of generalist bacterial genera is less typical for HCH contamination than for colonization of newly occurred free niches following the excavation process (Shrestha et al., 2007; Li et al., 2014).

4.3. Root and root-associated bacterial communities adaptation to HCH contamination and plant age effect

Following the trend we observed at the transition from bulk to rhizosphere soil, the only factor influencing the composition of *Lotus tenuis* root-associated bacterial communities was the plant development stage, regardless of HCH contamination. Overall bacterial richness (α- diversity) was not affected by HCH contamination in the roots of the plants growing on undisturbed plots. Nevertheless, bacterial richness in young roots of *L. tenuis* (from excavated plots) was altogether lower than in roots of mature plants from undisturbed plots. This is probably a reflection of the early succession stages of plant microbiome development, when the increase in copiotroph (r- strategists) abundance is supported by the non-limiting nutrient-rich new environment but species richness is low due to weak competition (Shrestha et al., 2007).

Lotus tenuis is a model plant for legumes known to have a great potential for adaptation to abiotic stress, making it the perfect candidate for dune re-vegetation or heavy-metal contaminated soils (Escaray et al., 2012). As we previously identified *L. tenuis* as both an early colonizer and HCH tolerant at the Turda production facility (Balázs et al., 2018), we considered it for restoration of HCH

contaminated sites. Plants collected from the excavRef, excavPoll and undistPoll plots had similar fresh biomass (~1g), while the mean fresh biomass of the plants collected from the undistRef plot was almost 30 times higher. This reveals a clear negative effect of long-term HCH contamination on *L. tenuis* growth and development. The effect of HCH or lindane on early growth and development of legumes had previously been studied on soybean (Tu, 1977) and *Phaseolus vulgaris* (Pereira et al., 2010). Both studies reported a certain degree of resistance of plants in this family to HCH, as their early growth was not significantly affected by the contaminant. These results are supported by the fresh biomass estimates of young *L. tenuis* roots, which had similar values regardless whether they grew on clean or contaminated excavated plots. In addition, the inhibited late growth of *L. tenuis* in high HCH contamination conditions is consistent with data of Tripathi et al. (2014), showing that lindane in high concentrations in soil reduces growth and yield of *Vigna radiata*. We further observed a strong nodulation on *L. tenuis* roots collected from the undistRef plot, hardly any or no nodules on the plants from the undistPoll plot and no nodules on young plants from excavated plots (personal observation). The C : N ratio of *L. tenuis* was nevertheless constant, regardless of HCH contamination and despite the lack of nodulation in roots of plants growing at the undistPoll plot. Khan et al. (2006) showed that certain herbicides decrease nodulation and biomass of nodules in chickpea, leading to a decrease in N content of the grains as well. Yet, in our case *L. tenuis* seems to have adapted to that impediment by taking up nitrogen from soil to maintain its C and N balance, possibly even in the form of ammonium which was more abundant in the rhizosphere of plants from both undistPoll and excavPoll plots. Supporting this idea, Rogato et al. (2010) reported a high affinity ammonium transporter in a short-root wild phenotype of *L. japonicus*, which presumably modulated root growth in conditions of potentially toxic external ammonium concentration. However, as this happened at an ammonium concentration higher than 10mM, which is not our case, we regard this rather as an adaptation of *Lotus tenuis* to contamination conditions, which prevent efficient nodulation and thus symbiotic nitrogen fixation, than to toxic ammonium concentrations.

HCH did not have significant effects on general root microbial composition. However, we found several particularities in roots of *L. tenuis* from undistPoll contaminated plot which could indicate HCH tolerant bacteria, with or without benefits for the host plants. Similar to rhizosphere soil, there was a higher abundance of ASVs from the Sphingomonadaceae family inhabiting undistPoll roots but not undistRef ones. The latter were characterized by a greater abundance of differential Burkholderiaceae genera like *Massilia*, *Douganella*, *Variovorax* and *Rhizobacter*. This highlights the high potential of taxa within the Burkholderiaceae to establish close interactions with plants, of both beneficial (Han et

al., 2010; Haack et al., 2016), neutral (Ofek et al., 2012) or pathogenic (Goto, 2015) nature in non-contaminated conditions.

Genera characteristic to the root-associated microbiome of plants growing at the undistPoll contaminated plot, were *Sphingomonas*, *Devosia*, *Brevundimonas* and *Rhodanobacter*. *Sphingomonas* and *Devosia* have been mentioned as non-nodulating bacteria in legume roots (Tariq et al., 2013). While *Sphingomonas* is a common legume endophyte frequently selected by the host as an antagonist to pathogens (Pini et al., 2012; Hartman et al., 2017), *Devosia* species were often isolated from soils contaminated with high amounts of HCH where they were able to tolerate the contaminant but not to degrade it (Talwar et al., 2020). As several *Devosia* species are capable of nitrogen fixation in legumes (Rivas et al., 2002; Hoque et al., 2011), they might have been selected by *L. tenuis* roots to fulfill the nitrogen fixation function in nodule-impaired old roots along *Allorhizobium- Neorhizobium-Pararhizobium-Rhizobium*. Although microbial composition in roots was generally driven by plant age, our results indicate that *L. tenuis* actively acquires beneficial bacteria for N fixation or rhizobacteria with plant growth promoting abilities that are at the same time capable of tolerating HCH. As such, *Massilia, Pantoea* and *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* were the most abundant genera characteristic for *L. tenuis* roots in the excavPoll plot. *Massilia* is a copiotroph characteristic for early microbial succession in roots (Ofek et al., 2012), while *Pantoea* and *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* can both fix nitrogen, and even degrade xenobiotics as in the case of *Pantoea* (Stacey, 2001; Walterson and Stavrinides, 2015).

4.4. HCH contaminated soil as inoculum for bioaugmentation procedures in microbe-assisted phytoremediation

We identified several HCH tolerant bacteria which might be transferred from the undistPoll bulk soil to the excavPoll bulk soil through excavation and through the rhizosphere to the roots of young plants germinating on the excavated soil. Two of the identified bacteria, which could serve as an inoculum, are *Mesorhizobium loti* and *Neorhizobium galegae*. Both rhizobia are capable of forming nitrogen-fixing symbiosis with legume roots and therefore successfully used to sustain the growth of the early colonizer *Lotus tenuis* in the recovery process of HCH contaminated soil environments. Furthermore, bacteria like *Mesorhizobium loti* growing on HCH contaminated soils encode haloalkane dehalogenases, enzymes involved in the assimilation of organochlorine pesticides (Fetzner and Lingens, 1994; Sato et al., 2005). Although *M. loti* cannot degrade nor survive on HCH as sole carbon source, the dehalogenases it encodes are still an interesting aspect to take into consideration for bioaugmentation projects of HCH contaminated soil.

Novosphingobium lindaniclasticum is another bacterium we identified as common to both contaminated bulk soils, to the excavPoll rhizosphere and at the same time to the root-associated bacterial communities of young roots growing in the excavPoll plot. *Novosphingobium lindaniclasticum* is an HCH degrading bacterium, first isolated from an HCH dumpsite in Lucknow, India (Saxena et al., 2013). The metabolic versatility of *Novosphingobium* strains allows them to colonize a wide range of habitats from soil and water to plant surfaces, rendering them perfect candidates for microbe-assisted phytoremediation and bioaugmentation procedures.

Pantoea agglomerans is a bacterium featuring plant growth promoting traits such as nitrogen fixation and phosphate solubilization and at the same time, it is a plant pathogen antagonist (Lim et al., 2014). Its additional ability to degrade DDT and γ-HCH (lindane) (Karagoz et al., 2016) makes it therefore another inoculum that might be well suited for bioremediation of HCH contaminated sites.

Two other species of the genus *Lysobacter* (*Lysobacter sp.* clone T28265 and *Lysobacter bugurensis*) were transferred from contaminated old bulk soil to young *L. tenuis* roots in excavated plots. Although there is not sufficient data regarding the behavior of these taxa in HCH contaminated environments, they are still of interest since bacteria from this genus have plant pathogen antagonistic capabilities. Furthermore, another species of this genus, *Lysobacter tolerans*, was isolated from HCH contaminated sites where it is known to degrade the compound (Expósito et al., 2015; Rani et al., 2016).

4.5. HCH effect on the potential nitrogen fixation in soil and roots of Lotus tenuis

Potential nitrogen fixation in bulk and rhizosphere soil and in the *L. tenuis* root-associated microbiome was generally not affected by HCH contamination. The single exception consisted in the rhizosphere of young plants growing at the excavPoll plot, where we observed lower *nifH* gene copy numbers as compared to excavRef rhizospheres. The lower diazotroph abundance in the excavPoll rhizosphere did not reflect either in the plant C: N ratio, nor in the average plant fresh biomass, which was similar to the young plants growing in non-contaminated soil. Fox et al. (2001) showed that DDT, a pesticide with similar chemical structure to HCH, caused negative effects on potential nitrogen fixation and nod-exposition by disrupting the plant- *Rhizobium* signaling. Nevertheless, we observed no difference in nodulation between young plants, regardless whether they were growing in contaminated or non-contaminated plots. Furthermore, there was no significant difference in the abundance of Rhizobiaceae in rhizospheres of young plants. It is well known that members of the Rhizobiaceae family are not the only organisms capable of fixing nitrogen (Vitousek et al., 2002) and that in later growth stages, specific nitrogen fixing populations which can tolerate HCH application may be selected and stimulated by the rhizosphere (Patnaik et al., 1996). Therefore, the temporarily lower potential nitrogen

fixation in excavPoll rhizosphere might be just a short-term HCH effect on the initial microbial succession, inhibiting the growth of early colonizing diazotrophs.

5. Conclusions

Our results show that soil and root-associated bacterial communities may be affected in different degrees by HCH contamination and by excavation as a soil clean-up measure. In bulk soil HCH was the main factor influencing bacterial community composition, while rhizosphere microbiome was shaped by both HCH and plant developmental stage. The bacterial community composition from both long-term contaminated and excavated plots provided information about microbial succession in HCH contamination conditions, which are key aspects to be considered in field bioaugmentation procedures where HCH is not the only factor influencing the success of microbe-assisted phytoremediation. Furthermore, we identified several bacteria which were common to the undisturbed HCH contaminated bulk soil, to the excavated HCH bulk soil, and to the rhizosphere and roots of young plants growing in the excavated HCH hotspot. Altogether, these taxa point towards a possible inoculum with HCH tolerant bacteria (H1), which are transferred from contaminated bulk soil to rhizospheres and roots of *L. tenuis* with beneficial effects for microbe-assisted phytoremediation and bioaugmentation procedures (H2). Finally, potential nitrogen fixation was not affected by HCH, except for the contaminated rhizosphere of young plants growing on the freshly excavated plots (H3). This highlights the high adaptability of native bacterial communities to perform important ecosystem functions in various field conditions regardless of HCH contamination.

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Figure 1. Relationship between bacterial richness and HCH concentration in the bulk soil, rhizosphere and root compartments. Dots and solid lines represent samples from undisturbed plots. Triangles and dashed lines represent samples from excavated plots. Regression significance: excavated bulk soil ($p < 0.01$; $R^2 = 0.6$); undisturbed rhizosphere ($p < 0.01$; $R^2 = 0.6$). All other regressions were not significant ($p > 0.05$).

Figure 2. CAP ordinations of bacterial communities showing differences induced by HCH contamination and excavation in **(a)** bulk soil, **(b)** rhizosphere soil and **(c)** roots of *Lotus tenuis*. (**a)** Model significance:**(a)** $p < 0.001$; Pseudo-$F_{1,18} = 1.39$; **(b)** $p < 0.001$, Pseudo-$F_{4,15} = 1.45$; **(c)** $p < 0.05$, Pseudo-$F_{3,16} = 1.14$. Axis significance: **(a)** CAP1: $p < 0.001$, and Pseudo-$F_{1,18} = 1.39$; **(b)** CAP1: $p < 0.001$, Pseudo-$F_{1,15} = 2.42$; **(c)** CAP1: $p > 0.05$, Pseudo-$F_{1,16} = 1.33$. Dots represent undisturbed plots; triangles represent excavated plots. Light blue color represents low HCH concentration and black represents high HCH concentration. Ellipses delineate 95% confidence ellipses. Red arrows mark the co-explanatory variables: OM (organic matter), ammonium and total N content.

Figure 3. Mean relative abundance of the most important responders at genus level to HCH contamination in **(a)** bulk soil, **(b)** rhizosphere soil and **(c)** roots of *Lotus tenuis*. The colors of the bars represent the disturbance status of the plots: light gray – undisturbed plots; dark gray– excavated plots.

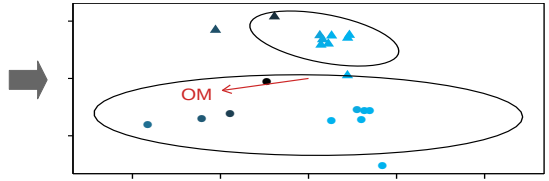
Graphical Abstract

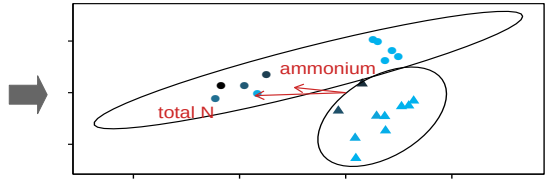
only HCH effect

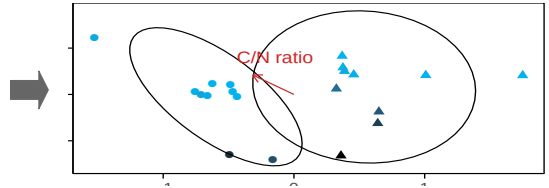
excavation and HCH effect

only excavation effect

Effects of HCH and excavation on **bacterial community composition**







Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

- HCH contamination shaped bulk soil bacterial communities
- Rhizosphere microbial communities are affected by HCH contamination and plant age
- *Lotus tenuis* growth and development is negatively affected by HCH contamination
- Lower nitrogen fixation potential in freshly excavated contaminated rhizosphere
- Bioaugmentation might improve and accelerate HCH removal in soils

Post-reclamation microbial diversity and functions in hexachlorocyclohexane (HCH) contaminated soil in relation to spontaneous HCH tolerant vegetation

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Abstract

Given the toxicity, volatility and persistence of the organochlorine pesticide hexachlorocyclohexane (HCH), reclamation of contaminated areas is a priority for the health and welfare of neighboring human communities. Microbial diversity and functions at field scale, in relation to spontaneous vegetation in post-excavation situations, are essential indicators to consider when bioaugmentation or microbe-assisted phytoremediation strategies are developed. Thus, the present study aimed to evaluate the effects of long-term HCH contamination on soil and plant-associated microbial communities, and whether HCH contaminated soil has the potential to act as a bacterial inoculum in post-excavation bioaugmentation and microbe-assisted phytoremediation strategies. To scrutinize the role of vegetation, the potential nitrogen fixation of free-living and symbiotic diazotrophs of the legume *Lotus tenuis* was assessed as a measure of nutrient cycling functions in soil under HCH contamination. Potential nitrogen fixation was generally not affected by HCH contamination. The single exception was a temporary lower nifH gene count in the excavated contaminated rhizosphere which is most probably a short-term HCH effect on early bacterial succession in this compartment. HCH was the main shaping factor of the microbial communities in long-term contaminated bulk soil, where we identified possible HCH tolerating genera such as *Sphingomonas* and *Altererythrobacter*. In *L. tenuis* rhizosphere, microbial community composition was influenced by both HCH contamination and plant growth stage. *Sphingobium* and *Massilia* were the bacterial genera characteristic for HCH contaminated rhizospheres. *L. tenuis* growth and development was negatively affected by long-term HCH contamination. The root-associated bacterial community composition however was driven solely by plant age, whereas the HCH effect was negligible. In contamination conditions, *L. tenuis* seems to acquire potentially HCH tolerant bacteria which could at the same time offer plant growth promoting (PGP) benefits for the host, such as the *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* clade, *Sphingomonas, Massilia* or *Pantoea*. Finally, we identified an inoculum with possibly HCH tolerant PGP bacteria transferred from the contaminated bulk soil to *L. tenuis* roots through the rhizosphere compartment, consisting of *Mesorhizobium loti, Neorhizobium galegae, Novosphingobium lindaniclasticum, Pantoea agglomerans* and *Lysobacter bugurensis*.

Keywords:

Hexachlorocyclohexane; Soil clean-up; Soil functions; Bacterial community; Bioremediation

1. Introduction

Hexachlorocyclohexane (HCH) was one of the most widely used organochlorine pesticides in agriculture between the 1950s and 1980s where a total of nearly 8 million tons of toxic and persistent waste was deposited worldwide at former production sites (Willett et al., 1998). Due to inadequate disposal techniques, HCH has been flagged for regulatory intervention and elimination to limit its dispersion via air or water and avoid accumulation into plant and animal food resources (Vijgen et al., 2011; Ogbeide et al., 2016). Since many countries lack funding for proper clean-up and restoration, excavation remains the cheapest conventional clean-up approach for HCH contaminated sites (Caliman et al., 2011; Morillo and Villaverde, 2017). However, there are often no follow-up interventions (*e.g.* landfilling or phytoremediation approaches) to prevent further dispersion of contaminant left-overs via air transport to neighboring residential areas.

Microbial diversity and functions at field scale, in relation to spontaneous vegetation in post-remediation situations, are essential criteria for the development of customized strategies to enhance and speed up the reclamation of brownfields representing a potential danger to local human communities. There are, however, very few studies of microbial diversity and functions as ecological indicators in HCH polluted soil (Dadhwal et al., 2009; Bala et al., 2010), and only one which tackles these aspects in post-reclamation situations (Balázs et al., 2020). Nonetheless, the latter study handles only lindane as a contaminant, and it was limited to controlled greenhouse conditions.

The partial excavation of the HCH waste deposits from Turda (Romania, Suppl. Mat. Fig. 1) offered an opportunity to study how soil and plant associated bacterial communities adapt to long-term pollution and remediation attempts. As HCH tolerant bacterial consortia proved to decrease HCH levels in soil at both pot and field scale (Garg et al., 2016), we hypothesized that post- excavation traces of contaminated soil might serve as an inoculum with native, HCH- tolerant bacteria for subsequent bioaugmentation strategies. Such inocula could prove more efficient than artificial consortia, given that native bacterial communities are already adapted to contamination and to the local physico-chemical conditions.

In addition, microbe-assisted phytoremediation studies showed that soil inoculation with HCH tolerant/degrading strains and plant growth promoting rhizobacteria could enhance HCH dissipation in rhizospheres and increase plant performance and tolerance to toxicity (Becerra-Castro et al., 2013a,b; Alvarez et al., 2015). Plants grown in contaminated soils become an important sink for organochlorine compounds via soil-plant and or air-plant route, as they are capable of accumulating high amounts of HCH (Pereira et al., 2006). Given that *L. tenuis* is growing spontaneously at the Turda site, we hypothesized that contaminated soil may potentially serve as an inoculum with HCH tolerant bacterial

strains for the plant, and that these strains might support *L. tenuis* growth and performance as first colonizer, as well as its function in nitrogen fixation.

Finally, recovery of microbial functions after excavation is a critical step in achieving soil restoration and a functional ecosystem. Since the excavated soil had already been colonized by the legume *Lotus tenuis*, potential nitrogen fixation was one of the most important bacterial functions at this stage of ecological succession. Hence, our third objective was to assess effects of HCH on potential nitrogen fixation in bulk and rhizosphere soil, as well as at root level of the early colonizer *Lotus tenuis*, as a measure of post-reclamation ecosystem functionality.

To summarize, the present field study aimed to address the following hypotheses: (H1) HCH contaminated soil acts a bacterial inoculum in post-reclamation bioaugmentation strategies; (H2) such an inoculum can improve plant performance and tolerance to HCH in early vegetation succession stages; and (H3) HCH affects potential nitrogen fixation of free-living and symbiotic diazotrophs. For this purpose, we measured soil physico-chemical parameters, plant fitness, and characterized soil and root-associated bacterial communities by means of 16S rRNA gene amplicon sequencing in long-term HCH- contaminated soil and their subsequent response to excavation procedures.

2. Materials and methods

2.1 Site description

The former chemical plant (46°55´N, 23°78´E) is located within the precincts of Turda city, Romania, near the river Arieş (Suppl. mat. 1). Between 1954 and 1983, approximately 15,000 tons of waste HCH were inadequately deposited within that enclosure (Prodan et al., 2011). The chemical plant shut down in 1998, but the close proximity to residential areas and easy access of unauthorized persons on the grounds represent a continuous pollution source and health concern for the local community. The factory area includes two HCH hotspots: one is a waste heap on the right bank of the river Arieş, and the other corresponds to the former lindane production department (Balázs et al., 2018). In spring 2018, an area including the former production department HCH hotspot and several neighboring low-contamination plots were excavated and leveled out, as a measure of soil reclamation (Suppl. mat. 1). The sampling campaign took place two months after the excavation operation, at the end of May 2018.

2.2 Sampling design and procedures

To assess the presence of a possible bacterial inoculum and the effect of post-reclamation HCH levels on nitrogen fixation and plant performance, we compared the previously identified HCH hotspots with two neighbouring low contaminated plots (Balázs et al., 2018). One of the HCH hotspots and one low contaminated plots were freshly excavated, while the other two were undisturbed and covered with spontaneous vegetation (Suppl. mat. 1). The undisturbed plots are further named “undistPoll” (high contamination), “undistRef” (low contamination) while the freshly excavated and leveled plots were named “excavPoll” (high contamination) and “excavRef” (low contamination). All plots were colonized by *Lotus tenuis* WALDST. & KIT. ex WILLD., but with different coverage. *Lotus tenuis* was chosen for this field study as it already grew spontaneously on the premises in HCH hotspots (Balázs et al., 2018). As an early perennial colonizer and member of the Fabaceae family, *L. tenuis* has an important role in creating optimal conditions for the further development of a spontaneous vegetation cover. Furthermore, it provides important ecosystem services such as nitrogen fixation, erosion control, build-up of soil organic matter or development of a diverse microbial community in the soil.

For optimal coverage, a five sampling point pattern corresponding to five biological replicates was randomly set within the limits of each of the four plots. Each of the equally spaced five sampling points consisted of a circular area with one meter radius. Five bulk soil sub-samples were collected at random from within the limits of each sampling point and mixed into one composite sample, resulting in five composite biological replicates for each of the four plots: “undistPoll”, “undistRef”, “excavPoll” and “excavRef”. Rhizosphere and plant samples were subsequently collected from within the circular sampling points established for bulk soil.

Bulk soil was collected from the upper 10 cm, mixed into composite samples for each sampling point, sieved through 2 mm mesh, filled into sterile tubes and temporarily stored on ice. Samples meant for DNA extraction were stored at -80°C and samples for physico-chemical measurements were stored at 4°C. Samples meant for HCH quantification were freeze-dried and subsequently stored at 4°C.

To collect rhizospheres, soil was loosened around the plant roots which were then carefully pulled out with adherent rhizosphere soil. Plants collected from one sampling point were pulled together into a composite sample, were shaken on top of a sieve equipped with a tray and brushed to retrieve as much rhizosphere material as possible. The soil was then sieved and stored in the same manner as the bulk soil. The plant material was washed with tap water, followed by autoclaved distilled water, separated into roots and shoots and stored at -80°C until further use. Before the plant material was separated, several plants from each sample were weighed to estimate mean fresh biomass.

2.3 Soil physico-chemical parameters, microbial biomass carbon and plant C/N ratio

Microbial biomass carbon (C_{mic}) was determined according to DIN ISO 14240-2:2011-09 after chloroform fumigation and subsequent extraction with 0.01M $CaCl_2$ solution (1:4 (w/v)). Dissolved organic carbon and nitrogen (DOC, DON) as well as ammonium, nitrate and nitrite concentrations were extracted in the same manner, excluding the fumigation step. All extractions were done in triplicates and stored at -20°C for later measurements. Organic C and N were quantified on a Total Carbon Analyzer (Shimadzu TOC 5050, Tokyo, Japan). C_{mic} was calculated as the difference of total C between fumigated and non-fumigated samples, using a fraction of 0.45 as extractable part of microbial biomass carbon (Joergensen, 1996). Total nitrogen and ammonium were determined using a continuous-flow photometric analyser (CFA-SAN Plus; Skalar Analytik, Germany). Soil pH was measured according to the ISO 10390:2005-02 method, in 0.01M $CaCl_2$ (soil: solution ratio of 1:5 (w/v)) after a 2 h incubation time. Soil organic matter content (OM) was determined after drying the soil for 24 hours at 65° C followed by heating at 450°C for 5 hours in a muffle oven. OM content was determined by weighing before and after heating samples to 450°C, the difference in weight corresponding to the incinerated soil OM.

Plant material (roots and leaves) was dried at 65° C for 48 h and ground to a fine powder with a Tissue LyserII (Qiagen GmbH, Germany). Approximately 1.5 mg of the powder was weighted into 3.5 mm × 5 mm tin capsules (HEKAtech GmbH, Wegberg, Germany). Total carbon and nitrogen contents in *L. tenuis* roots and leaves were determined using an Elemental-Analysator 'Euro-EA' (Eurovector, Milano, Italy).

2.4 HCH extraction and analysis from bulk soil

The extraction of HCH isomers from soil followed the QuEChERS method described by Fernandes et al. (2013). HCH was extracted from 5 g of dried sieved bulk soil hydrated with 3 mL MiliQ water after one hour incubation. 10 mL of acetonitrile were added to the solution, followed by vigorous vortexing and shaking. A powder mixture of 4 g anhydrous $MgSO_4$, 1 g NaCl, 1 g Na_3citrate dihydrate and 0.5 g Na_2citrate sesquihydrate was added to the acetonitrile solution, subsequently vigorously shaken and vortexed, sonicated for 5 minutes, ending with a 5 minute centrifugation at 3000 rpm (Avanti J-25, Beckman Coulter, USA). 1.5 mL of supernatant was cleaned-up with 150 mg $MgSO_4$, 50 mg C18 and 50 mg PSA. The mixture was vortexed, shaken vigorously, and centrifuged for 5 minutes at 4000 x g. 1mL of supernatant was spiked with hexachlorobenzene (HCB) as internal standard (10 ppm end concentration in the solution), and subsequently evaporated to dryness at 40° C under nitrogen gas flow and reconstituted with 1 mL n-hexane. The identification and quantification of α-, β- and γ-HCH isomers was done using an Agilent 6890N gas chromatograph equipped with a 7686B series injector,

both from Agilent Technologies (CA, USA) and coupled with a IRMS detector (Delta plus Advantage, Thermo Finnigan, Waltham, Massachusetts). Isomers were separated on a DB-5 column (30m x 0.25mm x 0.25µm, P/N 122-5032, J&W Scientific from Agilent Technologies) with helium (99.999 % purity) used as carrier gas, at a flow of 1mL/min. The column oven temperature was programmed to 80°C for 10 min, increased to 175° C at 20° C/min, followed by a 1° C/min increase until 185°, until it reached 300° at a rate of 35° C/min and then held for 2 min. A volume of 5 µL was injected in splitless mode with a 40-mm length needle (P/N 5181-1267, Agilent Technologies) into the injection port at 250°C. The HCH isomer concentrations were calculated based on a linear five-point calibration curve, ranging from 5 ppm to 20 ppm. Integrations were done using the Isodat-Gas Isotope ration MS software (version 3.0) from Thermo Scientific. HCH values are synthesized in Suppl. mat 2.

2.5 16SrRNA gene library preparation

Total DNA was extracted from 0.5g of soil and 0.3g of ground root material (both fresh weight), using the NucleoSpin Soil Kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's protocol. Empty sterile extraction tubes were prepared for each soil compartment and root DNA extraction, to serve as negative extraction controls. The concentration of DNA extracts was quantified with a 5200 Fragment Analyzer System (Agilent, US-CA), using the Genomic DNA 50Kb Analysis Kit. Ten ng of the DNA extracts were used to amplify the V3-V4 region of the 16S rRNA gene, using the primer pair 338F (5'- GCTGCCTCCCGTAGGAGT- 3') /789R (5' -GGAATCCTCTCTCACCACATTGCCCAGG CAGACC- 3') with Illumina adapter sequences, which was reported to exclude chloroplast amplification (Dorn-In et al., 2015). PCR reactions were carried out in three technical replicates using the NEBNext High-Fidelity 2× PCR Master Mix (New England Biolabs, Ipswich, US-MA) with the addition of tetramethyl ammonium chloride at 36mM final concentration in the PCR reaction. Non target control (NTC) and positive controls containing the target gene were also performed in triplicates. PCR conditions were: initial denaturation step, 30 s at 98 °C; 25 cycles (10 s denaturation at 98 °C; 30 s annealing at 60 °C; and 30 s elongation at 72 °C); and the final elongation step at 72 °C, 5 min. The technical replicates of the PCR products were checked on 1% agarose gel, and afterwards pooled for purification using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel), according to the manufacturer´s protocol. The purified product underwent quality check and quantification with the 5200 Fragment Analyzer System (Agilent, US-CA), using the NGS Fragment Kit (1-6000bp). 10 ng of the purified 16S rRNA gene amplicon were indexed using the Nextera XT Index Kit v2 (Illumina Inc., San Diego, US-CA) for multiplexed short-read sequencing. PCR conditions were: initial denaturation step, 30 s at 98 °C; 8 cycles (10 s denaturation at 98 °C; 30 s annealing at 55 °C; and 30 s elongation at

72 °C); and the final elongation step at 72 °C, 5 min. The indexed product was checked on 1% agarose gel and purified with NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel). Quality and concentration of the indexing product were assessed using the above mentioned kit from Agilent. The samples were diluted to 4nM and pooled equimolarly. 10 pmol DNA were sequenced with a MiSeq System (Illumina, San Diego, US-CA) using the MiSeq Reagent Kit v3 (600 cycle) for paired end sequencing with PhiX (Illumina, San Diego, US-CA) as a spike-in positive control.

2.6 Quantification of nitrogen fixation potential

DNA extracts used for 16S rRNA gene library preparation were also used to quantify the *nifH* gene (coding for a nitrogenase) copy numbers by means of real time quantitative PCR (qPCR). The *nifH* gene abundance was quantified using the primer pair *nifH*-f (5'-AAA GGY GGW ATC GGY AAR TCC ACC AC-3') and *nifH*-r (5'-TTG TTS GCS GCR TAC ATS GCC ATC AT-3') (Rösch et al., 2002). The source organism for the nitrogenase reductase standard was *Sinorhizobium meliloti* 30136, and the mean PCR reaction efficiency ± SD was 2.04 ± 0.1 for bulk soil, 2.04 ± 0.09 for rhizosphere and 1.88 ± 0.1 for plant samples. Serial dilutions of 1:8, 1:16, 1:32 and 1:64 of the DNA extracts were tested for DNA polymerase inhibitors. The dilution showing the best PCR efficiency (1:64 v/v) was used for quantification, using the PowerSYBR® Green PCR Master Mix (Life Technologies, Warrington, UK) according to the manufacturer's instructions. Bovine serum albumin (BSA) was added to all reactions at 0.06% final concentration to reduce inhibitory effects of polyphenolic compounds co-extracted from soil. To maintain similar PCR conditions throughout sample types, BSA was added in the same concentration to root samples as well.

Samples were measured in three technical replicates on a 7300 Real-Time PCR System (Applied Biosystems, Foster City, US-CA). The PCR program consisted of the following steps: initial denaturation 95°C, 10 min; amplification 40 cycles (denaturation 45s at 95°C, annealing 45s at 55°C, elongation 45s at 72°C); melting curve (15s at 95°C, 30s at 60°C and 15s at 95°C). The specificity of the PCR reactions was checked by melting curve analysis. Initial fluorescence values obtained for each sample were converted to gene copy numbers per gram of dry soil / dry root biomass, using standard curves created with 10-fold dilutions of the above mentioned standard with known copy number. The baseline was subtracted and normalized, followed by data analysis using the package qpcR (Spiess, 2018) applying the mechanistic cm3 model. This model is insensitive to low PCR efficiencies and minimizes effects of PCR inhibitors (Carr and Moore, 2012). Kinetic outliers were discarded automatically.

2.7 Data analysis

2.7.1 Bioinformatics analysis

The raw sequencing data were demultiplexed on the MiSeq system. Subsequently, primer and adapter sequences were removed using AdapterRemoval v. 2.1.7 (Lindgreen, 2012). 6.52 million raw reads were generated in total, with 97,242 ± 36,417 (mean ± SD) reads per sample. Rarefaction plots were constructed to assess sequencing coverage. There was saturation of the sequence coverage in all samples (data not shown). Sequencing run quality and the amount of trimming necessary for further processing of the sequences was assessed using quality plots using the R package DADA2 v. 1.10.1 (Callahan et al., 2016). All reads were trimmed by 10 bp at the beginning, while forward reads were trimmed after 290 bp and reverse reads after 210 bp. After removing remaining PhiX sequences, quality filtering and trimming, 99.3% of the initial reads remained for analysis after this step. The forward and reverse reads were denoised separately (91.3% from the forward reads and 93.2% of the reverse reads remaining) and merged (66.6% of the total reads remaining) using the R package DADA2. Amplicon Sequence Variants (ASVs) were generated, followed by removal of chimeric sequences (54.5% of reads remaining, i.e. 3,548,214 reads or 24,482 ASVs). DADA2 infers sample sequences which are resolved at differences of one nucleotide and generates ASVs as opposed to operational taxonomic units. The ASVs were taxonomically annotated using the SILVA database v. 128 (Quast et al., 2012). This data set was then imported in R and further filtered using the R package phyloseq v.1.26.1 (McMurdie and Holmes, 2013). ASVs assigned to kingdoms other than Bacteria, to chloroplasts or mitochondria were removed. Contaminant ASVs were identified and removed using the R package decontam (Davis et al., 2017), accounting for a total of 0.29% of the initial number of reads. Bacterial richness was estimated at this step from the remaining 24,407 ASVs as the number of observed ASVs per sample using the R function *estimate_richness()* from the phyloseq package.
For further analysis of the data set, a prevalence filter was applied to sort out the ASVs that were only present in a single sample. This abundance cut-off attempts to reduce the high number of ASVs that account for only a few reads, thus minimizing the influence of rare ASVs on the statistical models. The final data set contained 39.33% of the initial raw reads (3,010,093 reads, accounting for 8,334 ASVs). The nucleotide sequence data reported are available in the SRA database under the BioProject ID PRJNA648690.

2.7.2 Statistical analysis and identification of potential bacterial inocula

Constrained analysis of principal coordinates (CAP) on generalized UniFrac distances was conducted to assess effects of HCH contamination and excavation on the microbial community composition in

bulk soil, rhizosphere and root samples. Explanatory variables of the model were soil physico-chemical parameters or plant C/N ratio. Environmental parameters significantly influencing β-diversity were highlighted by means of an automatic model selection procedure, using the ordistep function of the R package vegan v. 2.5.5 (Oksanen et al., 2019), based on permutation p-values. The initial model for bulk and rhizosphere samples was based on the following variables: total HCH, disturbance status (excavated or undisturbed), pH, OM, gravimetric water content, ammonium, total inorganic N, DOC and DON. For root samples, in addition to the above mentioned rhizosphere soil parameters, we considered root C/N ratios as explanatory variables. The significance of the final CAP model, of the axes and of the selected variables was tested using the R function anova.cca() with 5,000 permutations. ASVs were subsetted according to Schmid et al. (2020) to reveal those ASVs present exclusively in a single of the four conditions (undistPoll, undistRef, excavPoll, excavRef). The "undist" plots were undisturbed, while the freshly excavated plots were called "excav". Subsetting aimed to identify bacterial taxa, specific for HCH contamination that could serve as an inoculum with HCH tolerant strains in soil clean-up procedures. Comparing the undistRef and the undistPoll plots allowed us to identify those bacterial taxa which are characteristic to long-term HCH contaminated bulk and rhizosphere soil, and to the root-associated bacterial communities of *L. tenuis*. Plants growing on these plots were of similar age. By comparing the undistPoll plot against the excavPoll plot, we assessed which HCH tolerant taxa initially adapt to soil disturbance and which ones settle only later on. We additionally identified HCH tolerant bacterial taxa that are first acquired by young *L. tenuis* plants after excavation, and those acquired in later growth stages. Finally, we compared excavPoll and excavRef rhizosphere and root-associated bacterial communities to evaluate which factor has greater influence on the development of these communities: the age of plants or HCH contamination. The five most frequently occurring ASVs (addressed as families and genera) in each of these comparisons were further analyzed as those taxa which reacted strongest to HCH contamination. Unclassified taxa and those which appeared in only one condition were not considered for further analysis. To assess whether the main responding families were also the most abundant ones throughout the samples, we used the simple ranking of mean relative abundance per family, per sample in bulk, rhizosphere and roots. The subsetting method was also used to identify possible bacterial inoculants to serve as HCH tolerant bacteria with beneficial properties for the plant, acquired by the roots of *Lotus tenuis* from the surrounding soil. The inoculation path considered was undistPoll bulk soil via excavPoll bulk, excavPoll rhizosphere, with the final destination, the roots of the young plants growing on the freshly excavated HCH contaminated plot (excavPoll). The bacterial taxa were identified at species level using BLASTN (Zhang et al., 2000).

Robust two-way analysis of variance (ANOVA) was used to test the effect of HCH and excavation on the bacterial taxa identified by means of subsetting as main responders to HCH contamination. The *t2way()* function was used to assess overall statistical significance (Wilcox, 2017). Differences in mean relative abundance of the specific comparisons mentioned above (undistPoll vs. undistRef, undistPoll vs. excavPoll and excavPoll vs. excavRef) were tested through pairwise comparisons using the function *yuenv2*. The p-values of multiple comparisons were corrected using the Benjamini-Hochberg method. Ordinary linear regressions in semi-log space were conducted to test effects of HCH on soil pH, OM, DOC, DON, soil ammonium concentration, bacterial richness, C_{mic}, plant C/N ratio and nitrogen fixation potential (*nifH* gene copy numbers) in soil and plant samples. Statistical data analysis was performed in R v.3.5.2 (R Core Team, 2018). All plots were generated using the package ggplot2 v.3.2.0 (Wickham, 2016).

3. Results

3.1 Plant performance and soil physico-chemical parameters and potential nitrogen fixation

The fresh biomass of plants collected from excavRef, excavPoll and undistPoll plots had similar average values (1.2g, 0.9g and 0.8g respectively), while that of plants collected from the undistRef plot was 27g. Regardless of the fresh biomass estimates, the C/N ratio of *L. tenuis* roots and leaves remained unaffected by HCH (data not shown). HCH concentrations detected in bulk soil ranged between 392 and 22068 ppm in the highly contaminated plots and between 3.1 and 403 ppm in the low contaminated plots (Suppl. Mat. 2). HCH significantly affected the pH of undisturbed bulk soil ($p < 0.05$; $R^2 = 0.6$) and excavated rhizosphere ($p < 0.001$; $R^2=0.9$) which in both cases declined from 7.7 to 7.4 with increasing HCH concentration (data not shown). There was a general significant positive relationship between HCH concentration and OM, DOC and DON content in bulk and rhizosphere soils (Suppl. mat. 3). Ammonium values increased significantly with HCH concentration in undisturbed ($p < 0.05$; $R^2 = 0.5$) and excavated ($p < 0.05$; $R^2 = 0.4$) rhizosphere soils (not shown).

We observed a general positive relationship between *nifH* gene abundance and HCH concentration in the undisturbed bulk and rhizosphere soil, as well as in the roots of mature *L. tenuis* plants (from undisturbed plots). Nevertheless, this effect was only marginal, as potential nitrogen fixation was not significantly affected by HCH contamination. HCH had a significant negative effect on *nifH* gene abundance only in rhizospheres of young *L. tenuis* plants ($p < 0.05$; $R^2 = 0.4$) (data not shown).

3.2 Microbial biomass carbon and bacterial diversity

Microbial biomass carbon (C_{mic}) increased significantly in undistPoll bulk soil (Suppl. mat. 5), while in excavated bulk soil and undisturbed rhizosphere, HCH had a similar but marginal effect. α- diversity (*i.e.* species richness) decreased significantly with increasing HCH concentration in excavated bulk soil and in undisturbed rhizosphere of *L. tenuis* (Fig. 1). The trend was similar but not significant at 5% level, in the case of root-associated bacterial communities of young plants growing on excavated plots.

3.3 Main responders to HCH contamination

HCH was the main shaping factor for bacterial community composition in bulk soil. HCH concentration was strongly correlated with OM content as co-explanatory variable ($p < 0.001$; Pseudo-$F_{1,18} = 1.39$) (Fig. 2a). ASV distribution in the rhizosphere was shaped mostly by the growth stage of plants ($p < 0.05$, Pseudo-$F_{1,15} = 1.35$), while HCH had a smaller effect, just above the 5% significance level ($p = 0.064$, Pseudo-$F_{1,15} = 1.24$). HCH was positively correlated with ammonium ($p < 0.05$, Pseudo-$F_{1,15} = 1.31$) and total N content ($p < 0.01$, Pseudo-$F_{1,15} = 1.64$) (Fig. 2b). The composition of the root-associated bacterial communities was determined solely by the age of plants ($p < 0.01$, Pseudo-$F_{1,16} = 1.30$), with no effect of HCH contamination detected at this level (Fig. 2c).

In bulk soil, main responders to HCH contamination were ASVs belonging to the Burkholderiaceae, Nitrosomonadaceae and Xanthomonadaceae bacterial families, and to the genera *Sphingomonas* and *Altererythrobacter* (both part of the Sphingomonadaceae family), and *Lysobacter* (fam. Xanthomonadaceae) (Suppl. mat. 4). HCH had a significant effect on the mean relative abundance of Burkholderiaceae ($p < 0.01$; Suppl. mat. 6). Further pairwise comparisons showed significantly higher abundance of this family in excavPoll soil as compared to undistPoll ($p < 0.05$) and in undistRef soil as compared to undistPoll ($p < 0.01$) (Suppl. mat. 6 and 7). Nitrosomonadaceae were negative responders to HCH, pairwise comparisons between undistPoll and undistRef plots showing a significantly lower abundance in the first condition ($p < 0.05$) (Suppl. mat. 6 and 7). Xanthomonadaceae were more abundant in excavPoll bulk soil, as compared to undistPoll soil ($p < 0.05$; Suppl. mat. 7).

Genus *Lysobacter* was significantly affected by HCH contamination in bulk soil, as it was more abundant in excavPoll bulk soil as compared to undistPoll soil ($p < 0.01$), but higher in undistPoll bulk soil when compared to undistRef soil ($p < 0.05$) (Fig. 3; Suppl. mat. 7). Genus *Altererythrobacter* was a positive responder to HCH contamination ($p < 0.05$), with slightly higher abundance in the undistPoll plot than in all other conditions. However, this effect was not further detected in pairwise comparisons (Fig. 3; Suppl. mat. 7).

In the rhizosphere, the most abundant responders at family level were Burkholderiaceae, Nitrosomonadaceae and Sphingomonadaceae. At genus level, the most common differential ASVs corresponded to the genera *Massilia* (Burkholderiaceae), *Sphingobium* and *Sphingomonas* (both Sphingomonadaceae) (Suppl. mat. 4). Similar to bulk soil, Nitrosomonadaceae were negative responders to HCH contamination, pairwise comparisons between undistPoll and undistRef plots showing a significantly lower abundance of this family in the first condition ($p < 0.001$) (Suppl. mat. 6 and 7). As second main responder to contamination, there was a significantly higher abundance of Burkholderiaceae in excavPoll rhizosphere as compared to undistPoll ($p < 0.05$) and in undistRef rhizosphere as compared to undistPoll soil ($p < 0.01$) (Suppl. mat. 6 and 7). HCH ($p < 0.01$) and the excavation process ($p < 0.05$) had a significant effect on the mean relative abundance of Sphingomonadaceae in rhizosphere soil (Suppl. mat. 6 and 7). However, the HCH effect was only marginal, as it was not further detected in pairwise comparisons.

Genus *Sphingobium* was a significant responder to HCH contamination in the rhizosphere. Pairwise comparisons showed a significantly higher abundance of the genus *Sphingobium* in the undistPoll rhizosphere than in undistRef one ($p < 0.01$). Similarly, *Sphingobium* was more abundant in excavPoll rhizosphere than in undistPoll rhizosphere ($p < 0.05$) or in excavRef rhizosphere ($p < 0.01$) (Fig. 3; Suppl. mat. 7). Plant age had a notable effect on the abundance of *Sphingobium*, which was significantly higher in excavated rhizosphere plots, regardless of contamination ($p = 0.001$). Genus *Massilia*, was significantly affected by both HCH and excavation in rhizospheres (Fig. 3; Suppl. mat. 7). Pairwise comparisons revealed a significantly higher abundance of this genus in excavPoll soil when compared to undistPoll rhizosphere ($p < 0.05$) as well as in comparison to the excavRef soil ($p < 0.05$) (Fig. 3; Suppl. mat. 7).

In roots of *L. tenuis*, the most frequently occurring differential ASVs belonged to Burkholderiaceae, Rhizobiaceae, and Sphingomonadaceae (Suppl. mat. 4). At genus level, the most frequent responders were *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* (Rhizobiaceae) and *Sphingomonas* (Sphingomonadaceae). Burkholderiaceae from the root-associated microbiome were significantly affected by HCH. Pairwise comparisons showed higher abundance of this taxon in the undistPoll as compared to excavPoll roots ($p < 0.05$) and in undistRef roots as compared to undistPoll roots ($p < 0.05$)(Suppl. mat. 7). Additionally, Burkholderiaceae were marginally more abundant in the excavPoll roots than in the excavRef roots ($p = 0.06$). Rhizobiaceae were identified as negative responders to HCH in the root-associated bacterial communities ($p < 0.01$) (Suppl. mat. 6 and 7). This effect was however not strong enough to be further detected in the pairwise comparisons. Plant age had a clear effect on the abundance of Sphingomonadaceae in root-associated bacterial communities ($p < 0.05$).

They were more abundant in the old roots, regardless of the contamination level (Suppl. mat. 6 and 7). Furthermore, this family was positively affected by HCH, as they were significantly more abundant in undistPoll roots ($p < 0.01$), and nearly significant when compared to undistRef roots ($p > 0.05$). The *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* clade was a strong positive responder to HCH in the root compartment of *L. tenuis*, pairwise comparisons showing higher abundance of this clade in undistPoll roots, as compared to excavPoll roots ($p < 0.01$). Genus *Sphingomonas* had significantly higher abundance in undistPoll roots when compared to excavPoll roots ($p = 0.001$) (Fig. 3; Suppl. mat. 7).

All identified main responders were also among the most abundant taxa at family level in our bulk, rhizosphere and root samples (Suppl. mat. 8). Further, we identified those HCH tolerant bacteria which were common to undistPoll bulk soil, excavPoll bulk soil, excavPoll rhizosphere and to roots of young plants germinating on excavPoll plot (Suppl. mat. 9). Bacteria transferred from bulk soil to the root-associated microbiome were identified as *Mesorhizobium loti* (100% similarity), *Neorhizobium galegae* (100% similarity), *Novosphingobium lindaniclasticum* (similarity 99%), *Pantoea agglomerans* (prev. *Enterobacter agglomerans*) (similarity 100%), *Lysobacter sp.* clone T28265 and *Lysobacter bugurensis* (99% similarity) (Suppl. mat. 9).

4. Discussion

4.1. Bulk soil bacterial communities adaptation to HCH contamination and excavation procedures

Our results showed that HCH and OM alone were the most significant environmental descriptors for the differentiation of the bulk soil microbial community. Because bacterial α-diversity (richness) was lower in the excavPoll plot than in all other three conditions, soil disturbance combined with HCH contamination may have led to a slower recovery of species richness in freshly excavated bulk soil. On the other hand, microbial growth (expressed here as C_{mic}) appeared to be sustained in both excavated and undisturbed highly contaminated bulk samples by higher DOC and OM contents. Similar to previous findings of Kalbitz and Popp (1999) and Balázs et al. (2020), high soil organic matter content was positively correlated with HCH concentrations present in both excavated and undisturbed plots. Kalbitz and Popp (1999) attributed higher OM and DOC concentration to either drought in summer or litter accumulation in autumn. Since our sampling campaign took place at the beginning of June when local temperatures were relatively high, this might be a plausible explanation for the OM peak in bulk soil, triggering the adsorption of HCH to soil particles rich in OM, resulting in mobilization of the

contaminant, followed by HCH uptake in plants (Wahid and Sethunathan, 1979; Kalbitz and Popp, 1999).

Finding ASVs from the Sphingomonadaceae and Burkholderiaceae families as main responders to long term HCH contamination is not surprising at all, as they all include versatile taxa found in a wide range of habitats. As sphingomonads are well known to degrade recalcitrant xenobiotics and polyaromatic compounds of both anthropogenic and natural origin (Glaeser and Kämpfer, 2014), we expected to find taxa from this bacterial group that are specific for HCH contamination at the Turda site. However, possibly because of their ubiquitousness, which allows these chemoorganotrophs to inhabit various ecosystems (Amils, 2014; Glaeser and Kämpfer, 2014), HCH did not affect mean relative abundances of Sphingomonadaceae in bulk soil. Burkholderiaceae are a very versatile group as well, and well known hydrocarbon degraders (Castorena et al., 2006). Nevertheless, they appear to thrive on easy degradable hydrocarbons rather than more recalcitrant compounds such as diesel oil (Bell et al., 2013) or HCH in our case, which could explain the higher occurrence of this group in clean bulk soil. Although ammonia-oxidizing bacteria have been proposed for the remediation of chlorobenzene contaminated soil (Sayavedra-Soto et al., 2010), the Nitrosomonadaceae in our study were in general negative responders to HCH. This supports results of Ibiene and Okpokwasili (2011), which showed that the type genus of the family, *Nitrosomonas* sp., is sensitive to lindane contamination. Excavated HCH bulk soil samples were much richer in Bulkhorderiaceae, Xanthomonadaceae and Sphingomonadaceae ASVs than undistPoll bulk soil. Xanthomonadaceae are known hydrocarbon degraders with many applications in bioremediation of oil / petroleum or trifluralin (herbicide) contaminated soil (Popp et al., 2006; Chang and Zylstra, 2010; Du et al., 2018). The fact that they were generally more abundant in the excavated conditions regardless if clean or contaminated, suggests that these taxa are fast-growing generalists and early colonizers. Thus, despite their metabolic capabilities which allowed them to tolerate and degrade HCH, they merely take advantage of free niches left available by previously established bacterial communities.

At genus level we found two taxa to be considered as HCH tolerant in long term contamination: *Sphingomonas* and *Altererythrobacter*, which is consistent with our previous findings in lindane contaminated soil (Balázs et al., 2020). There are several examples of *Sphingomonas* strains isolated from HCH contaminated soils found capable to tolerate and mineralize HCH isomers (Pal et al., 2005; Singh and Lal, 2009; Teramoto et al., 2010; Tonon et al., 2014). Our results show that HCH did not have any notable effect on the general abundance of this genus, highlighting the ability of *Sphingomonas* to tolerate HCH and the possibility of employing them in *in situ* bioremediation projects. *Sphingomonas* and *Lysobacter* were antagonistic responders to the excavation of HCH

contaminated soil. *Sphingomonas* was negatively affected by the excavation process, consistent with observations of Dong et al. (2017) and Shi et al. (2017), who reported lower abundance of this genus in tilled agricultural soil or land subsidence. Nevertheless, *Sphingomonas* was replaced by another known HCH degrader in excavPoll bulk soil, the xanthomonad *Lysobacter* (Rani et al., 2016; Margesin et al., 2018), suggesting quick adaptation of bacterial communities to HCH contamination after excavation procedures.

4.2. Rhizosphere soil bacterial communities adaptation to HCH contamination and the interaction with plant developmental stage

Rhizosphere is a transition compartment between bulk soil and the root environment. Accordingly, we expected the bacterial communities in this zone to reflect both soil HCH contamination and age of *L. tenuis*, as plants can select different subsets of microbes at different stages to fulfill age-specific functions (Chaparro et al., 2013). In line with this, the age of the rhizosphere had a significant influence on bacterial community composition, followed by HCH contamination. As such, HCH shaped the microbial community in the undisturbed rhizosphere, while excavated rhizosphere samples showed a high degree of similarity regardless of contamination, thus highlighting the importance of plant growth stage in acquiring a specific rhizosphere microbiome. Ammonium and total N content were the most important environmental descriptors in rhizosphere soil, and positively correlated with HCH concentration. This is consistent with results of Blondel et al. (2017) in lindane and chlordecone contaminated maize rhizospheres where the plants enabled efficient C- and N- turnover and maintained a normal ammonification process as opposed to lindane contaminated soil without plants. OM, DOC and DON concentrations in rhizosphere followed the same pattern as in bulk soil, where they were positively correlated with HCH concentration in undisturbed plots and possibly triggered an increased microbial carbon biomass. Higher C_{mic} content and at the same time a lower bacterial richness in the undistPoll rhizosphere suggests that contamination might offer suitable growth conditions for only few HCH tolerant taxa, which are able to thrive under these conditions.

The Sphingomonadaceae were positive responders to HCH in the rhizosphere compartment. This might have occurred either due to root exudates which could promote their growth (el Zahar Haichar et al., 2008), or by following a selection process of the rhizosphere microbiome by plants as stress response to contamination. Chapelle et al. (2016) showed that sphingomonads were enriched in the rhizosphere of sugar beet as a stress response upon fungal infection, while Tsavkelova et al. (2007) suggested that these bacteria are selected by orchid plant roots as they produce the growth hormone indole-3-acetic acid. The strongest positive responder to HCH contamination in rhizosphere from this bacterial family

was *Sphingobium*, well known for its HCH degrading capabilities (Lal et al., 2008; Sangwan et al., 2014). Burkholderiaceae are degraders of a vast range of aromatic compounds (Goldfarb et al., 2011), and the presence of the copiotrophic genus *Massilia* in the early stages of succession of the rhizosphere and root microbiome when supply of labile organic carbon in soil is high, is not surprising (Shrestha et al., 2007; Li et al., 2014). Being copiotrophic organisms, *Massilia* are good abiotic stress tolerants (*e.g.* to high HCH concentration in the excavated rhizosphere) but not particularly tolerant to biotic stress (Shrestha et al., 2007; Abou-Shanab et al., 2010). Considering that lower species richness (α- diversity) in the excavPoll rhizosphere also means lower competition between species, this low biotic stress is probably the reason for the dominance of *Massilia* in this condition, as opposed to the excavRef compartment.

In undistPoll rhizosphere, sphingomonads *Altererythrobacter* and *Sphingobium*, were the most abundant differential genera, all of which have known HCH tolerant representatives (Pal et al., 2005; Singh and Lal, 2009; Teramoto et al., 2010). The excavPoll rhizosphere however, showed higher diversity of bacterial families, dominated by *Massilia* (Burkholderiaceae), *Sphingomonas* (Sphingomonadaceae) and *Lysobacter* (Xanthomonadaceae). This configuration of generalist bacterial genera is less typical for HCH contamination than for colonization of newly occurred free niches following the excavation process (Shrestha et al., 2007; Li et al., 2014).

4.3. Root and root-associated bacterial communities adaptation to HCH contamination and plant age effect

Following the trend we observed at the transition from bulk to rhizosphere soil, the only factor influencing the composition of *Lotus tenuis* root-associated bacterial communities was the plant development stage, regardless of HCH contamination. Overall bacterial richness (α- diversity) was not affected by HCH contamination in the roots of the plants growing on undisturbed plots. Nevertheless, bacterial richness in young roots of *L. tenuis* (from excavated plots) was altogether lower than in roots of mature plants from undisturbed plots. This is probably a reflection of the early succession stages of plant microbiome development, when the increase in copiotroph (r- strategists) abundance is supported by the non-limiting nutrient-rich new environment but species richness is low due to weak competition (Shrestha et al., 2007).

Lotus tenuis is a model plant for legumes known to have a great potential for adaptation to abiotic stress, making it the perfect candidate for dune re-vegetation or heavy-metal contaminated soils (Escaray et al., 2012). As we previously identified *L. tenuis* as both an early colonizer and HCH tolerant at the Turda production facility (Balázs et al., 2018), we considered it for restoration of HCH

contaminated sites. Plants collected from the excavRef, excavPoll and undistPoll plots had similar fresh biomass (~1g), while the mean fresh biomass of the plants collected from the undistRef plot was almost 30 times higher. This reveals a clear negative effect of long-term HCH contamination on *L. tenuis* growth and development. The effect of HCH or lindane on early growth and development of legumes had previously been studied on soybean (Tu, 1977) and *Phaseolus vulgaris* (Pereira et al., 2010). Both studies reported a certain degree of resistance of plants in this family to HCH, as their early growth was not significantly affected by the contaminant. These results are supported by the fresh biomass estimates of young *L. tenuis* roots, which had similar values regardless whether they grew on clean or contaminated excavated plots. In addition, the inhibited late growth of *L. tenuis* in high HCH contamination conditions is consistent with data of Tripathi et al. (2014), showing that lindane in high concentrations in soil reduces growth and yield of *Vigna radiata*. We further observed a strong nodulation on *L. tenuis* roots collected from the undistRef plot, hardly any or no nodules on the plants from the undistPoll plot and no nodules on young plants from excavated plots (personal observation). The C : N ratio of *L. tenuis* was nevertheless constant, regardless of HCH contamination and despite the lack of nodulation in roots of plants growing at the undistPoll plot. Khan et al. (2006) showed that certain herbicides decrease nodulation and biomass of nodules in chickpea, leading to a decrease in N content of the grains as well. Yet, in our case *L. tenuis* seems to have adapted to that impediment by taking up nitrogen from soil to maintain its C and N balance, possibly even in the form of ammonium which was more abundant in the rhizosphere of plants from both undistPoll and excavPoll plots. Supporting this idea, Rogato et al. (2010) reported a high affinity ammonium transporter in a short-root wild phenotype of *L. japonicus*, which presumably modulated root growth in conditions of potentially toxic external ammonium concentration. However, as this happened at an ammonium concentration higher than 10mM, which is not our case, we regard this rather as an adaptation of *Lotus tenuis* to contamination conditions, which prevent efficient nodulation and thus symbiotic nitrogen fixation, than to toxic ammonium concentrations.

HCH did not have significant effects on general root microbial composition. However, we found several particularities in roots of *L. tenuis* from undistPoll contaminated plot which could indicate HCH tolerant bacteria, with or without benefits for the host plants. Similar to rhizosphere soil, there was a higher abundance of ASVs from the Sphingomonadaceae family inhabiting undistPoll roots but not undistRef ones. The latter were characterized by a greater abundance of differential Burkholderiaceae genera like *Massilia*, *Douganella*, *Variovorax* and *Rhizobacter*. This highlights the high potential of taxa within the Burkholderiaceae to establish close interactions with plants, of both beneficial (Han et

al., 2010; Haack et al., 2016), neutral (Ofek et al., 2012) or pathogenic (Goto, 2015) nature in non-contaminated conditions.

Genera characteristic to the root-associated microbiome of plants growing at the undistPoll contaminated plot, were *Sphingomonas*, *Devosia*, *Brevundimonas* and *Rhodanobacter*. *Sphingomonas* and *Devosia* have been mentioned as non-nodulating bacteria in legume roots (Tariq et al., 2013). While *Sphingomonas* is a common legume endophyte frequently selected by the host as an antagonist to pathogens (Pini et al., 2012; Hartman et al., 2017), *Devosia* species were often isolated from soils contaminated with high amounts of HCH where they were able to tolerate the contaminant but not to degrade it (Talwar et al., 2020). As several *Devosia* species are capable of nitrogen fixation in legumes (Rivas et al., 2002; Hoque et al., 2011), they might have been selected by *L. tenuis* roots to fulfill the nitrogen fixation function in nodule-impaired old roots along *Allorhizobium- Neorhizobium-Pararhizobium-Rhizobium*. Although microbial composition in roots was generally driven by plant age, our results indicate that *L. tenuis* actively acquires beneficial bacteria for N fixation or rhizobacteria with plant growth promoting abilities that are at the same time capable of tolerating HCH. As such, *Massilia, Pantoea* and *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* were the most abundant genera characteristic for *L. tenuis* roots in the excavPoll plot. *Massilia* is a copiotroph characteristic for early microbial succession in roots (Ofek et al., 2012), while *Pantoea* and *Allorhizobium- Neorhizobium- Pararhizobium-Rhizobium* can both fix nitrogen, and even degrade xenobiotics as in the case of *Pantoea* (Stacey, 2001; Walterson and Stavrinides, 2015).

4.4. HCH contaminated soil as inoculum for bioaugmentation procedures in microbe-assisted phytoremediation

We identified several HCH tolerant bacteria which might be transferred from the undistPoll bulk soil to the excavPoll bulk soil through excavation and through the rhizosphere to the roots of young plants germinating on the excavated soil. Two of the identified bacteria, which could serve as an inoculum, are *Mesorhizobium loti* and *Neorhizobium galegae*. Both rhizobia are capable of forming nitrogen-fixing symbiosis with legume roots and therefore successfully used to sustain the growth of the early colonizer *Lotus tenuis* in the recovery process of HCH contaminated soil environments. Furthermore, bacteria like *Mesorhizobium loti* growing on HCH contaminated soils encode haloalkane dehalogenases, enzymes involved in the assimilation of organochlorine pesticides (Fetzner and Lingens, 1994; Sato et al., 2005). Although *M. loti* cannot degrade nor survive on HCH as sole carbon source, the dehalogenases it encodes are still an interesting aspect to take into consideration for bioaugmentation projects of HCH contaminated soil.

Novosphingobium lindaniclasticum is another bacterium we identified as common to both contaminated bulk soils, to the excavPoll rhizosphere and at the same time to the root-associated bacterial communities of young roots growing in the excavPoll plot. *Novosphingobium lindaniclasticum* is an HCH degrading bacterium, first isolated from an HCH dumpsite in Lucknow, India (Saxena et al., 2013). The metabolic versatility of *Novosphingobium* strains allows them to colonize a wide range of habitats from soil and water to plant surfaces, rendering them perfect candidates for microbe-assisted phytoremediation and bioaugmentation procedures.
Pantoea agglomerans is a bacterium featuring plant growth promoting traits such as nitrogen fixation and phosphate solubilization and at the same time, it is a plant pathogen antagonist (Lim et al., 2014). Its additional ability to degrade DDT and γ-HCH (lindane) (Karagoz et al., 2016) makes it therefore another inoculum that might be well suited for bioremediation of HCH contaminated sites.
Two other species of the genus *Lysobacter* (*Lysobacter sp.* clone T28265 and *Lysobacter bugurensis*) were transferred from contaminated old bulk soil to young *L. tenuis* roots in excavated plots. Although there is not sufficient data regarding the behavior of these taxa in HCH contaminated environments, they are still of interest since bacteria from this genus have plant pathogen antagonistic capabilities. Furthermore, another species of this genus, *Lysobacter tolerans*, was isolated from HCH contaminated sites where it is known to degrade the compound (Expósito et al., 2015; Rani et al., 2016).

4.5. HCH effect on the potential nitrogen fixation in soil and roots of Lotus tenuis

Potential nitrogen fixation in bulk and rhizosphere soil and in the *L. tenuis* root-associated microbiome was generally not affected by HCH contamination. The single exception consisted in the rhizosphere of young plants growing at the excavPoll plot, where we observed lower *nifH* gene copy numbers as compared to excavRef rhizospheres. The lower diazotroph abundance in the excavPoll rhizosphere did not reflect either in the plant C: N ratio, nor in the average plant fresh biomass, which was similar to the young plants growing in non-contaminated soil. Fox et al. (2001) showed that DDT, a pesticide with similar chemical structure to HCH, caused negative effects on potential nitrogen fixation and nod-exposition by disrupting the plant- *Rhizobium* signaling. Nevertheless, we observed no difference in nodulation between young plants, regardless whether they were growing in contaminated or non-contaminated plots. Furthermore, there was no significant difference in the abundance of Rhizobiaceae in rhizospheres of young plants. It is well known that members of the Rhizobiaceae family are not the only organisms capable of fixing nitrogen (Vitousek et al., 2002) and that in later growth stages, specific nitrogen fixing populations which can tolerate HCH application may be selected and stimulated by the rhizosphere (Patnaik et al., 1996). Therefore, the temporarily lower potential nitrogen

fixation in excavPoll rhizosphere might be just a short-term HCH effect on the initial microbial succession, inhibiting the growth of early colonizing diazotrophs.

5. Conclusions

Our results show that soil and root-associated bacterial communities may be affected in different degrees by HCH contamination and by excavation as a soil clean-up measure. In bulk soil HCH was the main factor influencing bacterial community composition, while rhizosphere microbiome was shaped by both HCH and plant developmental stage. The bacterial community composition from both long-term contaminated and excavated plots provided information about microbial succession in HCH contamination conditions, which are key aspects to be considered in field bioaugmentation procedures where HCH is not the only factor influencing the success of microbe-assisted phytoremediation. Furthermore, we identified several bacteria which were common to the undisturbed HCH contaminated bulk soil, to the excavated HCH bulk soil, and to the rhizosphere and roots of young plants growing in the excavated HCH hotspot. Altogether, these taxa point towards a possible inoculum with HCH tolerant bacteria (H1), which are transferred from contaminated bulk soil to rhizospheres and roots of *L. tenuis* with beneficial effects for microbe-assisted phytoremediation and bioaugmentation procedures (H2). Finally, potential nitrogen fixation was not affected by HCH, except for the contaminated rhizosphere of young plants growing on the freshly excavated plots (H3). This highlights the high adaptability of native bacterial communities to perform important ecosystem functions in various field conditions regardless of HCH contamination.

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Figure 1. Relationship between bacterial richness and HCH concentration in the bulk soil, rhizosphere and root compartments. Dots and solid lines represent samples from undisturbed plots. Triangles and dashed lines represent samples from excavated plots. Regression significance: excavated bulk soil ($p < 0.01$; $R^2 = 0.6$); undisturbed rhizosphere ($p < 0.01$; $R^2 = 0.6$). All other regressions were not significant ($p > 0.05$).

Figure 2. CAP ordinations of bacterial communities showing differences induced by HCH contamination and excavation in **(a)** bulk soil, **(b)** rhizosphere soil and **(c)** roots of *Lotus tenuis*. (**a)** Model significance:**(a)** $p < 0.001$; Pseudo-$F_{1,18} = 1.39$; **(b)** $p < 0.001$, Pseudo-$F_{4,15} = 1.45$; **(c)** $p < 0.05$, Pseudo-$F_{3,16} = 1.14$. Axis significance: **(a)** CAP1: $p < 0.001$, and Pseudo-$F_{1,18} = 1.39$; **(b)** CAP1: $p < 0.001$, Pseudo-$F_{1,15} = 2.42$; **(c)** CAP1: $p > 0.05$, Pseudo-$F_{1,16} = 1.33$. Dots represent undisturbed plots; triangles represent excavated plots. Light blue color represents low HCH concentration and black represents high HCH concentration. Ellipses delineate 95% confidence ellipses. Red arrows mark the co-explanatory variables: OM (organic matter), ammonium and total N content.

Figure 3. Mean relative abundance of the most important responders at genus level to HCH contamination in **(a)** bulk soil, **(b)** rhizosphere soil and **(c)** roots of *Lotus tenuis*. The colors of the bars represent the disturbance status of the plots: light gray – undisturbed plots; dark gray– excavated plots.

Figure 1

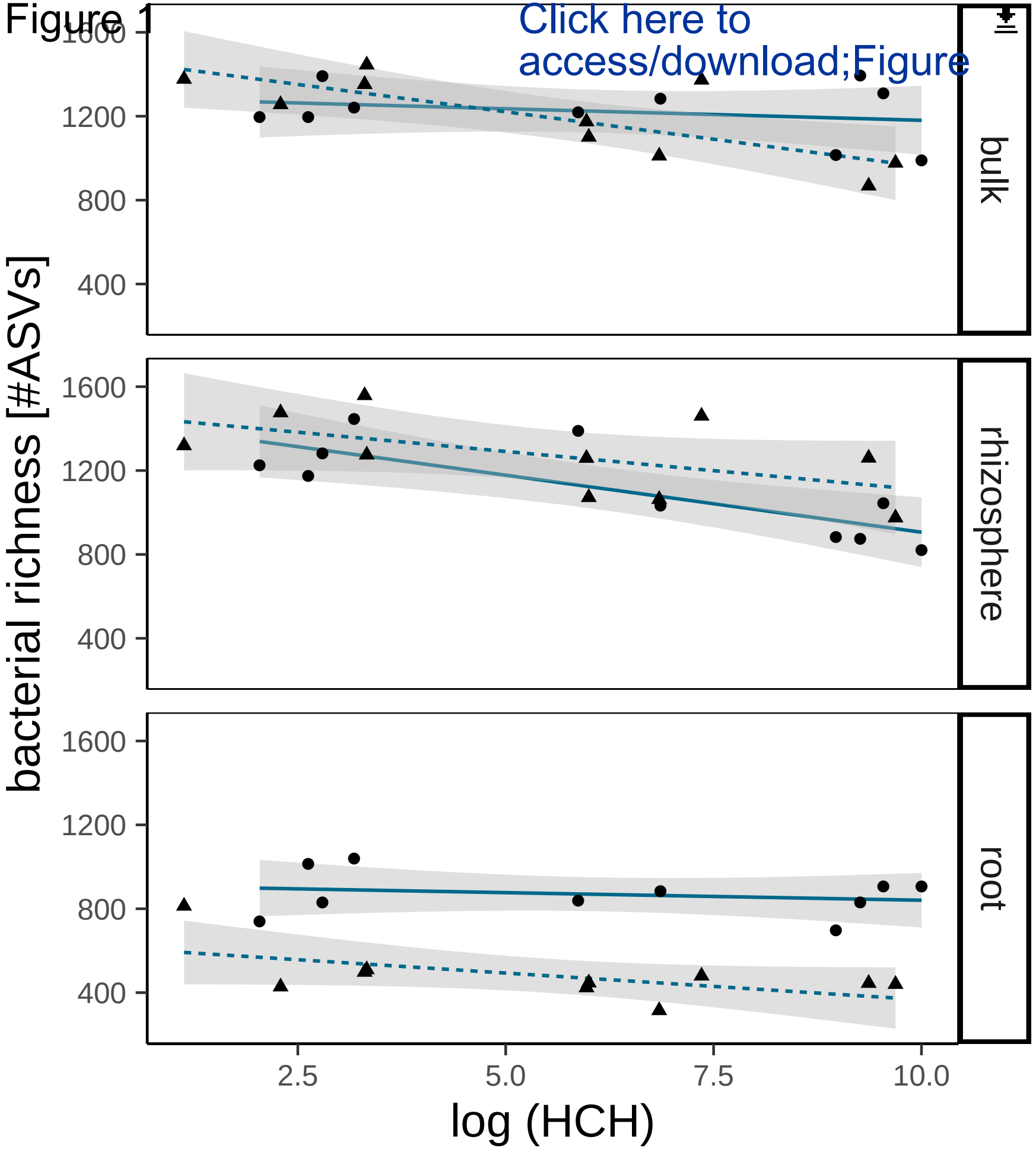


Figure 2

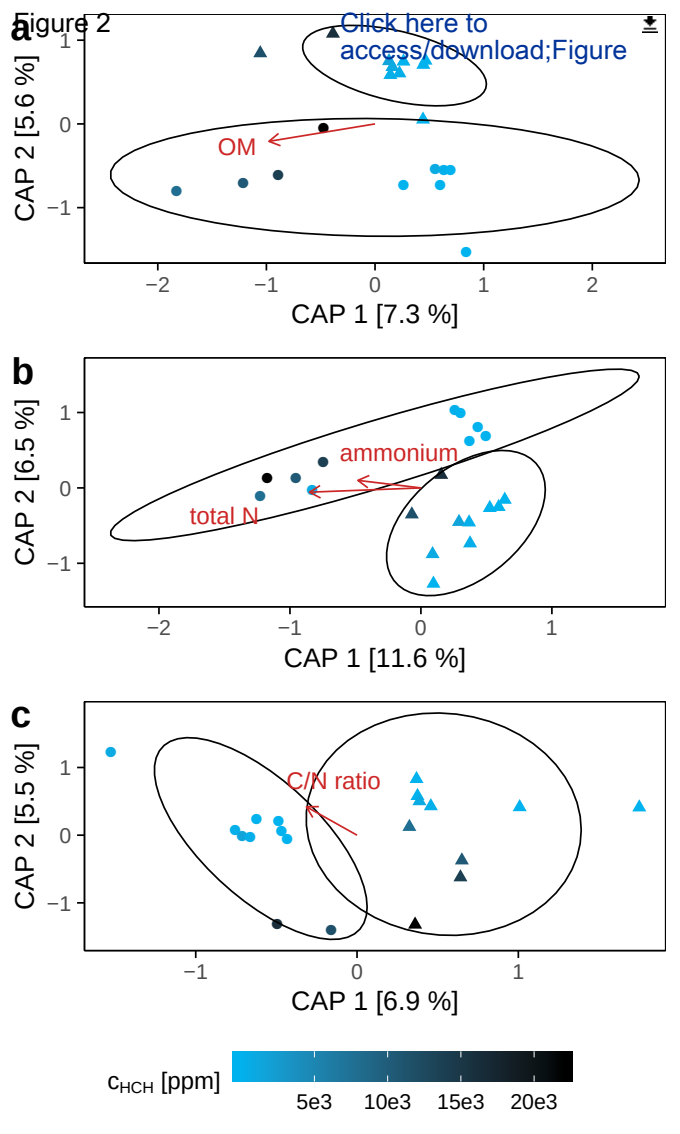
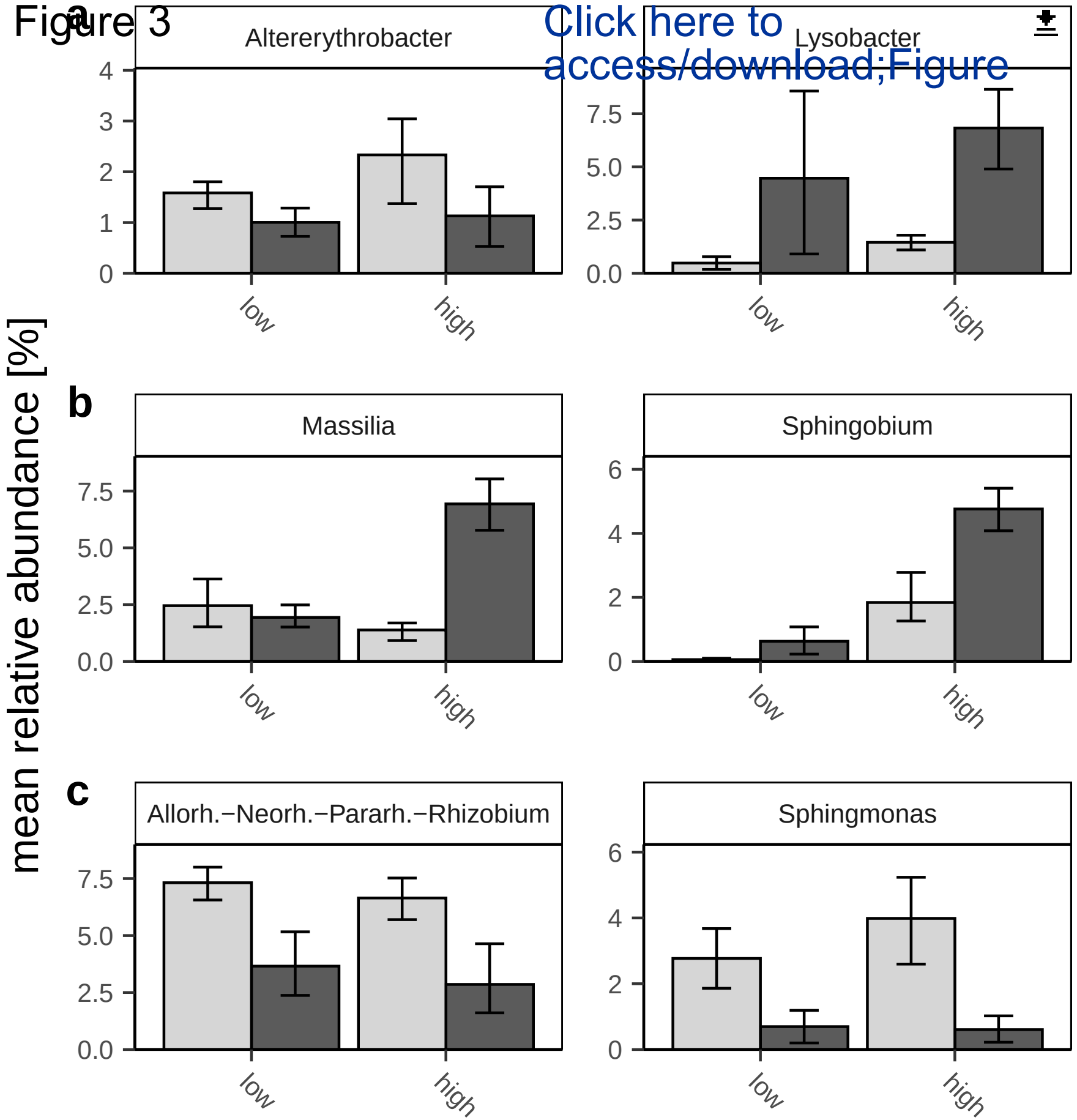
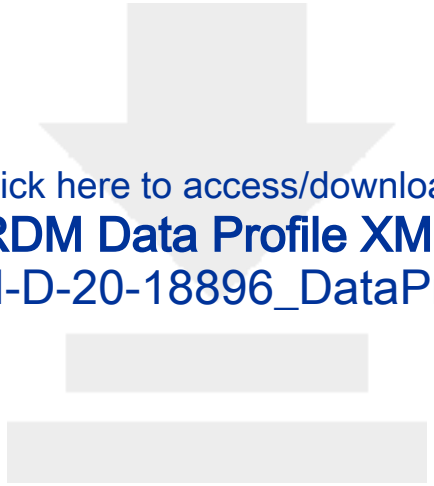

a
Click here to access/download;Figure
OM
CAP 2 [5.6 %]
CAP 1 [7.3 %]
b
ammonium
total N
CAP 2 [6.5 %]
CAP 1 [11.6 %]
c
C/N ratio
CAP 2 [5.5 %]
CAP 1 [6.9 %]
c_HCH [ppm]
5e3
10e3
15e3
20e3

Figure 3





Credit Author Statement

HB performed the experiments, evaluated the data and wrote the manuscript
CS evaluated the microbial data and designed the figures
CC analyzed the HCH and provided data with statistics
DP collected the pants and rhizospheres
PS performed the site description and collected plants and soil samples
FB analyzed the soil samples
GH analyzed the soil samples
VR gave microbiological advice and evaluated the data
PS designed and edited the manuscript, and provided funding for the experiments

*Declaration of Interest Statement

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: