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Long-term exposure of activated sludge in chemostats leads to changes in microbial communities composition and enhanced biodegradation of 4-chloroaniline and N-methylpiperazine

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HIGHLIGHTS

- Activated sludge was exposed to test chemicals in chemostat.
- Exposed and unexposed activated sludge was used as inoculum in ready biodegradability tests.
- Long term exposure in chemostat can lead to the biodegradation of N-methylpiperazine in biodegradability tests.
- 4-Chloroaniline is eliminated in biodegradability tests by the unexposed activated sludge microbial communities.
- Biodegradability test outcome is influenced by the inocula exposure history to the test compound.

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ABSTRACT

Exposure history and adaptation of the inoculum to chemicals have been shown to influence the outcome of ready biodegradability tests. However, there is a lack of information about the mechanisms involved in microbial adaptation and the implication thereof for the tests. In the present study, we investigated the impact of a long-term exposure to N-methylpiperazine (NMP) and 4-chloroaniline (4CA) of an activated sludge microbial community using chemostat systems. The objective was to characterize the influence of adaptation to the chemicals on an enhanced biodegradation testing, following the OECD 310 guideline. Cultures were used to inoculate the enhanced biodegradability tests, in batch, before and after exposure to each chemical independently in chemostat culture. Composition and diversity of the microbial communities were characterised by 16s rRNA gene amplicon sequencing. Using freshly sampled activated sludge, NMP was not degraded within the 28 d frame of the test while 4CA was completely eliminated. However, after one month of exposure, the community exposed to NMP was adapted and could completely degrade it. This result was in complete contrast with that from the culture exposed for 3 months to 4CA. Long term incubation in the chemostat system led to a progressive loss of the initial biodegradation capacity of the community, as a consequence of the loss of key degrading microorganisms. This study highlights the potential of chemostat systems to induce adaptation to a specific chemical, ultimately resulting in its biodegradation. At the same time, one should be critical of these observations as the dynamics of a microbial community are difficult to maintain in chemostat, as the loss of 4CA biodegradation capacity demonstrates.

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1. Introduction

The biodegradability of organic compounds is one of the most important properties that determine the environmental risk assessment of chemicals. Biodegradability can be determined using

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different tests and guidelines, such as the guidelines from the Organisation for Economic Cooperation and Development (OECD) (OECD, 2014). The results of these tests allow a fast categorization of the tested compounds from readily biodegradable to persistent, or recalcitrant, in the environment (ECHA, 2017). Ready biodegradability is an arbitrary classification of chemicals which have passed a specified type of screening tests for ultimate biodegradability (mineralization). Compounds identified as readily biodegradable are considered to be rapidly and completely biodegraded in aquatic environments under aerobic conditions (Comber and Holt, 2010; ECETOC, 2013; OECD, 2014). However, the commonly used ready biodegradability tests suffer from several well-known problems that can affect their outcome (Dick et al., 2016; Federle et al., 1997). One of these is the variability of the lag phase period, which is the time required for a community to start degrading a compound (Toräng and Nyholm, 2005). In some cases, specific factors such as the temperature or the occurrence of aerobic or anaerobic conditions could explain these variabilities. In many cases, however, the causes for the differences in biodegradation rate or lag-phase period are unknown (Greskowiak et al., 2017).

Adaptation of a microbial community that is used as inoculum to degrade the tested compound is one of the factors that potentially influences the lag-phase period (Itrich et al., 2015; Kowalczyk et al., 2015). A pre-adaptation step prior to a biodegradation test has been shown to decrease the number of false-negative results of the test (Ingerslev and Nyholm, 2000) and to reduce the lag-phase period (Toräng and Nyholm, 2005). It has been proposed to include adaptation to chemicals in enhanced biodegradability tests (ECETOC, 2007), however, pre-exposure prior any standard RBT is not allowed under the current ECHA guidelines. Enhanced biodegradability tests use the same methodology than the RBT, however, with variation in their duration and inoculum density (ECETOC, 2007; Ott et al., 2019). The objective of enhanced biodegradability tests is to improve to the reliability of tests and to decrease the number of false negative and positive results. In general, however, there is a lack of information about the microbial community dynamics and evolution during adaptation periods, both in laboratory systems or in the environment (Birch et al., 2017). Chemostat systems may be used to mimic the natural adaptation process in the laboratory under defined and controlled conditions. In the present study, we investigated the influence of long-term exposure of bacterial communities to chemicals in a chemostat on the biodegradation testing outcome along with the adaptive responses of the bacterial community composition. For this purpose, we exposed an activated sludge community to two different chemicals that show inconsistent behaviour in biodegradability testing.

The first one of these chemicals, 4-chloroaniline (4CA), is an intermediate product formed during the production of various organic chemicals and polymers, such as pharmaceuticals, pesticides, dyes and others. Chloroanilines are known to be relatively toxic for humans and wildlife (Argese et al., 2001), but also for microorganisms (Gosetti et al., 2010). It has been shown that the toxicity of chloroanilines for activated sludge microbial communities can affect and inhibit the degradation of other organic chemicals (Zhu et al., 2011). 4CA was reported to be present in the low $\mu\text{g/l}$ range in Dutch surface waters (Heugens and Verbruggen, 2009; Wickham, 2016), however, due to technical limitations, the concentration in activated sludge was not measured in this project. 4-chloroaniline shows erratic behaviour in ready biodegradability testing (Comber and Holt, 2010) as it may either pass or completely fail the test (Ingerslev and Nyholm, 2000). Hence, 4CA has been proposed as a reference chemical that would normally pass an enhanced ready biodegradability test but would fail any other standard tests (Comber and Holt, 2010).

N-Methylpiperazine (NMP), the second test compound in this study, is member of the family of piperazines, which are widely used industrial building blocks for pharmaceuticals, plastics and other products (Cai et al., 2013). As the extent of use and occurrence of NMP is unknown, many uncertainties exist about its biodegradability, though unpublished results, from the ECHA database, indicate that NMP is recalcitrant to biodegradation (ECHA, 2013). Significant degradation was, however, observed after adaptation of the community to this compound in a semi-continuous activated sludge system (ECHA, 2013).

In the present study, the biodegradation capacities for 4CA and NMP of unexposed activated sludge communities and those exposed to the chemicals in chemostat cultures were compared using the OECD 310 ready biodegradability testing protocol (OECD, 2014) and an enhanced version of the test. Differences between the tests output were evaluated in the light of the changes in communities composition in time followed by 16s rRNA gene Illumina sequencing.

2. Materials and methods

2.1. Sampling sites

The activated sludge used in this project was sampled in June 2016 from the aeration tank of the Amsterdam West wastewater treatment plant (WWTP) of the city of Amsterdam (The Netherlands). This facility operates since 2005 and has a hydraulic capacity of $8400\text{ m}^3/\text{hours}$ in dry weather flow and $30,000\text{ m}^3/\text{hours}$ in wet weather flow. Activated sludge was maintained under constant shaking (150 rpm) at $22\text{ }^\circ\text{C}$ ($\pm 1\text{ }^\circ\text{C}$) until use and for a maximum of 1 week, in order to allow the inoculum to acclimate to the laboratory conditions, according to the OECD 310 guideline (OECD, 2014). After one week of incubation under constant shaking, the dry matter content of the sludge sample was determined by filtration of a known volume through a $1.2\text{ }\mu\text{m}$ glass microfibre filter GF/C (Whatman), after which the filters were dried overnight in an oven at $70\text{ }^\circ\text{C}$ and weighed.

2.2. Chemicals

N-methylpiperazine (CAS Number 109-01-3) and 4-chloroaniline (CAS Number 106-47-8) were purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) with a $>99\%$ purity grade. Working and standard solutions were prepared in ultra-pure water (ELGA MK2-Analytic, Ede, the Netherlands) and stored at $4\text{--}8\text{ }^\circ\text{C}$. Methanol and acetonitrile used for sample pre-treatment and chromatography had an analytical or LC-MS grade and were purchased either from Biosolve, Valkenswaard, the Netherlands.

2.3. Chemostat reactor

To cultivate and expose the activated sludge community, we used three chemostat systems of 0.5 L volume each. Chemostats contained a similar sterile growing medium and one additional test compound. Both systems exposed to either 4CA or NMP were fed with medium containing a nominal concentration of 1.5 mg/l of the test compound, while the third one was used as a control and was only fed with growth medium without added substance. A chemical concentration of 1.5 mg/l was chosen in order to represent a stable food source without competing with the other carbon and energy sources.

This medium was composed of: 128 mg/l KH_2PO_4 ; 326 mg/l K_2HPO_4 ; 501 mg/l $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$; 19.5 mg/l NH_4Cl ; 2.63 mg/l $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$; 0.34 mg/l ZnCl_2 ; 0.24 mg/l $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$; 0.09 mg/l $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$; 0.03 mg/l H_3BO_3 ; 0.24 mg/l $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$; 36.4 mg/l

CaCl₂·2H₂O; 22.5 mg/l MgSO₄·7H₂O; 0.33 mg/l pyridoxine HCl; 0.17 mg/l riboflavin; 0.17 mg/l thiamine HCl; 0.17 mg/l cyanobalamin; 0.17 mg/l nicotinamide; 0.17 mg/l *p*-aminobenzoic acid; 0.17 mg/l lipid acid; 0.17 mg/l pantothenic acid; 0.07 mg/l d-biotin; 0.07 mg/l folic acid; 40 mg/l C₂H₃NaO₂; 1.5 mg/l yeast extract.

Fresh medium (10 L) was prepared every 10 d. Stock solutions of vitamins, 4CA or NMP were prepared using ultra-pure water and added under sterile condition to the autoclaved medium via a 0.2 µm silicone membrane filter (Whatman plc). System vessels for media, cultures and effluents were made of glass, tubes were made of stainless steel, air tubes of silicones, and pump tubes of silicon. The system was autoclaved (121 °C, 211 kPa, 21 min) before incubation. During the experiment, the pH (7.4) of the effluent was regularly measured with a pH probe. A constant bubbling system using compressed air (filtered through a 0.2 µm PTFE membrane) provided oxygen to the culture. All three chemostat systems were inoculated at the same time with the same activated sludge volume (5 ml) and were maintained in the dark at 22 °C (±1 °C) under constant stirring (300 rpm). Pumps were immediately activated after inoculation. Flow of the medium influent (20 ml/h) was controlled by a Gilson Minipuls-3 peristaltic pump to maintain a dilution rate of 0.04 h⁻¹. Samples of the culture were taken from the sampling port under sterile conditions.

2.4. Biodegradability testing

The biodegradation tests were performed following the OECD 310 ready biodegradability protocol (OECD, 2014) for the unexposed community and an enhanced version of the test for the exposed communities. During these tests, primary biodegradation was followed by the direct quantification of the compounds by LC-MS/MS analysis. Tested substances were dissolved at a nominal concentration of 20 mg/l in the mineral medium buffered at pH 7.4. The mineral medium was a mix of diverse salts and ammonium chloride as nitrogen source: 85 mg/l KH₂PO₄; 217.5 mg/l K₂HPO₄; 334 mg/l Na₂HPO₄·2H₂O; 5 mg/l NH₄Cl; 36.4 mg/l CaCl₂·2H₂O; 22.5 mg/l MgSO₄·7H₂O; 0.25 mg/l FeCl₃·6H₂O.

Bottles were directly inoculated by adding 10⁴–10⁵ CFU/mL (colony forming units) of activated sludge or chemostat samples. CFU numbers were determined on nutrient agar by serial dilution, after 48 h of incubation at room temperature. In parallel, abiotic degradation was assessed by inoculating a test with an autoclaved inoculum, diluted to the same level as the other inocula. Bottles were sealed with rubber seals and aluminium caps and headspace was replaced by synthetic air, composed of 80% N₂ and 20% O₂. Incubation was performed in the dark at 22 °C (±1 °C) and under constant shaking (150 rpm).

Data analyses of the biodegradation data was performed using Excel and R studio. When possible, models were used to determine the time necessary to reach 10%, 50% and 90% of degradation. The DRC R package (Ritz et al., 2015, p.) was used to fit a three-parameters log-logistic models to the data and to determine the different degradation times.

2.5. LC-MS/MS

To quantify 4CA and NMP in liquid samples, two LC-MS/MS methods were established using a Shimadzu LC20 instrument (Shimadzu, 's-Hertogenbosch, the Netherlands), equipped with a SIL-20ACXR auto sampler and connected in series with a Sciex 4000 QTRAP MS/MS system (AB SCIEX, MA, USA). Separation was performed on a reversed phase C18 column (1.6 µm, 50 × 2.0 mm, Shim-Pack, Shimadzu) with a security guard ultra-cartridge as guard column for 4CA and a HILIC column (1.7 µm, 50 × 2.1 mm,

amide BEH, Waters, Etten Leur, the Netherlands) for NMP. For both methods, the column oven was set at 35 °C and the injection volume was 20 µl. Two pumps (model LC-20ADXR) were used to create a gradient of two solvents. The mobile phase of the reversed phase method, used to quantify 4CA, consisted of (A) ultra-pure water + 0.1% acetic acid, and (B) methanol. The flow rate of the mobile phase was of 0.3 ml/min. After injection, the B concentration was increased from 10% to 100% within 2.5 min, then B held at 100% for 2 min before returning to the initial concentration of 10% for 5 min before injection of the next sample. For NMP, the flow rate was 0.4 ml/min. The mobile phase consisted of (A) ultrapure water and (B) acetonitrile, both buffered with 5 mM of ammonium formate and 0.075% (v/v) of formic acid. Samples were ionized using electrospray ionization (ESI, Turbospray) operating in positive mode. Multiple-reaction monitoring mode (MRM) was applied with the first transition (TR1) used for quantification and a second transition (TR2) used for qualitative confirmation. All of the MRM parameters can be found in Table 1. The following parameters were used for the mass spectrometer: Collision gas: 6 l/h, curtain gas: 10 l/h, ion-spray voltage: 4000 °C, Temperature: 500 °C, Ion source gas 1: 40 l/h and ion source gas 2: 50 l/h.

Before injection, the samples were diluted in the relevant solvents (e.g. 4CA: water; NMP: acetonitrile) and filtered using a 0.2 µm polypropylene filter (Filter-BIO, China). Calibration standards of both compounds were prepared and injected in order to quantify the substances in the samples. These standards were prepared under the same conditions as the samples. Data acquisition and analysis were performed with AB SCIEX Analyst software (Ver. 1.5.1).

2.6. Physiological profiling of bacterial communities

Physiological profiling of the microbial communities was done with Biolog EcoPlates™, which are 96 wells plates that contain 31 different carbon and free energy sources in triplicate. Briefly, microbial communities derived from chemostat or activated sludge were diluted to 10⁻³ CFU/ml. EcoPlates were directly inoculated with 150 µl of diluted sample per well, and were incubated at 22 °C in the dark for one week. Absorbance at 590 nm was measured with a BMG labtech SPECTROstat Nano absorbance microplate reader (Isogen life Science, The Netherlands). The average well-colour development (AWCD) was calculated as described by (Garland and Mills, 1991) and following this formula (AWCD = $\sum A_{590i}/31$) in which A₅₉₀ is the well colour development minus that of the control. The AWCD was calculated for each plate and for each of the six substrate groups: carbohydrates, carboxylic, amines, amino acids phenolic compounds and polymers. The Shannon-Weaver index (H'), was calculated as following: H' = $-\sum \pi_i (\ln \pi_i)$ in which π_i is the normalized value of well development (Gryta et al., 2014). Normalization of the absorbance data were performed to reduce the variability due to differences in inocula densities. Measured absorbance of each well, at each time point, were divided by the initial absorbance of the corresponding carbon source (Hackett and Griffiths, 1997; Muñoz et al., 2014). All statistical analyses were performed with R (v3.5.1) (R Core Team, 2016).

2.7. DNA extraction and PCR amplification

DNA samples were extracted and purified using the PowerSoil kit (QIAGEN Benelux B.V.), following the supplier's protocol. Samples were stored at -80 °C until further analysis. DNA concentrations were determined using the Qubit dsDNA HS Assay kit (Thermo Fischer Scientific, Waltham, MA USA) and the Qubit fluorometer (Thermo Fischer Scientific, Waltham, MA USA). Used

Table 1
Multiple reaction monitoring (MRM) parameters and precursor/product ion pair for the analyses of 4CA and NMP.

Chemical	Precursor ion (<i>m/z</i>)	Declustering potential (V)	Quantifier (TR1)		Qualifier (TR2)	
			Product ion (<i>m/z</i>)	Collision energy (V)	Product ion (<i>m/z</i>)	Collision energy (V)
4-Chloroaniline	128	60	93	27	75	48
N-Methylpiperazine	101	116	58	27	44	33

primers (8 forward primers and 16 reverse primers) (Appendix A) target the V3–V4 region of bacterial 16S rRNA genes and were yield at ~550 bp fragment during PCR amplification. PCR products were purified using an Agencourt AMPure XP magnetic bead (Beckman Coulter Nederland B.V.). Agarose gel electrophoresis was used to control the purification step. Samples were diluted to 1 ng/μl prior to pooling and subsequent analysis by the Illumina MiSeq platform.

2.8. Illumina MiSeq sequencing and data analyses

The MiSeq sequencing data were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) pipeline (<http://qiime.org/>) (Kuczynski et al., 2011). Sequences were aligned using the SINA aligner (<https://www.arb-silva.de/>). Further, sequences were clustered into Operational Taxonomic Units (OTUs) with the UPARSE method and taxonomy assignments to every OTU at 97% sequence identity was achieved using Uclust. Chimeras were filtered using SILVA DATABASE 99% SIM. Finally, the OTU table and the taxonomic table were exported in a single BIOM file. This BIOM file and the phylogenetic tree produced by QIIME were imported to R (ver. 3.5.1) (R core team, 2016) using the R Phyloseq package (ver. 1.26.0) (McMurdie and Holmes, 2013). Subsequent analyses and data visualization were then performed using the R packages Phyloseq (ver. 1.26.0) (McMurdie and Holmes, 2013), Vegan (ver. 2.5-3) (Oksanen et al., 2017) and ggplot2 (ver. 3.1.0) (Wickham, 2016).

Alpha (Shannon H, inverse Simpson, Observed) and beta diversity analyses (Unifrac and Bray-Curtis distance) were carried out using the Vegan package (Oksanen et al., 2017) of R (R Core Team, 2016). The Shannon diversity index (H) (Surovtseva et al., 1985) is a measure of diversity combining both abundance and evenness. Further, data were normalized using the variance-stabilizing transformations, using the R packages Phyloseq and DeSeq2 (ver. 1.22.1) (Love et al., 2014) and following examples from (McMurdie and Holmes, 2014). Dissimilarities of community composition were assessed by non-metric multidimensional scaling (NMDS) using the Bray-Curtis dissimilarity on the normalized data. Principal component analysis (PCA) and Unifrac (Lozupone and Knight, 2005) was also used on normalized data to compare the communities by incorporating phylogenetic information. Permutational multivariate analysis of variance (PERMANOVA) were performed using the Adonis function from the R package Vegan (Oksanen, J., et al., 2017). Finally, differential abundance analyses were performed with Phyloseq and DeSeq2. Differential abundance is defined as the difference in mean relative abundances between groups or conditions (McMurdie and Holmes, 2014). Only groups showing a significant difference ($p_{adj} < 0.01$) with the original inoculum, in each community, were displayed.

3. Results

3.1. Biodegradation in OECD 310 tests

The biodegradation profile in time of NMP, measured by LC-MS/MS according to the OECD 310 biodegradability guidelines, is shown in Fig. 1A&B. The colours of the lines indicate the exposure

time of the inoculum to NMP in the chemostat. The compound was not degraded after 28 d of incubation in the abiotic control, indicating that abiotic degradation did not take place. Test batches incubated with the original activated sludge were able to degrade approximately 10% of the original NMP content after 28 d, indicating that NMP can be considered as recalcitrant to biodegradation in this test. Moreover, the activated sludge community cultivated in chemostat culture without being exposed to NMP showed no sign of significant biodegradation activity, not even after 9 months (Fig. 1B). In contrast, after 1 month of exposure in the chemostat, the microbial community was able to completely degrade the original concentration of NMP within 10 d. We observed the same result after three and nine months of exposure. The inoculum exposed for nine months took only 6.63 ± 1.81 d to reach 90% degradation of NMP (Appendix B). It should be noted that the density of the inoculum, measured in CFU/ml, was similar in every test.

The biodegradation of 4CA in OECD 310 biodegradability tests, using inocula from activated sludge communities exposed to 4CA in chemostat culture, is shown in Fig. 1C and the results using unexposed inocula in Fig. 1D. We can observe that the results from the abiotic control do not correspond to the three-parameter log-logistic model and that the degradation pattern fluctuated over time with high standard deviations. However, tests with activated sludge communities exposed for one month as inocula reached 90% of degradation within 28 d. In contrast, communities exposed for three and nine months could only degrade 45% of the 4CA added before the end of the test, indicating loss of the capacity to degrade 4CA.

3.2. Community dynamics

The Illumina MiSeq platform produced 3,488,627 sequences in total and an average of 61,203 reads per samples, that belong to 17,650 operational taxonomic units (OTUs). OTUs with a number of reads lower than 3 were discarded, in order to filter single and artefact OTUs, reducing the total number to 5666. The abundance and taxonomic data were used to produce the different alpha and beta diversity indices display in Fig. 2.

Alpha diversity indices are used to follow the community evolution in each chemostat over time. The observed richness represents the number of unique species observed in our samples, while the Shannon diversity index (H) combines both richness and the evenness of the communities. The Simpson diversity index also includes both richness and evenness of the community. However, we used the inverse Simpson index since this diversity index is not influenced by the sampling effort in contrast to the regular Simpson. The chemostats were inoculated at the same time using the same sample of activated sludge, which is confirmed by similar extents of alpha diversity at the start of culturing. We then observe that their diversity quickly diverges in time, and even fluctuates. Only the chemostats exposed to 4CA and NMP seem to stabilize at the end of the experiment. However, ANOVA and Kruskal Wallis tests, show that these indices do not differ significantly over time.

Based on the Bray Curtis dissimilarity (Fig. 2B), it appears that the cultivated communities are different from each other. The

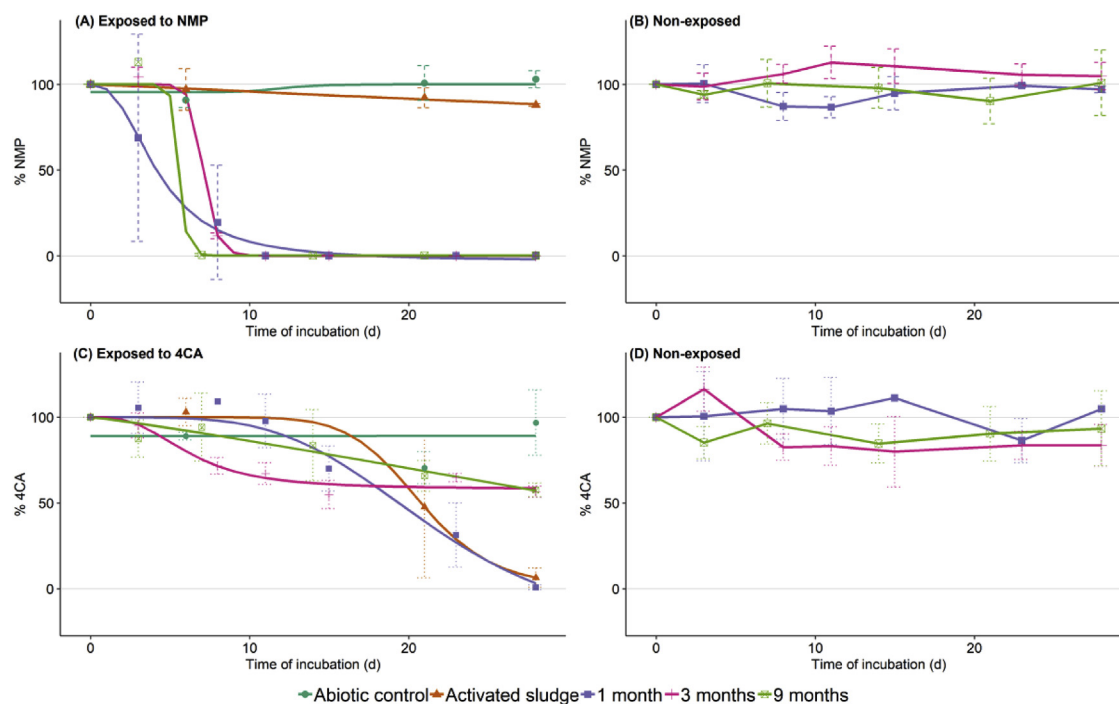


Fig. 1. Biodegradation of NMP (A&B) and 4CA (C&D) in OECD 310 biodegradation tests (measured by LC-MS/MS) using an activated sludge community cultivated in chemostat culture exposed to either NMP or 4CA. Graphs A & C show the fits (three-parameter logistic) for each condition, while graphs B & D represent the average percentage of elimination, as logistic model could not be fitted. Points represent the average percentages of degradation for each condition and error bars correspond to the standard deviation of the three replicates used to calculate the average biodegradation profile.

microbial community structure of each chemostat at the first day of the experiment, just after inoculation, showed considerable similarity, as can be observed from the cluster in the higher right-hand corner of the NMDS plot in Fig. 2B. Subsequently, the communities quickly started to diverge. This result is confirmed by the output from the PERMANOVA Adonis test (Appendix C). The three chemostat communities do not share the same centroid and the permutation test for homogeneity of multivariate indicates that the Adonis results are not due to group dispersions. We can therefore conclude that the communities are indeed different. Furthermore, based on the PCA in Fig. 2C, we observe differences over time in each chemostat. As expected, cultivating a microbial community in a chemostat with artificial media changes its composition. These results suggest that the exposure to 4CA or NMP, as well as the cultivating conditions, are influencing the community's evolution.

Microorganisms able to use 4CA as sole source of carbon and free energy have been isolated and identified since the 1980s (Surovtseva et al., 1985). They include bacteria from the genera *Delftia* (Zhang et al., 2010), *Comamonas* (Boon et al., 2001), *Acinetobacter* (Hongswat and Vangnai, 2011; Vangnai and Petchkroh, 2007), *Pseudomonas* (Loidl et al., 1990; Nitisakulkan et al., 2014; Surovtseva et al., 1985; Vangnai and Petchkroh, 2007) and *Klebsiella* (Vangnai and Petchkroh, 2007).

Some members of these genera were found in our samples. *Acinetobacter* and *Pseudomonas* together represented approximately 30–40% of the total community at the start (Fig. 3), while after 1 month of exposure in chemostat 4CA, species from the genus *Delftia* became more abundant until they represented approximately 40% of the community. Furthermore, these observations are supported by the data in Fig. 4, which displays every OTU in chemostat 4CA, whose abundance significantly ($p_{adj} < 0.01$) differs from the original inoculum. An OTU with a negative log₂-FoldChange (Fig. 4A) indicates that the abundance significantly decreased (Fig. 4B), while a positive one indicates an increase

(Fig. 4C). Several OTUs from the genera *Acinetobacter* and *Pseudomonas* are amongst the numerous OTUs whose abundance decreased. While one OTU from the genus *Delftia* increased over time, its contribution to the biodegradation of 4CA, in our experiment, is unknown.

Our data do not enable the identification of NMP-degrading microorganisms. However, the results shown in Figs. 2 and 5 show a shift in the community composition over time. The NMP community is different to the other chemostats and the initial activated sludge used. Fig. 5 shows that some OTUs from the genera *Variovorax*, *Terrimonas*, *Delftia*, *Mycobacterium*, an uncultured bacterium and an OTU from the order *Burkholderiales* increased in abundance after one months of exposure. Among these genera, *Delftia* and *Terrimonas* increased significantly in abundance in the unexposed chemostat as well (Appendix D). Fig. 5B represents the OTUs that were outcompeted from the community exposed to NMP.

3.3. Community physiological profiling

Biolog Ecoplates can provide extensive information regarding the general use of carbon sources by the cultivated communities. We did not perform any kinetic analyses on our data as this can be influenced by the biomass, which was not controlled in this experiment. However, to compare the catabolic diversity among communities, the data were normalized, as described in the materials and methods section, in order to reduce the variability due to differences in inocula densities (Hackett and Griffiths, 1997; Muñoz et al., 2014). The Shannon Weaver index (H) was used to calculate the physiological diversity of each community.

Analysis of the variance (ANOVA), performed on the calculated Shannon-Weaver indexes (Table 2), indicates that the chemostat communities significantly differed from the original activated sludge after 1 month of cultivation ($p < 0.001$), with the exception

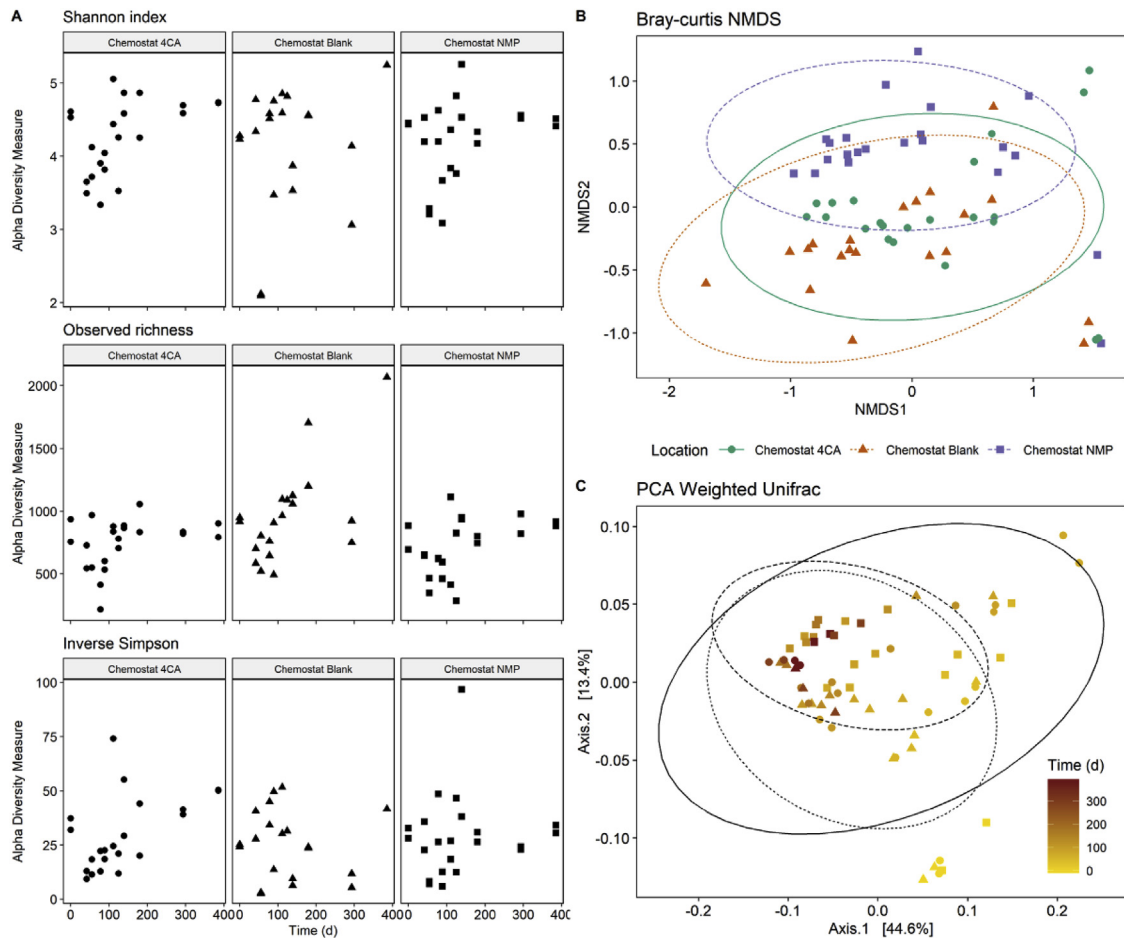


Fig. 2. (A) Alpha diversity index over time (d) for each chemostat. Observed richness represents the number of observed OTUs in the samples, while Shannon and inverse Simpsons are diversity indices (left panel). (B) NMDS ordination of the Bray-Curtis dissimilarity between communities. Confidence ellipses level at 0.95 (right panel). (C) PCA ordination of the different communities over time, using a weighted Unifrac distance method. The colour gradient shown in the legend represents the time (d) since the inoculation of the chemostats with activated sludge. Confidence ellipses level at 0.95 (right panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

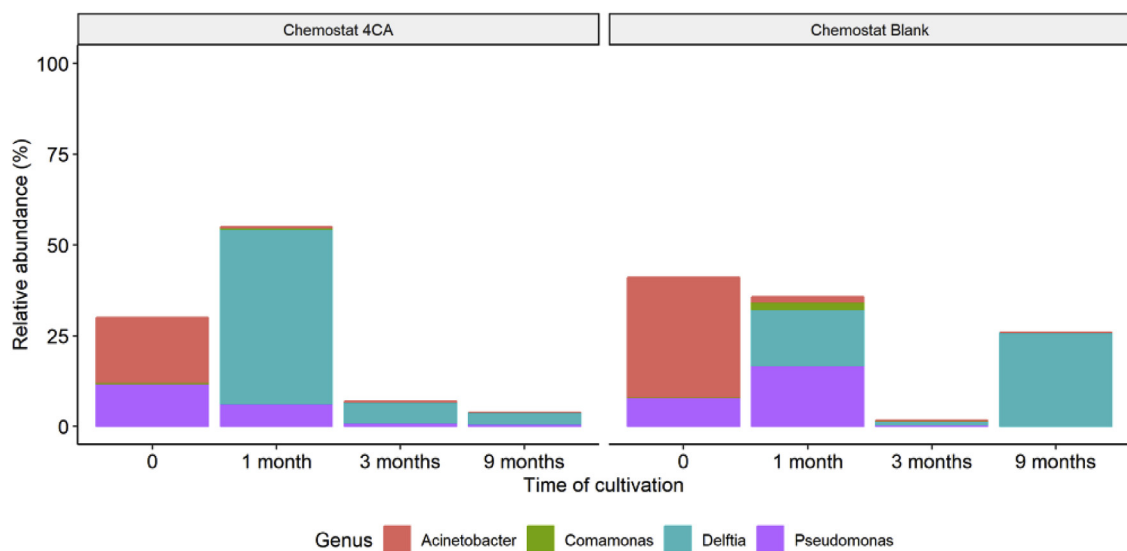


Fig. 3. Relative abundance (%) of the known 4CA-degrading genera in chemostats over time. Relative abundance calculated based on the whole community abundance. Left: community exposed to 4CA; Right: community that was not exposed to 4CA.

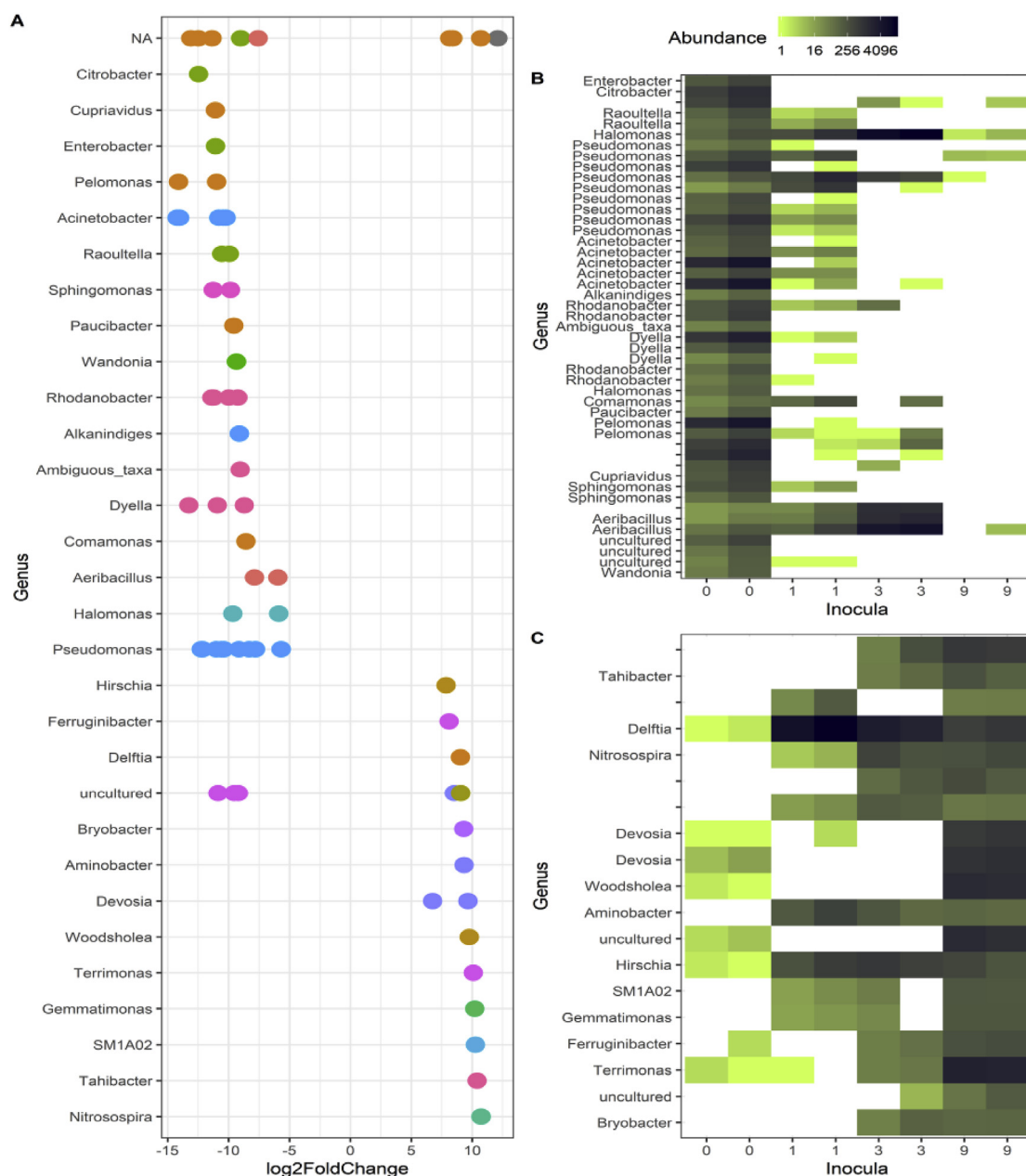


Fig. 4. (A) Differential analysis representing each OTU whose abundance significantly ($p < 0.01$) changed over time from the original inoculum (0), in chemostat 4CA over time. Log₂FoldChange is the log-ratio of observed differences between the tested sample and the original inoculum. Genera with a Log₂FoldChange > 0 were enriched in comparison to the original inoculum while a Log₂FoldChange < 0 indicates that the abundance of the genera decreased. The colour of the dots represents the family corresponding to each displayed OTUs (B) Heatmap representing the abundance of each OTU displayed in (A) with a Log₂FoldChange < 0. (C) Heatmap representing the abundance of each OTU displayed in (A) with a Log₂FoldChange > 0. Inocula represents the time of exposure used in biodegradation testing (0, 1 month, 3 months, 9 months). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of chemostat blank, that significantly differed after 3 months. However, no significant differences could be measured between the chemostats, with the exception of the chemostat exposed to NMP after 9 months of exposure and the chemostat exposed to 4CA after 1 and 3 months.

Cluster analysis of the AWCD for each substrate group shows different patterns that may be shared between chemostats (Fig. 6). For example, after 3 months of exposure the NMP and 4CA chemostats are statistically not different from each other, based on ANOVA and the post hoc Tukey's test. Overall, carbohydrates were the less well degraded group and activated sludge was the worst performing community, showing the least AWCD development. It

seems that the presence or absence of NMP and 4CA did not affect the community's physiological profile in a distinctive way. Exposed and unexposed communities could be significantly different at one time point, but this may no longer be the case at the next time point.

4. Discussion

Adaptation of microbial communities to chemicals being persistent in laboratory systems is a known and documented process (Itrich et al., 2015; Poursat et al., 2019a). Despite chemostats being suitable to study the adaptive evolution of microorganisms

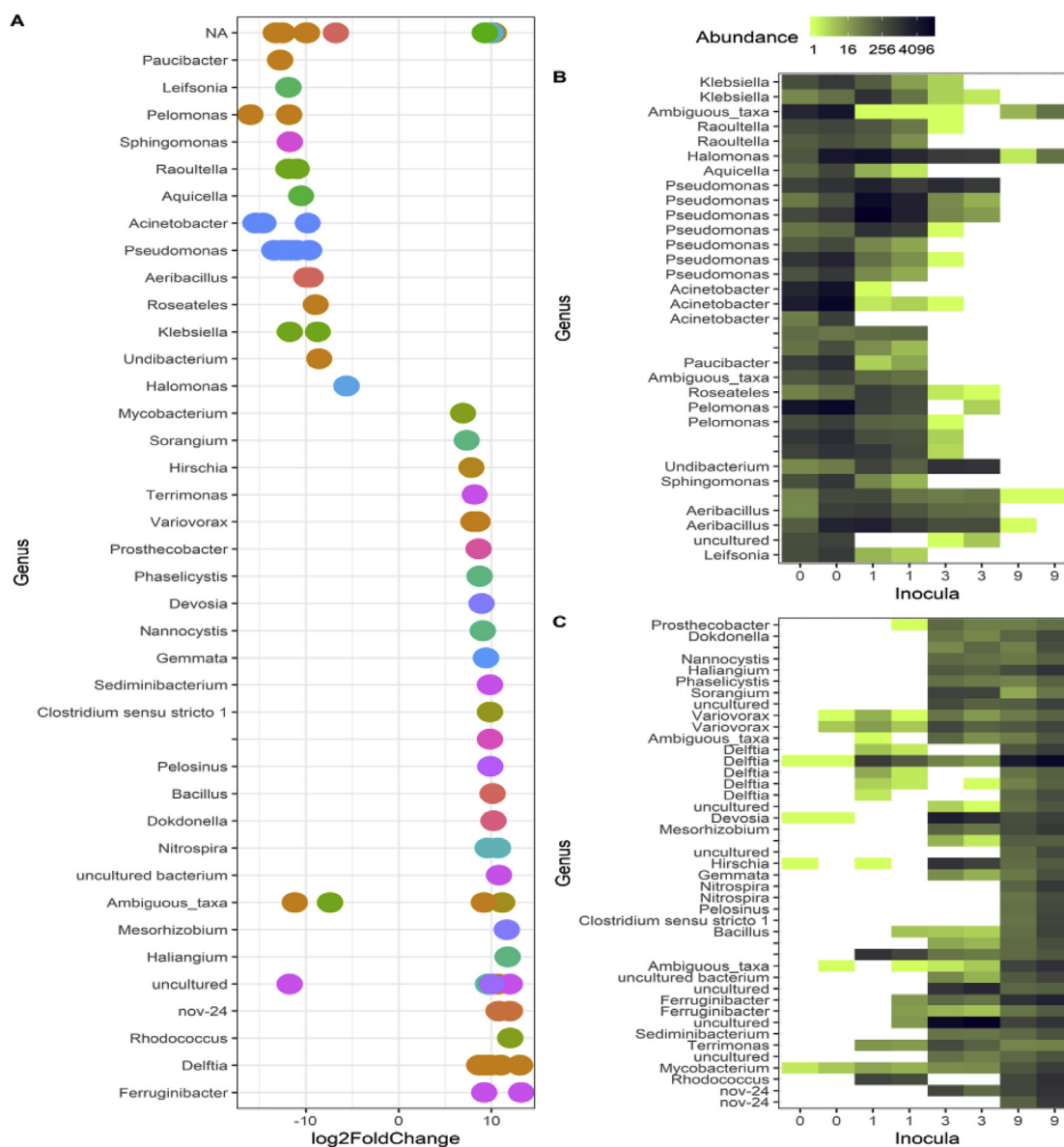


Fig. 5. (A) Differential analysis representing each OTU whose abundance significantly ($p < 0.01$) changed over time from the original inoculum (0), in chemostat NMP over time. The colour of the dots represents the family corresponding to each displayed OTUs (B) Heatmap representing the abundance of each OTU displayed in (A) with a Log₂FoldChange < 0. (C) Heatmap representing the abundance of each OTU displayed in (A) with a Log₂FoldChange > 0. Inocula represents the time of exposure used in biodegradation testing (0, 1 month, 3 months, 9 months). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2

Shannon Weaver indexes (H) for each community based on the average well carbon development (AWDC).

	1 month	3 months	9 months
Activated sludge	2.70 ± 0.14		
Chemostat 4CA	2.94 ± 0.06	3.04 ± 0.06	3.29 ± 0.01
Chemostat NMP	3.14 ± 0.01	3.13 ± 0.07	3.14 ± 0.04
Chemostat blank	3.07 ± 0.03	3.14 ± 0.04	3.32 ± 0.02

(Gresham and Hong, 2014), very few studies used them as model system to induce adaptation. Previous investigation showed that communities exposed in chemostat could adapt and influence the results of biodegradability tests (Thouand and Block, 1993). Adapted inocula enhanced the reproducibility of biodegradability

tests, decreased the lag time and increased the biodegradation rate of the test chemicals (Thouand and Block, 1993). Based on our results, we can also confirm that exposure of microbial communities in chemostat systems modify their biodegradation abilities. Moreover, it should be noted that the community cultivated in the unexposed chemostat, and only fed with acetate, yeast extract and micronutrients, was unable to degrade NMP and completely lost its ability to degrade 4CA. Loss or changes of biodegradation potential by cultivated activated sludge community, in absence of the test chemical, have been observed in the MITI tests (OECD 301C and 302 C) (Vázquez-Rodríguez et al., 2011) and in chemostat systems (Thouand and Block, 1993, Poursat et al., 2019b). Long term culturing in the absence of the test chemical, without sludge recycling, will lead to changes in the community structure, which might result in a loss of function. Moreover, community diversity in culture system is strongly affected by changes in the operating

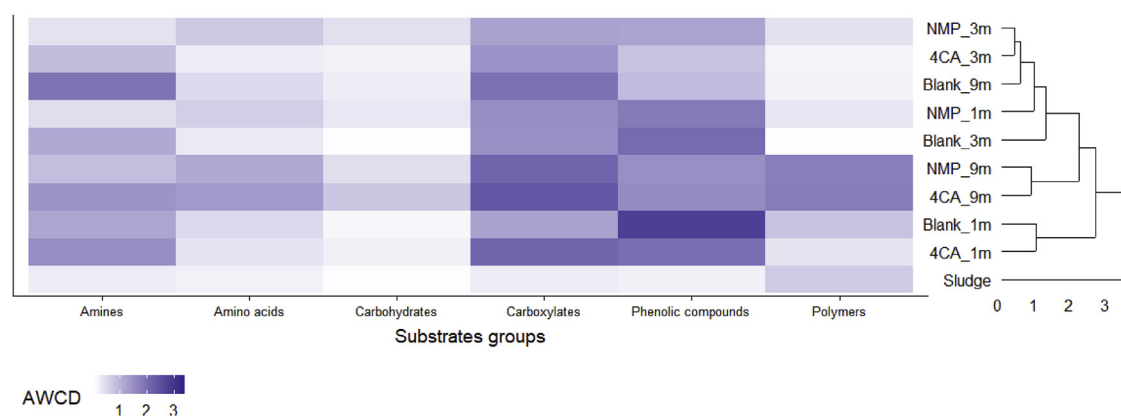


Fig. 6. Cluster analysis of the average well carbon development (AWCD) of each community, using the Euclidean distance. Carbon sources are grouped in six different substrate groups.

parameters. Configuration of the system has been shown to have the strongest effect on the community diversity, above loading rates and medium composition (Pholchan et al., 2010).

4CA has been reported to show inconsistent behaviour in ready biodegradability testing with variable outcomes and in many cases no degradation at all (Comber and Holt, 2010). Exposure to 4CA has been shown to promote its biodegradation and to greatly reduce the lag phase (Torång and Nyholm, 2005). Using batch and semi-continuous pre-exposure procedure (SCEP) to expose surface water microbial communities to low concentrations (0.1–100 µg/l) of 4CA resulted in a reduction of the lag-phase from 88 d, for the original river water, to 9 d after a 5 week exposure period (Torång and Nyholm, 2005). However, the semi-continuous system allowed the biomass to be retained, while in a chemostat, microorganisms with a generation time lower than the dilution rate will be washed out from the system. Growth in chemostats affected the taxonomic and physiological diversity of the communities, without there being a clear distinction in biodegradation capacity between the chemostats. Differential abundance analysis indicates that the loss of microorganism from the genera *Pseudomonas* (Loidl et al., 1990; Nitisakulkan et al., 2014; Surovtseva et al., 1985; Vangnai and Petchkroh, 2007) and *Acinetobacter* (Hongswat and Vangnai, 2011; Vangnai and Petchkroh, 2007), which have been identified in the literature as 4CA degraders, might have been the reason for changes in the biodegradation capacity of the community. Interspecies competition for resources led to selection of a community able to degrade the easily biodegradable carbon sources, such as sodium acetate and peptides and to the low 4CA concentration (Ferenci, 2007).

NMP is used as a starting material for the synthesis of pharmaceuticals (Dorokhova et al., 1974) and the fate of this compound upon emissions to the environment is unknown. Biodegradation of NMP has not been reported in the scientific literature, as this compound is rarely studied in environmental science. No data are available on the occurrence and fate of NMP in wastewater and the environment. The parent compound piperazine has been reported to have a lag phase for biodegradation up to 50 d (Cai et al., 2013). The exposed community could adapt to NMP and completely degrade it, however, factors influencing and regulating the adaptation to this compound remain unclear and need further investigation. The presence of the chemical itself, or a structural analogue, is a crucial condition to induce community adaptation (Alidina et al., 2014; Birch et al., 2017; Oh et al., 2013; Pfaender et al., 1985; Thouand et al., 1996). To fully understand the mechanisms behind the adaptation to NMP, we should investigate the microbial interactions within the community, the catabolic potential of the

whole community, and the influence of the environmental factors on the community. A population found in NMP a stable substrate, leading to the development of a degrading population for this ecological niche, and therefore to an adaptation at the community level (van der Meer, 2006). However, these changes observed in the taxonomic diversity could not be linked to the changes of the functional diversity. Functional diversity analysis only points out that the cultivated communities are different from the original activated sludge used as inoculum, as demonstrated by the taxonomic diversity analyses. Abundance in activated sludge of NMP-degrading microorganisms must have been too low to efficiently remove NMP under the stringent conditions of the RBT. The ECHA database on the safety data sheets (SDS) of NMP also point out this hypothesis to explain the absence of biodegradation by activated sludge, while an adapted community could quickly remove the compound (ECHA, 2013). The supplier of the SDS observed that NMP was degraded within a week in an enhanced biodegradation test (OECD 301) by an inoculum exposed for four weeks to 10 and 100 µg/l in a semi continuous activated sludge system (SCAS). Furthermore, similar results were obtained by using sessile microorganisms collected from glass beads during flow through of river water containing µg/l of NMP (ECHA, 2013). Hence, this paper confirms the previously observed mechanism that was not published in the scientific literature.

Differences in response between the communities exposed to NMP and 4CA show that biodegradation capacity in an open system is the result of a fine balance between the increase of competent species (i.e, adaptation) and loss of these strains. The presence of the test compound, the relative abundance of key degraders, inter- and intra-species competition and the presence of additional energy sources are some of the different parameters that will influence the capacity of a community to biodegrade substances. 4CA is a well-known environmental pollutant (Zhu et al., 2011), to which microbial communities may already been exposed and subsequently adapted. The environmental concentrations of NMP is unknown but its use as intermediate (ECHA, 2013) suggest that this compound is not prevalent in wastewater, which indicates that microbial communities might not be naturally exposed.

Our results confirm the importance of characterising inocula and their exposure history before using them in biodegradation testing, as this can affect the outcome of the test (Birch et al., 2017; Kim et al., 2017; Vázquez-Rodríguez et al., 2011). Including information about the exposure history of the inoculum would enhance the OECD biodegradation guidelines, however, it is challenging to obtain this type of information. Long term exposure in chemostats is one method, with its advantages and disadvantages, that can be

used to investigate microbial adaptation and its implication for ready biodegradability testing. Chemostats offer the possibility to adapt a community in a controlled and reproducible environment. However, as described in this study, they can lead to a loss of biodegradation capacity (Poursat et al., 2019b), as they cannot fully mimic environmental conditions, and they can be expensive and difficult to handle in laboratory. Moreover, long term exposure may lead to the development of a highly artificial community, that does not represent the environment. However, it should be noted that results of the regular RBTs, without previous exposure, did not express a high results variability or false negatives, as sometime reported in the literature (Dick et al., 2016; Federle et al., 1997), hence, use of an enhanced test did not reduce the expected test variability or number of false negatives. For these reasons, we do not advise to use highly adapted communities from chemostats as standard inocula in ready biodegradation test, as more investigation should be conducted before one can truly understand and therefore include adaptation in the tests. However, other systems with biomass retention may avoid some of these disadvantages and therefore be more suitable. Short-term pre-exposure, which is not allowed under the ECHA guidelines, seems to be a more reliable technique to produce a standardized inoculum for the tests, and deserve to be investigated. As proposed in a previously published work (Poursat et al., 2019a), we would recommend to perform pre-exposure, as an add-on, in parallel with the standard tests. In one test, the inoculum would be pre-exposed in a closed system, while in the second test, the inoculum will not be pre-exposed, and only processed according to the guideline. However, characterising the inoculum, by using multi-omics approaches (Kowalczyk et al., 2015), is a first step to reduce test outcome variations and false negative results.

5. Conclusions

This study highlighted the potential of chemostat systems to

induce microbial adaptation to specific chemicals. The present study is another example of exposure leading to adaptation and is a first step to understand the community mechanisms and dynamics involved in this process, in order to implement the adaptation of microbial communities to the ready biodegradability testing guidelines. The two test chemicals, 4-chloroaniline and N-methylpiperazine, were respectively completely eliminated and recalcitrant to biodegradation in the OECD 310 biodegradation test, using activated sludge as inoculum. However, after one month of exposure, the community adapted and was able to completely remove n-methylpiperazine from the batch degradation test within 10 days. On the other hand, cultivation in chemostat in the presence of 4-chloroaniline, led to a partial loss of biodegradation capacity for this compound after 3 months of exposure. Results of this study confirmed the importance of characterizing and standardizing the inocula used in ready biodegradability testing. Indeed, exposure to a chemical, in either the laboratory or in the environment, clearly changes the community behaviour in biodegradation testing. Investigating the implications of the chemical exposure on the test outcomes, will ultimately lead to a better prediction of the environmental fate of organic chemicals.

Acknowledgments

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Appendix A

Forward primers

Primer name	Adapter	i5	pad-forward	link-V3f	V3-16sf
v3.SA501	AATGATAGGCGACCACCGAGATCTACAC	ATCGTACG	TATGGTAATT	GG	CCTACGGGNGGCWGCAG V3.SA501F AATGATACGGCGACCACCGAGATCTACACATCGTACGTATGGTAATTGGCCTACGGGNGGCWGCAG
V3.SA502	AATGATACGGCGACCACCGAGATCTACAC	ACTATCTG	TATGGTAATT	GG	CCTACGGGNGGCWGCAG V3.SA502F AATGATACGGCGACCACCGAGATCTACACACTATCTGTATGGTAATTGGCCTACGGGNGGCWGCAG
v3.SA503	AATGATACGGCGACCACCGAGATCTACAC	TAGCGAGT	TATGGTAATT	GG	CCTACGGGNGGCWGCAG v3.SA503F AATGATACGGCGACCACCGAGATCTACACTAGCGAGTTATGGTAATTGGCCTACGGGNGGCWGCAG
v3.SA504	AATGATACGGCGACCACCGAGATCTACAC	CTGCGTGT	TATGGTAATT	GG	CCTACGGGNGGCWGCAG v3.SA504F AATGATACGGCGACCACCGAGATCTACACTGCGTGTATGGTAATTGGCCTACGGGNGGCWGCAG
v3.SA505	AATGATACGGCGACCACCGAGATCTACAC	TCATCGAG	TATGGTAATT	GG	CCTACGGGNGGCWGCAG v3.SA505F AATGATACGGCGACCACCGAGATCTACACTCATCGAGTATGGTAATTGGCCTACGGGNGGCWGCAG
v3.SA506	AATGATACGGCGACCACCGAGATCTACAC	CGTGAGTG	TATGGTAATT	GG	CCTACGGGNGGCWGCAG v3.SA506F AATGATACGGCGACCACCGAGATCTACACCGTGAGTGTATGGTAATTGGCCTACGGGNGGCWGCAG
v3.SA507	AATGATACGGCGACCACCGAGATCTACAC	GGATATCT	TATGGTAATT	GG	CCTACGGGNGGCWGCAG v3.SA507F AATGATACGGCGACCACCGAGATCTACACGGATATCTTATGGTAATTGGCCTACGGGNGGCWGCAG
v3.SA508	AATGATACGGCGACCACCGAGATCTACAC	GACACCGT	TATGGTAATT	GG	CCTACGGGNGGCWGCAG v3.SA508F AATGATACGGCGACCACCGAGATCTACACGACACCGTTATGGTAATTGGCCTACGGGNGGCWGCAG

Reverse primers

Primer name	Adapter	i7	i7-reverse-complement	pad-reverse	Link-V4r	V4-16Sr
v4.SA701	CAAGCAGAAGACGGCATAACGAGAT	AACTCTCG	CGAGAGTT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA701R CAAGCAGAAGACGGCATAACGAGATAACTCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
V4.Sa702	CAAGCAGAAGACGGCATAACGAGAT	ACTATGTC	GACATAGT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT V4.Sa702R CAAGCAGAAGACGGCATAACGAGATACTATGTCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA703	CAAGCAGAAGACGGCATAACGAGAT	AGTAGCGT	ACGCTACT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA703R CAAGCAGAAGACGGCATAACGAGATAGTAGCGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA704	CAAGCAGAAGACGGCATAACGAGAT	CAGTGAGT	ACTCACTG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA704R CAAGCAGAAGACGGCATAACGAGATCAGTGAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA705	CAAGCAGAAGACGGCATAACGAGAT	CGTACTCA	TGAGTACG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA705R CAAGCAGAAGACGGCATAACGAGATCGTACTCAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA706	CAAGCAGAAGACGGCATAACGAGAT	CTACGCGAG	CTGCGTAG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA706R CAAGCAGAAGACGGCATAACGAGATCTACGCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA707	CAAGCAGAAGACGGCATAACGAGAT	GGAGACTA	TAGTCTCC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA707R CAAGCAGAAGACGGCATAACGAGATGGAGACTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA708	CAAGCAGAAGACGGCATAACGAGAT	GTCGCTCG	CGAGCGAC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA708R CAAGCAGAAGACGGCATAACGAGATGTCGCTCGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA709	CAAGCAGAAGACGGCATAACGAGAT	GTCGTAGT	ACTACGAC	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA709R CAAGCAGAAGACGGCATAACGAGATGTCGTAGTAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA710	CAAGCAGAAGACGGCATAACGAGAT	TAGCAGAC	GTCGTGTA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA710R CAAGCAGAAGACGGCATAACGAGATTAGCAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA711	CAAGCAGAAGACGGCATAACGAGAT	TCATAGAC	GTCATATGA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA711R CAAGCAGAAGACGGCATAACGAGATTCATAGACAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SA712	CAAGCAGAAGACGGCATAACGAGAT	TCGCTATA	TATAGCGA	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SA712R CAAGCAGAAGACGGCATAACGAGATTCGCTATAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SB701	CAAGCAGAAGACGGCATAACGAGAT	AAGTCGAG	CTCGACTT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SB701R CAAGCAGAAGACGGCATAACGAGATAAGTCGAGAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SB702	CAAGCAGAAGACGGCATAACGAGAT	ATACTTCC	CGAAGTAT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SB702R CAAGCAGAAGACGGCATAACGAGATATACTTCCAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SB703	CAAGCAGAAGACGGCATAACGAGAT	AGCTGCTA	TAGCAGCT	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SB703R CAAGCAGAAGACGGCATAACGAGATAGCTGCTAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT
v4.SB704	CAAGCAGAAGACGGCATAACGAGAT	CATAGAGA	TCTCTATG	AGTCAGTCAG	CC	GGACTACHVGGGTWTCTAAT v4.SB704R CAAGCAGAAGACGGCATAACGAGATCATAGAGAAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT

Sequencing primers

V3F_seqprim F	TATGGTAATTGGCCTACGGGNGGCWGCAG
V4F_seqprim R	AGTCAGTCAGCCGACTACHVGGGTWCTAAT
V4P7_index	ATTAGAWACCCBDGTAGTCCGGCTGACTGACT

Appendix B

Estimated time to reach 10%, 50% and 90% of degradation (DT10, DT50 and DT90) of N-methylpiperazine by each inoculum. Degradation times were estimated using a three-parameters log-logistic model.

Inoculum		Estimated degradation time (d)		
		DT10	DT50	DT90
Activated sludge	1 month exposed to NMP	17.92 ± 12.01		
	3 months exposed to NMP	1.74 ± 0.42	4.25 ± 0.58	10.34 ± 2.98
	9 months exposed to NMP	6.94 ± 0.37	7.47 ± 0.26	8.04 ± 0.22
		6.11 ± 1.68	6.36 ± 1.74	6.63 ± 1.81

Appendix C

Results from PERMANOVA Adonis test
Permutation: free Number of permutations: 999
Terms added sequentially (first to last)

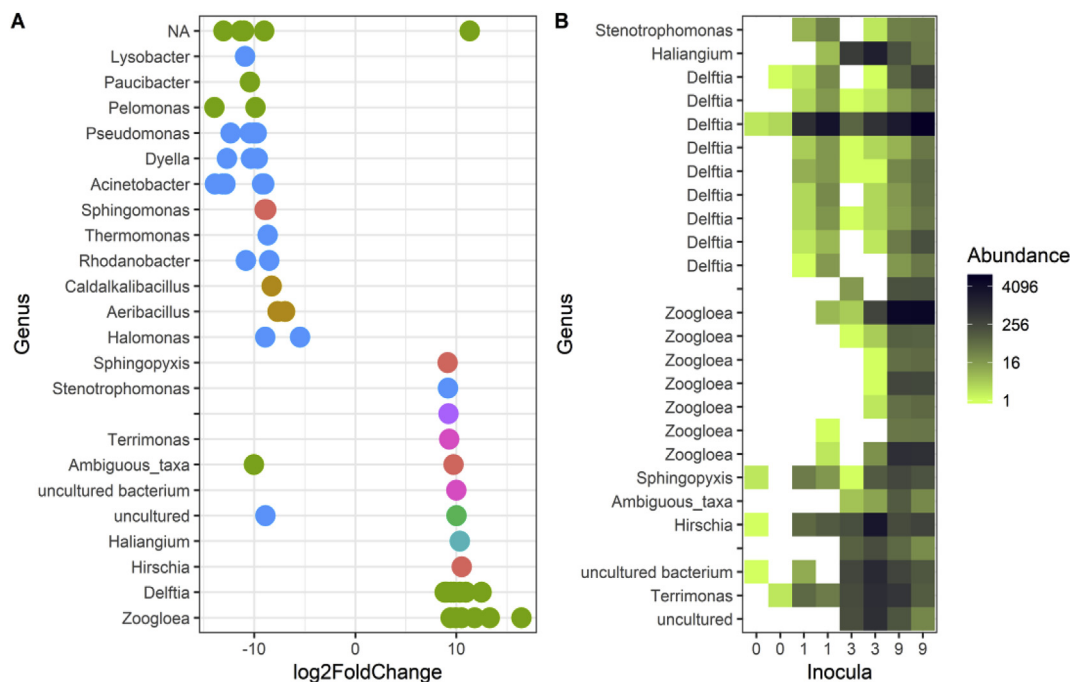
	Df	SumsOfSqs
Location	2	2.7766
Residuals	63	21.1139
Total	65	23.8904
		MeanSqs F. Model
Location	1.38828	4.1424
Residuals	0.33514	
Total		
		R2 Pr (>F)
Location	0.11622	0.001 ***
Residuals	0.88378	
Total	1.00000	
—		
Signif. codes:		
0 '****' 0.001 '***' 0.01		
'**' 0.05 '.' 0.1 ' ' 1		

Permutation test for homogeneity of multivariate dispersions
Permutation: free
Number of permutations: 999
Response: Distances

	Df	Sum Sq	Mean Sq	F N. Perm Pr (>F)
Groups	2	0.00105	0.0005275	0.0618 999 0.954
Residuals	63	0.53772	0.0085353	

Appendix D

Results from differential analysis in chemostat blank.



A) Differential analysis representing each OTU whose abundance significantly ($p < 0.01$) changed over time from the original inoculum (0), in chemostat Blank over time. Log₂FoldChange is the log-ratio of observed differences between the tested sample and the original inoculum. Genera with a Log₂FoldChange > 0 were enriched in comparison to the original inoculum while a Log₂FoldChange < 0 indicates that the abundance of the Genera decreased. (B) Heatmap representing the abundance of each OTU displayed in (A) with a Log₂FoldChange > 0. Inocula represents the time of incubation in the chemostat used in biodegradation testing (0, 1 month, 3 months, 9 months).

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