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Studies on the bioaugmentation of *Mycolicibacterium aubagnense* HPB1.1 in aerobic granular sludge from a WWTP: Adaptability of native prokaryotes and enhancement of paracetamol intermediate metabolites biodegradation

Jorge D. Carlier[a,*], Alba Lara-Moreno[a,c], Benjamin Igbodo[a,b], Maria C. Costa[a,b,**]

[a] Centre of Marine Sciences (CCMAR/CIMAR LA), Campus de Gambelas, Universidade do Algarve, Faro 8005-139, Portugal
[b] Faculty of Sciences and Technologies, University of the Algarve, Gambelas Campus, Faro 8005-139, Portugal
[c] Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville 41012, Spain

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ABSTRACT

This study aimed to evaluate for the first time the bioaugmentation of *Mycolicibacterium aubagnense HPB1*.1 in Sequencing Batch Reactors (SBRs) with Aerobic Granular Sludge (AGS), and its effect on the biodegradation of Paracetamol, also known as Acetaminophen and N -acetyl-para-aminophenol (APAP). The bioaugmentation was effective and persisted for at least nine days after five inoculations performed in 24 days (relative abundance of *M. aubagnense* was 0.13 ± 0.05 % in the test reactors and 0.0079 ± 0.0008 % in the control reactors) and for eight days after seven inoculations performed in 40 days (relative abundance of *M. aubagnense* was 0.04 ± 0.02 % in the tests and 0.0005 ± 0.0005 % in the controls). In what concerns APAP biodegradation, the results showed a faster removal of its transformation products Hydroquinone (HQ), 2,5-dihydroxy-1,4-benzoquinone (2,5-HO-BQ) and 1,4-benzoquinone (BQ) in the bioreactors bioaugmented with the bacterial strain *M. aubagnense* HPB1.1 (59 % or 85 % of HQ, 67 % or 85 % of 2,5-HO-BQ and 75 % or 82 % of BQ removals, respectively for assay 1 or assay 2) in comparison to the non-bioaugmented bioreactors (15 % or 31 % of HQ, 36 % or 63 % of 2,5-HO-BQ and no removal of BQ, also for assy 1 and 2, respectively). Regarding the effect on organics and nutrients treatment, overall, the SBR conditions favored ammonia, nitrites, and organics removal. Yet, the conditions did not allow complete denitrification nor higher assimilation than release of PO_4^{3-}.

1. Introduction

Over the last two decades, several synthetic and semisynthetic chemicals started to be detected in environmental waters, raising concerns regarding their potential impact on aquatic organisms and on possible broader effects on the respective ecosystems. Therefore, they became known as contaminants of emerging concern (CECs) and have been gradually included in lists of compounds regulated under environmental laws.

* Corresponding author.
** Corresponding author at: Centre of Marine Sciences (CCMAR/CIMAR LA), Campus de Gambelas, Universidade do Algarve, Faro 8005-139, Portugal.
E-mail addresses: jcarlier@ualg.pt (J.D. Carlier), mcorada@ualg.pt (M.C. Costa).

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Pharmaceuticals (Phs) and pharmaceutical transformation products (PhTPs) resulting from organisms' metabolic pathways or from abiotic reactions are becoming a major group of CECs in aquatic environments (e.g. Ibáñez et al., 2021; Styszko et al., 2021). They are released to the environment through several ways, such as treated or untreated wastewaters from municipalities, hospitals, and industries, as well as landfill leachates, illegal disposals and runoff waters from agriculture and livestock areas; nevertheless, municipal wastewater is considered the major source of this type of CECs to aquatic environments. Indeed, a total daily mass of Phs and personal care products (PCPs) discharge of 7.35–20.160 g/day was estimated in wastewater treatment plants (WWTPs) with secondary treatment and 4.8–10.602 g/day in WWTPs with additional tertiary/advanced treatment (Adeleye et al., 2021). For example, in a study in which 142 CECs (including Phs, PCPs and pesticides) were monthly monitored for a year in WWTPs at both urban and rural locations in Ireland: (i) the highest concentrations were measured for Phs in all types of samples (influent, effluent and receiving surface waters) and (ii) the compounds detected in effluent waters with frequencies over 70 % were twenty three Phs, one PhTP and three chemicals used in agriculture (Rapp-Wright et al., 2023).

There are several treatment techniques that can be applied to improve the removal of Phs from wastewater. For example, Pavithra et al. (2017) reported a review on the sources and forms of Phs in the environment as well as a detailed discussion on the advantages and disadvantages of various treatments. As conclusion, these authors gave their preference to nanotechnology because of its high efficiency in pollutants' removal. However, the use this strategy for in situ remediation must be very well evaluated since the nanomaterials may themselves be considered pollutants. Indeed, in wastewater treatment these techniques usually imply the use of additional infrastructures, such as reactors and decanters. Strategies that aim to improve the treatment capacity of biological processes are an alternative with great potential, even for highly recalcitrant organics. For example, Shaji et al. (2024) in a comprehensive review on the environmental effects, disposal, and biodegradation of plastic debris concluded that microbes can be used to develop microbial consortia to biodegrade polymers, and their enzymes can also be used in degradation processes. Moreover, Karishma et al. (2024) reported a review on emerging strategies for enhancing microbial degradation of petroleum hydrocarbons, while Saravanan et al. (2023) reported a specific example with immobilized *Lasiodiplodia theobromae* biomass successfully tested for the biodegradation of oil-contaminated medium.

The concept of bioaugmentation of activated sludge with selected microbial strains to improve biological treatment processes is not new and has gained increasing prominence. Indeed, bioaugmentation has been explored at various scales to enhance the biodegradation of persistent pollutants in activated sludge systems. While many studies have been conducted at the laboratory scale, there are some works on pilot-scale and full-scale applications. For example, successful treatment of refinery spent-sulfidic caustic was achieved in activated sludge system bioaugmented with three specific bacterial strains (*Bacillus thuringiensis*, *Bacillus cereus*, and *Acidovorax ebreus*) at both laboratory and pilot scale (Sun et al., 2016). In another example, the shock resistance and efficiency of an industrial scale activated sludge sequencing batch reactor treating heavy oil refinery wastewater was enhanced by a solid bioaugmentation agent of mixed bacteria (*Bacillus subtillis* and *Brucella* sp.) and glucose (Cui et al., 2021). In one example focused on PHs, Domanovac et al. (2019) have shown enhancement of the biodegradation process of high-strength PHs wastewater through bioaugmentation of activated sludge with bacterial isolates identified as *Aeromonas hydrophila* and *Pseudomonas putida*.

APAP (used as analgesic and antipyretic) is viewed as a safe drug because its consumption limit to be harmful is very high; therefore it is much more widely used than other drugs of the same family, and its disposal into the aquatic environment is also relatively higher (Lee et al., 2020). It is for long known that APAP removal in WWTPs with activated sludge can reach values from 80 % to 99.9 %, with HQ and 4-aminophenol as its intermediate metabolites of biodegradation, and several APAP degrading strains have been isolated from sludge biomass or effluents. For example, two bacterial strains identified as *Delftia tsuruhatensis* and *Pseudomonas aeruginosa* were identified by De Gusseme et al. (2011), three bacterial strains assigned to species of the genera *Stenotrophomonas* and *Pseudomonas* were identified by Zhang et al. (2013), and two *Pseudomonas* strains were identified by Poddar et al. (2022). Yet, APAP detection in receptor ecosystems suggests that in general the disposal in sewage overwhelms its removal in WWTPs (Peake et al., 2016; Parolini, 2020). Moreover, usually the APAP intermediate metabolites are not targets in works monitoring CECs in WWTP or receiving waters, and the works on their biodegradation are mainly focused on the adsorption/degradation rate and kinetic model or on improving biodegradation based on acclimatization strategies. For example, the biodegradation of dihydroxybenzene isomers (catechol, resorcinol, and HQ) has been studied under anaerobic and anoxic conditions (e.g. Latkar et al., 2003; Subramanyam and Mishra, 2008), under aerobic conditions (e.g. Pramparo et al., 2012) and also under the anaerobic-anoxic-oxic conditions of SBRs (Zhang et al., 2022). Nevertheless, bacterial strains capable of degrading HQ and other aromatic compounds have already been identified; for example: *Arthrobacter ureafaciens* strain CPR706 (Bae et al., 1996), *Pseudomonas putida* strain MTCC 1194 (Kumar et al., 2005) and *Pseudomonas putida* strain N6 (Guzik et al., 2011), have been documented. Moreover, efficient degradation of HQ by a metabolically engineered strain has already been shown possible: a strain unable to degrade HHQ (*Pseudarthrobacter sulfonivorans* strain Ar51) became HQ-degrading after incorporation of two subunits of HQ dioxygenase from one HQ degrading strain (*Sphingomonas* sp. strain TTNP3) (Sun et al., 2022). In summary, all this evidence is proof of the great potential of biotechnological approaches to improve the removal of APAP and its transformation products in biological wastewater treatment systems.

Bioaugmentation, the introduction of specific microbial strains or consortia into a biological system, is an effective method to improve and optimize biological treatment or bioremediation with promising application prospects in various aspects, such as promoting the degradation of toxic substances, improving removal efficiency of nutrients, adapting to adverse environmental conditions and quickly starting or upgrading bioreactors (Ma et al., 2022; Chettri et al., 2024). The most common way to apply the bioaugmentation approach to improve the transformation of contaminants into less dangerous compounds is to directly add microorganisms that have specific degradation capabilities for the target contaminants, which implies the need to obtain one or more high-efficiency microbial strains able to use the target contaminants as the carbon source and energy source (Huang and Ye, 2020). In the case of APAP and its transformation products, several bacterial degrading strains have already been identified, as exemplified above. Moreover, it has already been shown that bioaugmentation with specific strains can improve the removal of this PH. For example, Marchlewicz et al. (2023) reported a study on the biodegradation of non-steroidal anti-inflammatory drugs (NSAIDs) in SBRs bioaumented with *Bacillus thuringiensis* B1(2015b) and *Pseudomonas moorei* KB4 strains, in which the 10 mg/L of APAP dose in each bioreactor cycle of 7 days was always degraded within 24 h on all analyzed cycles after bioaugmentation. Nevertheless, a number of factors should be taken into consideration for the success of bioaugmentation process, such as the ability of newly added microbial strains to survive to the competition with native microorganisms, the presence of predators, and to adapt to the receiving abiotic factors (Kumari et al., 2022). Thus, the higher the number of isolated strains capable of biodegrading a drug and/or their intermediate transformation products and able to adapt to sludge ecosystems, the more tools will be available for the scientific community and for the WWTP industry to develop strategies and methods for improving the removal of these compounds from both the effluent (discharged to environmental waters) and the sludge (used largely in compost production for agriculture soils).

Recently, the team of the laboratory of Environmental Technologies of the Centre of Marine Sciences of the Algarve has isolated the bacterial strain *Mycolicibacterium aubagnense* HPB1.1, which is able to remove both APAP and HQ from liquid mineral medium having any of these compounds as the sole carbon and energy source (Lara-Moreno et al., 2024a). Indeed, when this strain was selected (Lara-Moreno et al., 2024a), an adsorption/absorption study was conducted to rule out possible adherence/accumulation in the strain's bacterial cells, and neither APAP nor any transformation product was detected in the cells, corroborating the hypothesis that *M. aubagnense* HPB1.1 is able to biodegrade these compounds. Moreover, in another recent study, it was shown that after bioaugmentation of *Pseudomonas extremaustralis* CSW01 and *Stutzerimonas stutzeri* CSW02 together in conventional floccular WWTP, just below 30 % of APAP in solution was mineralized, but when these two strains were added in consortia with *M. aubagnense* HPB1.1, mineralization significantly increased: up to 74 % and 58 % for CSW01 + HPB1.1 and CSW02 + HPB1.1, respectively (Lara-Moreno et al., 2024c).

Members of *Mycolicibacterium* were previously included in genus *Mycobacterium* but were separated in a different genus by Gupta et al. in 2018. Several authors have reported the ability of this genus to degrade aromatic compounds, such as pyrene (e.g. Yang et al., 2021) and fuel oxygenates (e.g. Zsilinszky et al., 2022); yet, to our knowledge, *M. aubagnense* HPB1.1 is the first member of this genus reported as able to degrade Phs. In fact, in addition to its capacity to biodegrade APAP and its transformation products, it was also successfully tested for Ibuprofen bio-removal from water, and its biodegradation capacity for this drug was suggested (Lara-Moreno et al., 2024a, 2024b, 2024c).

Regarding previous studies on bioaugmentation with other *Mycolicibacterium* species, a search in the Online Knowledge Library b-on (https://www.b-on.pt/en/) using the key words "*Mycolicibacterium*" and "bioaugmentation" resulted in sixty-one articles in scientific journals. Most of the studies found are focused on bioaugmentation of environmental samples to develop bioremediation strategies for contaminated ecosystems. For example, the results reported in the article by Ren et al. (2021) indicate that bioaugmentation of *Mycolicibacterium phocaicum* RL-HY01 in seawater and marine sediment samples is an efficient strategy to eliminate phthalic acid esters. In another example, Laothamteep et al. (2022) reported that *Mycolicibacterium* was a key player in enhancing crude oil removal from contaminated sandy soil microcosms after bioaugmentation with a consortium containing *Mycolicibacterium* strains PO1 and PO2, *Novosphingobium pentaromativorans* PY1 and *Bacillus subtilis* FW1. Moreover, in one example with freshwater, Naloka et al. (2021) reported that a consortium of *Mycolicibacterium parafortuitum* J101, *Mycolicibacterium austroafricanum* Y502 and *Rhodococcus ruber* S103 immobilized on plastic balls achieved over 50 % removal efficiency of fuel oil removal from natural freshwater to which 3000 mg L^{-1} of fuel oil was added. Then, a further search adding the key word "WWTP" to "*Mycolicibacterium*" and "bioaugmentation", resulted in just eight articles in scientific journals. Three of these articles refer to our previous works with the HPB1.1 strain (Lara-Moreno et al., 2024a, 2024b, 2024c), while the other five articles just refer *Mycolicibacterium* as an example of bacteria resistant to, or degrading, emerging organic contaminants (Ivshina et al., 2022; Sharma et al., 2023, 2024; Shah et al., 2024; Alidoosti et al., 2024). This highlights the innovative nature of the work reported in this article on the bioaugmentation of a *Mycolicibacterium* species in WWTP sludge with a view to improving the bio-treatment of Phs in sewage water.

The aim of the new work reported in this article was to study the bioaugmentation of *M. aubagnense* HPB1.1 in WWTP AGS by its direct addition to laboratory scale SBRs, and to study the respective effect on the removal of APAP and its transformation products from spiked synthetic wastewater. The study is a first step in analysing the effect of *M. aubagnense* HPB1.1 bioaugmentation on the removal of APAP from the water entering the bioreactors, since it was focused on two single drug spiking moments on different days rather than a continuous drug supplement throughout the entire bioaugmentation experience. Nevertheless, the presented positive results open the way and motivate the interest in performing further bioaugmentation studies aiming the biotreatment of CECs with this strain. Massive DNA sequencing of 16S rRNA genes was used to study the prokariotic comunitiy in the initial granular sludge and its dynamics during the bioaugmentation experiment, incuding the relative abundance of the added strain. HPLC analysis was used to detect and measure the removal of APAP and its transformation products, while UV–visible standard methods were applied to monitor rotine WWTP parameters.

2. Materials and methods

2.1. Bioreactors

Four SBRs with working capacities of about 1.3 L in volume each were built using acrylic pipes. The dimensions of each reactor were 6.2 cm in internal diameter and 44 cm in height while the discharge point was at mid height for an exchange rate of 50 % in each cycle. The oxygen was supplied using two Vultron 4000 air pumps for the four reactors (each air pump with two air supplies). Each reactor had an air supply connected to a hard PVC tube with two exits (< 1 mm) at the bottom of the reactors to supply air, resulting in a superficial air velocity of 0.8 cm/s.

The SBRs started with automatic operations using sequential time switches in cycles of 6 hours with four different phases: anaerobic feed from the bottom, aeration, settling, and effluent removal. However, due to failures in the automatic discharges (after discharge the outlet pumps became unprime), the operation was adapted to manual discharges first with cycles of 12 hours and then with cycles of 24 hours (details in Table 1).

Each reactor was fed with synthetic sewage prepared from four separated supply solutions that became mixed in a common tube receiving the four solutions through independent peristaltic pumps (Table 2), so in each of the four supply bottles there were always some nutrients missing to avoid the proliferation of contaminant microorganisms. The mineral composition of synthetic sewage was decided after comparing compositions in works reported by Beun et al. (2002), de Kreuk et al. (2005), Kong et al. (2013), Long et al. (2015), Adler and Holliger (2020), Castellanos et al. (2021) and Silva et al. (2022). Regarding the carbon sources, Acetate and D-glucose were used as major compounds, but a supplement of Glycerol was also added, making a total theoretical Chemical Oxygen Demand (COD) of 1.1 g O_2 per L, which is the value of COD used by Silva et al. (2022) in one of their SBRs with good results. The theoretical COD was calculated according to the equation reported by van Haandel and van der Lubbe (2007).

The operation started with 150 mL of mature granular sludge per reactor. Therefore, to have high selectivity for the fast-settling mature granules, a high minimal imposed settling velocity of 13.2 m/h was used; calculated using the equation described by Liu et al. (2005):

Minimal imposed settling velocity $= \frac{L}{t_s}$

Where L is the distance to the discharge port (m) and t_s is the settling time (hour).

2.2. Granular sludge characterization

2.2.1. Morphological attributes

The granular sludge used in this work, kindly provided by the company Águas do Algarve, was obtained from the Faro/Olhão WWTP located in the south of Portugal and immediately characterized and added to start the SBRs operation.

Sludge Volume (SV) was measured by pouring 1 L of mixed liquor into a graduated cylinder, leaving the sludge to settle for 5 and 10 minutes, and the volumes recorded respectively for each time interval (SV_5 and SV_{10}).

Sludge Volume Index (volume occupied by 1 g of sludge after settling for a certain time) was calculated for 5 and 10 minutes (SVI_5 and SVI_{10}) using the equation:

$$\mathrm{SVI}(\mathrm{mL}\big/\mathrm{g}) = \frac{\mathrm{SV(mL\ settled\ sludge\ per\ L\ of\ Mixed\ Liquor)}}{\mathrm{Mixed\ Liquor\ Suspended\ Solids}\left(\frac{g}{L}\right)}$$

where Mixed Liquor Suspended Solids (MLSS) were determined as described below.

The MLSS was measured according to Kasper et al. (2018), but with modifications since the study is with granular sludge: a filter with pore sizes of 20 – 30 μm (Whatman Grade 8 Ruled Qualitative Filter Paper Circle – 75 mm diameter) was used instead of a filter with 0.4–1 μm retention. The initial filter weight (W1) was measured using an analytical balance, 100 mL of mixed liquor was filtered, the filter with wet suspended solids was placed in the oven at 70 ° C for 3 days, the final filter weight with dry material (W2) was measured, and the MLSS was calculated using the equation:

$$\mathrm{MLSS}(\mathrm{g}\big/\mathrm{L}) = \frac{(\mathrm{W2(g)} - \mathrm{W1(g)})}{\mathrm{Volume\ filtered(L)}}$$

The size and shape of granules were evaluated using a magnifier for visual observation of mixed liquor poured into a petri dish placed over millimetric paper. The criteria defined by Beun et al. (2002) were evaluated on a simplified scale: diameter size (mm), particle surface (smooth, half-hairy, hairy), and roundness of particle (round, elongated, irregular).

2.2.2. Prokaryotic community

To study the sludge native prokaryotes a metataxonomic approach was applied based on massive sequencing of 16S rRNA gene amplicons followed by bioinformatic analysis to obtain taxonomic assignments and respective relative abundances. For that, genomic DNA was extracted from granular sludge immediately after collection, and the DNA was sent to a specialized company in next-

Table 1
Time, in minutes, of each phase during the SBRs cycles in the different operation stages.

	Stage I (Day 1 – 4)	Stage II (Day 5 – 7)	Stage III (Day 8 – 57)
Phase	**4 cycles / day**	**2 cycles / day** **daily cycle - night cycle**	**1 cycle / day**
Anaerobic feed	120	150 - 150	150
Aerobic incubation	237	387 - 747	1287
Settling	1	1 - 1	1
Effluent removal	2	2 - 2	2

Table 2
Synthetic sewage preparation and composition.

Compounds	Stock solution stored at 4°C (g/L)	Dil. factor to prepare solutions in the supply bottles	Dil. factor by mixing the four solutions - according to each pump flow	Total dil. factor	Influent (g/L)
Carbon sources and $MgSO_4$ solution					
Sodium acetate	90	31.9	4.7	150	0.6
D-glucose	55.5				0.37
Glycerol	24				0.16
$MgSO_4.7H_2O$	13.5				0.09
Macronutrients solution 1					
NH_4Cl	80	140.9	2.8	400	0.2
$CaCl_2.2H_2O$	12				0.03
Macronutrients solution 2					
K_2HPO_4	256	174.0	2.3	400	0.64
KH_2PO_4	60				0.15
KCl	16				0.04
Trace elements solution					
$FeCl_3.6H_2O$	9	220.5	4.5	1000	0.009
H_3BO_3	0.9				0.0009
$CuSO_4.5H_2O$	0.18				0.00018
KI	1				0.001
$MnCl_2.4H_2O$	0.72				0.00072
$Na_2MoO_2.2H_2O$	0.36				0.00036
$ZnSO_4.7H_2O$	0.72				0.00072
$CoCl_2.6H_2O$	0.9				0.0009
EDTA	60				0.06

generation DNA sequencing (Appgenomics, Lda., Faro, Portugal).

DNA was extracted using the NZY Soil gDNA isolation kit (nzytech, Lisbon, Portugal), and the concentration and the quality of eluted DNA were analyzed using a spectrophotometer (NanoDrop3300, Thermo Fisher Scientifc, USA). The methodology followed by the company Appgenomics, Lda. for PCR amplification of 16S rRNA genes, DNA sequencing, base-calling, trimming and filtering of reads, taxonomic assignments, taxa abundance estimations, and diversity analysis is described in Supplementary Material A.

2.3. Bioaugmentation

The bacterial strain *M. aubagnense* HPB1.1 used in this bioaugmentation experiment was isolated from the wall of a cave at the Poderosa mine in Spain (in the Iberian Pyrite Belt), during previous work on the selection of APAP degrading bacteria (Lara-Moreno et al., 2024a). The *M. aubagnense* HPB1.1 strain was approved for deposit into the collection of the Agricultural Research Service Culture Collection (NRRL) in the United States (https://nrrl.ncaur.usda.gov/), with the assigned accession B-65706.

The idea in this initial study of bioaugmentation with *M. aubagnense* HPB1.1 in bioreactors containing WWTP sludge was to evaluate whether this strain has the potential to improve the biodegradation of APAP in biological water treatment systems. Therefore, this work did not include a plan for optimizing the efficiency of bioaugmentation processes with this strain. Despite this, it was planned to carry out six initial inoculations every two days before the first biodegradation assay. However, due to a contamination issue in the *M. aubagnense* HPB1.1 cultures, only four inoculations were performed in that period. Subsequently, two additional inoculations were carried out at longer intervals (10 and 12 days) to verify whether this was sufficient to maintain the bioaugmentation and its potential effect.

Two test bioreactors (R1 and R2) were inoculated seven times (at operational days 2, 4, 6, 12, 24, 33, and 40) with *M. aubagnense* HPB1.1 strain, while two control bioreactors (R3 and R4) were maintained in equal conditions but not inoculated. Each inoculation was as follows: (1) a culture of the selected strain was grown in 400 mL LB medium supplemented with glycerol (5 mL /L) until reaching 1.0–1.5 $OD_{600\ nm}$, (2) the cells were collected by centrifugation (2500 g for 15 minutes at room temperature) in 8 centrifuge tubes of 50 mL, (3) the medium was discarded and the cells of each tube resuspended in 20 mL of synthetic sewage, and (4) the suspensions distributed equally to reactors R1 and R2.

To evaluate the bioaugmentation in the SBRs, the same metataxonomic approach used to study the native sludge prokaryotes was applied. For that, genomic DNA was extracted from granular sludge collected from the test and control bioreactors throughout the experiment until one week after the last inoculation. Then, the DNA samples were sent (together with the initial sample of DNA extracted before any inoculation for bioaugmentation) to the specialized company in next-generation DNA sequencing referred to above (Appgenomics, Lda, Faro, Portugal).

2.4. APAP bioremoval

Since this work is a first study on the evaluation of bioaugmenting *M. aubagnense* HPB1.1 in SBRs to improve the removal of APAP and/or its transformation products, as a first approach, a relatively high concentration of APAP was used (comparing to what can be found in real municipal wastewaters) to avoid the need of concentrating samples for analysis, thus reducing analytical errors and facilitating the observation of APAP and/or its transformation products removal.

Assay 1 - Immediately after the fourth inoculation of reactors 1 and 2 with *M. aubagnense* HPB1.1, at operational day 12, APAP was added to the four reactors to make a concentration of ~50 mg/L on the filled volume. Then, samples were collected to analyze APAP and its transformation products immediately when aeration started and along the aeration period (times 0, 2.5, 5, 7.5, 10 and 22 h) of that same operation cycle (cycle 1) and along the aeration period (0, 3, 12 and 22 h) of the subsequent cycle (cycle 2).

Assay 2 - One week after the sixth inoculation of reactors 1 and 2 with *M.aubagnense* HPB1.1, at operational day 47, APAP was again added to the four reactors to make a concentration of ~50 mg/L on the filled volume, and samples were collected during that operational cycle when aeration started and along the aeration period (times 0, 1, 2.5, 4, 6, 11.5, 15.5 and 18.5 h).

The samples collected from the test SBRs and control SBRs were immediately filtered with Politetrafluoretileno (PTFE) sterile syringe filters (pore size 0.22 μm) to 2 mL glass vials and analyzed to assess the evolution of APAP and its transformation products in solution by HPLC analysis.

The HPLC procedure was the one tested and used previously to detect the APAP biodegradation metabolites in the work leading to the isolation of *M. aubagnense* HPB1.1 (Lara-Moreno et al., 2024a). The analysis was performed with a XBridge C18 column of 4.6 × 250 mm with 5 μm particle size (Waters, USA) at 50ºC, using an isocratic method of 10 minutes at 1.3 mL/min flow with methanol/miliQ-H_2O - 10/90 (%) at pH ~3 (adjusted with orthophosphoric acid) as mobile phase. For that, an Ultra High Performance Liquid Chromatography Nexera system (SHIMADZU) was used with the following modules (SHIMADZU): a System Controller SLC-40; a Solvent Delivery Module LC-20AP combined with an FCV-200AL low-pressure gradient unit to perform gradient analysis using up to four mobile phases, a Degassing Unit DGU-405 an Autosampler SIL-10AP, a Column Oven CTO-40C, and a Photodiode Array UV-Vis Detector SPD-M40 with a Conventional Flow-Cell for SPD-M40.

APAP at ≥ 99.0 % purity (ref. A7085–100G, Sigma-Aldrich) and HQ also at ≥ 99.0 % purity (ref. H9003–100G, Sigma-Aldrich) were used to prepare calibration standards of 5, 10, 20, 30 and 50 mg/L, and one certified reference material of HQ, TraceCERT®, from Supelco (ref. 74347, Sigma-Aldrich) was used to confirm the retention time of this compound, to avoid confusion with the possible occurrence of its oxidized form BQ.

2.5. Wastewater parameters

To evaluate the organic load and nutrients' removals, the influent and the effluent from each of the four SBRs were sampled once a week, and the following parameters were analyzed: pH, COD, total nitrogen, ammonia nitrogen, nitrate nitrogen, and phosphate phosphorus. The nitrites nitrogen was estimated by mass balance using the measured concentrations of total nitrogen, ammonia nitrogen, and nitrate nitrogen.

The pH was measured using a GLP 21 (CRISON) pH meter with an SJ 223 electrode (VWR). Chemical oxygen demand, total nitrogen, ammonia nitrogen, nitrate nitrogen, and phosphate phosphorus) were quantified via colorimetric methods of spectroscopy with kit reagents from HACH company GmbH (Dusseldorf, Germany), using a DR2800 spectrophotometer (Hach-Lange). The general procedure was: (1) build standard calibration curves within the reported linearity concentration ranges; (2) calculate the Limits of Detection (LOD) and of Quantification (LOQ); (3) measure the reference materials Low Range Reference Solution (LCA- 721, HACH) and High Range Reference Solution (LCA- 721, HACH), both traceable to Standard Reference Materials of NIST, to estimate parameters of precision and accuracy; (4) measure the absorbances of samples and use the calibration curves to determine concentrations.

Ammonia nitrogen was measured with salicylate ammonia nitrogen powder-pillow reagents (method 8155, HACH) using calibration standards prepared from a fresh ammonium chloride (NH_4Cl) stock solution according to the ISO 7150/1–1984 ammonia standard preparation procedure of section E. The absorbance wavelength measured was 655 nm.

Total nitrogen was analyzed using the persulfate digestion reaction (method 10072, HACH), in line with ISO 29441:2010. The calibration standards were prepared as described above for the ammonia nitrogen analysis. The absorbance was measured at 410 nm.

Nitrate nitrogen was analyzed using the cadmium reduction powder-pillows reagents (method 8039, HACH) using calibration standards prepared with potassium nitrate (KNO_3), all procedures in line with the reference guidelines for nitrate determination from the American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WEF) standard method SM 4500 – NO_3^- - B. The absorbance was measured at 500 nm.

Phosphate-phosphorus was determined using the ascorbic acid powder pillows reagents (method 8048, HACH) using calibration standards prepared with potassium dihydrogen phosphate (KH_2PO_4), in line with the guidelines for phosphorous determination from the APHA, the AWWA, and the WEF standard method SM 4500 – P - E. The measurement wavelength was 880 nm.

Chemical Oxygen Demand was analyzed using the potassium dichromate digestion (method LCI 400, HACH) with calibration standards prepared with potassium hydrogen phthalate (KHP) [C_6H_4(COOH)(COOK)], according to the ISO – 15705:2002 (E). The absorbances were obtained at wavelength 605 nm.

To evaluate precision and accuracy, each method was applied to obtain six absorbance reads and the respective six found concentrations were used. The standard deviation and the random error of the mean for the six concentrations were used to evaluate the precision, while the relative bias in percentage related to the known concentration was used to evaluate the accuracy.

2.6. Statistics tests

To study the significance of results on the adaptability of most abundant genera during the bioaumentation experiment and on the degradation efficiency of APAP and its intermediate metabolites, the significance of differences between averages was tested by the single factor analysis of variance (ANOVA) considering a significance threshold level of 5 %. Then, when ANOVA revealed significant differences among treatments, post-hoc Tukey Kramer's tests (Tukey, 1949) were carried out (also for 5 % significance level).

3. Results and discussion

3.1. Granular sludge characterization

3.1.1. Morphological attributes

The sample of granular sludge mixed liqueur collected for this work was transported to the laboratory for 15 minutes in a 5 L plastic bottle and the settled sludge was used to start the SBRs (at day 0). At that time the MLSS was 4,99 g/L, while the SV_5 and SV_{30} were 250 mL/L and 200 mL/L, respectively, thus making an SVI_5 of 50,12 mL/g and an SVI_{30} of 40,10 mL/g. For the morphological characterization of the sampled granular sludge, 147 granules were observed with the aid of a magnifier. The observations revealed mainly smooth and round or elongated granules with sizes varying from 2 to 6 mm, with an average of 3.4 ± 0.9 mm, and a variation fitting a normal distribution for a 1 % probability (α=0.01) in a Kolmogorov-Smirnov test. All these characteristics are, as expected, typical for mature aerobic granular sludge (e.g. Barrón-Hernández et al., 2022).

3.1.2. Prokaryotic community

A metataxonomic analysis using 16S rRNA gene sequences was used to study the main Prokaryotic communities in the initial granular sludge and during the bioaugmentation experiment with *M. aubagnense* HPB1.1. The base-calling generated 7.4 million sequence reads with a mean read quality of 11.3 and a mean read length of ~1700 bp (raw fastq reads were uploaded to the NCBI database with BioProject accession PRJNA1173003 and SRA Accessions SAMN44286944 to SAMN44286987). After trimming and filtering, 3.4 million reads with a mean read quality of 16.9 and a mean read length of ~1500 bp were used for taxonomic assignment using Kraken2 and 99.9 % of the reads were classified in 8217 unique taxa. Afterward, rarefaction curves for species richness showed on all samples an initial fast-growing trend followed by a gradual flattening (first figure in supplementary material B: Figure SB.1), which indicates that the amount of sequencing data in the samples is reasonable to analyse the evolution of the most abundant taxa.

The 8 phyla with higher abundances were *Actinomycetota*, previous *Actinobacteria* (1.5–5.1 %), *Bacillota*, previous *Firmicutes* (1.0–7.3 %), *Bacteroidota*, previous *Bacteroidetes* (12–27 %), *Chloroflexota* (or *Chloroflexi*) (1.2 % - 4.1 %), *Myxococcota*, previous members of the class *Deltaproteobacteria* which have been proposed to be better classified as *Myxococcota* phyl. nov. (Waite et al., 2020) (0.6–3.0 %), *Planctomycetota*, previous *Planctomycetes* (2.3–6.4 %), *Pseudomonadota*, previous *Proteobacteria* (52–75 %), and *Verrucomicrobiota*, previous *Verrucomicrobia* (0.5–1.9 %) (Fig. 1).

Aerobic granules with stable structure and function can be obtained with a range of different wastewaters, seeded with different sources of sludge, incubated at different operational conditions, and their microbial communities may be different (Wilén et al., 2018). Therefore, it is not surprising that the 8 most common phyla in the sludge used in this work are not the same as in the sludge used in other works. Despite this, 4 of the most abundant phyla in this work (*Actinomycetota, Bacteroidota, Bacillota, Pseudomonadota*) are generally among the most abundant in other granular sludge samples, while the other 4 phyla (*Chloroflexota, Myxococcota,*

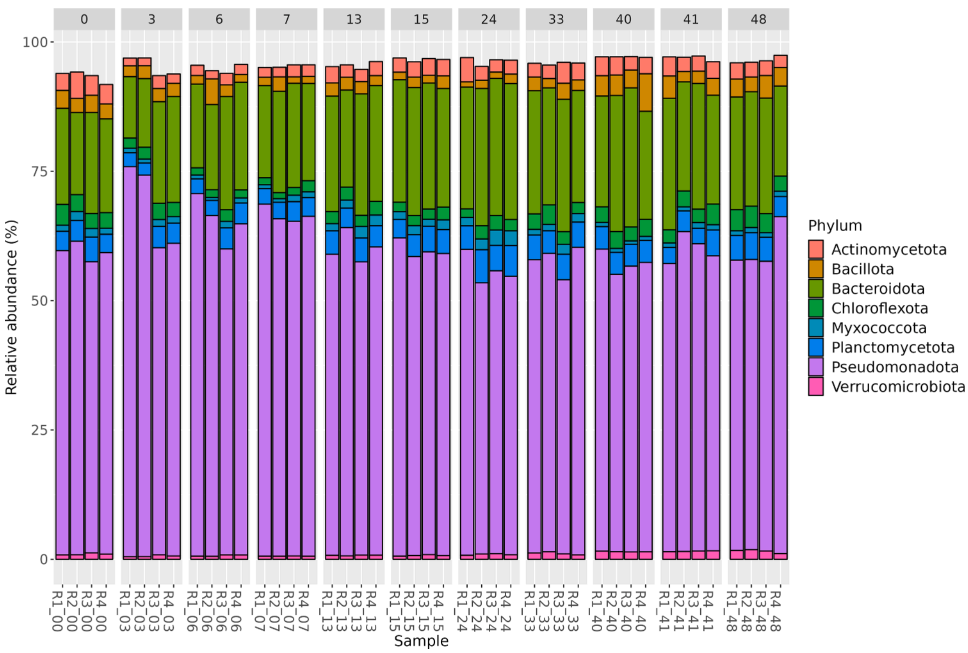

Fig. 1. Relative abundances of the eight highest abundant phyla in the 44 granular sludge samples organized by sampling time. Numbers on the top correspond to the operational days. R1_00 indicates Reactor 1 sampled at day 0 and R1_03 indicates Reactor 1 sampled at day 3, etc.

Planctomycetota, Verrucomicrobiota) are also generally represented but not always among the most abundant (e.g. Amorim et al., 2018; Barrón-Hernández et al., 2022; Kleikamp et al., 2023).

In what concerns the genera, 7 of the 8 most abundant were from phylum *Pseudomonadota*: *Aeromonas* (0.06–24 %), *Dokdonella* (0.4–8.5 %), *Lysobacter* (0.1–9.7 %), *Propionivibrio* (0.2–8.7 %), *Rhabdochromatium* (0.4–8.9 %), *Simplicispira* (0.8–5.7 %), and *Thiocystis* (1.1–10.1 %). The other was *Flavobacterium* (1–5 %), from phylum *Bacteroidota* (Fig. 2).

Looking in detail at each of these 8 most abundant genera, it can be said that together they have typical characteristics of what is expected in stable aerobic granular sludge and which can confer the capacity of guaranteeing wastewater treatment with efficient removal of organic compounds as well as nitrogen and phosphorus nutrients:

- *Aeromonas* genus (belonging to the family *Aeromonadaceae* and class *Gammaproteobacteria*) is often present in activated floccular sludge and has been detected in high abundances (4–14 %) in AGS (Fan et al., 2018). Moreover, it has been shown that it is possible to strengthen aerobic sludge granulation by the addition of certain *Aeromonas* spp. strains since they promote the secretion of extracellular polymeric substances (EPS), and improve the removal of COD, total nitrogen, and total phosphorus (Gao et al., 2019).

- *Dokdonella* genus (family *Rhodanobacteraceae* and class *Gammaproteobacteria*), is also usually present in activated sludge and has been found among the most abundant genera in AGS at relatively high abundances (~2.5 %) (Liu et al., 2020). *Dokdonella* spp. have been classified as putative denitrifiers since they were commonly detected in denitrifying bioreactors with aerobic or anoxic media, implying their tendency to take up both nitrate and nitrite (Pishgar et al., 2019). Moreover, a metagenomics study of denitrifying bacteria in anammox consortia based on the functional genes of denitrification pathways present in each genome allowed to identify 77 putative denitrifying bacteria mainly distributed in seven genera (*SCN-69–89, UTPRO2, Dokdonella, Ignavibacterium, UBA964, Rubrivivax,* and *Ottowia*), and identify five species: a *Dokdonella* sp., a *Ignavibacterium* sp., two *SCN-69–89* spp., and a *UTPRO2* sp. that dominated the community with abundances higher than 1.0 % for each species (Wang et al., 2022).

- *Lysobacter* genus (family *Xanthomonadaceae* and class *Gammaproteobacteria*) has been associated with the stability of AGS since their abundance increased when short settling time was successfully used to overcome the instability of AGS under nitrogen deficiency (Yin et al., 2019). Moreover, it has been reported that a *Lysobacter* sp. was among the microorganisms with a strong positive correlation with N-acyl-homoserine-lactone (AHL)-mediated quorum sensing (QS) production as well with External Polymeric Substances (EPS) production and the conversion of floccular biomass to highly structured granules (Tan et al., 2014).

- *Propionivibrio* genus (family *Rhodocyclaceae*; class *Betaproteobacteria*) is found among the most abundant bacteria in SBRs with AGS for municipal wastewater treatment (Ekholm et al., 2022). Most *Rhodocyclaceae* have the complete set of functional genes to synthesize poly-P from ATP and to hydrolyze poly-P, thus able to function as phosphorus-accumulating organisms (PAOs), and some can also perform denitrification thus able to function as denitrifying PAOs (DPAOs) (Hao et al., 2022). Interestingly, a study on SBRs operated to enrich for PAOs revealed co-dominant populations of a model PAO *Accumulibacter* and a novel GAO from the genus *Propionivibrio* with a genome closely related to the PAO genomes (Albertsen et al., 2016). Moreover, in a recent review article, this genus is included in a list of potentially identified DPAOs at the genus level (Diaz et al., 2022) since it is among the most abundant in both DPAO sludge and PAO sludge (Yun et al., 2019), and since studies of 4′-6–Diamidino-2–phenylindole (DAPI) staining and fluorescent in situ hybridization (FISH) using a selective probe demonstrated the participation of organisms in the *Rhodocyclaceae*

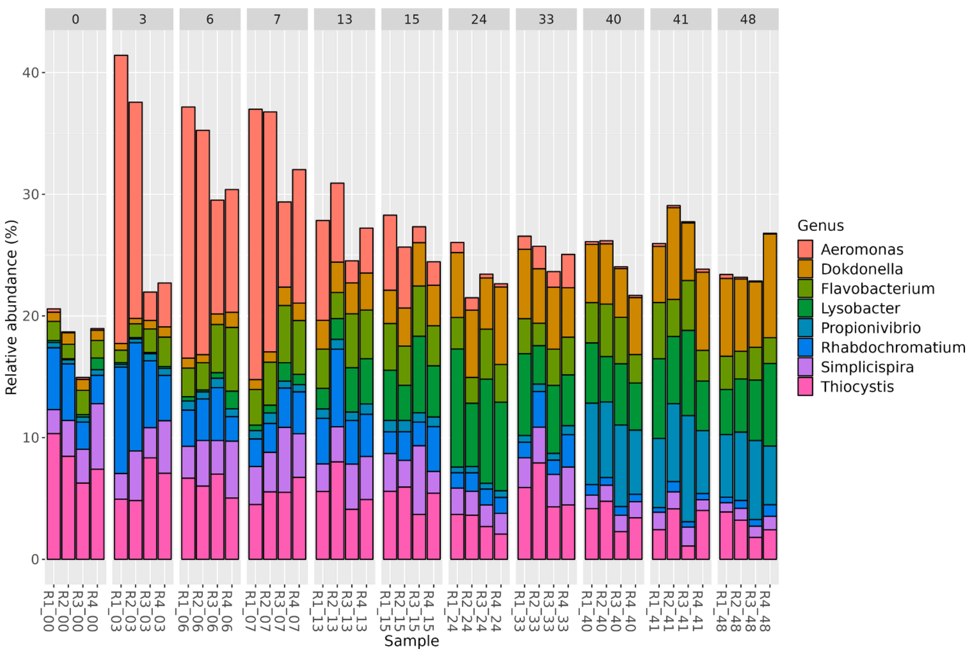

Fig. 2. Relative abundances of the eight highest abundant genera in the 44 granular sludge samples organized by sampling time. Numbers on the top for the operational day and R1_00 for Reactor 1 sampled at day 0 or R1_03 for Reactor 1 sampled at day 3, etc.

family as PAOs (Datta and Goel, 2010).

- *Rhabdochromatium* genus (family *Chromatiaceae*; class *Gammaproteobacteria*), has photrophic anaerobic purple sulfur bacteria (PSB) which were first found in mud samples of freshwater or salt marsh (Dilling et al., 1995). PSB bacteria can occur as blooms in stratified lakes if sulfate is present: sulfate-reducing bacteria in the sediments release sulfide, which diffuses into the water column forming a gradient that triggers the growth of these bacteria in specific zones where light and sulfide are optimal (Madigan and Jung, 2009). Studies in such blooms have shown that during the day, sulfide oxidation resulted in the intracellular accumulation of sulfur and glycogen, while at night the concentrations of glycogen and sulfur decreased concomitantly with the production of sulfide and poly-β-hydroxybutyrate (PHB) (van Gemerden et al., 1985). Interestingly, in the WWT industry, microorganisms employed for phosphorus removal, also known as PAOs, can synthesize poly-β-hydroxyalkanoates (PHA), including PHB, as their source of energy (Yuan et al., 2015; Seviour et al., 2003). Thus, the combination of this information suggests that the presence of members of this genus in granular sludge is important for treating wastewater where sulfates are present, as is the case with the WWTP subsystem where the sludge for this work was collected, which is in a coastal area and has evidence of saline inflows (Brito et al., 2022). Indeed, a metagenomic investigation on the microbial population in a lab-scale sequencing batch reactor operated in an enhanced biological phosphorus removal system to treat saline synthetic wastewater revealed PSBs from *Chromatiaceae* encoding the related sulfur conversion genes and the necessary genes for P removal, confirming they can be considered as potential S-related PAOs (Hao et al., 2022). Besides, it is known that the PSBs are within a major group of sulfur-oxidizing bacteria (SOB) that can be applied in the process called sulfur-driven autotrophic denitrification in wastewater treatment (Woo et al., 2022). Plus, a study on the nitrogen removal using activated sludge showed that carbon source supplements improved nitrogen removal and greatly increased the abundance of *Chromatiaceae* denitrifiers (Yasong et al., 2019), which supports the hypothesis that there are DPAOs in the *Chromatiaceae* family.

- *Thiocystis* genus (family *Chromatiaceae*, *Gammaproteobacteria*) is also a group of versatile phototrophic PSBs found in freshwater and brackish or marine waters (Imhoff, 2006). They are motile and can grow in darkness either as chemoorganotrophs or chemolithotrophs when the oxygen concentration is significantly reduced (Kampf and Pfennig, 1980). In our work, the dominant species of this genus was *Thiocystis minor* (formerly *Chromatium minus*). A study on the relations of phosphorus deficiency in the oxic–anoxic gradient with the distribution of planktonic photosynthetic populations (including *Thiocystis minor*) and with the activity of alkaline phosphatase (APA) along the vertical gradient of karstic sulfurous lakes, revealed capacity of sulfur phototrophic bacteria of thriving in nutrient-rich anoxic waters and an adaptation to face periods of phosphate limitation (Bañeras et al., 2010). These findings constitute further evidence that some PSB, and particularly *Thiocystis minor*, can function as PAOs. Interestingly, the granules of AGS have also oxic–anoxic gradients and are subjected to feeding and starving periods as occurring along the vertical gradient of karstic sulfurous lakes.

- *Simplicispira* genus (family *Comamonadaceae*, class *Betaproteobacteria*) includes aerobic bacteria and facultative anaerobic bacteria that can perform denitrification (Siddiqi et al., 2020), and it has been identified among the most abundant prokariotes in aerobic granular sludge which was enhanced to increase denitrification (Zhang et al., 2021). Interestingly, the species that most contributed to the high abundance of this genus in our work is *S. hankyongi*, which is a denitrifying bacterium discovered in sludge of a wastewater treatment plant (Siddiqi et al., 2020), and *S. limi*, which grows anaerobically by reducing nitrate to nitrite and was first time identified in activated sludge performing enhanced biological phosphorus removal EBPR (Lu et al., 2007).

- *Flavobacterium* genus (family *Flavobacteriaceae*, class *Flavobacteriia*) occurs frequently in AGS (Huang et al., 2021; Wilén et al., 2018). Species within this genus have been reported to promote the production of cyclic-diguanylate (c-di-GMP) which in turn stimulates the expression of genes responsible for EPS production, known to be associated with the granulation of WWTP sludge (Wan et al., 2015).

In addition to these 8 most abundant genera in the sludge used in this work, other genera commonly profuse in granular sludge were also present, in some cases with relatively high abundances (%): ammonia oxidizing bacteria - *Nitrosomonas* (0.2–3 %) and *Nitrosospira* (0 – 0.01 %); denitrifying bacteria able to secret EPS - *Rhodobacter* (0.6 – 2.3 %), *Thauera* (0.04 – 4 %), *Zoogloea* (0.02 – 4.9 %); EPS producers - *Acidovorax* (0.2 – 3.2 %), *Brachymonas* (0 – 0.05 %), *Paracoccus* (0.6 – 2.0 %), *Acinetobacter* (0.01 – 1.1); GAOs - *Defluviicoccus* (0.05 – 0.3 %); PAOs - *Tetrasphaera* (0.01 – 0.12 %), *Gemmatimonas* (0.08 – 0.6 %), *Dechloromonas* (0.002–0.3 %) (Wilén et al., 2018; Lv et al., 2014; Xia et al., 2018; Paulo et al., 2021 and Diaz et al., 2022, are some examples of works reporting these taxa in granular sludge). Moreover, though the genus *Candidatus Accumulibacter*, once considered to have the model PAOs, was not detected in our sludge, another genus from the same family (*Candidatus Competibacteraceae*) was represented: *Plasticicumulans* (0 – 0.2 %). This genus is also commonly present in AGS and it is known to accumulate large amounts of polyhydroxyalkanoates (PHA) (Zhang et al., 2021; Paulo et al., 2021).

3.2. Bioaugmentation

Looking at the evolution of phyla during the experiment, the first evidence is that there were two dominant phyla in the samples: *Pseudomonadota* with percentages of 52–75 %, and *Bacteroidota* with percentages of 12–27 %, while the other represented phyla had percentages < 8 %. Moreover, it is visible that the percentages of the 8 most abundant phyla in the two types of SBRs (control *vs* test) were similar throughout the experiment, except for a difference observed on day 3: the abundance of phyla *Pseudomonadota* was higher in the test SBRs than in the control SBRs, while the abundance of phyla *Bacteroidota* was lower in the test SBRs than in the control SBRs. Looking at the evolution at a lower taxonomic level, it is visible that the genus that most contributed to the differences observed on day 3 between the test and the control SBRs (described above) was *Aeromonas*, with a peak of 24 % on day 3 just in the test reactors. Apart from that, similar changes in the balance of the 8 most abundant genera occurred in all SBRs during the all experiment: the relative abundances of genera *Dokdonella*, *Lysobacter* and *Propionivibrio* increased over time, while the relative abundances of genera

Rhabdochromatium, *Simplicispira* and *Thiocystis* decreased. These dynamics indicate an ongoing adaptation of prokaryotic communities to the experimental conditions, which were different from those at the WWTP where the granular sludge was collected. This is not surprising since it has been demonstrated that microbial communities in granular sludge have sufficient plasticity to adapt to wastewater changes as well as operational failures and other incidents without loss of wastewater treatment capacity (Adler and Holliger, 2020; Zhang et al., 2022). The fact that at the end of the experiment there were no significant differences between the test reactors and the control reactors in the relative abundances of the 8 most abundant genera (Table 3) confirms that the final equilibriums in the prokaryotic communities were mainly the result of adaptations to the new operating conditions, rather than caused by the inoculations with *M. aubagnense* HPB1.1.

One of the differing conditions in the operation of the reactors was the addition of APAP on the days of biodegradation assays for this Ph, creating concentrations far higher than those typically found in the influents of municipal WWTPs. This could, in fact, be the reason for the increase in the relative abundances of the genera *Dokdonella*, *Lysobacter*, and *Propionivibrio*. Indeed, an increase in the relative abundance of the genus *Dokdonella* was previously observed following the addition of APAP in batch experiments with activated sludge (Palma et al., 2018). Moreover, though there are no proposed metabolic pathways for APAP in *Dokdonella* species, there are studies that show the functional potential of this genus for the biodegradation of persistent organic compounds. For example, Tusher et al. (2021), in a study that for the first time confirmed the involvement of a *Dokdonella* strain in the biodegradation of 1, 4-Dioxane, also confirmed the capacity of that strain to use as sole carbon and energy source phenol, a persistent compound with a similar structure to the metabolites detected in our bioreactors after APAP spiking. As for *Lysobacter* and *Propionivibrio*, to the best of our knowledge, there are no reported studies that specifically emphasize the role of these genera in the biodegradation of APAP. Despite that, there is information suggesting a potential connection. The *Lysobacter* genus comprises bacteria which typically produce exoenzymes that lyse and degrade other microorganisms, and *Lysobacter* spp. have been studied for their ability to degrade biofilms formed by microbes typically associated with device-related infections (Gökçen et al., 2014). This is *per si* an indication that *Lysobacter* spp. are able to degrade persistent compounds, and in fact *Lysobacter* strains have been described as able to degrade chlorinated Aromatic Compounds (Liang et al., 2011; Wang et al., 2011) and Ochratoxin A (Wei et al., 2020). In what concerns the *Propionivibrio* genus, for example, Brune et al. (2002) described a species able to degrade the hydroaromatic compounds quinic acid and shikimic acid. Thus, it is possible that *Propionivibrio* species may have the capability to degrade compounds with structural similarities such as the APAP intermediate metabolites detected in our work.

When the Chao 1 alfa-diversity index calculated with the absolute sequence read counts was used to make comparisons using the Kruskal-Wallis test, the hypothesis of equal diversities was not rejected in any of the tested comparisons: across the 44 samples, across samples merged by reactor, or merged by day, or merged by type of reactor. When the same tests were computed using Shannon's diversity metrics, they revealed similar conclusions except for the test with samples merged per type of reactor (control *vs* test), which gave a p-value lower than 0.05, meaning the rejection of equal populations for 95 % probability (Figures SB.2 to SB.6).

Since the Chao 1 index is more affected by the number of taxa – richness – (especially rare taxa) and the Shannon index is more affected by the evenness between samples (Xia and Sun, 2023), the fact that the test reactors and control reactors were just distinguished with the Shannon index confirms the significance of the differences on day 3 described above rather than indicating the incorporation of the inoculated strain in the sludge. Those differences were caused by the addition of LB growth medium in the test reactors in the first inoculation of *M. aubagnense* HPB1.1 on day 2, since in that inoculation the cells were added (by mistake) without a previous wash with the SBRs influent. All the subsequent inoculations were made with the cells first washed as described in the methods, and the taxa abundances in the subsequent sampling days returned to similar patterns in the test and the control reactors (Fig. 2).

To evaluate the incorporation of *M. aubagnense* HPB1.1 strain in the SBRs, the relative abundance of this species in the test and control reactors was plotted (Fig. 3). In the initial granular sludge, before inoculation with HPB1.1 strain, the relative abundance of *M. aubagnense* was ~0.007 %. Then, during the bioaugmentation experiment its relative abundance was always higher in the test reactors than in the controls. Looking more in detail at the changes of *M. aubagnense* relative abundance in the test reactors: first, a trend of growing occurred until day 13, a period in which there were four inoculations (at days 2, 4, 6, and 12); afterward, there was a peak of abundance in day 24, the only occasion samples for prokaryotic community studies were collected during an operation cycle with inoculation; finally, a decay followen by stabilization of this strain's abundance was observed in the remaining period, in which just two additional inoculations were made (at days 33, and 40). In the control reactors, there was some increase in the relative

Table 3
Relative abundances of the eight most abundant genera at the beginning and the end of the bioaugmentation experiment (averages with the same letters do not significantly differ at 0.05 level (ANOVA and Tukey Kramer's tests).

Genus	Day 0		Day 48	
	Test reactors	Control reactors	Test reactors	Control reactors
Aeromonas	0.1773 ± 0.1398 a	0.1497 ± 0.0144 a	0.2329 ± 0.1268 a	0.0646 ± 0.0107 a
Dokdonella	0.8498 ± 0.1247 a	0.8711 ± 0.0458 a	6.1653 ± 0.3203 b	6.9494 ± 2.2217 b
Flavobacterium	1.3764 ± 0.2680 a	1.7206 ± 0.3914 a	2.4939 ± 0.3218 a	2.4005 ± 0.4046 a
Lysobacter	0.1300 ± 0.0488 a	0.5767 ± 0.5427 a	4.0334 ± 0.4692 b	5.8717 ± 1.2871 b
Propionivibrio	0.3797 ± 0.0691 a	0.4372 ± 0.0333 a	5.3838 ± 0.3328 b	5.6517 ± 1.1832 b
Rhabdochromatium	4.8754 ± 0.3059 a	2.2833 ± 0.0633 b	0.5417 ± 0.1357c	0.7471 ± 0.2793c
Simplicispira	2.4481 ± 0.6933 a	4.0809 ± 1.8482 a	0.8674 ± 0.1446 a	1.0101 ± 0.1410 a
Thiocystis	9.3979 ± 1.3234 a	6.8328 ± 0.8013 a	3.5512 ± 0.4727 b	2.1163 ± 0.4419 b

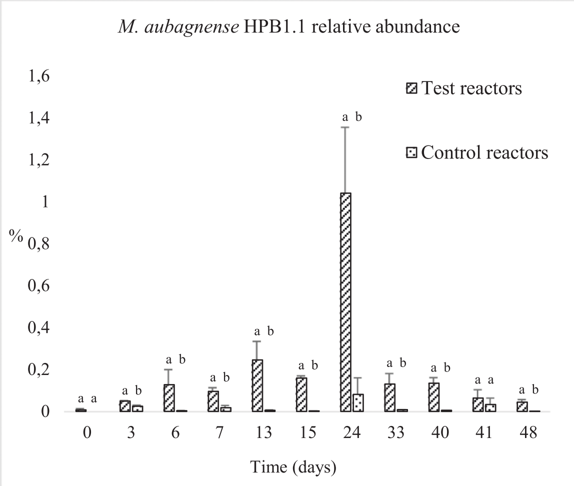

Fig. 3. Relative abundances of the bioaugmented strain *M. aubagnense* HPB1.1 in the SBRs based on the number of specific 16S rRNA sequences over the total 16S rRNA sequences. Averages with the same letters do not significantly differ at 0.05 level (ANOVA and Tukey Kramer's tests).

abundance of *M. aubagnense*, but just on three sampling times (days 3, 24, and 41) and always lower than in the test reactors. This was caused by problems that occasionally occurred at the time of discharging the effluent: when a reactor was discharged without closing (by mistake) the filling taps of the other reactors, there was transference of water from the full reactors to the one being discharged through the common filling pipe due to gravity. This was qwickly detected and immediately corrected, but it had some effect on the mentioned days. It is important to note that on the days of the APAP bioremoval assays (days 12 and 48) this did not occur, and those days the relative abundances of *M. aubagnense* were significantly higher in test reactors than in control reactors.

Regarding the duration of bioaugmentation, this work shows the following: (i) the relative abundance of *M. aubagnense* nine days after the fifth inoculation (performed in the first half-time of the experiment) was 0.13 ± 0.05 % in the test reactors and 0.0079 ± 0.0008 % in the control reactors; (ii) the abundance of *M. aubagnense* eight days after the seventh and last inoculation (performed in the second half-time) was 0.04 ± 0.02 % in the tests and 0.0005 ± 0.0005 % in the controls. Seen another way, the relative abundance of *M. aubagnense* in the day of the fifth inoculation in the test reactors was ~1,04 % and nine days after that was ~0,13 %, making a ratio of 8.Yet, the theoretical decrease in the abundance of the inoculated cells in a reactor after 9 discharges at half the height of the reactor is in the order of 1000 cells at the beginning to 1.95 at the end, which makes a ratio of 512. Moreover, the relative abundance of *M. aubagnense* in the day after the seventh inoculation in the test reactors was ~0063 % and seven days passed was 0044 %, making a ratio of 1.43.Yet, the theoretical decay of cells in 7 cycles is the magnitude of 1000–7.81, leading to a ratio of 128. This means that, despite some decrease in the relative abundance of the inoculated species, the sludge bioaugmentation was effective and persisted for at least nine days after five inoculations in 24 days and for eight days after seven inoculations in 40 days. In other words, although free specimens or specimens attached to small flakes of sludge have left the reactors during the discharges, some specimens have remained incorporated into larger agglomerates of sludge formed during the constant granulation process occurring in the SBRs.

Effective bioaugmentation of sludge freshly collected from a domestic wastewater treatment plant followed by some decrease in the relative abundance of the bioaugmented strain has already been reported for other species. For example, in a study on the bioaugmentation of sludge by a *Comamonas testosteroni* strain degrading 3-Chloroaniline in a lab-scale semicontinuous activated-sludge system, although it was shown that the inoculated strain maintained itself in the sludge for at least 45 days, the authors suggested that bioaugmentation is not permanent and will probably require regular supplementation (Boon et al., 2000). Nevertheless, in another study study conducted to evaluate the bioaugmentation of 1H-1,2,4-triazole (TZ) and tricyclazole (TC) degrading strains *Shinella sp. NJUST26* and *Sphingomonas* sp. NJUST37 in activated sludge it was shown that the strains increased to 2.2 % and 2.9 % of prokaryotes relative abundance after the bioaugentation inoculum and remained at 1.9 % and 2.3 % after 294 days, while the removals of these fungicides increased from ~14 % to > 95 % and remained at this level during that long-term operation time (Wu et al., 2018). Thus, only with more studies carried out over longer periods and with sludge kept in different conditions it will be possible to estimate models allowing predictions and establishing forms of operation in WWTPs. Even so, these initial works are important as they prove the feasibility of bioaugmentation with the species studied and pave the way for further experiments.

3.3. APAP bioremoval

Phs contaminants, such as APAP, significantly impact the environment, disrupting aquatic life, plant health, and overall ecosystem balance. APAP enters the environment through household waste, hospitals, and PH industries, where it is collected at WWTPs. However, the removal of APAP at these facilities is often incomplete, leaving residual amounts in treated effluents that are discharged into surface waters, which in some cases are then used for irrigation. Furthermore, some of the APAP is adsorbed onto sewage sludge, which is subsequently used as material to produce compost to fertilize agricultural soils (Grignet et al., 2022; Lara-Moreno et al., 2024c). As a result, APAP is detected not only in surface waters but also in groundwater systems, and it tends to accumulate in soil (Mejias et al., 2021). Even at low concentrations, its presence in these environmental matrices has been linked to negative effects and toxicity in various species due to its bioaccumulation (Nogueira and Nunes, 2021). Of particular concern is the accumulation of APAP in certain aquatic organisms, where it can cause reproductive, neurotoxic, and endocrine disorders (Guiloski et al., 2017). This work arises as a response to the pressing issue of environmental contamination, focusing on addressing these challenges through improvements in wastewater treatment processes and the adoption of green technologies, such as biodegradation, to mitigate the environmental burden of PH contaminants.

The HPLC analysis revealed the APAP peak at retention times (RTs) from 6.1 to 6.5 min and its calibration regression resulted in an R^2 of 0.9993, while the HQ peak was at RTs 4.2–4.5 min and its calibration regression had an R^2 of 0.9925. Moreover, two other peaks of putative transformation products were identified: one at RTs from 5.1 to 5.4 min and another at RTs from 6.7 to 6.9 min. Interestingly, some transformation products of HQ have different absorption spectra which can be used for identification and two of the compounds reported by Fónagy et al. (2021) have absorption spectra matching with the two de putative transformation products detected in our work: the peak at RTs 5.1–5.4 min has the typical absorption spectra of 2,5-HO-BQ, with a major characteristic peak at ~ 300 nm, and the peak at RTs of 6.7–6.9 min has the typical absorption spectra of BQ, with a major absorption peak at ~246 nm.

Assay 1 - In the experiment carried out immediately after the fourth inoculation of *M. aubagnense* HPB1.1, at operational day 12, the concentration of APAP decayed over 97 % during the aeration stage of the first cycle after the drug spiking, either in the inoculated test SBRs and in the control SBRs. During that period, two of the detected APAPs' transformation products (HQ and 2,5-HO-BQ) were generated in all reactors at the same time until they reached their maximum detected contents after ~7.5 h aeration. Interestingly, after the maximum detection of those compounds, they had a faster decay in the bioaugmented test SBRs than in the control SBRs. Moreover, the content of the third detected APAPs' transformation product (BQ) stayed stable after 7.5 h of aeration in the test reactors, while it continued raising in the control reactors (Fig. 4). When the aeration stage of the subsequent cycle started, APAP and all three studied transformation products were still detected in the control SBRs, while only 2,5-HO-BQ was detected in the bioaugmented SBRs. Nevertheless, during the aeration stage of this second cycle after APAP spiking all the analyzed compounds decayed to non-detected levels (Fig. 5).

Assay 2 - In assay 2, carried out (on day 47) one week after the last inoculation of *M. aubagnense* HPB1.1, the study was restricted to the first operational cycle after spiking the APAP since in assay 1 the major difference in the removals of APAP's transformation products occurred in the first cycle after the drug spiking. The results obtained in assay 2 are roughly like those in assay 1, thus showing that the bioaugmentation effect was maintained (Fig. 6). However, in this case, the difference between the test and the control SBRs regarding the removals of APAP's transformation products started to be evident after ~10 to ~15 h of aeration, that is ~2.5 to ~7.5 h later than in assay 1.

In general, similar removal of APAP was observed in all reactors (tests and controls) – decay in one operational cycle in both assays

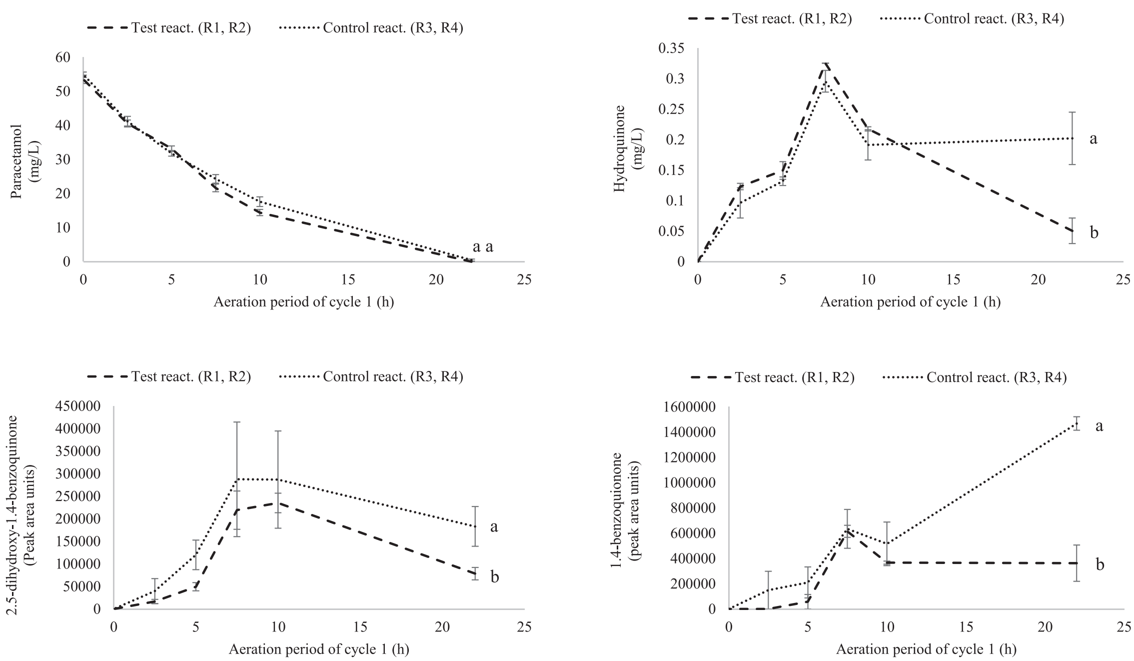

Fig. 4. Contents of Paracetamol, Hydroquinone (HQ), 2,5-dihydroxy-1,4-benzoquinone (2,5-HO-BQ) and 1,4-benzoquinone (BQ) in the liquid phase of test reactors and control reactors during the aeration stage of the first operational cycle after Paracetamol spiking in Assay 1 (1st assay, 1st cycle). Averages with the same letters do not significantly differ at 0.05 level (ANOVA and Tukey Kramer's tests).

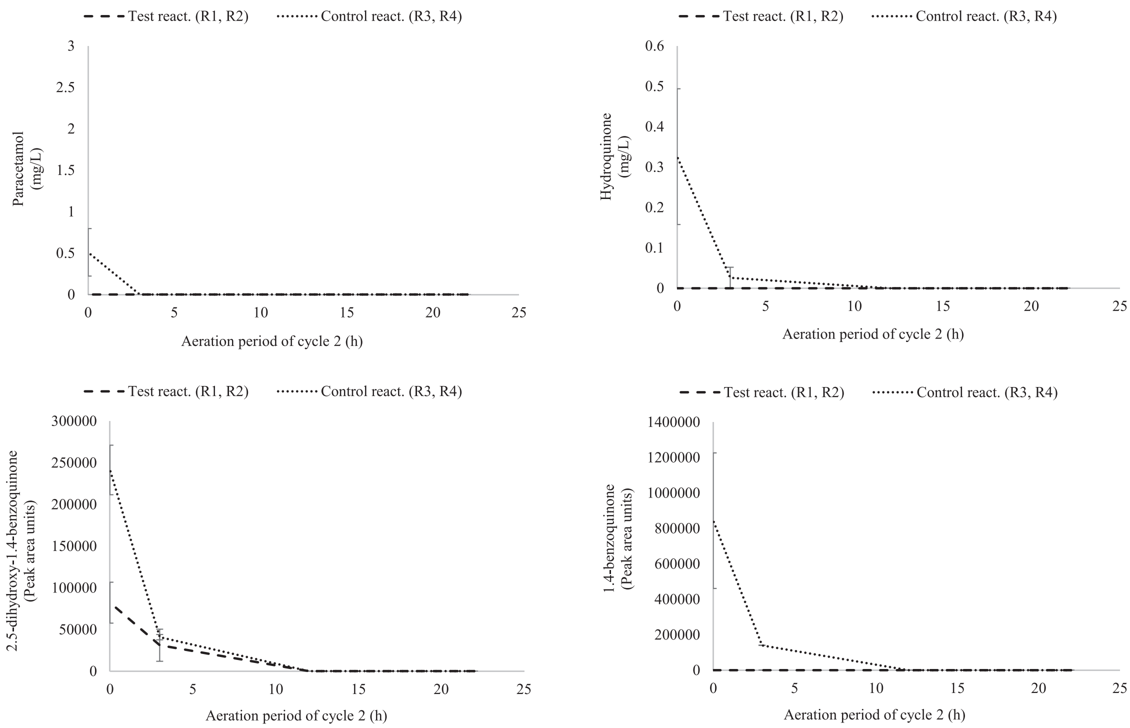

Fig. 5. Contents of Paracetamol, Hydroquinone (HQ), 2,5-dihydroxy-1,4-benzoquinone (2,5-HO-BQ) and 1,4-benzoquinone (BQ) in the liquid phase of test reactors and control reactors during the aeration stage of the second operational cycle after Paracetamol spiking in Assay 1 (1st assay, 2nd cycle).

1 and 2 was between 98 % and 99 % - showing that: (i) the achieved abundances of the bioaugmentated *M. aubagnense* HPB1.1 in the test SBRs (~0.35 % at the time of assay 1 and ~0.04 % at the time of assay 2) did not make a major difference in the removal of this drug, and (ii) the tested granular sludge had organisms with functional enzymes for the first catabolic reactions to breakdown this drug. Indeed, 2 of the 8 most abundant genera found in the granular sludge (*Dokdonella*: 2.67 % at time of assay 1 and 6.55 % at time of assay 2; *Flavobacterium*: 3.44 % at time of assay 1 and 2.45 % at time of assay 2) have been identified in a previous study as having an important role in the biodegradation of APAP (Palma et al., 2018). Moreover, *Pseudomonas* was also well represented in the granular sludge (0.21 % at the time of assay 1 and 0.17 % at the time of assay 2), and this genus is known to have an important role in the biodegradation of APAP (e.g. De Gusseme et al., 2011; Palma et al., 2018; Poddar et al., 2022).

Nevertheless, and interestingly, the bioaugmentation with *M. aubagnense* HPB1.1 caused a faster decay of APAP's intermediate metabolites. The maximum content achieved for two of these compounds (HQ and 2,5-HO-BQ) after APAP spiking was similar in all SBRs (a similar content of each compound was reached simultaneously in the four SBRs). Yet, subsequently, their removals percentages (based on the respective maximum achieved contents) were at the end of the first aeration stage higher in the test bioaugmented SBRs (HQ: 59 % or 85 %; 2,5-HO-BQ: 67 % or 85 %, decays respectively for the assay 1 or the assay 2) than in the control non-bioaugmented SBRs (HQ: 15 % or 31 %; 2,5-HO-BQ: 36 % or 63 %, decays also for the assay 1 or 2). In what concerns the third metabolite monitored (BQ), the difference was even more evident since in the control reactors the content of this compound did not decay, while its removal in the test reactors (% to the maximum achieved) was 75 % and 82 % in the assay 1 and assay 2, respectively.

Independently of bioaugmentation, the removal of APAP and its transformation products in the aeration phase of SBR reactors indicates that aerobic metabolisms in the granular sludge play a fundamental role in biodegrading persistent organic pollutants with aromatic rings in their structures. This was expected because other studies show this evidence. For example, it was reported that the decomposition of the 1H-benzotriazole in SBRs was five times higher in aerobic conditions than in the anaerobic phase (Struk-Sokołowska et al., 2022).

More importantly, this work, by showing the ability of *M. aubagnase* HPB1.1 to be incorporated in SBRs and to improve the bio-removal of APAP's main transformation products, together with our previous works that demonstrated the ability of pure cultures of this strain to biodegrade APAP and its main transformation products (Lara-Moreno et al., 2024a) and to perform the first steps of Ibuprofen biodegradation (Lara-Moreno et al., 2024b), as well as other works that showed the ability of different *M. aubagnense* strains to degrade polycyclic aromatic hydrocarbons (Darmawan et al., 2015; Naloka et al., 2021), highlight the high potential of this taxa in applications framed in biotreatment and bioremediation strategies aiming the biodegradation of a broad range of persistent organic pollutants, including Phs.

Our results are one more evidence highlighting the potential of biodegradation and bioaugmentation strategies to enhance the

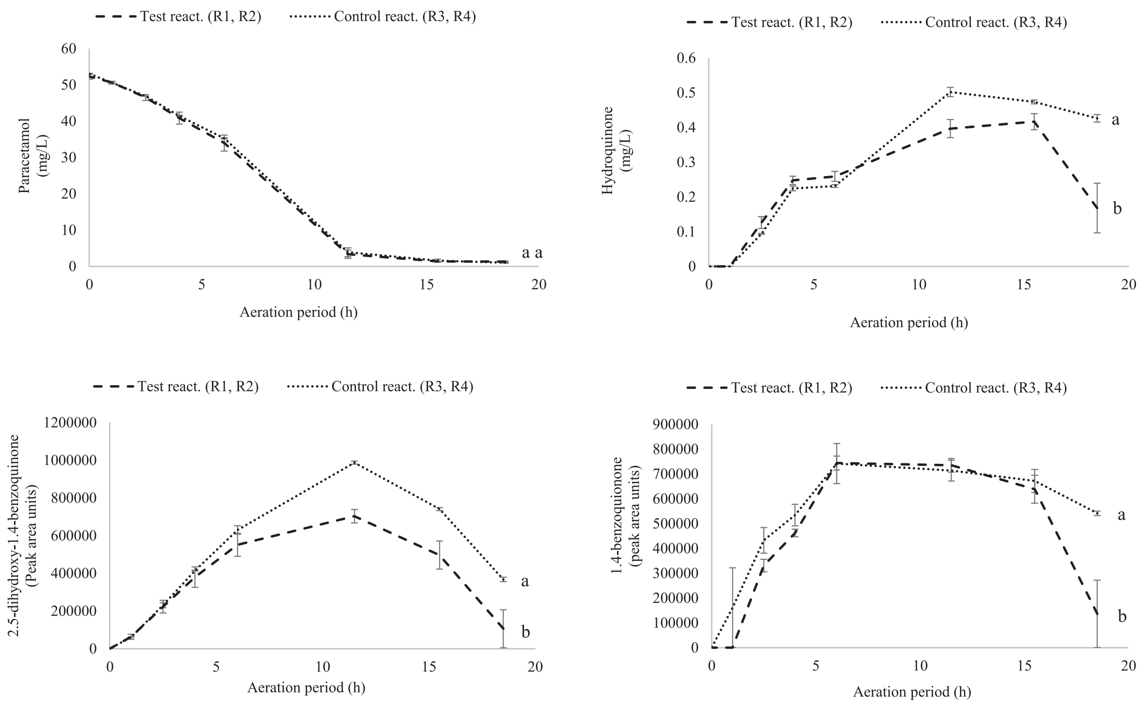

Fig. 6. Contents of Paracetamol, Hydroquinone (HQ), 2,5-dihydroxy-1,4-benzoquinone (2,5-HO-BQ) and 1,4-benzoquinone (BQ) in the liquid phase of test reactors and control reactors during the aeration stage of the first operational cycle after Paracetamol spiking in Assay 2 (2nd assay, 1st cycle). Averages with the same letters do not significantly differ at 0.05 level (ANOVA and Tukey Kramer's tests).

removal efficiency of Ph contaminants, particularly APAP, in WWTPs. Furthermore, the use of selected microorganisms is supported by a wide range of scientific studies that have proposed bioaugmentation as an effective approach for the removal of Ph compounds (Poddar et al., 2022; Lara-Moreno et al., 2024a). However, the number of studies focusing on drug removal in bioreactors through bioaugmentation remains very limited.

For instance, in the case of APAP, Gusseme et al. (2011) reported the complete removal of a 100 µg/L concentration of the drug within 15 days in a membrane bioreactor, attributed to the presence of *Delftia tsuruhatensis* and *Pseudomonas aeruginosa*. Additionally, they observed that HQ was temporarily formed as an intermediate during the removal process but was subsequently degraded. Similarly, Park and Oh (2020) investigated the bioaugmentation of activated sludge with a strain of *Pseudomonas* to enhance APAP biodegradation. Using a bioreactor fed with 100 mg/L of APAP, they demonstrated that bioaugmentation with *Pseudomonas* at proportions of 5 %, 25 %, and 50 % significantly increased transformation rates of APAP to 1.5, 1.9, and 2.3 d^{-1}, respectively, compared to 1.2 d^{-1} without bioaugmentation. In comparison, our study goes further by analyzing a broader range of metabolites beyond APAP and monitoring the bioaugmented species (*M. aubagnense*), to evaluate the effectiveness of this treatment. To our knowledge, no other published studies have investigated exogenous bioaugmentation for APAP degradation in bioreactors this way.

Additionally, studies on bioaugmentation for the removal of other Ph compounds are also scarce. For example, Chen et al. (2024) demonstrated that the use of *Sphingobacterium* sp. WM1 and a genetically engineered strain carrying the *tetX* gene significantly enhanced the degradation of tetracycline antibiotics (TC) in sequential batch reactors. Bioaugmentation also increased the abundance of the *tetX* gene, boosting TC degradation capacity and promoting its transfer to native bacterial species. Similarly, Fang et al. (2022) immobilized *Paraclostridium* sp. strain S2 and introduced it into a sulfate-reducing up-flow sludge bed bioreactor for 80 days. The bioaugmented bioreactor with immobilized strain S2 achieved an 18.7 % higher removal efficiency of ciprofloxacin (CIP) compared to the control bioreactor, even at high CIP concentrations (10,000 µg/L).

Mycolicibacterium species can be naturally found in the environment, both in soil and aquatic systems, and can cause opportunistic infections of the skin, soft tissues and occasionally the lungs, especially in immunocompromised patients or in the context of trauma, surgery or use of medical devices. invasive (Adékambi et al., 2006). However, according to this author's description of *Mycobacterium aubagnense* sp. nov., this species is susceptible to most antibiotics (amoxycillin, amoxycillin/clavulanate, cefoxitin, imipenem, minocycline, doxycycline, clarithromycin, erythromycin, azithromycin, amikacin, tobramycin, ciprofloxacin, ofloxacin, sparfloxacin and trimethoprim/sulfamethoxazole). The strain used in this work (HPB1.1) has not yet been tested for antibiotic resistance, but confirming this type of susceptibility could prove advantageous for its use in biotreatment and bioremediation strategies for persistent organic compounds. Moreover, even if this is not the case, the functional genomic information that could be obtained from additional studies on this strain has potential applications in genetic bioaugmentation. Indeed, genetic bioaugmentation to improve biodegradation of persistent pollutants is a proved concept (eg.Ke et al., 2022), and such type of strategies to improve the degradation of persistent pollutants can be viewed as a new Era in bioremediation (Jiménez-Díaz et al., 2022).

3.4. Wastewater treatment parameters

Differences in the effluents from the test SBRs and control SBRs were observed only on day 3, the day after the first inoculation with *M. aubagnense* HPB1.1 for the bioaumentation test, when cells were added still in LB growth medium (by mistake) without a previous wash with the synthetic sewage influent. This caused higher values of COD, total nitrogen, nitrates, and nitrites in the test reactors' effluents on that day (Figure SB.7). Afterward, the behavior of monitored parameters throughout the experiment was similar in all SBRs. The pH was ~7.1 and ~7.6 in the influent and effluent samples, respectively. The COD was ~1000 mg/L O_2 in the influent and ~55 mg/L O_2 in the effluents, indicating an effective consumption of organic compounds. The concentration of total nitrogen (TN) in the influent was ~45 mg/L; of which ~ 86.4 % corresponded to ammonia nitrogen (NH_4 - N), ~12.8 % to nitrite nitrogen (NO_2^- - N) and ~0.8 % to nitrate nitrogen (NO_3^- - N), while the concentration of TN in the effluents was ~ 31 mg/L, of which ~5.6 % was NH_4 - N, ~38.9 % was NO_2^- - N and ~55.5 % was NO_3^- - N, thus suggesting effective nitrification and partial denitrification in the SBRs. Finally, the concentration of phosphate phosphorus (PO_4^{3-} - P) was ~97 mg/L in the influent and ~93 mg/L in the effluent samples, indicating ineffective dephosphatation.

Looking in detail at the evolution of the analyzed parameters along the experiment, it is notable that the only days on which PO_4^{3-} - P was partially removed were: day 12, when there was a spike in COD in the influent (due to an error in the preparation of the carbon source solution on that week), and days 31 and 53, when there were peaks in ammonia and a drop in nitrites in the effluents (when after the anaerobic feeding, it was tested a longer incubation period without aeration - overnight - followed by shorter periods of aeration - 90 min).

These results together with the prokaryotic composition of granules described above suggest that the incomplete denitrification and ineffective dephosphatation, rather than being caused by a lack of denitrifiers and PAOs or DPAOs in the granular sludge, was due to inappropriate carbon sources dosage and/or dissolved oxygen and oxidation-reduction potential (ORP) conditions provided to the granules. In fact, these are two of the main parameters influencing P and N removals in WWTPs in general (Diaz et al., 2022), as well as in systems with granular sludge (de Kreuk et al., 2007; Bassin et al., 2012; Kehrein et al., 2020).

The results of parameters monitored along the operational cycle of day 16 (Figure SB.8) corroborate the idea of insufficient carbon sources dosage. The SBRs were operated in a laboratory at room temperature (23 ± 2 ºC), which was within a good range to accomplish simultaneous COD, nitrogen and phosphate removal is granular systems (Bassin et al., 2012). The pH was also within the optimal range (Kuba et al., 1997), despite the gradual rise from ~7.2 to ~7.6 along the cycle. On the other hand, after the anaerobic feed stage, the COD was ~260 mg/L O_2 and then dropped during the first hour of aeration to ~50 mg/L O_2 and stayed at this level, indicating that part of the carbon sources provided in the influent were still available at the beginning of the aerated period but then they were consumed in one hour. It is also visible that most of the ammonia was converted to nitrates in the anaerobic feeding phase, and then the remaining ammonia completely fell during the aeration phase. On the other hand, even if the nitrites gradually rose throughout the entire cycle, the nitrates decreased just during the first hour of aeration simultaneously with the last COD decay, which suggests denitrification occurred only when biodegradable-COD was still available.

Optimizing the removal of nutrients in the system where the granules were tested was an option. However, it is known that in granule systems lengthy experiments should be carried out to achieve steady-state optimized processes (de Kreuk et al., 2007), and the object of this study was not nutrient removal, but rather the bioaugmentation of a bacterial strain in granular sludge and its effect on the biodegradation of APAP.

4. Conclusions

The bioaugmentation of *M. aubagnense* HPB1.1 in laboratory-scale SBRs with AGSwas effective and persisted for at least nine days.

The bioaugmentation of *M. aubagnense* HPB1.1 in AGS can improve the removal of APAP's transformation productsHQ, 2,5-HO-BQ and BQ from wastewater.

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Author Statement

To whom it may concern, I, Jorge Daniel Dias Carlier, holder of Portuguese Citizen card N.er 9827289, on behalf of all the authors of the article entitled "Studies on the bioaugmentation of *Mycolicibacterium aubagnense* HPB1.1 in aerobic granular sludge from a WWTP: adaptability of native prokaryotes and enhancement of paracetamol intermediate metabolites biodegradation" for consideration of publication in the journal "Environmental Technology & Innovation" published by Elsevier, I declare that:

- the work described has not been published previously except in the form of a preprint, an abstract, a published lecture, academic thesis or registered report.
- the article is not under consideration for publication elsewhere.
- the article's publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out.
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Jorge Daniel Dias Carlier

CRediT authorship contribution statement

Costa Maria Clara: Writing – review & editing, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **Igbodo Benjamin:** Writing – review & editing, Methodology, Investigation. **Lara-Moreno Alba:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Carlier Jorge Daniel Dias:** Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Jorge Daniel Dias Carlier reports financial support was provided by Foundation for Science and Technology. Alba Lara-Moreno reports financial support was provided by European Union. Benjamin Igbodo reports financial support was provided by Erasmus MC. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2025.104073.

Data availability

raw fastq reads were uploaded to the NCBI database with BioProject accession PRJNA1173003 and SRA Accessions SAMN44286944 to SAMN44286987

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