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Selection of bacterial strains to bioaugment granular sludge and improve the removal of recalcitrant pollutants

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Abstract Several advantages are gained by the use of granular sludge in wastewater treatment plants (WWTP), such as higher biomass retention, faster settling properties, lower energy costs and smaller operational land space. However, relatively few studies have been carried out on the biodegradation of recalcitrant pollutants in these systems, compared to research performed on conventional WWTP with floccular sludge. For instance, bioaugmentation of bacterial strains with metabolic abilities of interest in biological reactors for wastewater treatment has been intensively studied in conventional WWTP systems, but not in systems with granular sludge. In the project "Bioaugmentation and conjugative plasmid transference using bacteria from extreme environments to enhance biodegradation of recalcitrant pollutants in WWTP granular sludge", funded by the Portuguese Foundation for Science and Technology, a group of bacterial strains showing potential capacity to metabolize recalcitrant pollutants (paracetamol, ibuprofen, fluoxetine and polyethylene terephthalate) was isolated and their genomes are being sequenced with the aim of identifying genes putatively involved in the metabolic pathways of interest. In addition, biodegradation experiments are being performed in laboratory-scale granular sludge bioreactors bioaugmented with selected isolates.
Keywords: Bioaugmentation, Bioremediation, Pharmaceuticals removal, Microplastics removal.

1. Introduction

Wastewater treatment plants (WWTPs) for urban sewage are important sources of persistent pollutants to the environment and concern on harmful environmental impact is growing due to chronic trace concentration exposure caused by WWTPs inability to completely degrade such type of pollutants.

Studies with activated sludge have revealed percentages of Paracetamol (PMOL) removal from 80% to 99.9%, revealing hydroquinone and 4-aminophenol as its intermediate metabolites, and allowed to isolate several PMOL degrading *Pseudomonas* strains by supplying synthetic WWTP with PMOL as the sole carbon source (De Gusseme et al. 2011; Zhang et al. 2013). Yet, its detection in receptor ecosystems suggests that the disposal in sewage overwhelms its removal in WWTPs (Peake et al. 2015). Moreover, it is possible to cultivate PMOL-degrading granules using this drug as the sole carbon and nitrogen source (Hu et al. 2012), but to our knowledge the biodegradation of this drug in bioreactors with granular sludge remains to be studied using realistic medium with more carbon sources.

Ibuprofen (IBU) is metabolized to 1-hydroxy-ibuprofen, 2-hydroxy-ibuprofen and carboxy-ibuprofen, and is removed in WWTPs with efficiencies of 90 to 100% (Ferrando-Climent et al. 2012; Larsson et al. 2014). However, high usage results in its presence in effluents (Gros et al. 2010 Pereira et al. 2015), and trace concentrations can have toxicological effects on aquatic organisms (Han et al. 2010). Bacterial strains able to degrade IBU have been isolated (Marchlewicz et al. 2017). Yet, a study on IBU degradation using granular sludge with synthetic wastewater revealed a removal efficiency of just 45% (Zhao et al. 2015).

Fluoxetine (FLX) and its metabolite norfluoxetine (NFLX) have been detected in raw and treated sewage as well as in environmental waters (Barclay et al. 2012; Yang et al. 2020). Both mimic the effects of serotonin and have toxicological effects on several aquatic organisms (Silva et al. 2012). Some studies indicated these drugs are removed from waters mainly through adsorption to sediments (including WWTP sludge), where they are persistent (Moreira et al. 2015; Yang et al. 2020). However, results of other works indicated that biodegradation has a major role in the removal (Moreira et al. 2014; Velázquez et al. 2017). Plus, Amorim et al. (2016) showed that a granular sludge system exhibited

preferential removal of the (R)-enantiomer of FLX and stated that biological-mediated processes occurred.

Polyethylene terephthalate (PET) is a polymer that makes up more than 50% of global fibre production. Research on the biodegradation of PET (e.g. Auta et al. 2017) and bio-based PET (e.g. Fernández de Villalobos et al. 2022) by bacterial strains and communities is on-going, but only slight changes in the structure of the polymer have been reported thus far. It is known that PET microfibers (< 5 mm) accumulate in activated sludge (Chen et al. 2020), and some types of bacteria frequently identified in WWTPs' bioreactors, such as different species of genus *Pseudomonas*, have been reported to have the capacity to metabolize different types of both pharmaceuticals and plastics (Cacciari et al. 1993; Nwachkwu et al. 2010). Yet, the biodegradation of microplastics in the sludge from granular systems remains to be studied.

Bioaugmentation of bacterium strains with metabolic abilities of interest in biological reactors for wastewater treatment is a known concept. It has been applied on conventional sludge systems, inclusively aiming to improve the removal of the drugs targeted in this project proposal. In systems with granular sludge, there are few studies applying this concept. Still, they prove the concept is viable also with granular sludge and pave the way for new applications. For example, a laboratory scale reactor with aerobic granular sludge unable to remove 2-fluorophenol (2-FP) exhibited a high removal performance after bioaugmentation with a 2-FP degrading strain, which was successfully retained by the granules (Duque et al. 2011).

The project "Bioaugmentation and conjugative plasmid transference using bacteria from extreme environments to enhance biodegradation of recalcitrant pollutants in WWTP granular sludge" has the goals of selecting bacterial strains from extreme environments (preferably carrying plasmids with genes putatively involved in degradative pathways) showing potential to degrade PMOL, IBU, FLX and PET, and to investigate the use of the selected strains to improve the biodegradation of such pollutants in bioreactors with granular sludge. The results so far obtained are encouraging and thus a summary of project objectives methods and current achievements is presented.

2. Materials and methods

The selection of degradative strains was divided in three phases: 1) enrichment culture, 2) microbial isolation and 3) confirmation of degradative abilities. The enrichment culture was made in liquid cultures using inorganic salt (IS) medium independently amended with the target pollutants and their known intermediate metabolites plus inoculum from different sources affected by recalcitrant drugs and also from diverse extreme environments. The isolation was performed by plating dilutions of the enriched liquid cultures in solid IS media amended with the target pollutants. To confirm the degradative abilities of strains, they were incubated in IS medium amended with the target drugs or microplastics and the medium was periodically sampled to analyze the target pollutants and their metabolites by High Performance Liquid Chromatography. Then, the selected strains were classified using 16S rRNA gene sequences and whole genome sequencing performed through the Pacific Biosciences (PacBio) technology with the aim of looking for genes putatively determinant of degrading capacities.

3. Results and discussion

From the enrichments using samples from mines in the Iberian pyrite belt, seven isolates potentially degrading PMOL were obtained, from the genera *Aeromonas, Bacillus* (two isolates), *Niallia, Paraburkholderia, Rhizobium and Variovorax*, as well as one isolate potentially degrading hydroquinone, from the genus *Mycolicbacterium*. The enrichments from benthic marine samples collected on reefs resulted in eight paracetamol-degrading isolates, four belonging to the genus *Paenibacillus*, one to *Micrococcus*, another to *Microbacterium*, while the remaining two could not be classified. From the same marine samples, two isolates belonging to the genus *Bacillus* showed potential to grow on PET films as sole carbon source. From the enrichments using marine sediments exposed to pollutants, three morphologically distinct colonies have been isolated from paracetamol plates, one colony from FLX plates and five colonies from IBU plates, which are being used in tests to confirm their degrading capacity. Moreover, up to now, whole genome sequencing was performed for three of the selected strains and genome assembling and genes annotation is ongoing. Plus, bioreactors with granular sludge bioaugmented with the isolated *Mycolicbacterium* strain are being evaluated for the biodegradation of paracetamol and the results so far obtained show best removal of this drug's metabolites hydroquinone and 4-aminophenol comparing with the non-bioaugmented control reactors.

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References

Amorim C L Moreira I S Ribeiro A R Santos L Delerue-Matos C Tiritan M E Castro P M L (2016) Treatment of a

simulated wastewater amended with a chiral pharmaceuticals mixture by an aerobic granular sludge sequencing batch reactor. International Biodeterioration & Biodegradation 115: 277 – 285. https://doi.org/10.1016/j.ibiod.2016.09.009

Auta HS, Emenike CU, Fauzia SH (2017) Screening of Bacillus strains isolated from mangrove ecosystems in Peninsular Malaysia for microplastic degradation. Environmental Pollution 231: 1552-1559. https://doi.org/10.1016/j.envpol.2017.09.043

Barclay VKH, Tyrefors NL, Johansson IM, Pettersson CE (2012) Trace analysis of fluoxetine and its metabolite norfluoxetine. Part II: Enantioselective quantification and studies of matrix effects in raw and treated wastewater by solid phase extraction and liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1227: 105–11. https://doi.org/10.1016/j.chroma.2011.12.084

Cacciari I, Quatrini P, Zirletta G, Mincione E, Vinciguerra V, Lupatelli P, Sermanni GG (1993) Isotactic polypropylene biodegradation by a microbial community: Physico-chemical characterization of metabolites produced. Applied and Environmental Microbiology 59: 3695-3700. https://doi.org/10.1128/aem.59.11.3695-3700.1993

Chen Z, Zhao W, Xing R, Xie S, Yang X, Cui P, Lü J, Liao H, Yu Z, Wang S (2020) Enhanced in situ biodegradation of microplastics in sewage sludge using hyperthermophilic composting technology. Journal of Hazardous Materials 384: 121271. https://doi.org/10.1016/j.jhazmat.2019.121271

De Gusseme B, Vanhaecke L, Verstraete W, Boon N (2011) Degradation of acetaminophen by *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* in a membrane bioreactor. Water Research 45(4): 1829-1837. https://doi.org/10.1016/j.watres.2010.11.040.

Duque, A.F.; Bessa, V.S.; Carvalho, M.F.; de Kreuk, M.K.; van Loosdrecht, M.C.M.; Castro, P.M.L. (2011) 2-Fluorophenol degradation by aerobic granular sludge in a sequencing batch reactor. Water Research 45: 6745-6752. https://doi.org/10.1016/j.watres.2011.10.033

Fernández de Villalobos NF, Costa M, Marín-Beltrán I (2022). A community of marine bacteria with potential to biodegrade petroleum-based and biobased microplastics. Marine Pollution Bulletin 185, Part A: 114251. https://doi.org/10.1016/j.marpolbul.2022.114251

Ferrando-Climent L, Collado N, Buttiglieri G, Gros M, Rodriguez-Roda I, Rodriguez-Mozaz S, Barcelo D (2012) Comprehensive study of ibuprofen and its metabolites in activated sludge batch experiments and aquatic environment. Science of the Total Environment 438: 404–413. https://doi.org/10.1016/j.scitotenv.2012.08.073

Gros M, Petrovic M, Ginebreda A, Barcelo D (2010) Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. Environment International 36: 15–26. https://doi.org/10.1016/j.envint.2009.09.002

Han S, Choi K, Kim J, Ji K, Kim S, Ahn B, Yun J, Choi K, Khim JS, Zhang X, Giesy J (2010) Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans *Daphnia magna* and *Moina macrocopa*. Aquatic Toxicology 98: 256–264. https://doi.org/10.1016/j.aquatox.2010.02.013

Hu J, Xhou L, Zhoug Q, Wei F, Zhang L, Chen J (2012) Biodegradation of Paracetamol by Aerobic Granules in a Sequencing Batch Reactor (SBR). Advanced Materials Research 441: 531-535. https://doi.org/10.4028/www.scientific.net/AMR.441.531

Iwamoto A, Tokiwa Y (1994) Enzymatic degradation of plastics containing polycaprolactone. Polymer Degradation and Stability 45: 205-213. https://doi.org/10.1016/0141-3910(94)90138-4

Larsson E, al-Hamini S, Jonsson JA (2014) Behaviour of nonsteroidal anti-inflammatory drugs and eight of their metabolites during wastewater treatment studied by hollow fibre liquid phase microextraction and liquid chromatography mass spectrometry. Science of the Total Environment 485–486: 300–308. https://doi.org/10.1016/j.scitotenv.2014.03.055

Marchlewicz A, Guzik U, Smułek W, Wojcieszyńska D. (2017) Exploring the Degradation of Ibuprofen by Bacillus thuringiensis B1(2015b): The New Pathway and Factors Affecting Degradation. Molecules 22(10): 1676. https://doi.org/10.3390/molecules22101676

Moreira IS, Amorim CL, Ribeiro AR, Mesquita RBR, Rangela AOSS, van Loosdrecht MCM, Tiritan ME, Castro PML (2015) Removal of fluoxetine and its effects in the performance of an aerobic granular sludge sequential batch reactor. Journal of Hazardous Materials 287: 93–101. https://doi.org/10.1016/j.jhazmat.2015.01.020

Moreira IS, Ribeiro AR, Afonso CM, Tiritan ME, Castro PML (2014) Enantioselective biodegradation of fluoxetine by the bacterial strain *Labrys portucalensis* F11. Chemosphere 111: 103-111. https://doi.org/10.1016/j.chemosphere.2014.03.022.

Nwachkwu S, Obidi O, Odocha C (2010) Occurrence and recalcitrance of polyethylene bag waste in nigerian soils. African Journal of Biotechnology. 9(37): 6096–6104. https://doi.org/10.5897/AJB10.364

Peake BM, Braund R, Tong A, Louis A (2015) The Life-Cycle of Pharmaceuticals in the Environment. Woodhead Publishing Series in Biomedicine. 1st edition. https://www.elsevier.com/books/the-life-cycle-of-pharmaceuticals-in-the-environment/peake/978-1-907568-25-1

Pereira A, Silva L, Meisel L, Lino C, Pena A (2015) Environmental impact of pharmaceuticals from Portuguese wastewaters: geographical and seasonal occurrence, removal and risk assessment. Environmental Research 136: 108–119. https://doi.org/10.1016/j.envres.2014.09.041

Silva LJG, Lino CM, Meisel LM, Pena A (2012) Selective serotonin re-uptake inhibitors (SSRIs) in the aquatic environment: an ecopharmacovigilance approach. Science Total Environment: 437: 185–95. https://doi.org/10.1016/j.scitotenv.2012.08.021

Velázquez YF, Nacheva PM (2017) Biodegradability of fluoxetine, mefenamic acid, and metoprolol using different microbial consortiums. Environmental Science

and Pollution Research 24: 6779–6793. https://doi.org/10.1007/s11356-017-8413-y

Yang H, Lu G, Yan Z, Liu J, Dong H, Bao X, Zhang X, SunY (2020) Residues, bioaccumulation, and trophic transfer of pharmaceuticals and personal care products in highly urbanized rivers affected by water diversion. Journal of Hazardous Materials 391: 122245. https://doi.org/10.1016/j.jhazmat.2020.122245

Zhang L, Hu J, Zhu R, Zhou Q, Chen J (2013) Degradation of paracetamol by pure bacterial cultures and their microbial consortium. Applied Microbiology and Biotechnology 97, 3687–3698 (2013). https://doi.org/10.1007/s00253-012-4170-5

Zhao X, Chen Z, Wang X, Li J, Shen J, Xu H (2015) Remediation of pharmaceuticals and personal care products using an aerobic granular sludge sequencing bioreactor and microbial community profiling using Solexa sequencing technology analysis. Bioresource Technology 179: 104–112. https://doi.org/10.1016/j.biortech.2014.12.002