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MULTI-STRAIN IMMOBILIZATION IN EXTRACELLULAR POLYMERIC SUBSTANCES FOR IMPROVING RECALCITRANT POLLUTANTS REMOVAL FROM WASTEWATER

by

BIDHATA K.C

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Thesis presented to Escola Superior de Biotecnologia of the

Universidade Católica Portuguesa to fulfill the requirements of Master of Science degree in

Applied Microbiology

by

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RESUMO

A descarga de resíduos industriais e domésticos em sistemas coletores de águas residuais leva a introdução de poluentes recalcitrantes que resistem ao tratamento pelas comunidades microbianas indígenas presentes nas estações de tratamento de águas residuais (ETAR). Esses poluentes recalcitrantes, tais como desreguladores endócrinos, compostos fluorados e clorados e produtos farmacêuticos podem afetar negativamente a saúde humana e a vida aquática. Uma possível solução para eliminar os poluentes recalcitrantes é através do uso de microrganismos degradadores especializados para bioaumentar sistemas de tratamento de águas residuais. Neste estudo, substâncias poliméricas extracelulares (EPS) extraídas de grânulos aeróbico (AGS) foram usadas como material de imobilização de origem natural para três estirpes bacterianas *Labrys portucalensis* F11, *Rhodococcus* sp. ED55 e *Rhodococcus* sp. S2, conhecidas por degradar fluorobenzeno (FB), 17β-estradiol (E2) e ácido 4-fluorocinámico (4-FCA), respetivamente. Foram realizados ensaios para avaliar a eficiência dos grânulos de bactérias imobilizadas para remover uma combinação de FB (1 mM), 4-FCA (0,5 mM) e E2 (0,018 mM) e comparar o desempenho destes relativamente aquele obtido pelas suspensões bacterianas das mesmas estirpes. Técnicas de cromatografia gasosa e cromatografia líquida de alta eficiência foram usadas para quantificar os tóxicos. Os grânulos de bactérias imobilizadas degradaram completamente E2 em 42,5 h, 4-FCA em 66,2 h e FB em 90 h. Embora os tóxicos sejam parcialmente adsorvidos pelos grânulos (FB 3,16 ± 2,49 $mM/g_{grânulos}$, 4-FCA 0,640 ± 0,073 $mM/g_{grânulos}$ e E2 0,0048 ± 0,0003 $mM/g_{grânulos}$), foi observada libertação estequiométrica de fluoreto, indicando a biodegradação completa de compostos fluorados. As suspensões bacterianas exibiram uma degradação mais rápida de E2 (<20 h), mas foram necessários tempos de remoção mais longos para remover 4-FCA e FB de 90,1 h e 231,5 h, respetivamente. Os resultados destacam que a co-imobilização de estirpes numa matriz de EPS pode aumentar a eficiência de remoção de compostos fluorados em comparação com as suspensões bacterianas. Adicionalmente, os resultados realçam o potencial uso de EPS extraído como um agente imobilizador de estirpes degradadoras para bioaumento, possivelmente melhorando a eficiência dos sistemas de tratamento de águas residuais.

Palavras-chave: grânulos aeróbios, substâncias poliméricas extracelulares, multi-imobilização de bactérias, compostos recalcitrantes.

Abstract

The increasing discharge of industrial and household waste into wastewater collection systems introduces recalcitrant pollutants that resist treatment by indigenous microbial communities present in wastewater treatment plants (WWTP). These recalcitrant pollutants, such as endocrine disrupting compounds, fluorinated and chlorinated chemicals, and pharmaceuticals can adversely affect human health and aquatic life. A possible solution to eliminate the recalcitrant pollutants and protect humans and ecosystems could be the use specialized degrading microorganisms to bioaugment wastewater treatment system. In this study, extracellular polymeric substances (EPS) extracted from aerobic granular sludge (AGS) were used as a natural carrier material for the immobilization of three bacterial strains *Labrys portucalensis* F11, *Rhodococcus* sp. ED55, and *Rhodococcus* sp. S2, known for degrading fluorobenzene (FB), 17β-estradiol (E2), and 4-fluorocinnamic acid (4-FCA) respectively. Batch assays were performed to assess the efficiency of the multi-immobilized bacteria granules to remove a combination of FB (1 mM), 4-FCA (0.5 mM), and E2 (0.018 mM), and compare their performance with the ones obtained by using bacterial suspensions of the same strains. Gas chromatography and high-performance liquid chromatography were used to quantify the toxics. The co-immobilized bacterial granules were able to completely degrade E2 within 42.5 h, while 4-FCA and FB were completely degraded by the end of 66.2 h and 90 h, respectively. Although the toxics adsorbed onto the multi-immobilized bacteria granules to some extent (FB 3.16 ± 2.49 mM / $g_{granules}$, 4-FCA 0.640 ± 0.073 mM / $g_{granules}$, and E2 0.0048 ± 0.0003 mM / $g_{granules}$), stoichiometric fluoride release was observed, which is indicative of complete biodegradation of fluorinated compounds. Bacterial suspensions exhibited a faster degradation of E2 (<20 h), but 4-FCA and FB required longer removal times of 90.1h and 231.5 h, respectively. These results highlight that the co-immobilization of strains in an EPS matrix can enhance the removal efficiency of fluorinated compounds in comparison to the bacterial suspensions. It also supports the potential use of extracted EPS as an immobilizing agent of degrading strains for bioaugmentation purposes, possibly improving wastewater treatment systems' efficiency.

Keywords: aerobic granular sludge, extracellular polymeric substances, multi-immobilized bacteria, recalcitrant compounds.

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1. Introduction

1.1. Water and its environmental challenges

Water is universally accepted as one of the major factors for livelihoods. Approximately 70% of the Earth's surface is covered by water, but only 1% is available for consumption. The human population is globally increasing which has significantly raised the amount of water consumption (Kumar et al., 2022). Nowadays, the supply of clean drinking water is becoming increasingly scarce. Consequently, about 2.2 billion people do not have access to safe drinking water worldwide (UNICEF/WHO, 2021).

The rapid growth of the population has led to increased demands for an enhanced quality of life, which, in turn, drives urbanization and industrialization. However, these developments have significant environmental consequences, and pollution by natural and anthropogenic factors (Figure 1) is affecting both surface water and groundwater (Akhtar et al., 2021). One of the most pressing issues is the improper disposal of sewage, industrial effluents, and wastewater from domestic uses. This mismanagement results in severe water pollution, characterized by high levels of heavy metals, organic matter, and various chemicals which often lead to serious environmental problems, including the deterioration of water quality and ecosystems in water bodies, eutrophication events, and human health issues. One of the most significant and challenging tasks of this century will be to meet the growing demand for freshwater (He et al., 2021).

Wastewater often contains excessive amounts of suspended solids, toxic chemicals, and heavy metals. Moreover, it could have high and variable salinity levels as well as micropollutants, such as endocrine disrupters, pharmaceuticals, illicit and non-controlled drugs, personal care products, flame retardants, nanoparticles, perfluorinated compounds, pesticides, or fuel additives, among others (Houtman, 2010). One of the main problems that wastewater treatment plants (WWTPs) are facing is the difficulty in treating emerging contaminants in wastewater. The term "emerging contaminants" refers primarily to contaminants for which there is currently no regulation requiring monitoring or public reporting of their presence in our water supply or wastewater. The concentration of emerging contaminants mostly ranges between ng/L to µg/L (Matamoros & Bayona, 2006). Various research studies have been performed to demonstrate their increasing concerns and effects (Morin-Crini et al., 2022).

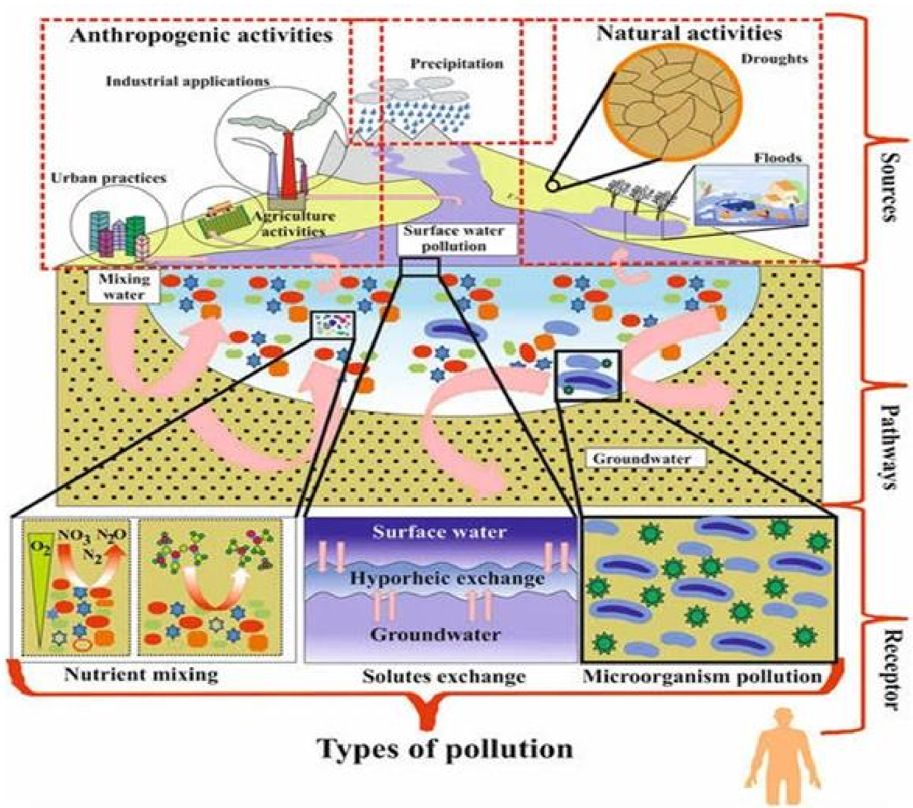

Figure 1 - Schematic representation of water contamination due to natural sources (droughts and floods) and anthropogenic sources (industrial, agriculture, and urban activities), their pathways, and receptors. Image from Akhtar et al., (2021).

1.1.1. Fluorinated aromatic chemicals

The water contamination resulting from fluorinated aromatic chemicals, which tend to be persisting organic compounds, is one of the biggest threats to both the environment and human health. The physicochemical properties of fluorine atoms in those compounds contribute to their recalcitrance. The smaller van der Waals radius (1.47 Å) of fluorine allows a more stable bond with carbon atoms. Additionally, the high electronegativity of fluorine (3.98) strengthens the C-F bond, making the fluorinated molecules highly stable (Murphy et al., 2009). This high stability makes fluorinated chemicals highly desirable for a wide range of commercial applications such as fluorocarbons in refrigerants and propellants, fluoroelastomers for sealing and gasket materials, and fluoropolymers for coatings and linings. Additionally, fluoroantimicrobials are employed in medical products, fluoroacids serve as catalysts in organic synthesis, and fluorinated surfactants are utilized in cleaning agents, lubrication, and fire-fighting products. (Key et al., 1997; Linclau et al., 2016). The knowledge of the biodegradation pathway of these chemicals can provide information on the molecule breakdown and potential removal processes.

One of these fluorinated compounds is 4-fluorocinnamic acid (4-FCA, Figure 2a), frequently employed in the manufacturing of fine chemicals, medicines, agrochemicals among others (Freitas dos Santos et al., 2001). Amorim et al., (2014) found that the bacterial strain *Rhodococcus* sp. S2 was able to completely mineralize 0.5 mM of 4-FCA by first converting it to 4-fluorobenzoate (4-FBA) within 216 h. However, when acetate (0.20 mM) was present as an additional source of carbon and energy, a faster biodegradation of 4-FCA was achieved in about 50 h (Amorim et al., 2014). The use of a mixed culture of two bacterial strains, *Arthobacter* sp. strain G1 and *Ralstonia* sp. strain H1, also provided complete degradation of the 4-FCA (10 mM). *Arthrobacter* sp. strain G1 was able to transform 4-FCA into 4-fluorobenzoate which can then be degraded by *Ralstonia* sp. strain H1 allowing for the mineralization of 4-FCA with stoichiometric fluoride release (Hasan et al., 2012).

Fluorobenzene (FB, Figure 2b) is used in the pharmaceutical, and chemical industries as a solvent, to produce insecticide and as a reagent to produce plastics (Carvalho et al., 2005). *Labrys portucalensis* F11 was reported to degrade FB (1 mM) as a single carbon and energy source by Carvalho et al. (2002, 2005). *Labrys portucalensis* F11 was also able to degrade other haloaromatic compounds including fluoroanilines, chlorobenzene and difluorobenzene (Amorim et al., 2013; Carvalho et al., 2005; Moreira et al., 2012a). F11 strain starts degrading FB through its partial breakdown forming catechol and 4-fluorocatechol, followed by an ortho cleavage (Carvalho et al., 2009). Strain F11 was also reported as the first microorganism to degrade FB in the presence of chlorobenzene (Moreira et al., 2012b). Another microorganism with the capacity to completely degrade FB (1.3 mM) is the

Burkholderia fungorum FLU100 which also completely degraded metabolites 3-fluorocatechol and 2-fluoromuconate (Strunk & Engesser, 2013).

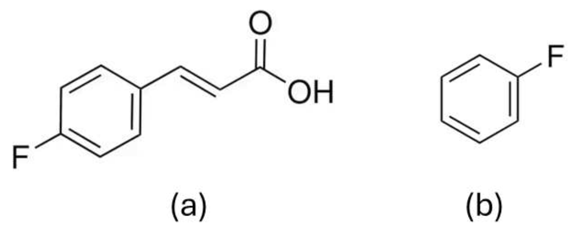

Figure 2 - Chemical structure of 4-fluorocinnamic acid (a) and fluorobenzene (b).

1.1.2. Endocrine disruptors

The presence of endocrine-disrupting compounds in wastewater poses significant risks to both human and aquatic life (Pelch et al., 2011). Natural estrogens, such as estrone (E1), 17β-estradiol (E2), and estriol (E3), are hormones produced by the humans' bodies and some animals. These compounds are found in the environment, reaching it by various routes such as animal faeces, human activities and sanitary and agricultural sewages (Huang et al., 2020; Johnson et al., 2013; Liu et al., 2012). Exposure to endocrine-disrupting compounds can disrupt the hormonal balance in both wildlife and humans, leading to a range of health issues (Tiedeken et al., 2017). Among the three natural estrogens, E2 is considered one of the more potent endocrine-disrupting compounds (Caliman & Gavrilescu, 2009). Endocrine-disrupting compounds are emerging contaminants which can be found in the environment at low concentrations, ranging between ng/L to μg/L. The concentration of E2 in groundwater was found below 1 ng/L (Shore & Shemesh, 2003). In a study by Wee & Aris (2019), the concentration of endocrine-disrupting compounds in tap water ranged from 0.2 to 5510 ng/L with the highest concentration of 28000 ng/L found in drinking water (wells) in India. Although they may be present in the environment at low concentrations, they can have harmful effects if they persist over time. The average concentration of E2 (Figure 3) in the surface water is less than 50 ng/l but this is significantly higher than the suggested Annual Average Environmental Quality Standard (AA EQS) that is 0.04 ng/l (Tiedeken et al., 2017).

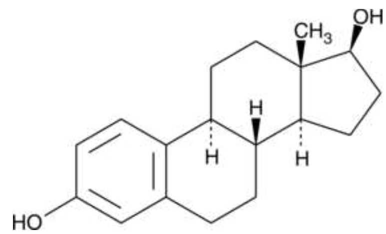

Figure 3 – Chemical structure of 17β-estradiol.

1.2. Wastewater treatment - a vital step to reduce pollution

Contaminants must be removed from wastewater, not only to prevent the deterioration of receptor ecosystems but also to enable water reuse and aid the transition to a more sustainable water management system. Wastewater treatment plants (WWTP) have been considered effective for the elimination of different contaminants present in the wastewater (Eggen et al., 2014). Various biological methods can be used to treat wastewater (Kumar et al., 2021) and the selection of the appropriate method is based on the precision of the technology required to increase the water quality to acceptable levels, the flexibility for supervision, and costs (Saravanan et al., 2021). Conventional WWTP combines physical, chemical, and biological processes to remove organic matter, solid particles, and nutrients. However, certain chemical pollutants are still present in the treated wastewater after treatment (Ranjit et al., 2021).

1.2.1. Aerobic granular sludge systems

Aerobic granular sludge (AGS, Figure 4) are aggregates of microbial origin, with a minimum diameter of 0.2 mm, which do not coagulate under reduced hydrodynamic shear and settle significantly faster than activated sludge flocs (Bathe et al., 2015; de Kreuk et al., 2007; Niermans et al., 2014). The microorganisms in the AGS are immobilized in a self-producing matrix called extracellular polymeric substances (EPS) forming a special kind of biofilm (Beun et al., 1999). The AGS has diverse microbial groups embedded in its biopolymer matrix that contribute to the formation of stable granules, as well as provide better operational results through the simultaneous occurrence of different biological processes (Nancharaiah & Sarvajith, 2019).

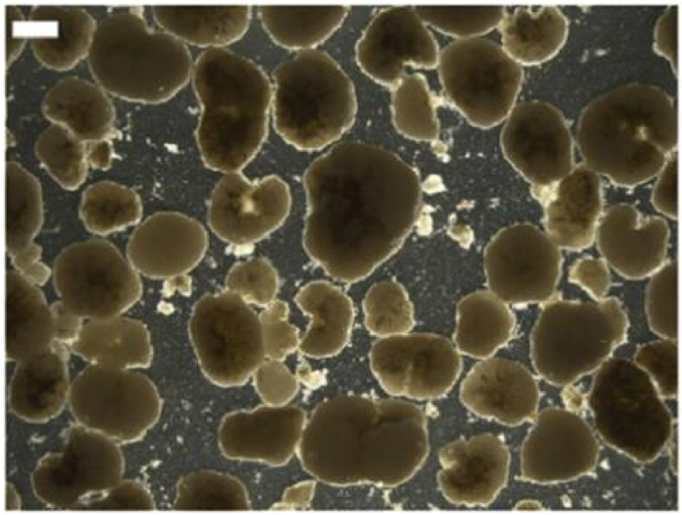

Figure 4 – Seawater-adapted aerobic granular sludge. The scale bar equals 1 mm. Image from de Graaff et al. (2020).

The AGS advantageous characteristics when compared to conventional activated sludge include its compact structure, fast settling velocity, strong load resistance, resistance to toxics, and simultaneous C, N and P removal (Lin et al., 2023). Although the AGS systems high efficiency, there is still the need for process performance enhancement through the optimization of current setups and the retrofitting of existing infrastructures. This is especially important to improve the removal of a range of more recalcitrant pollutants such as pharmaceuticals, personal care products, and endocrine disruptors (Mavinic et al., 2018).

In the transition to a circular economy, a range of resources can be recovered from AGS systems such as phosphorous, alginate, exopolymers, biodegradable plastic, biogas, among others (de Carvalho et al., 2021; Nancharaiah & Sarvajith, 2019; Oliveira et al., 2021b). Recently, EPS has emerged as one of the most valuable resources to be recovered. EPS are organic biopolymers with high molecular weight that are produced by microorganisms and play a crucial role in microbial aggregate systems by binding cells and other particulate matter together and attaching them to surfaces. The main components of EPS are organic substances such as polysaccharide, proteins, humic substances, extracellular DNA and other nucleic acids, and lipids (Figure 5) (Flemming et al., 2016; Frølund et al., 1996; Houari et al., 2008).

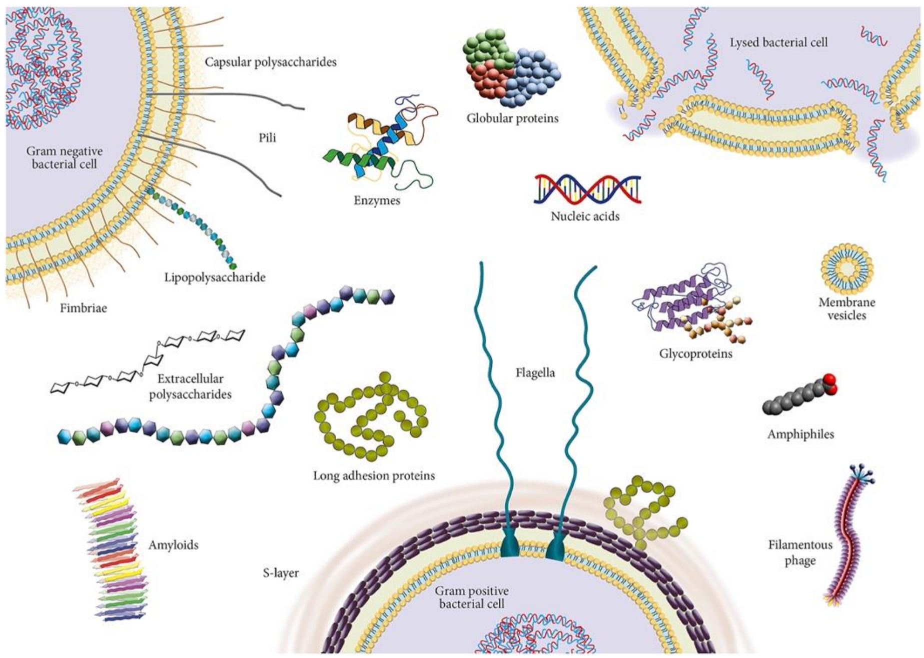

Figure 5 - Most common components usually found in EPS. These components have been found in a range of different biofilms, but not all matrices contain each of these components. Image from Seviour et al., (2019).

Oliveira et al., (2020) demonstrated that the recovery of EPS from waste AGS can be a feasible valorisation scheme although the EPS composition may differ according to the chemical variations in the wastewater influent streams composition. Recovery of EPS is in fact a key valorisation strategy for AGS. In 2021, the first EPS extraction plant started to operate in the Netherlands. The EPS is being commercialized under the brand name Kaumera Nereda® Gum, a novel bio-based material derived from AGS collected in the Nereda® wastewater treatment process (https://kaumera.com/english/). The EPS matrix has a complex structure and composition, having the ability to sorb compounds, to promote the interaction of the microbial community with nutrients and other chemicals in the near environment, and to provide mechanical strength to maintain the spatial arrangement of microorganisms for long periods. These EPS properties make them suitable for many applications such as sludge flocculation, settling, dewatering, metal binding and removal of toxic organic compounds (Klausen et al., 2004; Wingender et al., 1999). The large-scale recovery of EPS is increasing, encouraging the need to explore potential applications for these biopolymers. One approach for the valorisation of recovered EPS could be the entrapment of microorganisms for bioaugmentation purposes to improve the efficacy of recalcitrant pollutants' removal.

1.2.2. Bioaugmentation - an innovative approach to reduce pollutants' contamination

Although some pollutants can be biodegraded by naturally occurring microorganisms found in wastewater's biological treatment systems, others cannot. Low biodegradation can be due to low water solubility, high toxicity, and limited bioavailability of the pollutants to microorganisms (Sweetlove et al., 2016). For most recalcitrant substances, frequently microorganisms may not have adapted mechanisms to perform their biodegradation (Rios Miguel et al., 2020). Bioaugmentation can help to overcome the challenges faced in the removal of recalcitrant pollutants (Nzila et al., 2016). Bioaugmentation has been successfully applied in the removal of the toxic pollutant quinoline (3.87 mM) by adding a bacterial suspension of strain *Bacillus* sp. Q2 (Tuo et al., 2012). In a study conducted by Wang et al., (2009), bioaugmentation of an activated sludge system with *Pseudomonas* sp. HF-1 to treat tobacco wastewater resulted in complete nicotine degradation with more than 84% of chemical oxygen demand removal within 12 h. However, some limitations can hinder the success of bioaugmentation with bacterial suspensions, like the rapid reduction of the mass of the inoculated bacteria within the system (Goldstein et al., 1985), insufficient inoculation (Ramadan et al., 1990), starvation periods (Martín-Hernández et al., 2012), among others. To overcome those limitations the immobilization or entrapment of cells has been studied as a promising tool using natural or synthetic carriers, that can improve the survival and activity of the introduced microorganisms. Alginate, carrageenan, agar, agarose, and chitosan have been tested as natural carriers, while acrylamide, polyurethane, and polyvinyl resin were used as synthetic ones (An et al., 2008; López et al., 1997). Synthetic carriers are found to be more stable, but the use of natural carriers offers more advantages as they are non-toxic (de-Bashan & Bashan, 2010). In fact, the recovered EPS from surplus AGS has shown the ability to form a stable gel for bacterial strains entrapment which enables its use as an alternative natural biodegradable carrier (Oliveira et al., 2021b). Another successful bioaugmentation strategy was performed by Oliveira et al. (2021b) where the toxic pollutant 2-fluorophenol (2-FP) was completely degraded after adding the bacterial strain *Rhodococcus* sp. FP1 immobilised in an EPS matrix. In addition to the successful removal of the recalcitrant compound 2-FP, the bioaugmentation of an AGS system allowed for the improvement of phosphate and ammonium removal efficiencies. Moreover, it showed that the EPS granules with immobilized bacteria enabled the maintenance of the bioreactor's operational conditions and the viability of the bioaugmentation strain (Oliveira et al., 2020). Zhang et al., (2019) also reported that by using two kinds of bacteria immobilized in natural carriers namely modified pine bark and corn straw, the toxic compound phenol was removed from the wastewater.

1.3. Purpose of the study

Within the scientific context outlined in this introduction, along with the increasing interest in the improvement of wastewater treatment efficiency through bioaugmentation, this work aims to evaluate the efficiency of co-immobilized degrading bacteria in EPS for the removal of specific recalcitrant pollutants present in wastewater. The effectiveness of EPS granules with one bacterial strain has been tested and proven successful, but the simultaneous immobilization of multiple strains to produce multi-strain immobilization granules was never described. Three bacterial strains *Labrys portucalensis* F11, *Rhodococcus* sp. S2 and *Rhodococcus* sp. ED55 have been selected for this study due to their known capacity to degrade FB, 4-FCA and E2, respectively. The study aimed to ascertain the potential of the multi-bacterial strains immobilized in EPS for removing recalcitrant compounds, to be further applied as a potential bioaugmentation strategy to simultaneously remove multiple industrial recalcitrant pollutants and endocrine-disrupting compounds.

2. Materials and methods

2.1. Bacterial strains cultivation

The three strains *Labrys portucalensis* F11, *Rhodococcus* sp. ED55, and *Rhodococcus* sp. S2 were obtained from the Resources and Environment research group from the Centre of Fine Chemistry and Biotechnology at Universidade Católica Portuguesa (CBQF-UCP). Strains were grown in NB media agar plates, incubated at room temperature. Once the colonies were grown, one colony was taken with a loop wire, following the aseptic techniques, and inoculated into flasks filled with Luria Bertani (LB) media. These flasks were incubated using optimal growth conditions for the three bacterial strains (Table 1): overnight at 30 ºC, ± pH 7.0, 130 rpm.

Table 1 - Optimal growth conditions and colony characteristics of *Labrys portucalensis* F11, *Rhodococcus* sp. ED55, *Rhodococcus* sp. S2.

Microorganism	Optimal pH and temperature	Pollutants degraded	Colonies' characteristics
Labrys portucalensis F11	pH 4-8 30 ºC	Fluorobenzene (Carvalho et al., 2008)	White, dry, opaque
Rhodococcus sp. ED55		17β-estradiol (Moreira et al., 2022)	Red, dry, opaque
Rhodococcus sp. S2		4-Fluorocinnamic acid (Amorim et al., 2014)	Cream, mucoid, translucent

2.2. Extraction of EPS and characterization

The extraction of EPS was performed using a method described by Felz et al. (2016). Briefly, the AGS was sieved to remove excess water. Sodium carbonate 0.5% (w/v) was added to the AGS granules and the mixture was homogenized at 500 rpm, 80 °C for 35 min. After cooling down, the mixture was centrifuged 4000 rpm, 20 min at 4 °C. The supernatant was collected, and the resulting pellet was subjected to the same protocol for a second round of EPS extraction. The collected supernatants containing the extracted EPS were mixed. An EPS purification step by acidic precipitation was employed by adjusting the pH of the supernatants to 2.2 ± 0.5 by adding 1 M HCl. After this, the supernatants were centrifuged at 4 °C, 8500 rpm, for 30 min. The obtained supernatants were discarded, and the pellets containing the purified EPS were freeze-dried.

The biochemical characterization of the collected EPS in terms of protein and carbohydrates was performed using colorimetric methods. Carbohydrate quantification followed the anthrone method (Dubois et al., 1956) using glucose as standard. The Lowry method was used for protein estimation with bovine serum albumin as standard (Lowry et al., 1951). All the tests were performed in triplicates (n=3).

2.3. EPS granules production with immobilized strains

Freeze-dried EPS was dissolved in demineralized water to a final concentration of 11.8% (w/v). The pH was adjusted to 7.0 ± 0.5 using 1 M of NaOH, and bacterial suspensions of *Labrys portucalensis* F11, *Rhodococcus* sp. ED55, and *Rhodococcus* sp. S2 were added to a final optical density at 600 nm ($OD_{600\ nm}$) of 0.225. Once this mixture was completely homogenized, sodium alginate was added to a final concentration of 0.7% (w/v) to enhance the strength of the granules. With the help of a sterile Pasteur pipette coupled to a 10 µL tip, the mixture was slowly dripped into a 2.5% (w/v) calcium chloride ($CaCl_2$) solution. The produced EPS granules with the multiple immobilized strains were stored at 4 °C overnight in the $CaCl_2$ solution.

2.4. Batch Assays

Batch assays were performed to evaluate the capacity of the multi-immobilized bacterial strains in EPS granules as well as of the bacterial suspensions to remove the target pollutants under different experimental conditions as illustrated in Figure 6. Briefly, the following experimental test conditions were set in mineral media: (1) EPS granules with the 3 bacterial strains immobilised supplemented with the 3 pollutants; (2) EPS granules with the 3 bacterial strains immobilised previously autoclaved and the 3 pollutants; (3) the 3 bacterial strains suspensions and the 3 pollutants; (4) strain ED55 suspension and the 3 pollutants; (5) strain S2 suspension and the 3 pollutants; (6) strain F11 suspension and the 3 pollutants; (7) strain ED55 suspension and pollutant E2; (8) strain S2 suspension and pollutant 4-FCA; (9) strain

F11 suspension and pollutant FB; (10) the 3 pollutants. The final concentration of the bacterial suspensions is the same as in the EPS granules, OD $_{600\ nm}$ 0.225. The pollutants were supplemented considering a final concentration of 0.5 mM for 4-FCA, 1 mM for FB, and 0.018 mM for E2. The selected concentrations were chosen because they thoroughly coordinated those used in other studies, which effectively achieved the complete degradation of the toxic compounds and the detection of metabolites (Amorim et al., 2014; An et al., 2008; Moreira et al., 2012a). Each condition was run in triplicates (n=3), at 30 °C and 100 rpm for 14 days. Samples were collected every day for FB, 4-FCA and E2 quantification, fluoride quantification, and OD $_{600\ nm}$ measurement. At the end of the experimental period, the liquid in each flask was plated into NB media agar plates using the spread technique (20 µl) to confirm the presence of the introduced strains and possible contaminations.

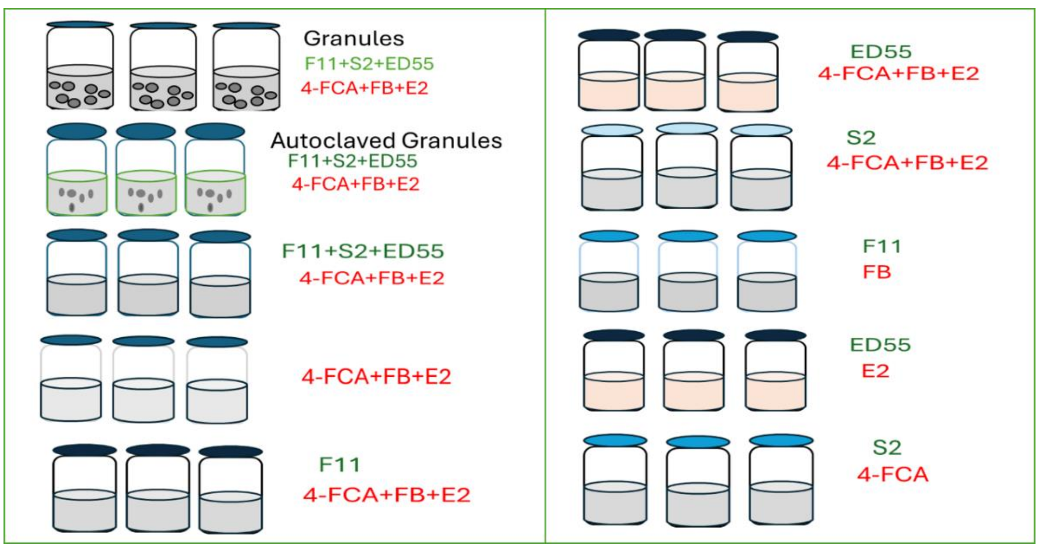

Figure 6 - Experimental design of the batch assay with different test conditions.

The adsorption capacity of the autoclaved granules for each of the target pollutants was calculated according to Equation 1.

Equation 1:

$$\text{Adsorption capacity}\left(\frac{mM}{g\ granules}\right)=\frac{initial\ pollutant\ concentration\ -minimum\ pollutant\ concentration}{autoclaved\ granules'\ dry\ weight}$$

The dry weight of autoclaved granules was calculated based on the equation obtained from the calibration curve of dry weight vs wet weight of granules (Equation 2), where a r-square value of 0.9814 was obtained.

Equation 2:

$$Granules'wet\ weight\ (g) = 13.05\ \times Granules'\ dry\ weight\ (g) - 0.01$$

2.5. Analytical methods

During the batch assays, samples were collected under aseptic conditions. Samples for quantification of 4-FCA, E2 and fluoride were further centrifuged at 14000 rpm for 5 min at room temperature to remove the biomass. The supernatants were collected were frozen at -20 ºC until further analysis.

2.5.1. Quantification of FB

FB was extracted from samples by mixing 4 ml of sample with 2 ml of diethyl ether with mesitylene. The mixture was vortexed for 1 min at maximum speed. Mesitylene was used as an internal standard for FB as it has a similar behaviour in terms of extraction efficiency with diethyl ether. The ether layer was collected for further analysis by gas chromatography using a Varian Chrompack CP 3800 GC gas chromatography with a CP-Wax 52 CB capillary column. The following temperature regimen was set: 50 °C for 2 min, increasing to 150 °C at a rate of 25 °C min^{-1} until reaching the final temperature of 250 °C at a rate of 50 °C min^{-1}. Injector and detector temperatures were 250 °C. Injection volume into the GC equipment was 1 μl. A calibration was performed using FB standards with concentrations ranging between 0.2 and 2 mM. The limit of detection for FB was identified as 0.015 mM. A calibration curve with high R-squared value (>0.999) was obtained (Figure 7).

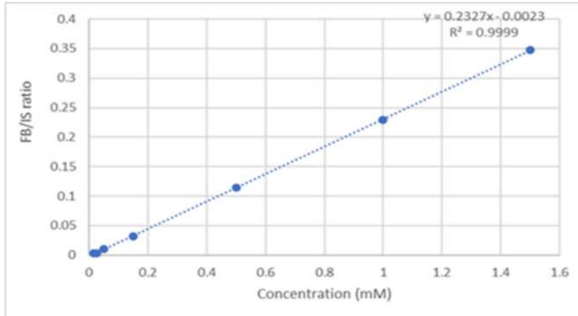

Figure 7 - Optimized calibration curve for the quantification of FB.

2.5.2. Quantification of 4-FCA and E2

Quantification of 4-FCA and E2 was performed using high-pressure liquid chromatography (HPLC), using a LiChrospher®100 RP-18 (5µm) column. A flow rate of 0.06 ml/min was set, with and injection volume of 20 µL, and running time of 30 minutes. For the quantification of 4-FCA an UV detector was used with the wavelength set to 230 nm. For E2 quantification a fluorescence detector was used with excitation wavelength set to 230 nm, and emission wavelength to 310 nm. The mobile phase was 60:40 (v/v) of acidified water with trifluoroacetic acid to pH 2.0 and acetonitrile. Calibration was performed using 4-FCA and E2 standards with concentrations ranging from 0.0015 mM to 0.6 mM and 9.18 x 10^{-8} to 0.044 mM, respectively. Standards of 4-FCA and E2 were prepared separately and mixed in a proportion 1:1 prior to HPLC analysis. Figure 8 (a) and Figure 8(b) show the optimized calibration curves obtained for the quantification of 4-FCA and E2, respectively. High R-squared values (>0.999) were achieved, which is indicative of a high level of correlation. The limit of detection was 0.0015 mM for 4-FCA and 9.18 x 10^{-8} for E2.

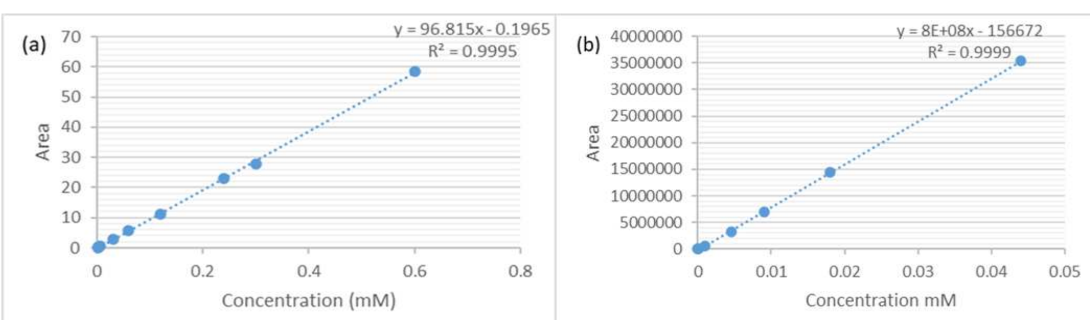

Figure 8 - Optimized calibration curve for the quantification of (a) 4-FCA and (b) E2.

The chromatogram obtained for the standards of 4-FCA, E2 and E1 showed well-separated peaks with a retention time of 10.9 min, 21.7 min and 29.0 min, respectively. Compound E1 was also in the mixture of standards and identified in the HPLC chromatograms since this is a common E2 degradation metabolite.

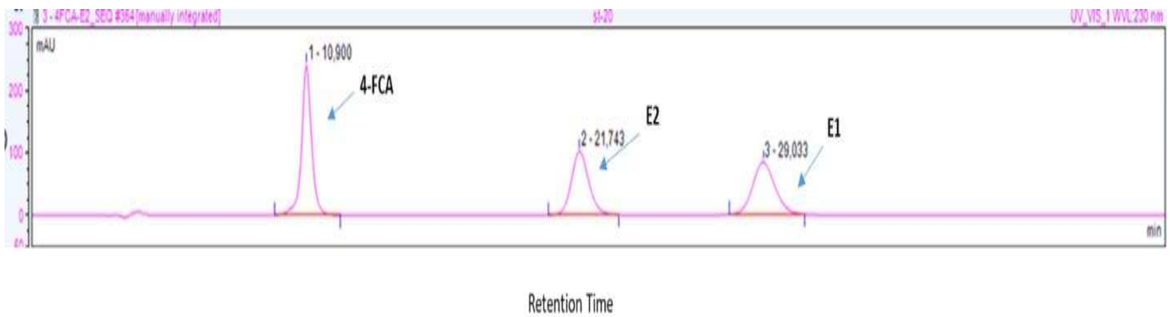

Figure 9 - Chromatograms showing 4-FCA, E2, and E1 peaks in the same run.

2.5.3. Quantification of fluoride ions

Fluoride ions concentration was measured using an ion-selective combination electrode (model CH-8902, Mettler-Toledo GmbH, Switzerland), which was calibrated with NaF (0.01 - 5 mM). To adjust the ionic strength of samples, a total ionic strength adjustment buffer (TISAB) was added to standard/samples in a proportion 1:1 (Moreira et al., 2012a). The TISAB buffer composition was as follows: NaCl 2 M, CH_3COOH 0.38 M, CH_3COONa 0.75 M and $Na_3C_6H_5O_7$ 0.002 M. Standards and samples were analysed in mV and fluoride concentration was calculated using the correlation established between the E (electrode potential in millivolt) and logarithmic function of fluoride ions concentrations in standards.

2.5.4. Optical density

The measurement of optical density (OD) was done in a spectrophotometer at a wavelength of 600 nm. The OD was measured to determine the bacterial concentration and its growth.

3. Results and discussion

3.1. EPS extraction and characterization

AGS collected in a full-scale WWTP (Figure 10 a) was used to recover EPS through alkaline and heating extraction followed by a purification step of acidic precipitation. This extraction method was chosen because it achieved a better solubilization of EPS and obtained a higher extraction yield than other extraction methods tested as stated in a previous study by Felz et al., (2016). The precipitation of the extracted EPS at low pH results in a gel-like material and after lyophilization, the EPS has the appearance of light-dried flocs or a powder (Figure 10b).

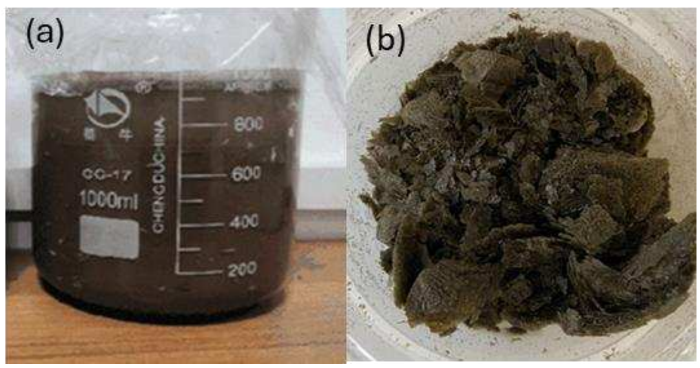

Figure 10 - AGS used in the extraction of EPS (a) and the extracted EPS after lyophilization (b).

The characterization of the EPS in terms of protein and carbohydrates. The concentration of carbohydrates in the extracted EPS was 26.04 ± 9.91 mg/g EPS and the concentration of proteins was 343.14 ± 6.57 mg/g EPS. Therefore, proteins exhibit a concentration ca. 13-fold higher than carbohydrates. Many researchers have found similar results where the concentration of proteins was always higher than other components like humic acids, carbohydrates, DNA, or lipids (Bathe et al., 2015; Oliveira et al., 2021a; Oliveira et al., 2020). The extracted EPS constituents' concentrations are in agreement with those reported by Oliveira et al., (2020) which found 298-485 mg/g VSS of proteins and 50-65 mg/g VSS of carbohydrates.

3.2. Degradation of FB, 4-FCA, and E2 by multi-immobilized bacterial granules

3.2.1. Development of the multi-immobilized bacterial granules

Co-immobilization of bacterial strains *Labrys portucalensis* F11, *Rhodococcus* sp. S2, and *Rhodococcus* sp. ED55 using the extracted EPS as a carrier was successfully achieved (Figure 11). The multi-immobilized bacteria granules were drop-shaped, black-brownish colour, and with an average size of 3-4 mm.

Figure 11 - Multi-immobilized bacteria granules produced through the co-immobilization of strains *Labrys portucalensis* F11, *Rhodococcus* sp. S2, and *Rhodococcus* sp. ED55 in an EPS matrix.

3.2.2. Degradation of combined pollutants by the multi-immobilized bacterial granules

A batch assay was set up to investigate the ability of the multi-immobilized bacteria granules to remove a combination of pollutant compounds FB (1 mM), 4-FCA (0.5 mM), and E2 (0.018 mM) present in wastewater. Pollutant E2 was completely degraded after 42.5 h, while 4-FCA was removed after 66.2 h, and FB after 90 h (Figure 12a). By the end of 90 h, the target pollutants were no longer present in the wastewater showing that the produced multi-immobilized bacterial granules can degrade the three compounds simultaneously. The initial concentration of FB analysed was approximately 0.7 mM, rather than the target 1 mM that was fed into the flask. This discrepancy is related to Henry's partition coefficient of FB. As FB is a volatile compound, this partition establishes the distribution of FB between the gas and liquid phases (Carvalho et al., 2005). A similar trend regarding FB initial concentration was observed in the other batch assay test conditions.

The E2 initial concentration was also lower than expected, ca. 0.0018 mM, which is ten times lower than the target amount of 0.018 mM in each flask. This difference in the concentration of the E2 could be credited to measurement errors during the preparation of the stock standard solution used to feed the flasks.

Concerning the concentration of fluoride ions, resulting from its release during fluorinated toxics (FB and 4-FCA) removal, an increasing pattern from 40 h to 136.5 h was observed, reaching a concentration of 0.95 mM ± 0.49 (Figure 12b). Then it fluctuated between 0.75 ± 0.11 mM and 0.89 ± 0.16 mM until the end of the experiment, which indicates that no more fluoride was released. Considering the concentrations of fluorinated compounds used at the beginning of the experiment and the number of fluorine atoms in each molecule, a release of 1.5 mM of fluoride ions would have to be observed to consider it a stoichiometric release. The maximum fluoride ions concentrations reached in this experiment was 0.95 mM to which a corresponding 0.49 mM standard deviation was observed. Taking the standard deviation value into consideration, this fluoride release is close to a stoichiometric release.

The optical density of the solution inside flasks was also analysed to infer on the bacterial concentration in the liquid phase along the experiment (Figure 12c). An increasing pattern of optical density was observed until 210 h reaching a maximum value of 2.19 ± 0.92, fluctuating thereafter and reaching 1.91 ± 0.60 until the end of the experiment. It is important to highlight that the optical density increase in the flasks of multi-immobilized bacterial granules did not occur solely due to bacterial growth, but also due to granules disintegration. From 43 h onwards, the EPS granules started to break down into smaller fragments and debris, releasing EPS that seemed to dissolve and render a brown colour to the solution (Figure 13).

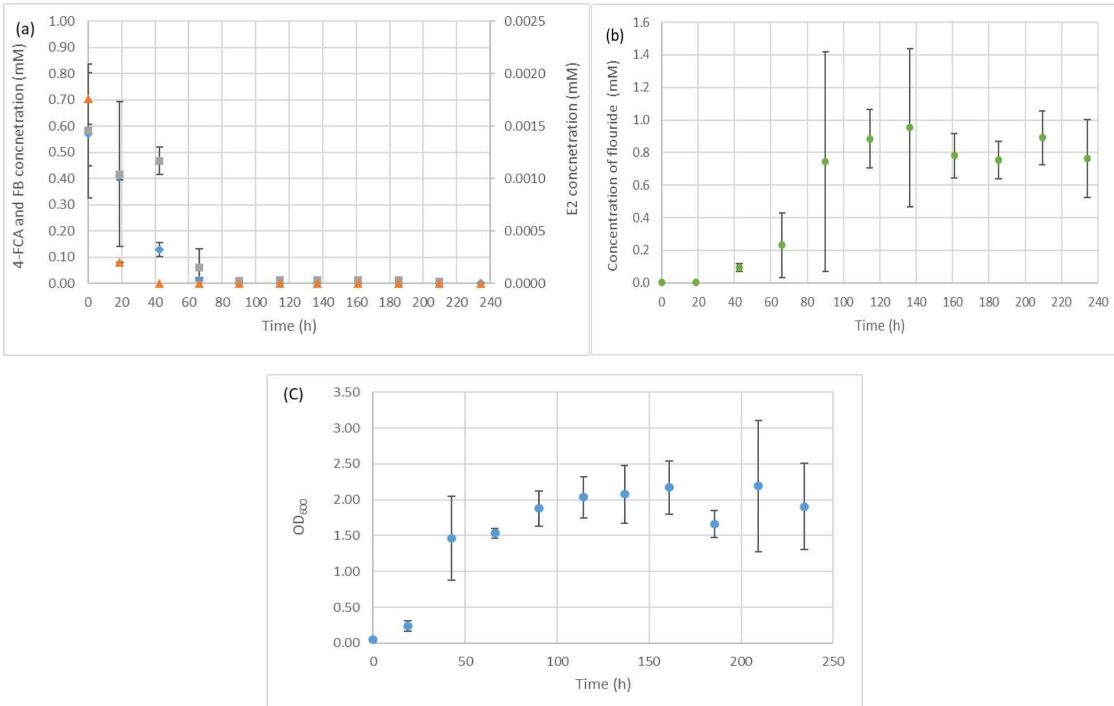

Figure 12 – Pollutants removal pattern by the multi-immobilized bacterial granules. Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●). Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

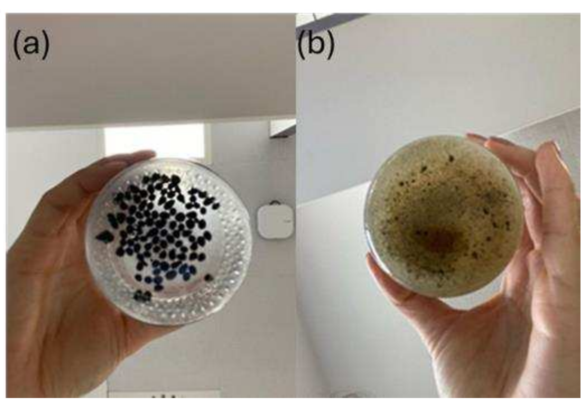

Figure 13 - Multi-immobilized bacteria granules in the beginning at 0 h (a), and end at 234 h of the batch experiment (b).

3.2.3. Adsorption of pollutants onto EPS granules

The variation of the pollutant's concentration using autoclaved multi-immobilized bacterial granules was assessed over time to verify the pollutants removal capacity of the granules without the contribution of live cells. It is expected that the autoclaved granules do not contain live microorganisms which can aid to prove if the decrease in the concentration of the pollutant compound is due to adsorption onto the granules rather than the metabolic activity of microorganisms (Figure 14a). The concentration of E2 has shown a decrease from 0.0020 ± 0.00004 to 0.0013 ± 0.00006 mM during the first 19 h, after which it stabilized at ca. 0.001 ± 0.00003 mM. The concentration of 4-FCA has shown a slight decrease in the concentration throughout the experiment, from 0.54 ± 0.04 to 0.43 ± 0.02 mM. The concentration of FB shows a gradual removal from 0.69 ± 0.38 mM to 0.31 ± 0.005 mM from the beginning until the end of the experiment. The results obtained allowed to calculate the adsorption capacities of granules for each pollutant compound: 3.16 ± 2.49 mM / $g_{granules}$, 0.640 ± 0.073 mM / $g_{granules}$, and 0.0048 ± 0.0003 mM / $g_{granules}$ for FB, 4-FCA and E2, respectively. In a study by Castellanos et al., (2021), it was found that 0.01% of E2 at an initial concentration of 7.34×10^{-5} mM was adsorbed onto the AGS, which is significantly lower than the 37.6% of E2 adsorption observed in this study. Also, the adsorption percentages for 4-FCA and FB were recorded at 20% and 51.3%, respectively, for the present study.

The mean concentration of fluoride ions (Figure 14b) shows negligible values (0.001 and 0.004 mM), corroborating the previous observation that no degradation of pollutants occurred if autoclaved multi-immobilized bacterial granules were used.

The optical density (Figure 14c) shows an increasing pattern until 161 h reaching a maximum value of 2.14 ± 0.19, which slightly decreases until the end of the experiment. The increasing optical density observed in this test condition could be due to structural changes in the granules, leading to their fragmentation and the release of soluble EPS (Figure 15).

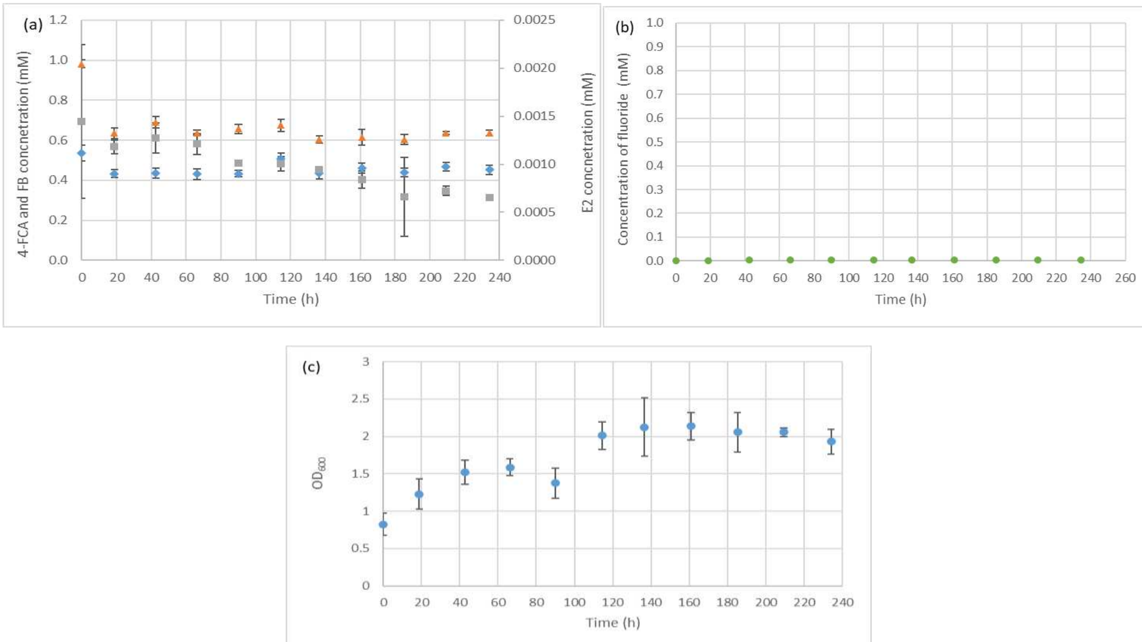

Figure 14 – Pollutants removal pattern by the autoclaved multi-immobilized bacterial granules. Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●). Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

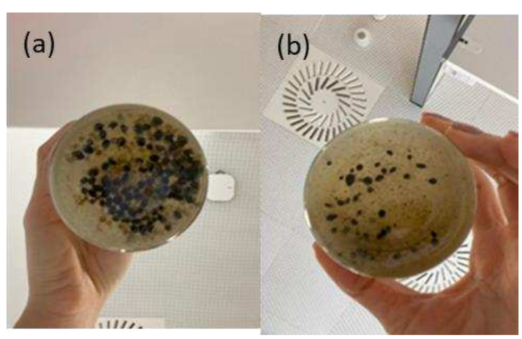

Figure 15 - Autoclaved multi-immobilized bacterial granules in the beginning at 0 h (a), and end at 234.25 h of the experiment (b).

3.2.4. Effect of combined pollutants on the degradation capacity of the mixed suspension of the three strains

To ascertain the capability of each bacterial strain in suspension to degrade its target pollutant in the presence of the other pollutants and strains in the medium, the variations of the concentrations of the three pollutant compounds during the batch assay were followed (Figure 16a). Variable degradation rates of each pollutant were observed. In the mixture of three bacterial suspensions, compound E2 was rapidly degraded in less than 20 h from the onset of the experiment, proving the fastest degradation, while 4-FCA was completely degraded after 90.1 h, and FB after 231.5 h. The immediate degradation of 0.018 mM of E2 was also observed in a study performed by Moreira et al. (2022). The degradation of 4-FCA and FB compounds appears to be more efficient with multi-immobilized bacterial granules compared to the suspended bacteria. Usually, when immobilised cells are used the degradation of pollutant is slower due to mass transfer limitation which restricts the diffusion of the toxic pollutants to the cells (Ehrl et al., 2019). The enhanced removal of pollutants with the multi-immobilized bacterial granules seen in the present study can be credited to the combined effects of the physical adsorption of the toxic compounds to granules and biodegradation. This synergistic interaction seems to accelerate the removal process compared to suspended bacteria.

A release of 1.5 mM of fluoride ions was expected to occur if a stoichiometric release of F^- was achieved (i.e. complete mineralization of fluorinated compounds). A maximum fluoride ion concentration of 1.83 ± 0.21 mM (Figure 16b) was detected, which is higher than the expected concentration. This suggests that the fluorinated compounds were degraded completely with stoichiometric release of fluorine.

The mean optical density of the solution inside flasks containing suspensions of bacterial strains F11, S2, and ED55 with the pollutants showed an increasing pattern until 134 h, when a maximum value of 1.04 ± 0.02 was reached, slightly decreasing thereafter until the end of the experiment (Figure 16c). The increasing pattern of optical density observed indicates the adaptive features of the microorganisms to the pollutants as they utilize them as carbon and energy sources. By employing the set of enzymes available, they were able to metabolize the pollutants, breaking them down into simpler forms that could be used for growth and energy production. Moreover, this condition demonstrates that there is no incompatibility between strains and the combination of pollutants has no significant negative effect on the metabolic activity of each strain (Figure 17).

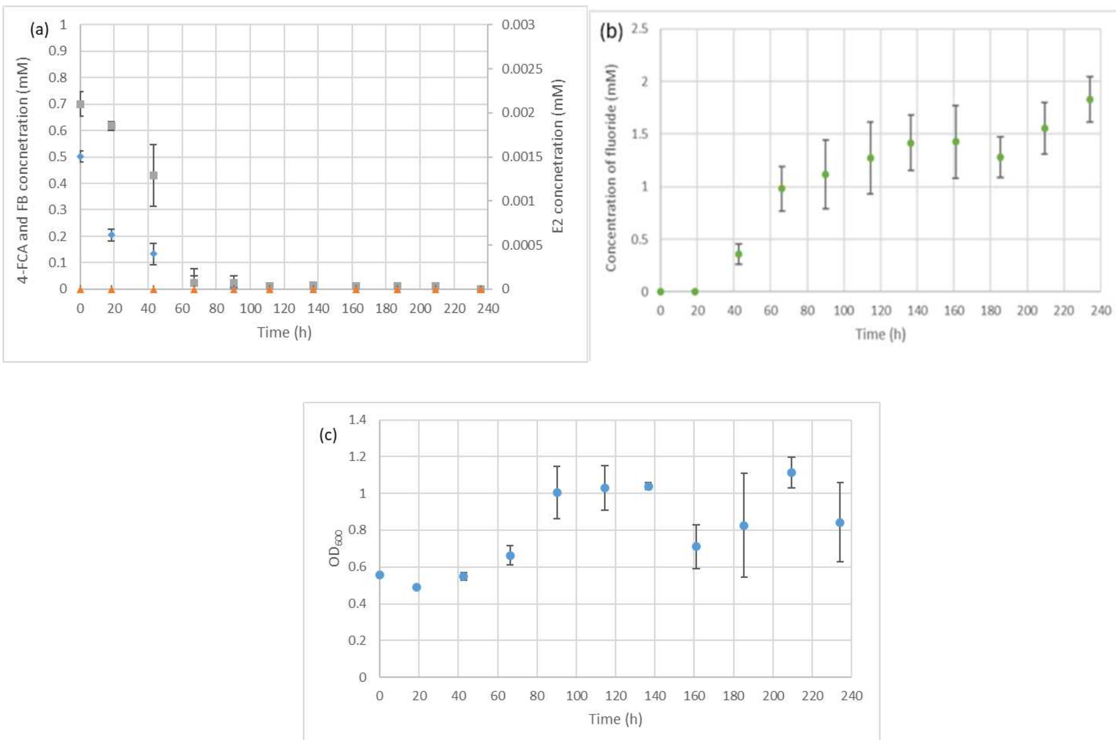

Figure 16 - Pollutants removal pattern by the multi-bacterial suspension. Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●). Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

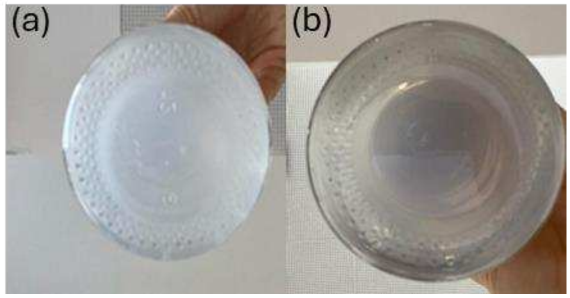

Figure 17 - Flask with bacterial strains F11, S2, and ED55 along with three pollutants in the beginning at 0 h (a), and end at 231.5 h of the experiment (b).

3.2.5. Effect of combined pollutants on the degradation capacity of individual bacterial strains suspensions

When the ED55 strain was inoculated with all the target pollutants, the bacterial strain was able to degrade the pollutant E2 from the onset of the experiment (Figure 18a). In the present study, E2 was fed to the flasks in a concentration lower than expected (ten times lower), which justifies the quick degradation of the compound that led it to be undetected even at the beginning of the experiment (t = 0h). Chromatographic analysis revealed the presence of compounds with similar chemical structure to E2, likely E2 metabolites, with peaks appearing near the E2 peak throughout the experiment (Figure 19). Additionally, complete degradation of 4-FCA at 67.5 h and the partial degradation of FB were observed. This might suggest that there could be the presence of other bacteria besides ED55 able to degrade these two pollutants, or strain ED55 is actually able to degrade them.

The release of fluoride ions exhibited an increasing trend from 0 hours to 90 hours, reaching a concentration of 0.61 ± 0.49 mM. Subsequently, the concentration fluctuated between 0.57 ± 0.48 mM and 0.80 ± 0.62 mM until the end of the experiment with high standard deviations. The high standard deviation occurred because one of replicas showed concentration values exceeding 1.0 mM after 8 days and remained above this threshold until the end of the experiment, while the other two replicas showed values between 0.06 mM and 0.76 mM. For the stoichiometric release of fluoride, 1.53 mM of fluoride ions was required. The maximum observed fluoride ion concentration was 0.80 ± 0.62, considering this maximum observed fluoride concentration with standard deviation confirms there was no stoichiometric release of fluoride (Figure 18b).

The optical density showed an increasing pattern between 0 h and 115.6 h reaching 1.21± 0.54 followed by fluctuations in the optical density between 1.01± 0.70 and 1.71± 0.63 until the end of the experiment (Figure 18c). The increase in optical density indicates that the microorganisms were utilizing the pollutants as a source of carbon and energy. This process is facilitated by enzymes present within microorganisms, which support the metabolism.

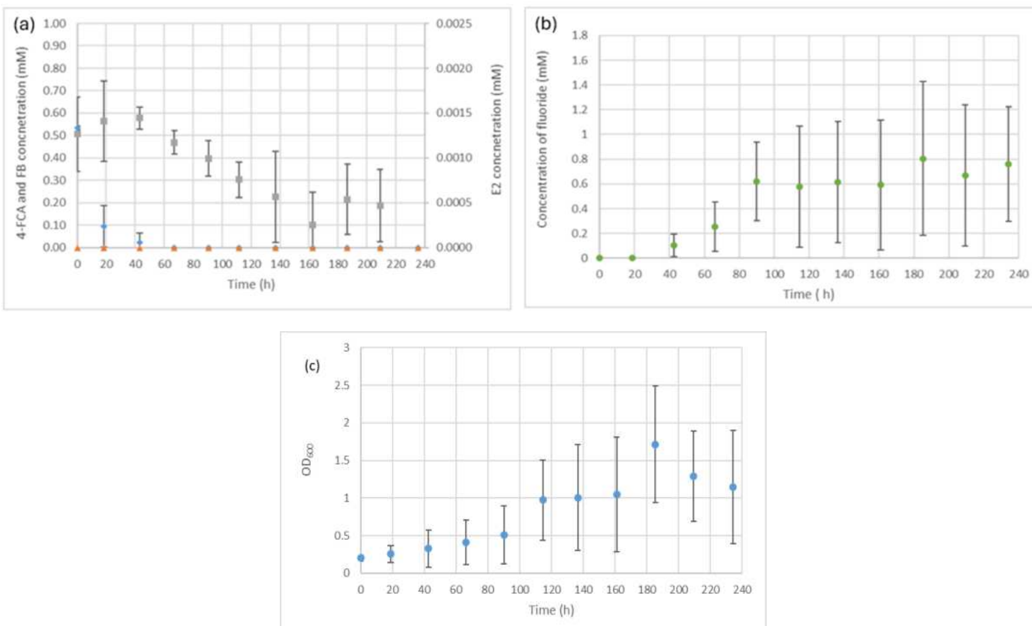

Figure 18 - Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain ED55 with three pollutant compounds. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

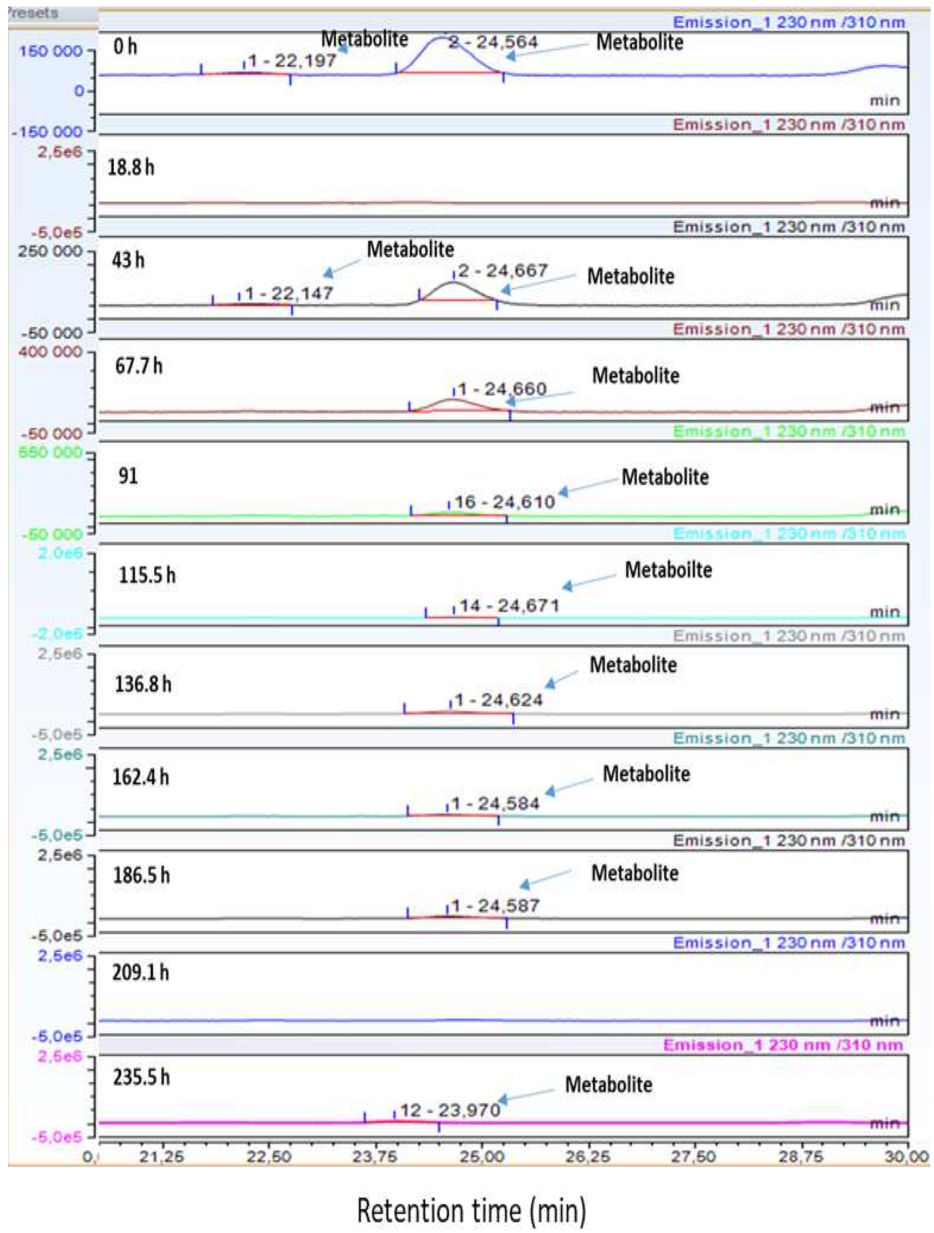

Figure 19 - Chromatograms showing metabolites peaks detected during experiments with the bacterial strain ED55 with three pollutants.

Under conditions where the S2 strain was exposed to three pollutant compounds, the total degradation of the pollutant 4-FCA by strain S2 occurred at 111.7 h (Figure 20a). The pollutant compound FB was completely degraded at 189.3 hours. The degradation of 4-FCA and FB was slower than in the EPS granules condition because the EPS granules might provide a protective environment which increased the stability and activity of degrading strains, leading to more efficient degradation of 4-FCA and FB. Moreover, as EPS granules allowed for toxics adsorption, perhaps that could also have contributed to improve degradation rate. The pollutants were degraded at 90 h and 66.2 h for 4- FCA and FB, respectively, in co-immobilized EPS granules. However, the degradation of 4-FCA and FB is faster in this condition compared to the condition with three bacterial suspensions and pollutant compounds, where degradation took 231.5 hours and 90.1 hours, respectively. Results showed that FB was degraded, and it is hypothesized that strain S2 can degrade FB due to its structural similarity to 4-FCA although this capability has not been previously supported. Both compounds are fluorinated, but FB is a simpler molecule compared to 4-FCA. Strain S2 likely has the necessary cellular machinery and enzymes to degrade fluorinated compounds which suggests that it can also break down FB thereby extending its known biodegradation capabilities. Another hypothesis is that a cross-contamination may have occurred with bacteria able to degrade FB. Compound E2 was not degraded.

To indicate the stoichiometric release of fluorine, a release of 1.49 mM of fluoride ions was required. The maximum amount of fluoride released was 2.21 ± 0.17 mM, proving the stoichiometric release of fluorine occurred (Figure 20b).

The optical density (Figure 20c) showed a uniform increasing trend from the beginning of the experiment to the end of the experiment, reaching the highest OD of 0.47 ± 0.05. Hence, the increasing trend of optical densities highlights microbial growth utilizing pollutants as the source of carbon and energy.

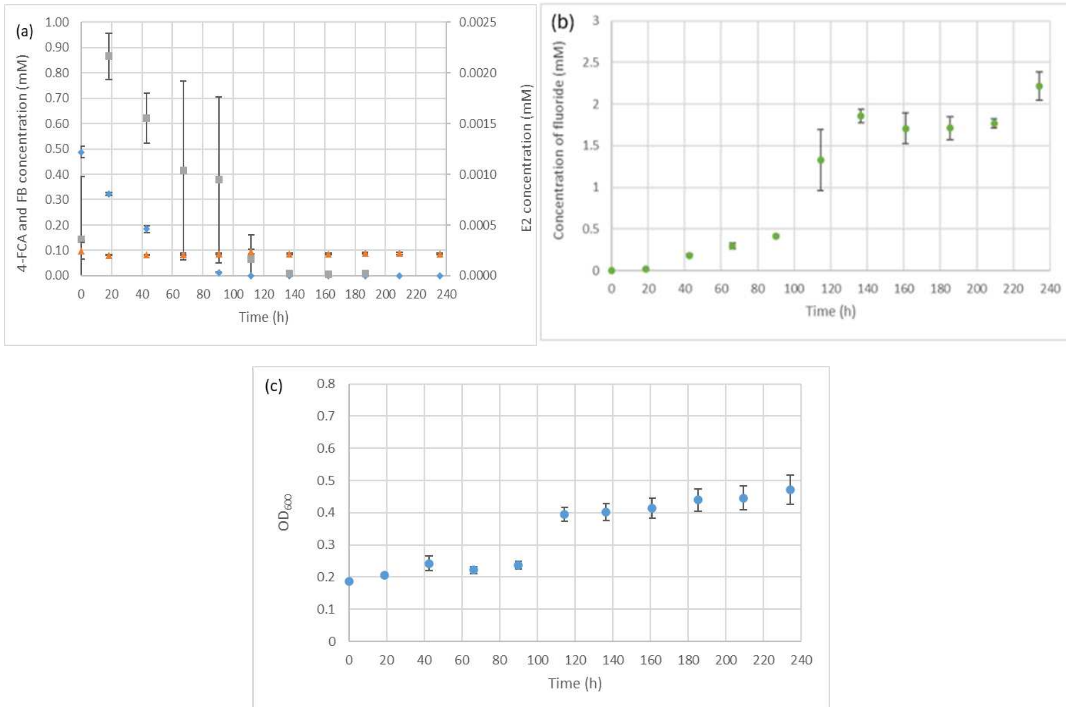

Figure 20 - Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain S2 with three pollutants. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

The exposure of the F11 strain to the three pollutants showed this strain is effective in breaking down FB but, it cannot fully degrade E2 and 4-FCA under the conditions tested. In Figure 21 (a), the complete degradation of FB by strain F11 at 137 h was observed. Partial degradation of 4-FCA from 0.58 ± 0.02 mM to 0.32 ± 0.17 mM and of E2 from 0.00158 ± 0.00024 mM to 0.00024 ±0.00005 mM occurred.

The release of fluoride ions showed a continuous increase over the period from 0 to 235.58 h, attaining a peak concentration of 1.30 ± 0.13 mM. The stoichiometric release of fluoride would require the release of 1.58 mM, and in this condition, the maximum fluoride obtained proves there was no stoichiometric fluoride release (Figure 21 b). Nevertheless, this high fluoride release is inconsistent with the degradation pattern of fluorinated compounds observed in Figure 21a, since complete FB and only partial 4-FCA removal was detected.

The reason for the inconsistency is related to the high standard deviation values observed for 4-FCA concentration.

The optical density exhibited a gradual increase to 0.50 ± 0.01 at 235.58 h. An increasing optical density in the presence of all three pollutants compounds suggests active metabolization and degradation of pollutants as carbon sources and energy (Figure 21 c).

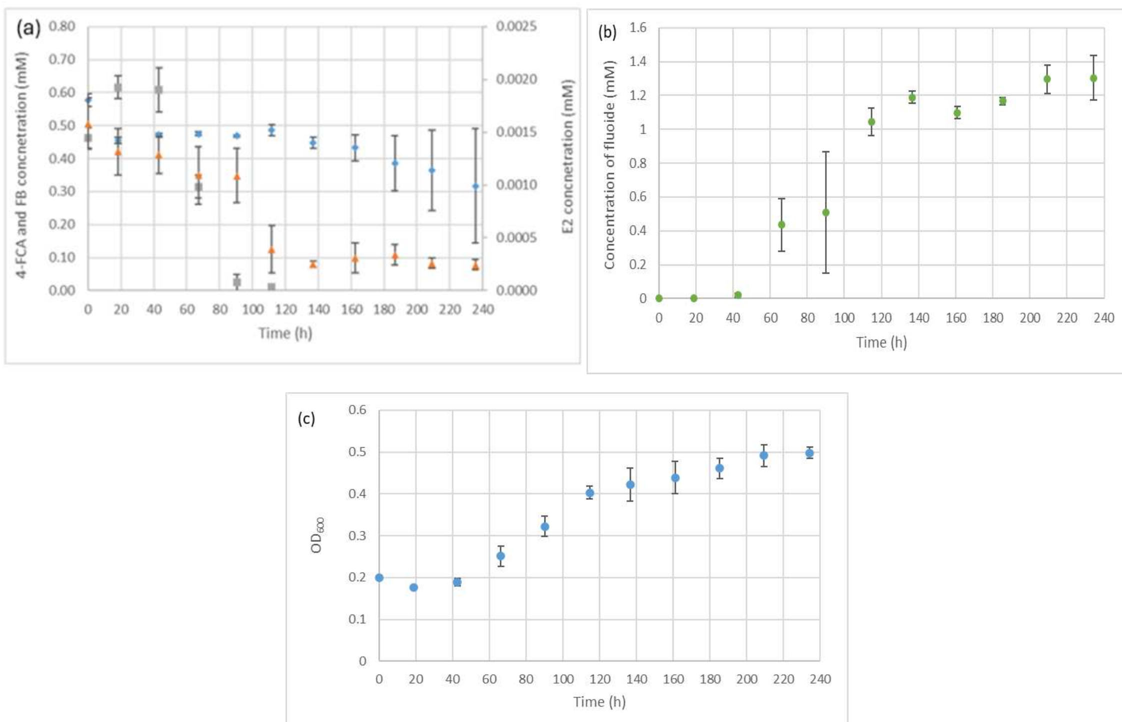

Figure 21 - Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain F11 with three pollutants compounds. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

3.2.6. Evaluation of degradation of each pollutant compound using specific microbial strains as positive controls

Figure 22a shows the mean concentration of E2 in the condition containing a suspension of strain ED55 with compound E2 as a positive control. Compound E2 was completely degraded at the beginning of the experiment, in less than 20 h. The observed peaks in the chromatograms confirmed the presence of E2 metabolites, which exhibited a pattern very similar to the emission of E2 peak over time. Nevertheless, the complete degradation proves the known capacity of strain ED55 to degrade the endocrine disruptor E2, which in a previous study, the bacteria took a total of 1.25h to remove it from real wastewater at a concentration of 0.0184 mM (Moreira et al., 2022).

Overall, small changes in the optical density were observed in this test condition (Figure 22 b) with a slight increase from the beginning to the end of experiment, from 0.19 ±0.04 to 0.28 ±0.06, respectively. The immediate degradation of E2, being that the only carbon source, could justify the low bacterial growth rate.

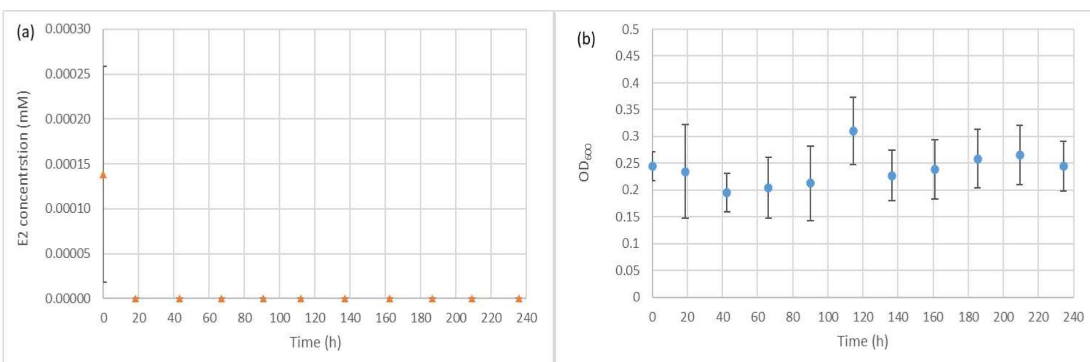

Figure 22 - Concentration in mM of (a) E2 (▲), and (b) optical density (●) in the flasks of bacterial strain ED55 with compound E2. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

The fluorinated pollutant 4-FCA was gradually degraded in the flasks containing the bacterial suspension of strain S2, and a complete degradation was achieved at 115.5 h (Figure 23 a). This proved the capacity of strain S2 to degrade 4-FCA. The release of fluoride ions showed an increasing trend, reaching a concentration of 0.49 ± 0.06 mM by the end of the experiment, which is indicative of stoichiometric fluoride release (Figure 23b).

The optical density inside flasks (Figure 23 c) shows a minor increase until 91 h with OD values at 0.24 ± 0.01, followed by a rise to values that remained steady around 0.38 ± 0.07 and 0.43 ± 0.03. These results corroborate previous findings that *Rhodococcus* sp. S2 can effectively degrade 0.5 mM of 4-FCA and grow using it as the only carbon source within 240 h (Amorim et al., 2014). In a previous study, strain S2 was able to degrade compound 4-FCA with a concentration 0.5 mM in the presence of 20 mM sodium acetate as an additional carbon source in 50 h (Amorim et al., 2014), which is less than half the time observed in the present experiment. In that specific case, the presence of an additional carbon source, that could be easily degraded by strain S2 could have contributed to accelerate the degrading process of the 4-FCA.

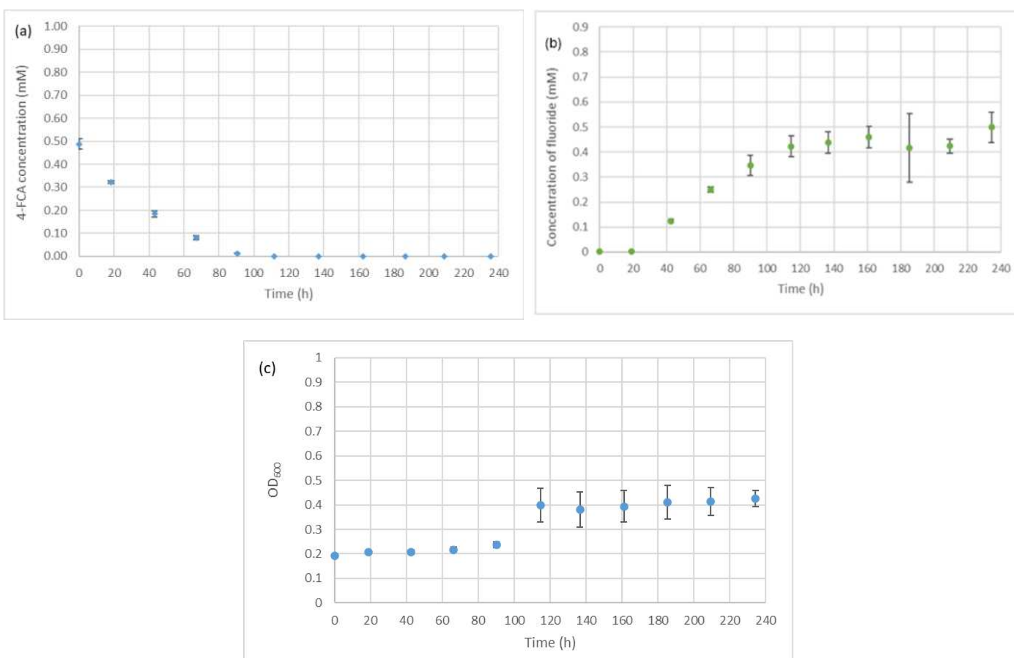

Figure 23 - Concentration in mM of (a) 4-FCA (◆), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain S2 with pollutant compound 4-FCA. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

Bacterial strain F11 was able to completely degrade the pollutant compound FB in the flask containing only that pollutant. The concentration of FB exhibited a declining trend achieving complete degradation after 114.6 h (Figure 24 - Concentration in mM of (a) FB (■), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain F11. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.a). In the study by Carvalho et al., (2006) , total degradation of FB occurred by the end of 13 h in the presence of 1 mM glucose as an additional carbon source and using pre-grown cells in FB.

The release of fluoride ions showed an increasing concentration until the end of the experiment, reaching a peak of 1.12 ± 0.03 mM (Figure 24 - Concentration in mM of (a) FB (■), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain F11. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.b). To achieve the stoichiometric release of fluoride there must be a release of 1 mM of fluoride ions. The maximum observed fluoride concentration was 1.12 ± 0.03, proving that the complete degradation of fluorinated compounds with stoichiometric release of fluorine. The optical density showed small fluctuations around 0.21 ± 0.02 and 0.22 ± 0.04 (Figure 24 - Concentration in mM of (a) FB (■), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain F11. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.. The difference in optical density observed between the beginning and the end of the experiment indicates that the bacterial strain F11 efficiently degraded the fluorobenzene leading to a stable population of bacteria that used that compound as the only carbon source.

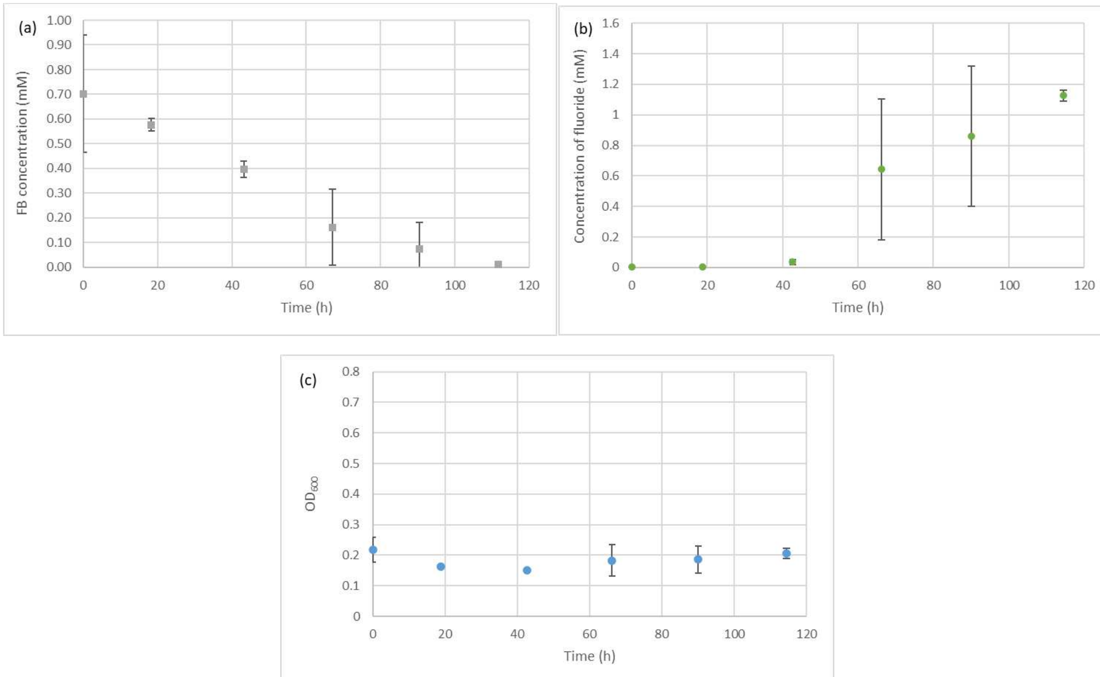

Figure 24 - Concentration in mM of (a) FB (■), (b) fluoride ions (●), and (c) optical density (●) in the flasks of bacterial strain F11. Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

3.2.7. Chemical stability of the three pollutant compounds in solution

An abiotic control was also included in the experiment to ascertain the contribution of other processes apart from degradation and adsorption for the pollutant's removal. The concentrations of the three pollutants did not change expressively (Figure 25a), and there was no release of fluoride ions (Figure 25b) which is indicative of the chemical stability of each compound in the medium. In addition, optical density remained zero throughout the experiment demonstrating that no bacterial growth occurred (Figure 25c).

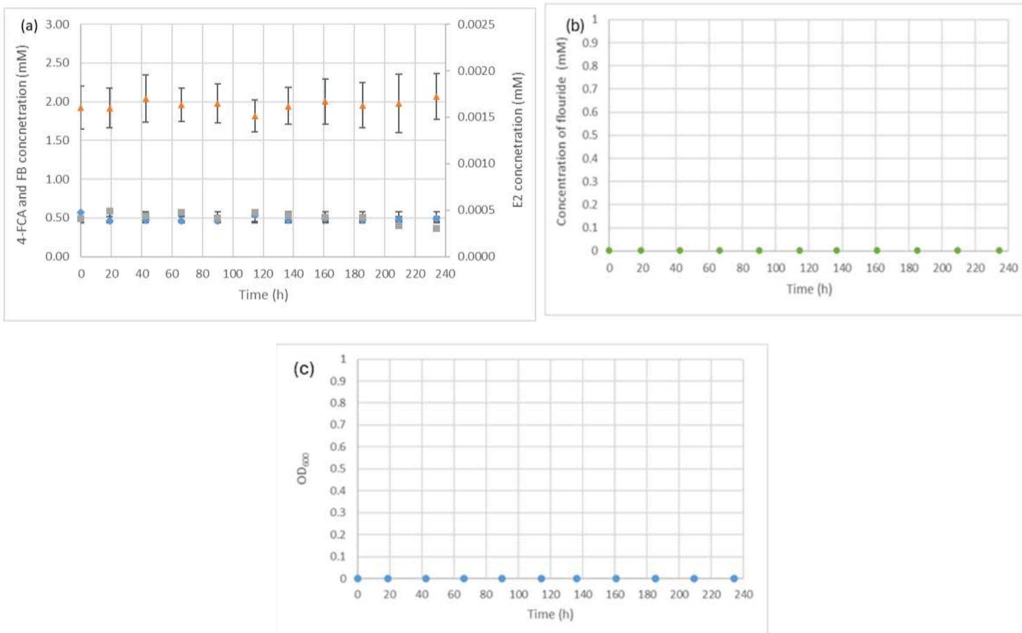

Figure 25 - Pollutants removal pattern in the abiotic control. Concentration in mM of (a) 4-FCA (◆), FB (■), and E2 (▲), (b) fluoride ions (●), and (c) optical density (●). Data points represent the mean of triplicates (n=3), and error bars represent the standard deviation.

To sum up, Table 2 shows the time it takes to degrade each pollutant in the tested experimental conditions and the results displayed in the literature. The study demonstrated several key findings. Improved degradation rates of the pollutant compounds were achieved with the immobilization of degrading strains, which significantly accelerated the removal of the pollutants. This also suggested that the EPS matrix provided a protective effect when the strains F11 and S2 were immobilized, potentially contributing to the enhanced degradation rates by shielding the bacteria from adverse conditions. Also, the EPS matrix has aided in the adsorption of pollutant compounds that could further enhance the degradation process by allowing pollutant compounds to contact with the degrading bacteria. However, E2 degradation was slower when strain ED55 was immobilized in the granules. This slower rate may be due to mass transfer limitations, which likely delayed the diffusion of E2 into the EPS matrix, making it more difficult for the bacteria to degrade the compound. When ED55 was tested with the three pollutant compounds, complete degradation of E2 was observed at the beginning of the experiment, while 4-FCA was fully degraded by 67.5 h. Additionally, partial degradation of FB was achieved during the experiment. In the study by Moreira et al., (2022) the pollutant compound E2 was degraded at 1.25 h when strain ED55 was added to the wastewater. In this study, E2 was immediately degraded at the beginning of the experiment (0 h) this could be due to the low initial concentration of the E2. Similarly, at 137 h, FB was completely degraded when strain F11 suspension was added to the medium containing the three pollutant compounds while 4-FCA and E2 got partially degraded. 4-FCA and E2 might have inhibitory effects on the enzymes or metabolic pathways responsible for their degradation. When the suspension of strain F11 was used with FB, it was completely degraded within 114.6 h which is slower than the study done by Carvalho et al., (2006) where pre-grown cells and the addition of 1 mM glucose were employed which could have accelerated the degradation process. The absence of these factors in this study accounted for the slower degradation rate observed. When strain S2 was exposed to the three pollutants or solely to 4-FCA, complete degradation of 4-FCA was achieved within 111.7 h and 115.5 h, showing a very similar result.

Table 2 - Time needed for total degradation of each pollutant in the tested experimental conditions in the present study and in the literature.

Experimental condition	FB	4-FCA	E2	Literature
EPS granules + pollutants	90 h	66.2 h	42.5h	This study
3 strains suspension + pollutants	231.5 h	90.1 h	0 h	This study
ED55 + pollutants	p	67.5 h	0 h	This study
ED55 + E2	n.a.	n.a.	< 20 h	1.25 h (Moreira et al., 2022)
F11 + pollutants	137 h	p	p	This study
F11 + FB	114.6 h	n.a.	n.a.	13 h (Carvalho et al., 2006)
S2 + pollutants	189.3 h	111.7h	n.	This study
S2 + 4-FCA	n.a.	115.5 h	n.a.	240 h (Amorim et al., 2014)

n.a.: non applicable
p.: partial degradation
n.: not degraded

3.3. Post-experimental bacterial isolates identification

To verify the presence of any bacteria other than the three strains F11, S2 and ED55 used in the study, and to assess for potential contamination during the batch assays or granules formation process post experimental bacterial cultures were isolated. The method used was liquid plate culture, where samples from all the conditions were plated on nutrient agar plates. The resulting colonies were then examined for morphological differences and compared against the known characteristics of the three expected bacterial strains (Table 3).

It was expected that all three strains F11, ED55, and S2 would be present in the experimental condition using the multi-immobilized bacterial granules and in the condition with the mixed bacterial strains suspensions. However, in both conditions, only colonies from strains F11 and S2 were recovered, while colonies with characteristics from ED55 were not detected. The absence of strain ED55 suggests two possible hypotheses: (i) the bacterial strains F11 and S2 presented competitive interactions against the strain ED55, effectively inhibiting the growth of strain ED55; (ii) strain ED55 rapidly degraded E2, present at very low concentrations, and in the absence of other biodegradable carbon source that this strain can metabolize, it may have faced difficulties sustaining and proliferating in the experimental conditions, leading to its absence. The degradation profile of E2 observed in those two mentioned conditions (EPS granules and the mixed bacterial strains suspensions) demonstrated complete degradation of the pollutant. This supports the second hypothesis, indicating that strain ED55 was present and all three bacterial strains coexisted but ED55 was not recovered by the plating technique by the end of the experiment.

The absence of all three bacterial strains in the condition of autoclaved granules, as well as in the abiotic control implies that the autoclaving process successfully eliminated the cultivable bacteria from the granules, and there was no cross-contamination in these conditions once the experiment began.

In the experimental conditions with strain F11 exposed to the three pollutants compounds and just exposed to FB, it was expected that only the F11 strain would be identified by the end of the experiment through culturing. However, colonies characteristic from strain S2 were also detected. Possibly a cross-contamination occurred during the inoculation of flasks or the sampling procedure, which could have been responsible for the partial degradation of 4-FCA in the condition with F11 fed with the three pollutants.

Strain S2 should have been the only bacterial species present in the experimental conditions with S2 and the three pollutants, and S2 with 4-FCA. However, strain F11 was also isolated from the suspensions of both conditions. This cross-contamination with strain F11 could have contributed to the complete degradation of FB in the flasks of S2 fed the three pollutants.

Lastly, the three strains were detected in the suspension of the experimental conditions that were supposed to contain strain ED55 and E2, and ED55 with the three pollutant compounds. This cross-contamination could be the cause of 4-FCA degradation in these conditions.

Table 3 - Post-experimental bacterial strain analysis by plate culture technique.

Post-Experimental Bacterial Strain Analysis by Plate Culture Technique				
Conditions	Expected strains	Observed strains		
		F11	S2	ED55
Granules+toxics	F11+S2+ED55	YES	YES	NO
Autoclaved Granules+toxics	-	NO	NO	NO
Abiotic Control + toxics	-	NO	NO	NO
ED55+toxics	ED55	YES	YES	YES
ED55+E2	ED55	YES	YES	YES
F11+FB	F11	YES	YES	NO
F11+toxics	F11	YES	YES	NO
S2+4-FCA	S2	YES	YES	NO
S2+toxics	S2	YES	YES	NO
All strains+ toxics	F11+S2+ED55	YES	YES	NO

4. Conclusion

The present work aimed at investigating the capability of co-immobilized specialized degrading bacteria within EPS to eliminate different recalcitrant pollutants present in wastewater. The co-immobilization of the bacterial strains *Labrys portucalensis* F11, *Rhodococcus* sp. S2, and *Rhodococcus* sp. ED55 to produce degrading granules allowed the simultaneous degradation of a combination of pollutants, namely, FB (1 mM), 4-FCA (0.5 mM), and E2 (0.018 mM), marking the first report on this issue.

Co-immobilized bacteria granules showed the ability to completely degrade pollutant FB and 4-FCA rapidly within 90 h and 66.2 h, which was faster if compared to degradation by strains F11 and S2 suspensions that took 231.5 h for FB and 90.1 h for 4-FCA, respectively. Nevertheless, E2 degradation by the multi-immobilized bacteria granules was slower, taking 42.5 h, when compared to a suspension of strain ED55 (< 20h). This complete degradation of the pollutants shows the effectiveness of the co-immobilized bacteria within EPS in degrading the pollutant compounds, proving that immobilization is an effective method for the degradation of these three pollutant compounds that can be found in wastewater. The longer degradation time for the pollutants to degrade in bacterial suspensions in this study proves that co-immobilized bacteria in EPS granules can be more effective for treating pollutant compounds effectively, especially fluorinated chemicals.

This study highlights the potential of multi-bacterial strain immobilization in granules for the removal of recalcitrant compounds, which could be a promising bioaugmentation strategy to simultaneously remove multiple industrial recalcitrant pollutants and endocrine-disrupting compounds from wastewater. Moreover, it emphasizes the use of recovered EPS as a natural and biodegradable immobilizing agent, aiding in the transition of WWTP to a circular economy model.

5. Future work

The successful use of co-immobilized specialized degrading bacteria within EPS was achieved allowing for the simultaneous removal of the target pollutants present in wastewater. However, additional research should be conducted with these co-immobilized granules to better ascertain their further applications. Since the target pollutant compound of ED55 degraded fast, immobilized granules could be further tested for consecutive batch assays to verify if all the bacterial strains persist and proliferate in the long run. Also, these granules could be used to bioaugment an AGS system to evaluate the stability and efficiency of the granules in such an environment. Likewise, these EPS granules can be evaluated to identify their effectiveness in the system to improve the degradation of the target pollutants increasing the overall performance of the reactor. Moreover, investigation of the possibility of horizontal gene transfer is another promising direction that could highlight genetic exchanges between the bacterial strains and the indigenous microbial community present in the AGS reactor through conjugation assays.

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