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Biodegradation of metformin and its transformation product, guanylurea, by natural and exposed microbial communities

Baptiste A.J. Poursat[a,b,*], Rob J.M. van Spanning[b], Martin Braster[b], Rick Helmus[a], Pim de Voogt[a,c], John R. Parsons[a]

[a] Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1098, XH Amsterdam, the Netherlands
[b] Department of Molecular Cell Biology, Vrije Universteit, de Boelelaan 1108, 1081, HZ Amsterdam, the Netherlands
[c] KWR Watercycle Research Institute, Nieuwegein, the Netherlands

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ABSTRACT

Metformin (MET) is a pharmaceutical product mostly biotransformed in the environment to a transformation product, guanylurea (GUA). In ready biodegradability tests (RBTs), however, contrasting results have been observed for metformin. The objective of this study was to measure the biodegradation of MET and GUA in RBTs, using activated sludge from the local wastewater treatment plant, either directly or after pre-exposure to MET, in a chemostat. The activated sludge community was cultivated in chemostats, in presence or absence of MET, for a period of nine months, and was used in RBT after one, three and nine months. The results of this study showed that the original activated sludge was able to completely remove MET (15 mg/l) and the newly produced GUA (50% of C_{0MET}) under the test conditions. Inoculation of the chemostat led to a rapid shift in the community composition and abundance. The community exposed to 1.5 mg/l of MET was still able to completely consume MET in the RBTs after one-month exposure, but three- and nine-months exposure resulted in reduced removal of MET in the RBTs. The ability of the activated sludge community to degrade MET and GUA is the result of environmental exposure to these chemicals as well as of conditions that could not be reproduced in the laboratory system. A MET-degrading strain belonging to the genus *Aminobacter* has been isolated from the chemostat community. This strain was able to completely consume 15 mg/l of MET within three days in the test. However, community analysis revealed that the fluctuation in relative abundance of this genus (< 1%) could not be correlated to the fluctuation in biodegradation capacity of the chemostat community.

1. Introduction

Pharmaceuticals, such as metformin (MET), are prevalent compounds in wastewater (Briones et al., 2016). Metformin is a product used in the treatment of type 2 diabetes and widely distributed in European wastewater and surface water. In wastewater, an average concentration of about 60 µg/l was measured in ten different wastewater treatment plants (WWTPs) in Germany (Scheurer et al., 2012, 2009; Tisler and Zwiener, 2018), while in fresh water, concentration from 1 to 8 µg/l were reported in Belgian and Dutch rivers (ter Laak et al., 2014). Metformin is commonly degraded by conventional biological treatment in WWTPs. However, biotransformation of metformin leads to the formation of a recalcitrant metabolite termed guanylurea (GUA) (Trautwein and Kümmerer, 2011). Furthermore, contrasting results in ready biodegradability testing (RBTs) have been reported for metformin. Tests used to determine the ready biodegradability of a compound, such as the guideline from the Organisation for Economic Cooperation and Development (OECD), allow a fast categorization of the tested chemical from readily biodegradable to persistent. A readily biodegradable compound is expected to be rapidly eliminated from the aquatic aerobic environment (ECETOC, 2013; OECD, 2014). However, a chemical that is classified based on these tests as readily biodegradable may in practice remain in the environment longer than expected, while one that has been classified as persistent may be eliminated. This is because biodegradation of a chemical in the environment is dependent on several environmental factors and on the presence and activity of degrading microbial communities (Painter, 2002), which are not mimicked in RBTs.

Hence, even if metformin can be biodegraded in WWTPs, it was still classified as not readily biodegradable in the Closed Bottle test (OECD 301D), while it showed signs of elimination in the Manometric Respiratory test (OECD 301F) with degradation up to 48.7% in only one

* Corresponding author. Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Science Park 904, 1098, XH Amsterdam, the Netherlands.
E-mail address: B.A.J.Poursat@uva.nl (B.A.J. Poursat).

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of three bottles and it was partially degraded in the Zahn-Wellens test (OECD 302B), with 51.3% and 49.9% of elimination in both replicates (Trautwein and Kümmerer, 2011). However, different sources of inoculum were used to perform these tests, which might have contributed to the variability of the results (Trautwein and Kümmerer, 2011). Variability in test results due to differences in the inocula is one of the well-known problems that affect the conclusion of biodegradability testing (Federle et al., 1997; Kowalczyk et al., 2015; Vázquez-Rodríguez et al., 2007). Metformin appears to be quickly degraded through biological treatment in WWTPs, and a recent study shows that guanylurea can be completely degraded as well (Briones et al., 2018). However, very little is known about the effect of long-term exposure to metformin on microbial communities and their ability to degrade metformin or guanylurea. It might be assumed that an inoculum that has been exposed to metformin or guanylurea may be more efficient in degrading these compounds compared to a non-exposed community. Adaptation through exposure to chemicals has been described in the literature (Itrich et al., 2015; Poursat et al., 2019). However, implications of these adaptation processes on the biodegradation abilities of heavily exposed communities remains poorly understood, and deserves further investigation (Blok, 2000). In the present study we investigated the effect of long-term exposure to metformin in a chemostat culture on the ability of a microbial community to degrade this compound. Continuous culture systems, such as chemostats, are used to mimic and reproduce the adaptation process, in a simplified and controlled environment. The capacities to degrade metformin by an activated sludge community either directly or after exposure to metformin, were compared using the OECD 310 guideline. Community dynamics were analyzed by 16s rRNA gene amplicon sequencing on the Illumina platform. This study will help to understand the implication of long term exposure of activated sludge microbial communities to emerging contaminants and their transformation products, and may help to develop a more robust RBTs. As mentioned previously, adaptation to environmental contaminants has been shown to modify the RBTs results, however, there is a lack of information about the community dynamics and evolution during this adaptation period. Furthermore, investigating the biodegradation of metformin and guanylurea by activated sludge microbial communities will help to understand their environmental fate and improve their treatment in WWTP. Finally, to our best knowledge, metformin degrading microorganisms remain unknown and need to be identified.

2. Materials and methods

2.1. Sampling site

Activated sludge was sampled from the aeration tank of the local wastewater treatment plant of the city of Amsterdam (Amsterdam West, The Netherlands) in November 2016. Before use, the sampled sludge was transferred to a shaker (150 rpm) at room temperature (22 ± 1 °C) for 1 week. Dry matter content of the sludge sample was determined by filtration of a known volume through a 0.2 µm membrane filter. The filters were dried overnight in an oven at 70 °C and the dry matter was determined by comparing the filter weight before and after filtration. The activated sludge sample used to inoculate the chemostats and the biodegradability tests had a dry matter content of 3.1 g/l, a pH of 7.34 and contained 2.37 * 10^{07} colony forming units (CFU)/ml (± 8.15 * 10^{06}).

2.2. Chemicals

Metformin hydrochloride (CAS Number 1115-70-4) (MET.HCl) and N-guanylurea sulfate salt hydrate (CAS Number, 207300-86-5) (GUA) were purchased from Sigma-Aldrich. Metformin was of pharmaceutical standard while guanylurea had a 97% purity grade. Working and standard solutions were prepared in ultra-pure ELGA water (ELGA MK2-Analytic, Ede, the Netherlands) and stored at 4-8 °C. All solvents used for sample pretreatment and chromatographic separations had an analytical or LC-MS grade and were purchased from Biosolve, Valkenswaard, the Netherlands.

2.3. Chemostat systems

To cultivate and expose the activated sludge community to metformin, two chemostat systems were operated for a total duration of 301 d. Both chemostat contained a similar sterile growing medium. The medium contained several salts, carbon and nitrogen sources and vitamins: 128 mg/l KH_2PO_4; 326 mg/l K_2HPO_4; 501 mg/l $Na_2HPO_4.2H_2O$; 19.5 mg/l NH_4Cl; 2.63 mg/l $FeCl_3.6H_2O$; 0.34 mg/l $ZnCl_2$; 0.24 mg/l $CoCl_2.6H_2O$; 0.09 mg/l $CuCl_2.2H_2O$; 0.03 mg/l H_3BO_3; 0.24 mg/l $Na_2MoO_4.2H_2O$; 36.4 mg/l $CaCl_2.2H_2O$; 22.5 mg/l $MgSO_4.7H_2O$; 0.33 mg/l pyrodoxine HCl; 0.17 mg/l riboflavine; 0.17 mg/l thiamine HCl; 0.17 mg/l cyanobalamine; 0.17 mg/l nicotinamide; 0.17 mg/l *p*-aminobenzoic acid; 0.17 mg/l lipoid acid; 0.17 mg/l pantothenic acid; 0.07 mg/l d-biotin; 0.07 mg/l folic acid; 40 mg/l sodium acetate; 1.5 mg/l yeast extract. The medium used to feed the chemostat exposed to metformin (chemostat MET), contained a nominal concentration of 1.5 mg/l MET.HCl. The second chemostat was used as a control and was only fed with growth medium without added substance (chemostat Blank). Stock solutions of vitamins, metformin and carbon sources were prepared using ultra-pure ELGA water and directly added to the autoclaved medium, under sterile condition, using 0.2 µm silicone membrane filter (Whatman plc). Cultures were maintained in the dark at 22 °C under constant stirring system (300 rpm). 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 of the effluent was regularly measured with a pH probe and maintained at 7.4. pH variation did not exceed 0.4 during the whole duration of the experiment. A constant bubbling system using filtered (0.2 µm PTFE membrane) compressed air provided oxygen to the culture. Chemostats were directly inoculated with 5 ml of secondary activated sludge, in order to obtain an inoculum density of 30 mg/l of dry matter. The flow of the medium influent (20 ml/h) was controlled by a Gilson Minipuls 3 peristatic pump to maintain a dilution rate of 0.04 h^{-1}. Every weeks, samples of the culture were taken from the sampling port, under sterile condition, using a 10 ml sterile syringe. Samples were immediately placed at −20 °C and stored until preparation and analysis. The microbial communities cultivated in chemostat were used as inoculum for a biodegradability test after 1 (34 d), 3 (95 d) and 9 months (280 d) of exposure.

2.4. Biodegradability testing

Biodegradation tests were performed in batch cultures following the OECD 310 ready biodegradability test protocol (OECD, 2014). During these tests, biodegradation was followed by the direct quantification of the compounds by LC-MS/MS. MET was dissolved at a nominal concentration of 15 mg/l in the mineral buffer medium buffered at pH 7.4. Mineral medium was prepared according to the guideline. Bottles were directly inoculated by adding 10^4–10^5 CFU/ml (colony forming units) of activated sludge or chemostat samples, after 1, 3 and 9 months of culture. CFU were determined on nutritive agar by serial dilution, after 48 h of incubation at room temperature. Biodegradation testing was performed in the dark at 22 °C and under constant shaking (150 rpm) for a period of 28 d.

Data analyses of the biodegradation tests were performed using Excel and R (R core team, 2016). 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 produced data and the

determine the different DT (degradation time) values, as described in Ritz et al. (2015).

2.5. LC-MS/MS analysis

To quantify metformin and guanylurea in liquid samples, a LC-MS/MS method was established using a Shimadzu LC20 instrument (Shimadzu, Kyoto, Japan), equipped with a SIL-20ACXR autosampler and connected in series with a Sciex 4000 QTRAP MS/MS system (AB SCIEX, MA, USA). Separation was performed on a HILIC column (1.7 µm, 50 × 2.1 mm, amide BEH, Waters B.V., Etten-Leur, the Netherlands) with a securityGuard ultra-cartridge as guard column (Waters B.V., Etten-Leur, the Netherlands). The column oven was set at 35 °C and injection volume was 20 µl. Two pumps (model LC-20ADXR) were used to create a gradient of two solvents and the flow rate was of 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. Ionization was performed using electrospray ionization source (ESI, Turbospray) operating in positive mode. Multiple-reaction monitoring mode (MRM) was applied with the first most sensitive transition (TR1) used for quantification and a second transition (TR2) used for qualitative confirmation. All of the MRM parameters can be found on 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 V, Temperature: 500 °C, Ion source gas 1: 40 l/h and ion source gas 2: 50 l/h.

For the preparation of liquid sample, 1 ml of medium was collected and immediately frozen at −20 °C in a 1.5 ml LC vial until use. Before analysis, samples were diluted 100 times in acetonitrile and then 10 times in ultra-pure ELGA water. Diluted samples were thenfiltered using a 0.2 µm polypropylene filter (Filter-BIO, China) and transferred to a 1.5 ml glass LC vial and store at 4 °C until injection. Calibration standards (0.5–20 µg/l) of both compounds were prepared and injected in order to quantify the substances in the samples. Standards were prepared identically to the samples. During the analysis, analytical blanks and calibration standards were injected before and in-between the samples at regular interval as quality control. Data acquisition and analyses were performed with AB SCIEX Analyst software (Ver. 1.5.1).

2.6. DNA extraction and PCR amplification

DNA samples were extracted and purified using the Mo Bio PowerSoil kit (QIAGEN Benelux B.V.), following the supplier's protocol. Samples were stored at −80 °C until analysis. DNA concentration was determined using the Qubit dsDNA HS Assay kit (Thermo Fischer Scientific, Waltham, MA USA) and a Qubit fluorometer (Thermo Fischer Scientific, Waltham, MA USA). The used primers target the V3-v4 region of bacterial 16S rRNA genes and yielded a ~550 bp fragment during PCR amplification. The PCR program used for amplification can be found in the supplementary material (Appendix A). PCR products were purified using an Agencourt AMPure XP magnetic bead (Beckman Coulter Nederland B.V.). Agarose gel was used to control the amplification and purification step. Samples were diluted to 1 ng/µl and pooled prior analyze by Illumina MiSeq.

2.7. 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 were assign to every OTU at 97% sequence identity using Uclust. Chimera were filter 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 phylogenic tree produced by QIIME were imported to R (R core team, 2016) using the R Phyloseq package (McMurdie and Holmes, 2013). Subsequent analyses and data visualization were then performed using the R packages Phyloseq (McMurdie and Holmes, 2013), Vegan (Oksanen et al., 2017), DeSeq2 (Love et al., 2014a) and Ggplot2 (Wickham, 2016).

Parameters and index of richness and diversity, such as the Shannon index and the observed richness were calculated using the R package Phyloseq (McMurdie and Holmes, 2013) and Vegan (Oksanen, J., et al., 2017). A variance-stabilizing transformation was applied to the data using the R packages Phyloseq (McMurdie and Holmes, 2013) and Deseq2 (Love et al., 2014b) before performing the cluster analyses (beta diversity) and differential abundance analysis. Beta diversity was measured using weighted Unifrac (Lozupone and Knight, 2005) and Bray Curtiss dissimilarity. In a second analysis, transformed data were used to measure the differential abundance of taxa over time. A taxon or OTU is considered as differentially abundant if its mean proportion is significantly different ($p < 0.05$ or $p < 0.01$) between sample classes (McMurdie and Holmes, 2014), in our case the time of cultivation in chemostat in comparison to the original community.

2.8. Isolation and identification of degrading bacteria

In addition to the biodegradability tests, microorganisms that were able to consume MET were isolated and identified using samples from chemostat MET as inocula for enrichment cultivation. Incubation flasks (300 ml) were autoclaved and filled, under sterile condition, with 90 ml of sterile MET mineral medium and 10 ml of inoculum. Mineral medium was prepared according to the OECD 310 guideline and MET was added, as sole source of carbon, in order to obtain a final concentration of 100 mg/l. The medium was sterilized through filtration (0.2 µm). Cultivation was performed aerobically in the dark at 22 °C and under constant shaking (150 rpm). Every 15 days, 10% of the enriched culture was inoculated into a fresh medium. Serial 10-fold dilutions were dispensed on a nutrient agar medium and incubated aerobically at 22 °C. The growing colonies were isolated by repeated streak culturing. After repeated selection, only one strain was able to grow in liquid medium in presence of MET as sole source of carbon. This strain was identified by amplification of the 16s rRNA gene sequences using the primers 8F (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1512R (5′-ACGGYTACCTTGTTACGACTT-3′). The PCR product was sequenced by Macrogen, Inc. (Amsterdam, The Netherlands). Obtained sequences were aligned and compared with the GenBank database using the BLAST program. Aligned sequences can be found in appendix C.

The biodegradation capacity of the isolated strain was assessed following a standard biodegradation test as described above. Each test

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

Chemicals	Precursor ion (m/z)	Declustering potential (V)	Quantifier		Qualifier	
			Product ion (m/z)	Collision energy (V)	Product ion (m/z)	Collision energy (V)
Metformin	130	47	71	32	60	19
Guanylurea	103	37	86	13	60	16

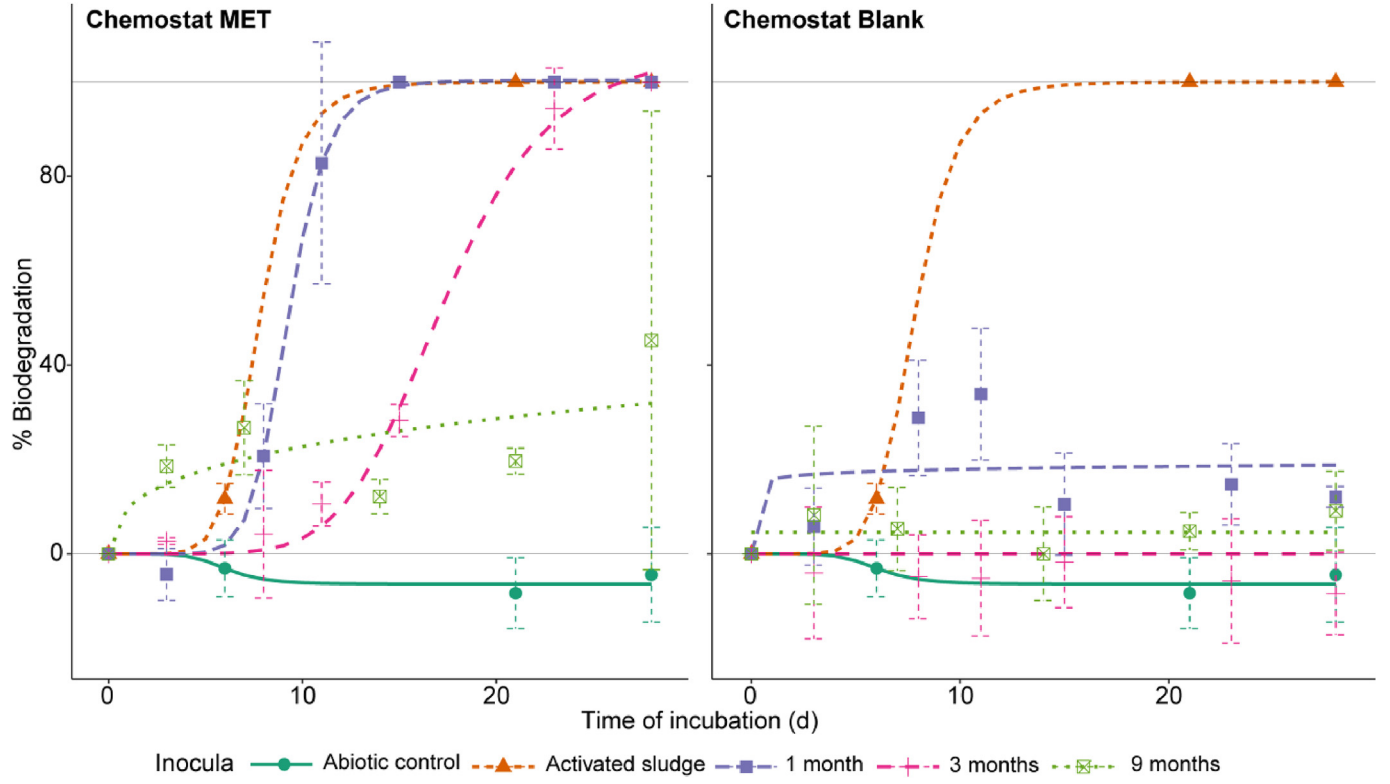

Fig. 1. Biodegradation of metformin (C/C0) by communities pre-exposed (left) and not pre-exposed (right) to MET in biodegradation tests (measured by LC-MS/MS). Activated sludge was not pre-exposed but is represented in both figure (dotted orange line), as well as the abiotic control (full green line), to facilitate comparison, Lines show the fitted model (three-parameter logistic) for each condition. Points and shapes represent the average elimination from each conditions and error bars correspond to the standard deviation of the three replicates used to calculated the average biodegradation in each condition.

flask (n = 3) was inoculated with a single fresh colony. MET degradation and GUA formation were followed by LC-MS/MS analysis.

3. Results

3.1. Biodegradation of metformin

In the RBTs, metformin was measured by LC-MS/MS and compared to the original measured concentration (15 mg/l) in order to calculate a percentage of biodegradation over time (Fig. 1). No elimination was observed in the sterile abiotic control, indicating that metformin was not transformed or degraded by an abiotic reaction. Complete primary degradation was found in every bottle inoculated with activated sludge, that was not pre-exposed, and with the chemostat community pre-exposed to metformin for one and three months (Fig. 1, left panel). Divergence among the test bottles (n = 3) was observed when inoculated with the community pre-exposed for nine months. Almost complete degradation was observed in one of the bottles (99.9%), while the second one reached 28% of biodegradation and the last one only 7%. Incomplete primary degradation was observed in the tests inoculated with the chemostat community that was not pre-exposed to metformin (Fig. 1 right panel). The measured concentrations indicated an average variation of 12 ± 2% (1 month), 8 ± 9% (3 months) and 9 ± 8% (9 months) after 28 days, in comparison to the original measured concentration. Hence, only the test performed after 1 month of cultivation showed indications of degradation (> 10% according to OECD guideline) at the end of the test.

The community from activated sludge was the fastest to completely degrade metformin (Table 2), with the shortest lag-phase (DT10 = 5.9 d). The next fastest inoculum was the chemostat community after one month of exposure, followed by the same community after three months of exposure. The large variability in the biodegradation test replicates inoculated with the community pre-exposed for nine months did not allow us to calculate the degradation time (DT) for 10%, 50% and 90% removal.

Table 2
Calculated times (days) to reach 10%, 50% and 90% of degradation, based on a log-logistic model with three parameters. DT values were only calculated for the pre-exposed communities.

	DT10	DT50	DT90
Activated sludge	5.9	7.3	9
1 month pre-exposed	7.3	9.3	11.8
3 months pre-exposed	12.2	17.3	24.6
9 months pre-exposed			

3.2. Transformation to guanylurea

For each sample, metformin and guanylurea were measured simultaneously as both compounds could be detected and quantified individually using the LC-MS/MS method. Invariably, every time metformin degradation was observed, guanylurea could be quantified in the test. On average, guanylurea production reached a peak of approximately 80% of the theoretical maximal production, confirming that guanylurea was the main biotransformation product of metformin (Fig. 2). With the exception of the test after 9 months of exposure, every other test showed signs of elimination of guanylurea when metformin was completely consumed. In the test performed with activated sludge as inoculum, both compounds completely disappeared from the three replicates after 28 d, while in the tests performed after one and three months of exposure, metformin and guanylurea were completely eliminated in only one and two out of the three incubation flasks, respectively. In the remaining flasks, guanylurea could still be quantified,

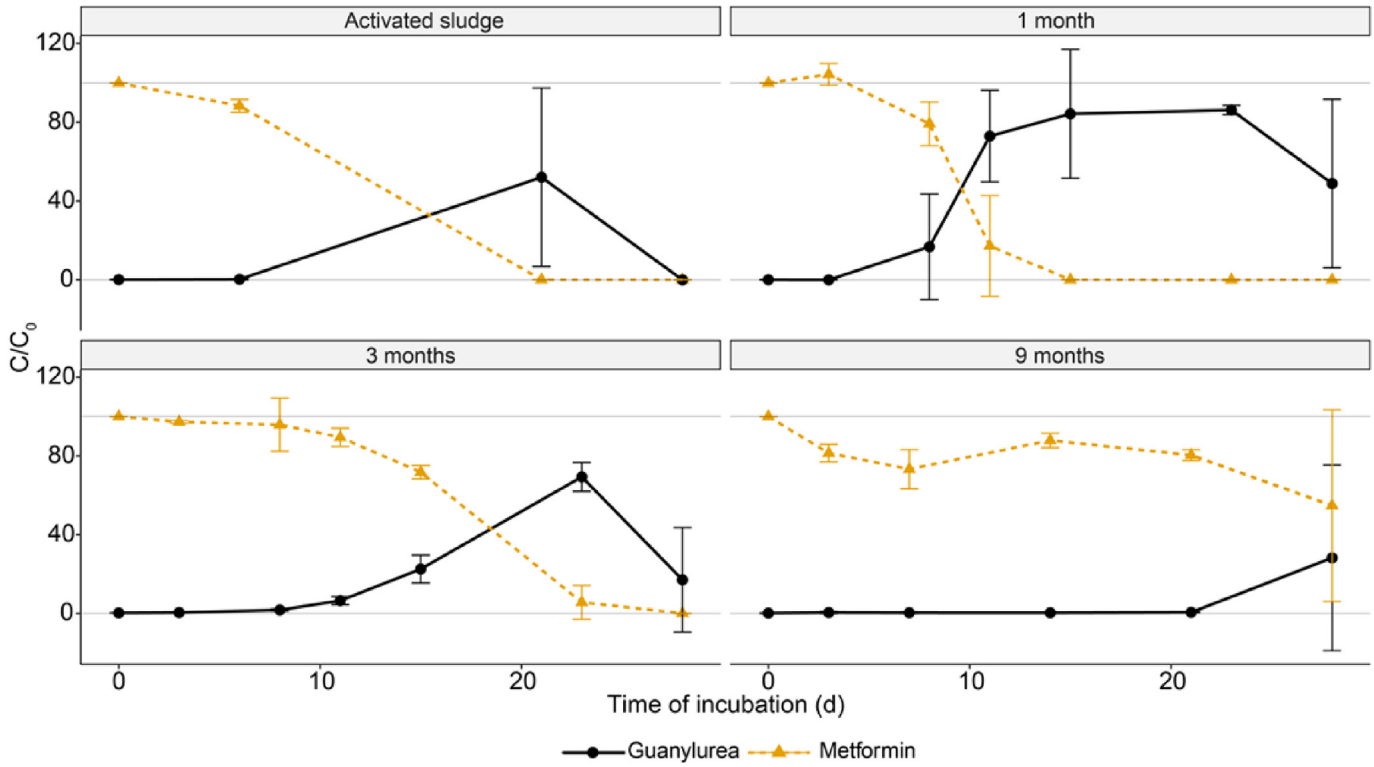

Fig. 2. Transformation of metformin to guanylurea. Metformin degradation (%) is based on the measured concentration in sample divided by the original measure concentration (C/C_0). Guanylurea percentage (%) is based on the theoretical maximal transformation of metformin to guanylurea, at the original measured concentration of metformin.

although partial elimination of the compound had occurred. The fate of the transformed GUA is unknown, as we did not attempt to measure mineralization or possible GUA transformation products by non-targeted or suspected target analyses.

3.3. Community dynamics

Operational taxonomic units (OTUs) were selected and filtered from the sequences produced by the Illumina MiSeq system. OTUs with read number lower or equal to 3 in the whole data set were discarded, reducing the total number to 5666 OTUs.

The Shannon index and observed richness were the two alpha diversity indexes used in this study (Appendix B). Anova and Kruskal Wallis tests that were performed to measure the variation of the alpha diversity indices of each chemostat over time do not report any significant ($p > 0.05$) temporal variation of each chemostat community.

Weighted UniFrac patterns (Fig. 3, left panel) clearly show that both communities vary over time and differ from each other, despite the fact that both chemostat were inoculated with the same inoculum. For the non-exposed chemostat Blank, no clear cluster can be observed, with the exception of the samples taken at the beginning of the experiment just after inoculation. The community started to diverge quickly after inoculation, without a clear pattern. Each sample from chemostat MET, clearly clusters over time, indicating a continuous evolution of the community. However, the last time points seem to group together, which might indicate a stabilization of the community after 200 d of growth in chemostat. These observations are supported by the NMDS (Fig. 3, right panel), displaying the Bray-Curtis dissimilarity of both communities. Communities cluster separately, and as is the case for the weighted UniFrac PCoA, chemostat Blank diverged from the original inoculum without a clear pattern, while chemostat MET continuously evolved.

PERMANOVA and ANOSIM analyses were used to confirm whether both communities were divergent from each other and in time. Significant differences ($p < 0.001$) were observed between the communities with both PERMANOVA and ANOSIM ($R = 0.3499$). Moreover, the dispersion test confirms that the divergences of centroid are not due to the data dispersion within the conditions. Furthermore, communities were tested individually to confirm that the temporal variations were significant. As mentioned above, each community diverged significantly over time ($p < 0.001$) (ANOSIM: chemostat Blank ($R = 0.9025$), chemostat MET ($R = 0.8174$), indicating a significant and unique evolution of communities over time.

The R packages DeSeq2 and Phyloseq were used to produce the data shown in Fig. 4A, which display the different taxa whose abundances significantly ($p < 0.05$) changed over time in chemostat MET. Taxa with a log2foldchange > 0 were enriched over time, while if log2foldchange < 0, they are considered as eliminated from the community, as their abundance significantly decreased.

Bacteria from the genera *Acinetobacter*, *Pelomonas* and an unknown genus from the *Gammaproteobacteria* class disappear over time from the community pre-exposed to MET (Fig. 4B). In contrast, all of the other taxa are more abundant after 9 months than during the rest of the experiment. Moreover, it should be noted that the p value use to identify taxa with a differential abundance is different for chemostat Blank as for chemostat MET no taxa are significantly divergent over time with a p value < 0.01. In chemostat Blank, a total of 61 OTUs have a negative or a positive differential abundance over the time of cultivation, based on the log2FoldChange ($p < 0.001$) shown in Fig. 5A. Among these OTUs, 27 OTUs that are enriched (log2FoldChange > 0), are mostly from the class *Betaproteobacteria*. Most of these OTUs, are absent or undetectable in the original inoculum, but they became predominant after three and nine months of cultivation in the chemostat (Fig. 5B). The 34 remaining OTUs are outcompeted over time (log2FoldChange < 0) (Fig. 5C), and are a majority from the class *Betaproteobacteria* and *Gammaproteobacteria*. In addition, among the *Gammaproteobacteria*, *Pseudomonas* and *Acinetobacter* are the two dominant genera that were outcompeted in the community. The other outcompeted

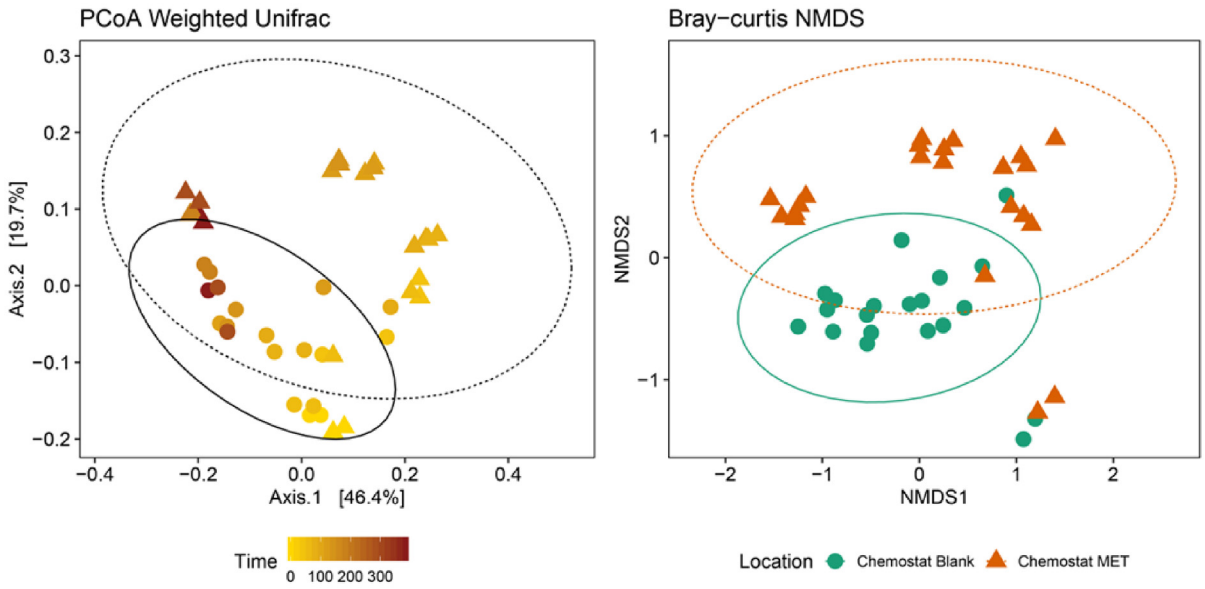

Fig. 3. (Left) PCoA ordination of the different communities over time, using a weighted Unifrac distance method. The gradient of color shown in the legend represents the time (d) since the inoculation of the chemostats with activated sludge. (right) NMDS ordination of the Bray-Curtiss dissimilarity between communities. Confidence ellipses level at 0.95.

OTUs are from the classes *Bacilli* and *Alphaproteobacteria*.

3.4. Isolation and identification of metformin-degrading bacteria

The chemostat pre-exposed to MET was the source from which a strain was isolated (see the methods section for details). This strain was cultivable on nutrient agar medium at 22 °C and was shown to be able to grow in liquid medium by using MET as sole carbon and energy source. Based on the comparison of the 16s rRNA gene sequence (Appendix C) with the GenBank database, this strain is identified as *Aminobacter anthyllidis* (98%). Other potential candidates producing significant alignment can be found in the supplementary material (Appendix C). All of these candidates are member of the family *Phyllobacteriaceae* and are gram-negative soil bacteria.

Biodegradation tests using the isolated strain as inoculum shows complete transformation of MET to GUA within 3 d (Appendix C). One colony was used as initial biomass during the inoculation and MET had an initial nominal concentration of 15 mg/l. MET was completely transform to GUA within 3 d, however, GUA is not further degraded and remained in the medium.

4. Discussion

Contrasting data are available in the literature about the biodegradability of MET in OECD tests (Markiewicz et al., 2017; Straub et al., 2019), with results ranging from no biodegradation at all to complete elimination within 28 d. In the present study, metformin was completely removed in batch OECD 310 RBTs by the activated sludge microbial community that we used as an inoculum. Similar results were reported in the literature (Briones et al., 2018; Scheurer et al., 2012; Trautwein and Kümmerer, 2011), which confirm that activated sludge microbial communities are competent to transform MET to GUA under laboratory condition. This culture was also able to degrade MET after 1 and 3 months of continuous culturing in a chemostat culture exposed to MET, but without significant enhancement of degradation rates. Moreover, loss of biodegradation ability was observed after 9 months of exposure in two out of the three replicate RBTs. In this last test, variation between the test replicates (n = 3) was higher than in the other test, as only one of the replicates reached 100% of elimination, while the two other remained below 20%. This absence of biodegradation activity by two of the replicates, after nine months, and the slower biodegradation rate observed after three months, indicate that the relative and/or absolute number of competent MET degraders progressively decreased over time in chemostat MET. Recent studies showed degradation of GUA using activated sludge as inoculum under both aerobic (Briones et al., 2018; Straub et al., 2019) and anaerobic conditions (Tisler and Zwiener, 2019), demonstrating that one or more degradation pathways have evolved in certain types of bacteria, while this compound was previously considered as persistent (Trautwein and Kümmerer, 2011). In the present study, GUA was eliminated from biodegradation tests inoculated with activated sludge and with the chemostat community exposed to MET. Efficient biodegradation of GUA occurred after all of the MET was consumed. As reported recently elsewhere (Straub et al., 2019), we observed that if the transformation of MET starts within the two first weeks of the biodegradation test, then GUA elimination will follow almost immediately after complete removal of MET. Currently, we cannot conclude whether GUA was metabolized to CO_2 or transformed to another product (Tisler and Zwiener, 2019), as the fate of the degraded GUA was not investigated in this study. Long-term exposure of the community to MET did not appear to influence the overall biodegradation of GUA in the tests. Nevertheless, based on a recent investigation, exposure of a microbial community from activated sludge to GUA could influence its biodegradability (Tisler and Zwiener, 2019).

As mentioned previously, the presence of competent degrading microorganisms is a key factor for the removal of organic chemicals. The original activated sludge community used in our project had already been exposed to MET and GUA. Although the exact level of contamination of the sampled activated sludge is unknown, other measurements of Dutch rivers reported concentrations up to 8 µg/l for MET and 50 µg/l for GUA (ter Laak et al., 2014), while concentration in Dutch wastewater treatment plant was predicted to reach 80 µg/l for MET and 46 µg/l for GUA (Oosterhuis et al., 2013). Hence, most activated sludge communities, from countries in which MET is consumed, are probably already adapted to these chemicals and even long-term exposure at non-environmentally realistic concentrations, as performed in our study, is unlikely to further enhance their biodegradation capacity in RBTs. In fact, we observed that long-term exposure may lead to

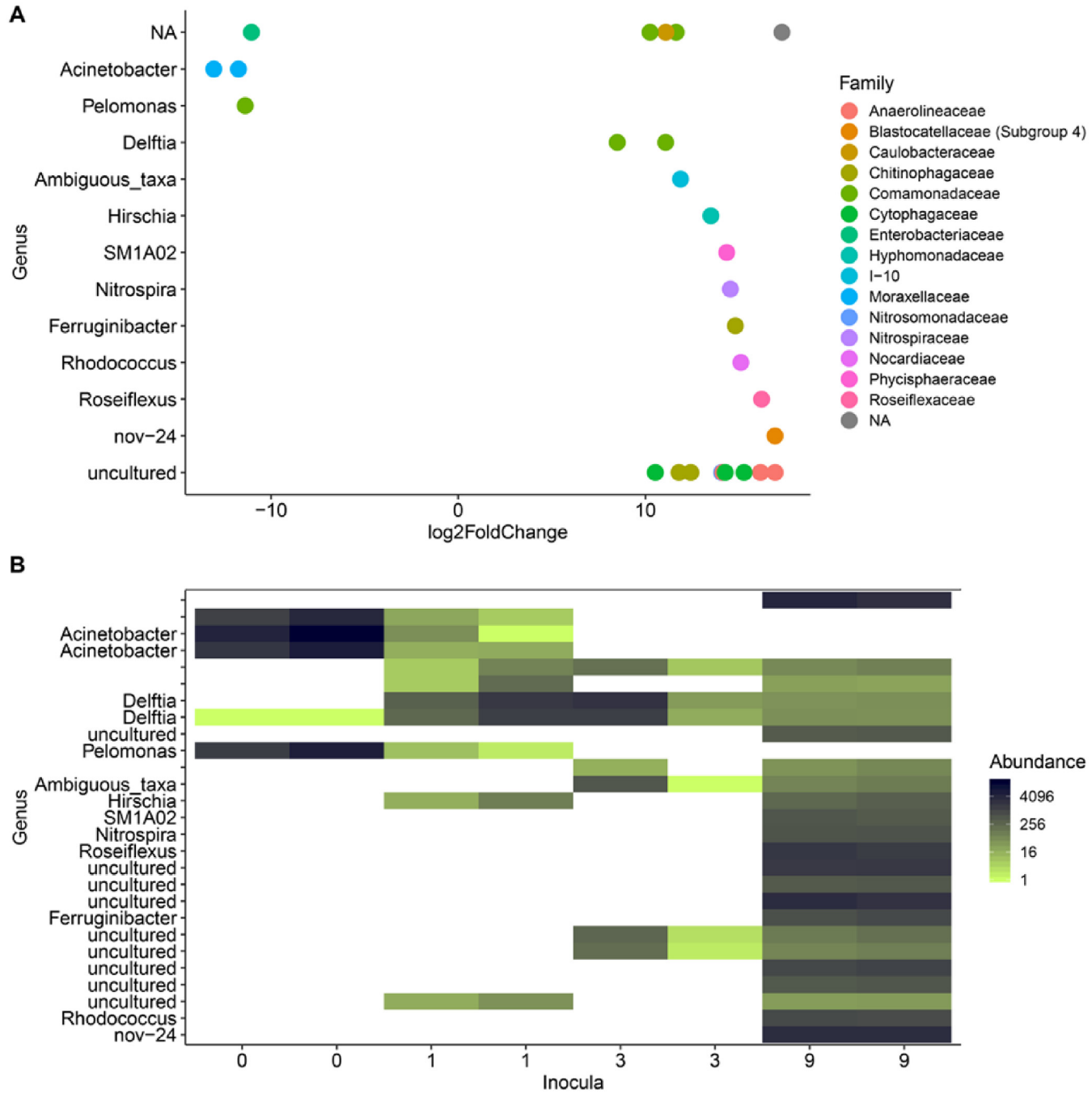

Fig. 4. (A) Differential analysis representing each genus whose abundance changed significantly ($p < 0.05$) over time from the original inoculum (0), in chemostat MET. Log2FoldChange is the log-ratio of observed differences between the tested sample and the original inoculum. Genera with a Log2FoldChange > 0 were enriched in comparison to the original inoculum while a Log2FoldChange < 0 indicates that the abundance of the genus decreased. Colors represent the class of the presented genus. (B) Heat map representing the abundance of each genus displayed in (A). Inocula represent the time of exposure used in biodegradation testing (0, 1, 3, 9 months).

loss of biodegradation capacity. This loss of biodegradation capacity can be the result of several interconnected mechanisms related to the nature of chemostat systems and inter-species competition. Differential analyses of OTUs from the chemostat after 9 months of exposure to MET revealed an enrichment of different taxa that may not be able to degrade MET in RBTs and may have out-competed MET-degraders, due to competition for the micro-nutrients.

The inoculum density and the fraction of efficient degraders introduced into the test are two parameters that may influence the outcome of biodegradation testing and the difference between replicates (Martin et al., 2017). In our study, bacterial density was determined by CFU counting, and approximately the same amount of CFUs was inoculated in the test bottle. However, the fraction of cultivable microorganisms in the community may have changed over time and thus modified the absolute number of efficient degraders in the community, thereby decreasing the chance of introducing them into the test bottles. Although our data do not allow us to draw any community structure-function relationship, the population clearly changed between the different communities in which the MET degradation function remained in the presence of MET as a potential extra carbon source. The absence of

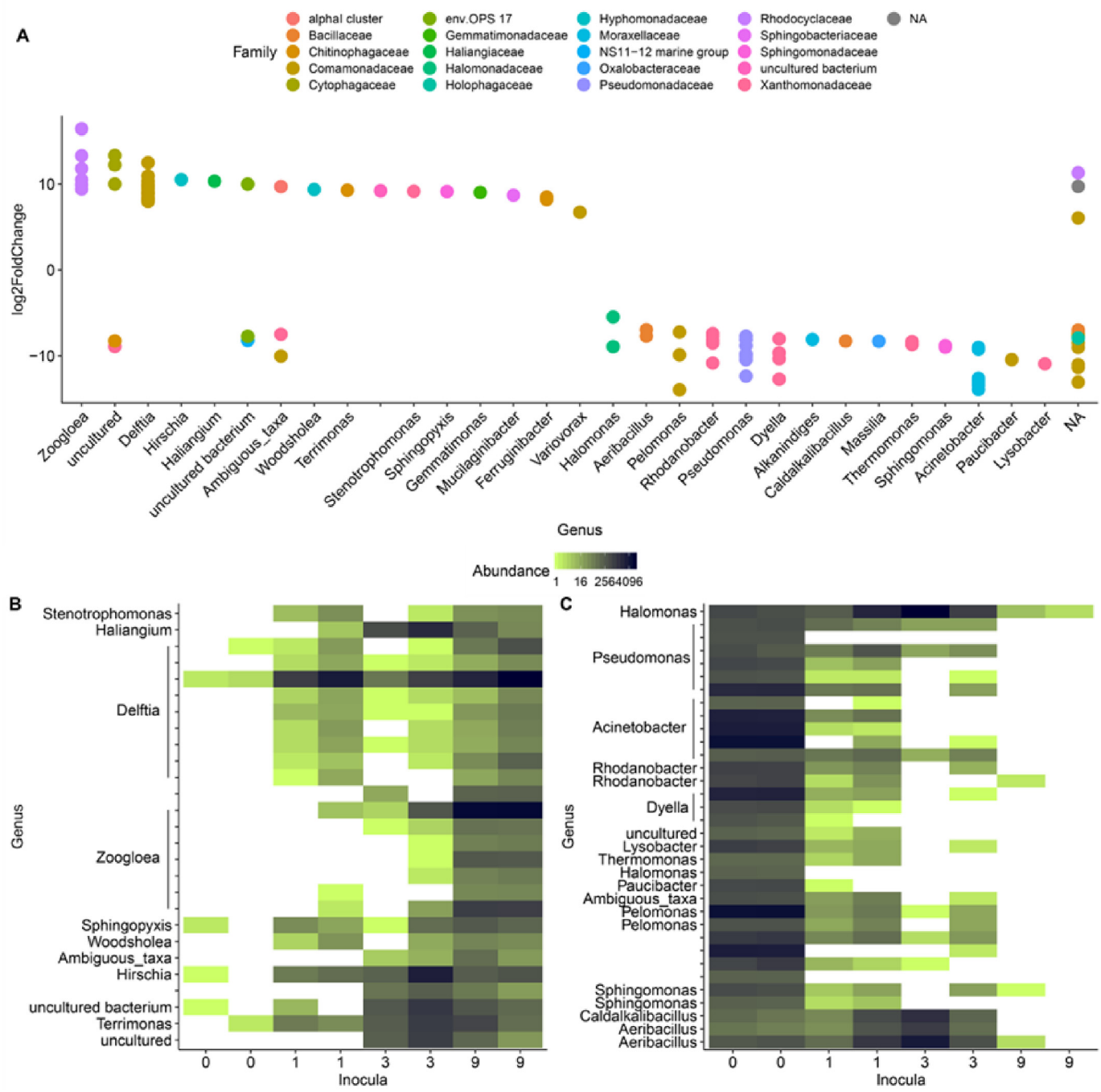

Fig. 5. (A) Differential analysis representing each genus whose abundance changed significantly ($p < 0.01$) over time from the original inoculum (0), in chemostat Blank. Log2FoldChange is the log-ratio of observed differences between the tested sample and the original inoculum. Genera with a Log2FoldChange > 0 were enriched in comparison to the original inoculum while a Log2FoldChange < 0 indicates that the abundance of the genus decreased. Colors represent the class of the presented genus. (B) Heatmap representing the abundance of each genus displayed in (A) with a Log2FoldChange > 0. (C) Heatmap representing the abundance of each genus displayed in (A) with a Log2FoldChange > 0. Inocula represent the time of exposure used in biodegradation testing (0, 1 month, 3 months 9 months).

MET in the medium resulted in a complete loss of the biodegradation capacity for MET after one month, apparently as a consequence of the shift of the community towards metabolism of remaining available carbon and free energy sources.

To our knowledge, there is no information in the literature on which microorganism or genes are involved in the transformation of MET. In the present study, a MET-degrading strain from the genus *Aminobacter* was isolated. This bacterium is able to completely transform MET to GUA, under RBT conditions, within 3 d. This strain belongs to the family *Phyllobacteriaceae*, some members of which are known for their nitrogen-fixing potential and for their biodegradation of micropollutants such as dichlorobenzamide (T'Syen et al., 2015). More sequencing should be conducted to truly identify and characterize this strain. Furthermore, based on our results, we cannot conclude whether only one taxon or a whole population was involved in the transformation of MET. Analysis of the community revealed that the fluctuation in relative abundance of this family could not be correlated to the fluctuation in biodegradation capacity, hence, other microorganisms may be involved in the transformation of MET as well.

There is a need to improve biodegradability tests to address the

unwanted variability and adaptation of microbial communities. Different cultivation techniques, such as semi-continuous culture and the use of omics techniques and non-pre-adapted communities, should be used to investigate the mechanisms of adaptation in the laboratory and in the field. A number of recent studies have already addressed some of these issues (Kowalczyk et al., 2015; Martin et al., 2017; Ott et al., 2019). However, pre-exposure in laboratory is probably not the most relevant method to include adaptation in biodegradability testing for chemicals that are present at high concentrations in the environment but may be suitable for investigating the processes involved in adaptation using novel omics techniques.

5. Conclusions

In the present study, we compared the capacity of an activated sludge microbial community from the Amsterdam West WWTP to degrade MET, and its main transformation product GUA, before and after long-term exposure in a chemostat system. The aim was to measure the biodegradation capacity of an activated sludge microbial community and to follow its evolution, in term of composition and function, after exposure to metformin. The original activated sludge was able to completely consume MET and GUA within 28 d under the OECD 310 biodegradability test conditions. (Pre-)Exposure in the chemostat led to a partial loss of the biodegradation function and after 9 months only one replicate out of three was able to completely transform MET to GUA. Cultivation in the chemostat in absence of MET quickly led to a complete loss of the biodegradation ability as well as a shift in the community composition and abundance compared to the exposed community. Adaptation to this compound is not only driven by its presence in wastewater, but also by various unknown environmental parameters, that will be difficult to mimic in laboratory test systems. A bacterial strain from the family *Phyllobacteriaceae*, and most likely from the genus *Aminobacter,* was isolated that was able to consume 15 mg/l of MET within 3 d. Extended work needs to be conducted to further identify and characterize this strain. Finally, the results of this study show that pre-exposure to a chemical already present at high concentrations in the environment does not enhance biodegradation in RBTs as activated sludge microbial communities are probably already adapted to such chemicals. Characterizing the inoculum, through multi-omic techniques, would be a more reliable method to predict and interpret the RBT's outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.109414.

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