

Effect of concentration and hydraulic reaction time on the removal of pharmaceutical compounds in a membrane bioreactor inoculated with activated sludge

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Summary

Pharmaceuticals are often not fully removed in wastewater treatment plants (WWTPs) and are thus being detected at trace levels in water bodies all over the world posing a risk to numerous organisms. These organic micropollutants (OMPs) reach WWTPs at concentrations sometimes too low to serve as growth substrate for microorganisms; thus, co-metabolism is thought to be the main conversion mechanism. In this study, the microbial removal of six pharmaceuticals was investigated in a membrane bioreactor at increasing concentrations (4–800 nM) of the compounds and using three different hydraulic retention times (HRT; 1, 3.5 and 5 days). The bioreactor was inoculated with activated sludge from a municipal WWTP and fed with ammonium, acetate and methanol as main growth substrates to mimic co-metabolism. Each pharmaceutical had a different average removal efficiency: acetaminophen (100%) > fluoxetine (50%) > metoprolol (25%) > diclofenac (20%) > metformin (15%) > carbamazepine (10%). Higher pharmaceutical influent concentrations proportionally increased the removal rate of each compound, but surprisingly not the removal percentage. Furthermore, only metformin removal improved to 80–

100% when HRT or biomass concentration was increased. Microbial community changes were followed with 16S rRNA gene amplicon sequencing in response to the increment of pharmaceutical concentration: *Nitrospirae* and *Planctomycetes* 16S rRNA relative gene abundance decreased, whereas *Acidobacteria* and *Bacteroidetes* increased. Remarkably, the *Dokdonella* genus, previously implicated in acetaminophen metabolism, showed a 30-fold increase in abundance at the highest concentration of pharmaceuticals applied. Taken together, these results suggest that the incomplete removal of most pharmaceutical compounds in WWTPs is dependent on neither concentration nor reaction time. Accordingly, we propose a chemical equilibrium or a growth substrate limitation as the responsible mechanisms of the incomplete removal. Finally, *Dokdonella* could be the main acetaminophen degrader under activated sludge conditions, and non-antibiotic pharmaceuticals might still be toxic to relevant WWTP bacteria.

Introduction

In the past years, sensitive mass spectrometry methods have enabled the detection of organic pollutants at very low concentrations in nearly all water bodies globally (Jameel *et al.*, 2020). These organic micropollutants (OMPs) include diverse chemicals such as pharmaceutical compounds, and they have detrimental effects on the development, behaviour and stress response of aquatic organisms (Gauthier and Vijayan, 2020). Pharmaceutical compounds are emitted into waste streams from different sources (i.e. hospitals, households, industries) and are transported via the sewer system to wastewater treatment plants (WWTPs) where they arrive at very low concentrations compared with more common pollutants such as ammonium and easily degradable organic matter. During the treatment, many of these pharmaceutical compounds are not eliminated from the water and end up in the environment, contaminating surface and groundwaters that may ultimately be used as drinking water sources for many organisms, including humans.

Most pharmaceuticals are hydrophilic compounds with low volatility and are removed to various degrees *via* microbial degradation. Other pharmaceuticals, such as

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fluoxetine, can also adsorb significantly to biomass (Fernandez-Fontaina *et al.*, 2012; Salgado *et al.*, 2012). Full biodegradation has only been reported in WWTPs for a few pharmaceuticals such as ibuprofen and acetaminophen, and several microorganisms have been implicated in their conversion (Žur *et al.*, 2018). On the other hand, many other pharmaceuticals are only partially or not removed at all in WWTPs (i.e. diclofenac, metoprolol). The removal differences between compounds are partially governed by the chemical structure's properties (Nolte *et al.*, 2018; Nolte *et al.*, 2020). For example, charged compounds are less biodegradable than neutral compounds probably due to a cellular uptake limitation (Nolte *et al.*, 2018; Nolte *et al.*, 2020). In addition to chemical properties, the higher the concentration of pharmaceuticals and other OMPs in the influent of WWTPs, the more these compounds seem to be removed (Nolte *et al.*, 2018; Wang *et al.*, 2020). The same compound can also have different removal rates/efficiencies in different WWTPs, which may depend on the operational parameters and the active microbial community present (Lautz *et al.*, 2017; Kassotaki *et al.*, 2018; Wang *et al.*, 2020). For example, nitrifying activities have been correlated with the biodegradation of several OMPs, which might be due to the promiscuity of the ammonia monooxygenase enzyme (Tran *et al.*, 2009; Fernandez-Fontaina *et al.*, 2012; Men *et al.*, 2017).

Currently, WWTPs are not very effective at removing OMPs because microorganisms do not share an evolutionary history with these compounds that have only been introduced into the environment in the last decades. Furthermore, evolution seems to be slow due to low concentrations and complexity in chemical structures. Recently, two studies described a putative evolution of acesulfame degradation in WWTPs all around the world (Kahl *et al.*, 2018; Kleinstauber *et al.*, 2019). Acesulfame was considered recalcitrant in WWTPs until 2014, when several studies started to observe good biodegradation of this compound. Since then, more reports showed the significant removal of this compound in WWTPs. In follow-up studies, microorganisms able to use acesulfame as carbon source were isolated (Kahl *et al.*, 2018; Kleinstauber *et al.*, 2019). This example demonstrates the ongoing adaptation of microorganisms towards OMP biodegradation in WWTPs. Mobile genetic elements (MGEs) have been previously suggested to be involved in microbial adaptation. MGEs may thus play a role in the biodegradation of xenobiotics or organic micropollutants in different environments such as soils and WWTPs (Top and Springael, 2003; Rios-Miguel *et al.*, 2020). Furthermore, previous studies found a correlation between pesticide exposure and MGE abundance in soils and agricultural WWTPs (Dunon *et al.*, 2013; Dealtry *et al.*, 2014). Consequently, MGE

abundance might also be correlated with the pharmaceutical concentration entering WWTPs and the subsequent adaptation to degrade OMPs.

Membrane bioreactors (MBRs) are known to perform well in the removal of pharmaceutical compounds (Prasertkulsak *et al.*, 2016; Goswami *et al.*, 2018), sometimes even better than conventional activated sludge systems (Reif *et al.*, 2011; Verlicchi *et al.*, 2012). MBRs are able to separate the solid retention time (SRT) and the hydraulic retention time (HRT), thus keeping slow-growing bacteria at high SRT while treating wastewater at regular HRT. In general, higher SRTs (with a maximum of 10–15 days) have been associated with better removal of pharmaceutical compounds (Clara *et al.*, 2005; Joss *et al.*, 2005; Achermann *et al.*, 2018; Nguyen *et al.*, 2020). For some compounds, however, extremely high SRTs are needed such as for diclofenac (more than 100 days) and metoprolol (60 days) (Clara *et al.*, 2005; Fernandez-Fontaina *et al.*, 2012; Gurung *et al.*, 2019). Previous studies also observed a positive correlation between HRT and the removal of some pharmaceuticals (i.e. fluoxetine) (Fernandez-Fontaina *et al.*, 2012). However, recent experiments and models suggested that pharmaceutical and other OMP removal might not improve beyond a specific HRT (Gonzalez-Gil *et al.*, 2018; Jiang *et al.*, 2018; Boonnorat *et al.*, 2019). Furthermore, Gonzalez-Gil *et al.* hypothesized that this might be due to an equilibrium in the transformation or sorption processes (Gonzalez-Gil *et al.*, 2018; Gonzalez-Gil *et al.*, 2019).

In order to get more insights about the (in)complete degradation of some pharmaceuticals in WWTPs, we have studied the effect of concentration and HRT on pharmaceutical removal in a MBR. We also determined the changes in the microbial community in response to higher pharmaceutical concentrations, and finally, we tested whether higher pharmaceutical concentrations resulted in higher relative abundance of specific MGEs previously correlated with pesticide occurrence and degradation. In our experimental setup, we inoculated a MBR with activated sludge from a Dutch WWTP and fed it with ammonium, acetate and methanol as main growth substrates. We kept all parameters constant except for the influent pharmaceutical concentration (4–800 nM) or HRT (1, 3.5 and 5 days). We monitored pharmaceuticals by liquid chromatography–mass spectrometry (LC-MS), microbial community changes by bacterial 16S rRNA gene amplicon sequencing and MGEs by qPCR.

Results and discussion

Bioreactor performance

The bioreactor performance was monitored throughout the whole experiment using a complementary array of

methods (Table S3). Total suspended solids (TSS) were measured every week (Fig. S1) and were stable at $0.2\text{--}0.3\text{ g l}^{-1}$ after the start-up period. Only when the HRT was reduced to 1 day, TSS increased to 0.9 g l^{-1} . Activated sludge was diluted around $100\times$ times when inoculated to the bioreactor so that volatile suspended solids (VSS) were similar to TSS (data not shown). Ammonium, acetate and methanol were fully consumed at all measured times (Fig. S2). Nitrate was measured in the effluent at concentrations between 500 and $800\text{ }\mu\text{M}$, indicating that ammonium was partly converted to nitrate during the experiment. The remaining ammonium was likely assimilated. When HRT was reduced to 1 day, nitrite slightly accumulated ($\sim 50\text{ }\mu\text{M}$) and nitrate concentration considerably increased in the effluent ($\sim 1.6\text{ mM}$). Since HRT was shortened from 5 days to 1 day in the short period of 6 days, the accumulation of nitrite and nitrate could indicate an imbalance between ammonia and nitrite oxidizers and a decreased use of nitrate for assimilation, respectively. Pharmaceuticals, 16S rRNA gene amplicons and mobile genetic element (MGE) monitoring are addressed in the next sections.

Pharmaceutical removal at increasing concentrations

After the start-up period, the bioreactor was close to a steady state where all variables remained stable throughout the operating period: temperature, dO_2 , pH, TSS, HRT, SRT and influent concentrations. When the steady state was reached, the concentration of the pharmaceutical compounds was step-wisely increased in the influent every two weeks, from 4 to 800 nM . Removal percentages of pharmaceuticals under different concentration regimes are shown in Fig. 1. Overall, each pharmaceutical had a different removal percentage. Acetaminophen was the only compound that was fully removed (100%). Metoprolol and diclofenac removal was between 20% and 40% , carbamazepine removal values varied between -1% and 20% , and metformin removal was between -10% and 30% . Fluoxetine removal was highly variable, between 25% and 70% , and the influent concentration was around 10 times lower than expected, probably due to its high sorption behaviour to several surfaces, including glass (Peake *et al.*, 2015). For pharmaceuticals with K_d (solid–water distribution coefficient) values lower than 0.5 l g^{-1} , sorption is considered insignificant in WWTPs (Ternes *et al.*, 2004; Wang *et al.*, 2020). For this reason, sorption was only considered as a removal mechanism for fluoxetine and it was calculated using Equation 3. The K_d coefficient value ($\sim 1.60\text{ l g}^{-1}$) was taken from a previous study where adsorbed fluoxetine levels were directly measured in nitrifying sludge (Fernandez-Fontaina *et al.*, 2012). An average value of 0.21 g l^{-1} of TSS was maintained in the

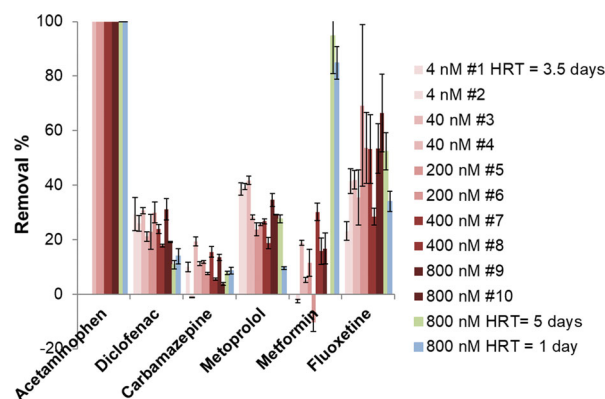


Fig. 1. Pharmaceutical removal percentage at different influent concentrations and hydraulic retention times (HRTs). Fluoxetine concentration was measured in the influent ten times lower than what it is shown in the graph. Each error bar is the standard deviation of the removal percentage calculated from the standard deviation of the technical triplicates. The symbol '#' followed by a number means the week the measurements were done since the starting of the concentration increase experiment.

bioreactor at a SRT = 15 days and HRT = 3.5 days. Thus, the amount of biomass generated per litre of medium treated was 0.049 g l^{-1} , which corresponds to 7% of fluoxetine sorption. Volatilization was not considered as a removal mechanism based on the low values of Henry's law constants of the selected pharmaceuticals (taken from the Hazardous Substances Data Bank) and based on previous studies (Suárez *et al.*, 2008).

The removal efficiencies reported in Fig. 1 are in line with previous studies, except for metformin, whose removal in WWTPs has been reported much higher (usually more than 80%) (Oosterhuis *et al.*, 2013; Briónes *et al.*, 2016). Acetaminophen is known to be removed very well, and carbamazepine is known for its high persistence in WWTPs all around the world (Joss *et al.*, 2005; Falás *et al.*, 2016; Wang *et al.*, 2020). Negative removals have previously been reported for carbamazepine (He *et al.*, 2019), but mostly when conjugated forms are present in the influent and they transform back to the parent compound in WWTPs. In our bioreactor, carbamazepine and metformin had some negative removals (Fig. 1). However, based on the low values (-1% , -2%) and the presence of an outlier in the measurement of -12% of metformin removal, we would need more replicates to truly confirm the negative removals. Metoprolol and diclofenac removal is variable in WWTPs, but their removal is often slightly higher than carbamazepine removal (Falás *et al.*, 2016; Wang *et al.*, 2020). Diclofenac removal during nitrification has been recently reported to be higher ($\sim 84\%$) than during heterotrophic processes or conventional activated sludge treatment ($\sim 50\%$) (Tran *et al.*, 2009; Wu *et al.*, 2020). However, earlier studies did not observe such high

removal of diclofenac in nitrifying sludge, only when solid retention time was extended to 150 days removal was improved to 70% (Fernandez-Fontaina *et al.*, 2012; Fernandez-Fontaina *et al.*, 2016). It might be that rare species present in the sludge proliferated during the high SRT and then started to contribute to metabolize diclofenac. This might explain the differences in removal under nitrifying conditions. Previous studies showed full removal of metoprolol after four days in activated sludge (Rubirola *et al.*, 2014; van Bergen *et al.*, 2020) and after 10–20 days in constructed wetland biomass (He *et al.*, 2018). Furthermore, the former study also observed an enhancement of metoprolol removal with more dissolved organic matter and a decrease in removal during nitrification. This was further confirmed by a different experiment with partial nitrification–anammox biomass where metoprolol removal was higher under aerobic heterotrophic conditions than under nitrifying conditions (Kassotaki *et al.*, 2018). Furthermore, the addition of allylthiourea (an inhibitor of ammonia monooxygenase and other monooxygenases) did not result in a significant decrease in metoprolol removal. Consequently, metoprolol was probably removed by heterotrophic bacteria in our bioreactor. Furthermore, the removal variability of diclofenac and metoprolol in WWTPs might be due to the differences in nitrification and heterotrophic microbial activities. Fluoxetine has a high K_d , and its sorption to biomass has a great contribution to the removal percentage in activated sludge (Fernandez-Fontaina *et al.*, 2012). Besides sorption, fluoxetine may also be degraded in nitrifying and heterotrophic cultures (Fernandez-Fontaina *et al.*, 2016; Velázquez and Nacheva, 2017). Therefore, fluoxetine removal in our bioreactor probably happened via sorption and transformation by nitrifiers and heterotrophic bacteria.

The removal percentage of each pharmaceutical did not correlate with the influent concentration (Fig. 1). Only small fluctuations in removal percentage were observed for each pharmaceutical. In the case of metoprolol, we observed that higher concentrations slightly decreased the removal percentage. However, when the highest concentration was added to the bioreactor (800 nM), metoprolol removal increased again. In summary, we did not find any effect of concentration on pharmaceutical removal percentage under the conditions tested in our MBR. These results are in contradiction to other studies reporting a correlation between influent concentration and removal percentage of some OMPs in WWTPs (Onesios-Barry *et al.*, 2014; Nolte *et al.*, 2018; Wang *et al.*, 2020). Onesios-Barry *et al.* reported a negative correlation between concentration and removal for some pharmaceuticals such as fluorouracil in laboratory columns inoculated with wastewater effluent. The lower removal values at higher concentrations might have

been related to toxicity due to the decrease in protein content that they measured at higher concentrations (Onesios-Barry *et al.*, 2014). On the other hand, Nolte *et al.* and Wang *et al.* reported a positive correlation between OMP concentration and removal. Their data came from several WWTPs with many different variables, whereas our experiment relied on a MBR in steady state. High OMP concentrations over long periods of time (years) in WWTPs may lead to microbial adaptation and, consequently, to an increase in removal. In our bioreactor experiment, we increased concentration every two weeks, so we did not expect microbial evolution to occur towards pharmaceutical utilization in this short time frame. Furthermore, the putative toxicity of some pharmaceuticals did not result in lower removal efficiencies probably because the microbial community composition changed during the increasing concentrations (Fig. 3) and mitigated this effect.

Our results suggest that the incomplete removal of pharmaceuticals is not dependent on concentration. Consequently, we could rule out hypotheses that rely on threshold concentrations as determinants of partial removal. One example is the cessation of transformation reactions due to product inhibition or toxicity, which would be concentration-dependent. As we did not observe consistent inhibition at one particular pharmaceutical concentration, but rather a similar removal percentage at all tested concentrations, it is unlikely that product inhibition or toxicity was an important factor for the pharmaceuticals in our experiment. The hypothesis of product inhibition for governing the incomplete degradation of OMPs was also discarded in a previous study using a mechanistic modelling approach (Gonzalez-Gil *et al.*, 2018). Another hypothesis for explaining the cessation of transformation reactions relies on catalytic constants of enzyme activity, such as enzyme affinity and corresponding reaction velocity. When a pollutant is transformed, its concentration decreases to a point that might be below the substrate threshold concentration for efficient catalysis. However, removal percentage would have increased with pharmaceutical concentration if this hypothesis was true. As a result, our experimental evidence does not support these hypotheses where the biodegradation efficiency is dependent on the influent pharmaceutical concentration under concentration regimes relevant to real-world scenarios.

Although no correlation was found between pharmaceutical influent concentration and removal percentage, the absolute amount of nmols removed increased proportionally with the concentration (Fig. 2). This indicates that enzyme saturation was not yet reached and that bacteria had the capacity to transform more pharmaceutical molecules per time even though they did not fully convert them at lower concentrations. Previous studies

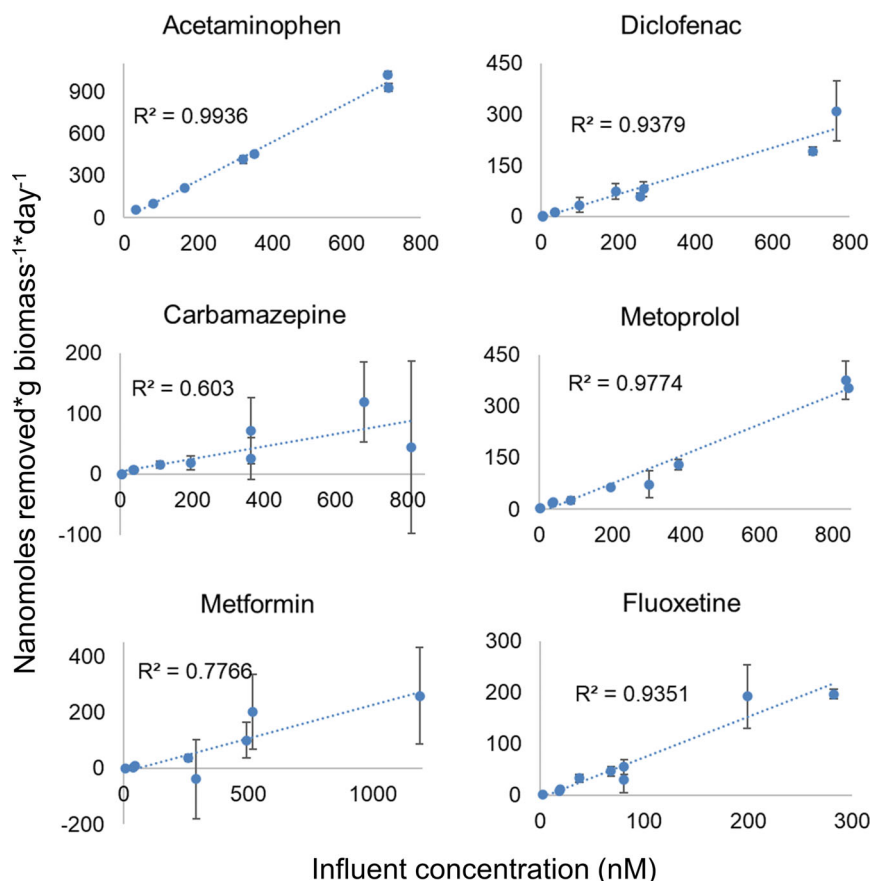


Fig. 2. Pharmaceutical removal rate at increasing influent concentrations. Each error bar is the standard deviation of the removal rate calculated from the standard deviation of the technical triplicates.

reported higher biodegradation rates at higher OMP concentrations (in the nM and μ M range) in activated sludge, biofilm reactors, pure enzymes and bacterial isolates (Leng *et al.*, 2016; Lonappan *et al.*, 2017; Svendsen *et al.*, 2020; van Bergen *et al.*, 2020). In many cases, they used the Michaelis–Menten model to describe the increase in biodegradation rates with concentration. This model corresponds to a linear relationship between initial concentration and velocity up until the system saturation, when biodegradation rates do not increase as fast as before and they finally reach a limit. In our bioreactor experiment, the system did not reach saturation probably because pharmaceutical concentrations were increased every two weeks, thus leaving time for the microbial community to shift and adapt.

Pharmaceutical removal at different hydraulic retention times

During a second experiment in the same bioreactor, the HRT was increased to five and decreased to one day to prolong/shorten the reaction time of the pharmaceuticals with the microbial community. Influent concentration of

pharmaceuticals was kept at 800 nM, and operational parameters in the bioreactor remained unchanged. However, biomass concentration increased 3 times to 0.9 g l^{-1} at HRT = 1 day due to the increased loading rate of growth substrates, mostly acetate and methanol (Fig. S2). Furthermore, higher nitrite and nitrate concentrations were observed at HRT = 1 day (Fig. S2). Figure 1 shows the removal percentage of the six pharmaceuticals at different HRT. Acetaminophen was always removed 100% with no effect of HRT on removal percentage, which means that total removal happened in the first 24 h. HRT had no effect on carbamazepine and fluoxetine removal efficiencies as they were in the same range as with HRT of 3.5 days. Diclofenac removal was slightly lower at HRT 1 and 5 days. Metoprolol removal did not change by increasing HRT to 5 days, but it was lower at HRT = 1 day. Therefore, 10% of the removal happened in the first 24 h and the remaining 20% in the following 2.5 days reaching a removal maximum of 30%. Metformin was the only pharmaceutical removed to a higher degree by increasing the HRT to 5 days (~95%), suggesting that the length of the reaction time was responsible for the incomplete removal of metformin in

our bioreactor. However, decreasing the HRT to 1 day maintained a high metformin removal percentage of 80%, which indicates that another factor was affecting the removal. Biomass concentration at HRT = 1 day was three times higher than in the other HRTs, so this might be the reason why removal was higher. These results suggest that metformin removal depends on the reaction time or HRT and the biomass concentration present in the bioreactor. Metformin is usually very well-degraded, so its removal efficiency did not increase with HRT in previous reports from WWTPs and constructed wetlands (Oosterhuis *et al.*, 2013; Auvinen *et al.*, 2017). Biomass concentration in WWTPs is usually higher than what we used in our experiments, indicating that the data obtained under HRT = 1 day with higher biomass concentrations are more representative of the *in situ* WWTP situation.

Previous studies reported higher removal percentages at higher HRTs for diclofenac and carbamazepine in constructed wetlands (from 2 to 6 days), for fluoxetine in nitrifying and activated sludge (from 0.75 to 1 and 5 days) and for metoprolol in activated sludge (from 1 to 24 h) (Maurer *et al.*, 2007; Fernandez-Fontaina *et al.*, 2012; Alvarino *et al.*, 2014; Auvinen *et al.*, 2017). However, other studies in activated sludge and anaerobic reactors did not observe an increase in diclofenac removal percentage at higher HRT (at 19–61 h and 5–11 days, respectively) (Queiroz *et al.*, 2012; Oosterhuis *et al.*, 2013). A recent study in bioaugmented activated sludge showed an increase in diclofenac and carbamazepine removal percentage from 12 to 18 h, but removal was no longer improved at 24 h (Boonnorat *et al.*, 2019). These results suggest that increasing the HRT of wastewater bioreactors might increase the removal efficiency of specific pharmaceuticals, but only up to a specific point. This indicates that there will always be a removal constrain not related to kinetics. For example, in our MBR this point was 3.5 days for metoprolol and 1 day or lower for carbamazepine. Furthermore, we observed a decrease in diclofenac removal percentage at higher HRT (5 days), which might be related to a desorption or back transformation from diclofenac reaction products.

The incomplete removal of diclofenac, carbamazepine, metoprolol and fluoxetine was not dependent on concentration nor HRT, so only few removal mechanisms are possible. One hypothesis is that (1) transformation or sorption reactions are reversible and reach an equilibrium between the parent and transformation products or between the adsorbed and dissolved form of the compound. In such a case, we would always observe the same removal percentage at different concentrations and no removal increases with time or HRT. This was recently proposed by Gonzalez-Gil *et al.* using a

mechanistic model based on the reversibility of bisphenol phosphorylation by two key enzymes in anaerobic digestion: hexokinase and acetate kinase (Gonzalez-Gil *et al.*, 2018; Gonzalez-Gil *et al.*, 2019). Furthermore, He *et al.* have reported higher carbamazepine concentrations in the effluent than in the influent of WWTPs due to reactions transforming conjugates back to carbamazepine (He *et al.*, 2019). Another plausible explanation of these results is that (2) removal percentage of an OMP is related to the amount of growth substrate added to the medium. Tran *et al.* reported that the removal percentage of diclofenac and carbamazepine was increased up to a maximum by adding higher concentrations of ammonium to activated sludge bottles (Tran *et al.*, 2009). In our bioreactor, the concentration of growth substrates in the medium did not change, which results in a specific and limited number of enzymes and cofactors able to transform the selected pharmaceuticals. Oxygenases are key enzymes for the biodegradation of OMPs under aerobic conditions and require reducing power (i.e. NAD(P)H, FADH) to transfer oxygen atoms to the respective substrate. When the substrate is used for growth, the cell first invests reducing power, but the subsequent catabolism of the transformation products restores the reducing power in the cell (Luo *et al.*, 2014). In the case of co-metabolism, OMPs undergo only the first reaction with input of reducing power, which is energy costly for the cell (Cornelissen and Sijm, 1996). A previous study used a kinetic model to demonstrate that external donor and acceptor concentrations affect the removal percentage of OMPs because they change the NADH/NAD⁺ ratio inside the cell (Bae and Rittmann, 1995). Thus, a specific growth substrate concentration might be able to support only partial biotransformation of pharmaceuticals in WWTPs. On the other hand, Kennes-Veiga *et al.* did not observe a difference in OMP removal efficiency by increasing acetate concentrations in a continuous bioreactor (Kennes-Veiga *et al.*, 2020). Instead, they only reported increasing biodegradation rate constants with increasing acetate loads. Furthermore, other studies observed different effects (increased, decreased or unchanged) of growth substrate concentrations on biodegradation rate constants depending on the OMP (Su *et al.*, 2015; Liang *et al.*, 2019; Zhang *et al.*, 2019). Consequently, the co-metabolic effect of growth substrates on pharmaceutical removal might be compound-specific and might reach a maximum or a decay phase when there is competition for the same enzyme.

Bacterial community changes

Biomass samples were taken from the inoculum and from the bioreactor at different time points reflecting different pharmaceutical micropollutant influent concentrations

(4 nM, 200 nM and 800 nM) for bacterial 16S rRNA gene amplicon sequencing. Two time points with 4 nM were selected to account for the bacterial community changes inherent to the bioreactor maintenance and not to the shift in pharmaceutical concentration. Furthermore, technical triplicates were independently analysed for each time point.

Figure 3 shows the relative 16S rRNA gene abundance at the phylum level in the inoculum and bioreactor. First of all, the inoculum had a different bacterial community composition than the biomass in the bioreactor, where *Nitrospirae* and *Planctomycetes* increased in abundance and the rest decreased (i.e. *Actinobacteria* and *Chloroflexi*) or remained similar (i.e. *Proteobacteria* and *Acidobacteria*). Furthermore, the increase in pharmaceutical concentration in the bioreactor also induced a community shift. Overall, we saw an increase in *Acidobacteria* and *Bacteroidetes* and a decrease in *Nitrospirae* and *Planctomycetes* phyla. However, samples taken at two different time points (July and August) but with the same influent pharmaceutical concentration (4 nM) show a decrease in *Nitrospirae*, which suggests that this phylum is highly sensitive to small fluctuations during the bioreactor operation. On the other hand, a great decrease of around 50% in *Nitrospirae* relative abundance at the highest pharmaceutical concentration points towards a putative toxic effect. Similarly, *Planctomycetes* also showed a slight shift in the two time points with the same concentrations, but ultimately, increasing pharmaceutical concentrations removed *Planctomycetes* to undetectable levels.

The relative abundance of ammonia-oxidizing bacteria (AOB) significantly decreased with increasing pharmaceutical concentrations (data not shown). AOB consisted

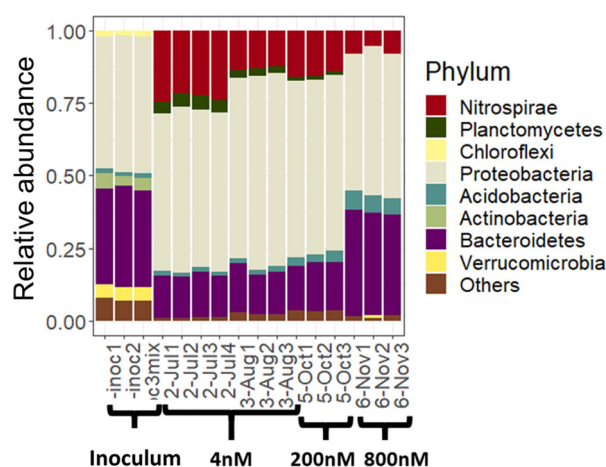


Fig. 3. Relative abundance of 16S rRNA genes in the inoculum and bioreactor at different time points and different pharmaceutical concentrations added in the influent.

mainly of members of the *Nitrosomonadaceae* family and they considerably decreased in relative abundance in the bioreactor compared with the inoculum, in contrast to *Nitrospirae*. Due to the low abundance of AOB (less than 0.3%), the *Nitrospirae* might include complete ammonia-oxidizing (comammox) species in addition to canonical nitrite oxidizers (van Kessel *et al.*, 2015). Since it is not possible to distinguish between comammox and nitrite-oxidizing *Nitrospira* using 16S rRNA gene sequencing methods (Koch *et al.*, 2019), a PCR was performed using specific primers for comammox *amoA* (Pjevac *et al.*, 2017). Primers targeting the clade A comammox *amoA* gene produced a single band of approximately 500 base pairs coinciding with the theoretical fragment length amplified by the primers. However, primers targeting clade B produced several bands not corresponding to the desired fragment. Therefore, we can conclude that clade A comammox may be present in the bioreactor and coexisted with AOB.

Previous studies reported a putative toxic effect of several pharmaceuticals towards *Nitrospira* and *Nitrosomonas* spp. (Dokianakis *et al.*, 2004; Kraigher *et al.*, 2008; Wang and Gunsch, 2011a,b; Bian *et al.*, 2020; Park and Seungdae, 2020). *Nitrosomonas* spp. resisted higher concentrations of paracetamol and triclocarban than *Nitrospira* spp. (Bian *et al.*, 2020; Park and Seungdae, 2020). Furthermore, heterotrophic activity for chemical oxygen demand removal was more robust than AOB activity in the presence of gemfibrozil and naproxen (Wang and Gunsch, 2011b). Wang *et al.* also reported that these two individual pharmaceuticals did not have an effect on ammonium removal, while the mixture of them inhibited nitrification and altered AOB community composition. In conclusion, each pharmaceutical has a different effect on *Nitrospira* and *Nitrosomonas* sp. and this effect is dose-dependent and stronger in a mixture. In our MBR, *Nitrospira* and *Nitrosomonas* spp. significantly decreased in relative abundance, but no effect was observed on nitrification. However, if there was inhibition it might not have been noticed due to the high HRT applied (3.5 days).

Acidobacteria and *Bacteroidetes* were the two phyla that increased in relative abundance with increasing pharmaceutical concentration. These phyla did not change in abundance at the two time points where the bioreactor had the same influent concentration of pharmaceuticals. Therefore, the relative abundance changes can be directly associated with the pharmaceutical concentration increase. Higher *Bacteroidetes* abundance has been previously associated with increased concentrations of pharmaceuticals and other OMPs in activated sludge reactors and river sediments (Drury *et al.*, 2013; Jiang *et al.*, 2017). Furthermore, there are several *Bacteroidetes* species able to

degrade complex organic molecules (Kwon *et al.*, 2019). Some studies have also associated *Acidobacteria* with micropollutant removal in soil and river sediments (Nogales *et al.*, 1999; George *et al.*, 2009; Njeru *et al.*, 2017; Posselt *et al.*, 2020). It is important to mention that an increase in abundance of these two phyla does not necessarily mean that they degrade pharmaceuticals. They could also be more resistant to toxic effects than other taxa giving them a growth advantage.

The genus *Dokdonella* (*Proteobacteria*) significantly increased in abundance from 0.5% to 17% of the total relative abundance in the microbial community at the highest pharmaceutical concentration in the bioreactor (800 nM) (Fig. S3). *Dokdonella* spp. consist of aerobic denitrifiers (Pishgar *et al.*, 2019), and in addition, they have been previously suggested to be responsible for the degradation of acetaminophen (Palma *et al.*, 2018). In another study with activated sludge, *Dokdonella* spp. increased in abundance after adding around 400 μ M of ibuprofen and 1 μ M of tetracycline, so they might degrade other pharmaceuticals as well (Abdelrahman *et al.*, 2018). Furthermore, this genus has been previously suggested as a pesticide and polycyclic hydrocarbon degrader (Bacosa and Inoue, 2015; Qi and Wei, 2017; Romero *et al.*, 2019). Many other taxa (i.e. *Pseudomonas*, *Bacillus*, *Flavobacterium*, *Ensifer*, *Delftia* spp.) have been isolated from activated sludge and soil and have been shown to degrade acetaminophen in pure and mixed cultures (Žur *et al.*, 2018; Akay and Tezel, 2020; Chopra and Kumar, 2020; Park and Oh, 2020). In our bioreactor, some of these genera were not present and others did not show an increase with higher pharmaceutical concentrations (data not shown). For this reason, we propose *Dokdonella* as the main acetaminophen degrader in our bioreactor experiment.

Alpha diversity was analysed with three different indices: Chao1, Shannon and Simpson (Fig. S4). The richness (number of different species), calculated with the Chao1 index, decreased in the bioreactor compared with the inoculum, and it further decreased with the highest pharmaceutical concentration. The main driver of this change could be the synthetic medium used to feed the bioreactor: we simplified the influent organic matter to acetate and methanol compared with the large variety of carbon sources usually found in the influent of WWTPs. Furthermore, a decrease in richness with pharmaceutical concentration could be explained by a toxicity effect. Shannon index measures the richness and evenness (a measure of how close the relative abundance of each taxa is) of a sample, whereas Simpson's index of diversity ($1 - \text{Simpson index}$) gives more weight to the abundance and evenness of species (Lemos *et al.*, 2011). Both indices considerably decreased in the bioreactor biomass compared with the inoculum. In the middle of

the experiment, they increased again and decreased during the highest pharmaceutical concentration. The samples taken at two consecutive time points at the same pharmaceutical concentration (4 nM) have very different evenness values, so this diversity characteristic cannot be associated with the pharmaceuticals. As seen in Fig. S2, there are small fluctuations in the influent growth substrate concentrations that might be responsible of these changes. Previous studies have correlated biodiversity (richness and evenness) with higher biotransformation rates of specific micropollutants (Johnson *et al.*, 2015; Torresi *et al.*, 2016; Stadler *et al.*, 2018). However, those studies did not correlate the diversity changes to the addition of micropollutants, which might increase diversity due to a putative cooperation of different taxa in order to degrade the chemicals (Vasiliadou *et al.*, 2018) or decrease diversity due to putative toxic effects (Chonova *et al.*, 2016).

Mobile genetic elements relative abundance

In this study, we tested whether increasing pharmaceutical concentrations in the bioreactor led to an increase in the relative abundance of IncP-1 plasmids and IS1071 insertion sequences as a measure for MGEs. We performed a qPCR of the *trfA* replication gene of different IncP-1 plasmids and the *tnpA* transposase gene of the IS1071 insertion sequence. As internal standard, the bacterial 16S rRNA gene was used. Interestingly, higher pharmaceutical concentrations did not significantly increase relative abundance of MGEs (Fig. S5). The studied MGEs remained stable in the bacterial community independent of the pharmaceutical concentration. This result does not necessarily mean that plasmids are not being transferred, as they can also remain in a dynamic equilibrium in the bioreactor. Yang *et al.* observed that the relative abundance of an external plasmid remained unchanged despite being transferred in a MBR (Yang *et al.*, 2013). The biomass density in the bioreactor presented in this study was ten times lower than a common WWTP activated sludge reactor. As conjugation events are more likely to happen at higher densities or in environments where cells are in close contact such as granules and biofilms (Rios-Miguel *et al.*, 2020), this experiment might underestimate the true role that MGEs play in the degradation of pharmaceutical micropollutants. Furthermore, previous studies reported higher relative abundances of MGEs in sites with higher OMP exposure over much longer periods of time (years) compared with our bioreactor experiment that lasted 2.5 months (Dunon *et al.*, 2013; Dealtry *et al.*, 2014). These might be reasons why MGE relative abundances were stable in our bioreactor.

Conclusions

This study contributes to a better understanding of pharmaceutical removal limitations in activated sludge processes. The observed relative abundance shifts of the microbial community suggest toxic effects on specific phyla relevant to WWTP functioning and point out several microbial groups potentially involved in degrading pharmaceuticals. The main conclusions from our experimental data are:

1. Selected pharmaceuticals (diclofenac, metoprolol, metformin, carbamazepine and fluoxetine) were not fully degraded under activated sludge conditions in a MBR. Only acetaminophen was fully removed.
2. Higher pharmaceutical influent concentration proportionally increased the removal rate of each compound.
3. The removal percentage was not correlated with influent concentration and remained relatively stable in the order: acetaminophen (100%) > fluoxetine (50%) > metoprolol (25%) > diclofenac (20%) > metformin (15%) > carbamazepine (10%).
4. Metformin removal increased to 80–100% when HRT or biomass concentration increased in the bioreactor. Removal % of the other pharmaceuticals could not be improved by extending the HRT nor increasing biomass concentration.
5. The microbial community changed in response to higher pharmaceutical concentrations: *Nitrospira* and *Planctomycetes* decreased and *Bacteroidetes* and *Acidobacteria* increased in relative abundance.
6. *Dokdonella* spp. could be the main acetaminophen degraders under activated sludge conditions.
7. Increasing pharmaceutical concentrations did not increase relative abundance of mobile genetic elements (IncP-1 plasmids and *IS1071*). The biomass density or the pharmaceutical concentration in the bioreactor might have been too low to increase conjugation.

Experimental procedures

Pharmaceutical selection and bioreactor operation

Six pharmaceuticals were selected based on (i) presence in priority lists, (ii) variability in primary biodegradation rate, and (iii) their commercial classification as non-antibiotics. The selected pharmaceuticals were acetaminophen, diclofenac, metoprolol, carbamazepine, metformin and fluoxetine. They were all purchased in solid phase from Merck (Darmstadt, Germany), dissolved in methanol and diluted in water to obtain a 1:1 methanol-water solution.

A 5L MBR (Applikon Biotechnology B.V., Delft, The Netherlands) was inoculated with 100 times diluted activated sludge from a municipal WWTP in Groesbeek, the Netherlands. The membrane was built at Radboud University Technical Center and consisted of an integral-immersed Zenon ZW-1 module with 0.04 μm pore size hollow fibres from Suez. It was never backwashed or replaced during the experiment. The bioreactor was fed with synthetic wastewater containing ammonium (~3 mM), acetate (~3 mM) and methanol (~1 mM) as main energy sources. The detailed composition of the medium can be found in supplement SI1. The medium was autoclaved, and then, pharmaceuticals were added. After an acclimatization period of 180 days at a hydraulic retention time (HRT) of 3.5 days and a solid retention time (SRT) of 15 days, the pharmaceutical concentration in the influent was increased every two weeks from 4 nM to 800 nM. These concentrations were chosen due to their environmental relevance. Methanol concentration in the influent did not change, as stock solutions with increasing pharmaceutical concentrations were used. During the process, pharmaceuticals were monitored by LC-MS/MS and the microbial community was analysed by 16S rRNA gene amplicon sequencing. Furthermore, the MBR was run in the dark at constant mechanical stirring (200 rpm), pH = 7, $\text{dO}_2 = 1 \text{ mg l}^{-1}$, room temperature ($20 \pm 1 \text{ }^\circ\text{C}$) and total suspended solids (TSS) = $0.2\text{--}0.3 \text{ g l}^{-1}$. Dissolved oxygen (dO_2) and pH sensor probes were connected to an ADI 1010 controller (Applikon Biotechnology B.V., Delft, The Netherlands) that activated a KHCO_3 base pump to control the pH and regulated the O_2/CO_2 entrance flow in the bioreactor. Nitrogen (ammonium, nitrite and nitrate) and carbon (acetate and methanol) balances were measured and calculated during the experiment. After this first experiment, pharmaceutical removal was measured at HRTs of 5 and 1 days keeping pharmaceutical concentration at 800 nM.

Carbon, nitrogen and total suspended solid measurements

Ammonium, nitrite and nitrate assays were performed with technical duplicates in 96-well microtitre plates using a plate reader as previously described in van Bergen *et al.*, (2020). Briefly, ammonium was measured colorimetrically at 420 nm after reaction with OPA reagent (0.54% (w/v) ortho-phthaldialdehyde [OPA], 0.05% (v/v) β -mercaptoethanol and 10% (v/v) ethanol in 400 mM potassium phosphate buffer (pH 7.3)). Nitrite was measured at 540 nm using the Griess assay. Afterwards, an incubation with vanadium chloride at 60 $^\circ\text{C}$ reduced all nitrate to nitrite and the sample was measured again at 540 nm. Methanol was measured colorimetrically with

the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay (Mangos and Haas, 1996 and <https://www.sigmaaldrich.com/technical-documents/protocols/biology/enzymatic-assay-of-alcohol-oxidase.html>). We adjusted the volumes to fit in a 96-well plate. First, a stock solution was prepared with the following composition: 14 ml ABTS solution, 100 μ l of peroxidase solution (1 mg ml⁻¹), 3 ml of 100 mM potassium phosphate buffer pH 7.5 and 100 μ l of alcohol oxidase solution (1 mg ml⁻¹). Afterwards, 170 μ l of the stock solution and 20 μ l of samples or standard were added to each well. The plate was incubated for one hour in the dark at room temperature and measured at 405 nm in the plate reader. Finally, acetate was measured by GC-MS using the protocol described in a previous study (in 't Zandt *et al.*, 2018). Briefly, 200 μ l of 100 mM pentafluorobenzyl bromide solution in acetone, 40 μ l of 0.5 M phosphate buffer pH = 6.8 and 40 μ l of sample/standard were mixed in an Eppendorf tube and incubated for one hour at 60 °C. Afterwards, 400 μ l of 0.1 mM methylstearate solution in hexane was added and the mixture was vortexed and centrifuged for one minute at maximum speed. The top layer was transferred to a GC vial and measured in a GC-MS.

TSS and VSS were measured as in Ref. (van Bergen *et al.*, 2020). Briefly, 40 ml of sample was passed through a 0.45 μ m pore size glass-fibre filter, dried overnight in the oven at 105 °C and then 4 h at 550 °C. Filters were weighed in the beginning and after each step (105 and 550 degrees) to calculate the number of TSS and VSS respectively.

Pharmaceutical measurements via LC-MS/MS and mass balances

Samples (2 ml) were taken every week from the medium (influent) and the bioreactor (effluent) in technical triplicates. Samples were centrifuged at maximum speed, and the supernatant was immediately treated and analysed as in Ref. (van Bergen *et al.*, 2020). Briefly, 0.5 ml of sample was used for liquid-liquid extraction of metformin. One ml of sample was used for solid phase extraction (Oasis HLB 3cc SPE cartridges, sorbent bed of 60 mg from Waters Corporation, Milford, USA) of the other five pharmaceuticals. The extracts were directly injected into an LC-MS system together with the calibration standards prepared in the same way as samples. Internal standards (deuterated compounds) were added to the samples and standards before the extractions. Only parent compounds were determined.

The removal efficiency or percentage of a compound was calculated using equation 1. C_i corresponds to the pharmaceutical concentration in the influent and C_e in the effluent of the bioreactor (nM).

$$\text{Removal (\%)} = \frac{C_i - C_e}{C_i} * 100 \quad (1)$$

The removal rate of a compound was calculated using equation 2, where TSS corresponds to the total suspended solids (g l⁻¹) inside the bioreactor. In our experiment, TSS corresponds to the biomass content.

$$\text{Removal rate} \left(\frac{\text{nmol}}{\text{day} * \text{g}} \right) = \frac{C_i - C_e}{\text{HRT} * \text{TSS}} \quad (2)$$

The sorption of fluoxetine was calculated using equation 3 (Ternes *et al.*, 2004), where K_d represents the solid-water distribution coefficient, and SS, the quantity of sludge generated per unit of wastewater treated. The K_d value used in this study was taken from Ref. (Fernandez-Fontaina *et al.*, 2012).

$$\text{Sorption (\%)} = \frac{K_d * \text{SS}}{1 + K_d * \text{SS}} * 100 \quad (3)$$

Molecular analysis

Sampling and DNA extraction. Samples (8 ml) were taken every week from the bioreactor in technical triplicates. The samples were centrifuged at maximum speed, and the pellet was stored at -20 °C until DNA extraction. DNA was extracted and purified using the DNeasy PowerSoil Kit (Qiagen Benelux B.V.) following the manufacturer's instructions. The DNA concentration was determined using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA USA) and a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA USA).

Bacterial 16S rRNA gene sequencing and data analysis. DNA samples were submitted to Macrogen (Seoul, South Korea) for amplicon sequencing of the bacterial 16S V3 and V4 regions using Illumina MiSeq Next Generation Sequencing. The primers used for amplification were Bac341F (5'-CCTACGGGNGGCWGCAG-3') and Bac785R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013). Subsequent analysis on the sequencing output files was performed within R version 3.4.1 (R Core Team, 2012). Pre-processing of the sequencing data was done using the DADA2 pipeline (Callahan *et al.*, 2016). Taxonomic assignment of the reads was up to the species level when possible using the Silva non-redundant database version 128 (Yilmaz *et al.*, 2014). Count data were normalized to relative abundances. Data visualization and analysis were performed using phyloseq and ggplot packages (Wickham and Wickham, 2007; McMurdie and Holmes, 2013). Chao1, Simpson and Shannon diversity indexes were calculated using the estimate richness

function of the phyloseq package. All sequencing data were submitted to the GenBank databases under the BioProject ID PRJNA670630.

Comammox amoA gene PCR. The primers and PCR conditions used to amplify the *amoA* gene of comammox bacteria are explained in Pjevac *et al.*, (2017). Two degenerate primer pairs were used to target the *amoA* gene from clade A and clade B comammox (Table S2). The oligonucleotide primers were obtained from Biologio (Nijmegen, The Netherlands). PCRs were performed in a total volume of 25 μl , which contained 1 μl DNA sample of 1 to 10 $\text{ng } \mu\text{l}^{-1}$, 0.5 μl of each degenerate primer solution of 20 μM , 12.5 μl of PerfeCTa Quanta Master Mix (Quanta Bio, Beverly, MA) and 10.5 μl of autoclaved Milli-Q water. The PCR protocol consisted of an initial denaturation step at 94 $^{\circ}\text{C}$ for 5 min, followed by 25 cycles of denaturation at 94 $^{\circ}\text{C}$ for 30 s, primer annealing at 52 $^{\circ}\text{C}$ for 45 s and elongation at 72 $^{\circ}\text{C}$ for 1 min. Finally, the last step was an elongation at 72 $^{\circ}\text{C}$ for 10 min.

Quantitative PCR. 16S rRNA genes were amplified using the above primers. Amplification of the *trfA* gene from IncP-1 α , β , ϵ , γ and δ plasmids and the *tnpA* gene from the IS1071 insertion sequence was performed with primers previously used in Ref. (Providenti *et al.*, 2006; Bahl *et al.*, 2009; Dunon *et al.*, 2013). A summary of all primers used in this study can be found in Table S2. All qPCRs were performed using 96-well optical PCR plates (Bio-Rad Laboratories, Veenendaal, the Netherlands) with optical adhesive covers (Applied Biosystems, Foster City, CA) in a C1000 Touch Thermal Cycler equipped with a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, the Netherlands). The qPCR total volume was 25 μl containing 1 μl DNA sample of 1 to 10 $\text{ng } \mu\text{l}^{-1}$, 0.5 μl of each primer solution of 20 μM , 12.5 μl of PerfeCTa Quanta Master Mix (Quanta Bio, Beverly, MA) and 10.5 μl of autoclaved Milli-Q water. Negative controls were added to each run by replacing the template with sterile Milli-Q water. All qPCR data were analysed using the Bio-Rad CFX Manager version 3.0 (Bio-Rad Laboratories, Veenendaal, the Netherlands). The qPCR protocol consisted of initial denaturation at 95 $^{\circ}\text{C}$ 3 min followed by 39 cycles of denaturation, annealing and extending (95 $^{\circ}\text{C}$ 30 s, 60 $^{\circ}\text{C}$ 30 s and 72 $^{\circ}\text{C}$ 30 s respectively), and two final steps at 65 $^{\circ}\text{C}$ 5 s and 95 $^{\circ}\text{C}$ 50 s.

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Conflict of interest

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

Author contributions

All authors started the project and contributed to the conceptual framework. ARM conducted the experiments and data analyses. ARM wrote the manuscript, and all authors contributed to improve versions of the manuscript.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Data availability statement

All sequencing data were submitted to the GenBank database under the BioProject ID PRJNA670630. The data that support the findings of this manuscript are available in Dans Easy with the following digital object identifier: <https://doi.org/10.17026/dans-zhx-nuux>.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Bioreactor medium composition.

Table S2. List of primers used in this study.

Table S3. Bioreactor monitoring.

Fig. S1. Total suspended solids values along the bioreactor acclimatization period and experiments.

Fig. S2. Carbon and nitrogen measurements in the influent (A) and effluent (B) along the bioreactor experiment.

Fig. S3. Relative abundance of the *Dokdonella* genus in the bioreactor at different time points and pharmaceutical concentrations.

Fig. S4. Alpha diversity in the inoculum and bioreactor at different time points.

Fig. S5. Mobile genetic elements genes versus bacterial 16S rRNA gene relative abundance in the bioreactor when pharmaceutical concentrations were 4 nM and 800 nM.