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Exploring new strategies to improve the bacterial defluorination of polyfluorinated pollutants

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Resumo

Os compostos orgânicos fluorados são poluentes persistentes disseminados na maioria dos ecossistemas e que podem resistir à maioria das tecnologias de tratamento devido à estabilidade das suas ligações C-F. Ainda assim, a degradação bacteriana continua a ser uma das poucas abordagens com potencial para degradar e mineralizar substancialmente estes poluentes, uma vez que algumas bactérias podem catalisar reações de defluorinação. No entanto, este processo catabólico é pouco comum e tende a ser altamente ineficiente. Encontrar novas formas de melhorar a produtividade da defluorinação bacteriana tem o potencial de abrir novas portas para a biorremediação destes poluentes ambientais críticos. Como tal, este trabalho teve como objetivo explorar a influência de diferentes estratégias e limitações na defluorinação bacteriana de diferentes compostos polifluorados por duas estirpes bacterianas modelo de defluorinação (*Delftia acidovorans* MFA5 e *Labrys portucalensis* F11, capazes de degradar compostos orgânicos fluorados alifáticos e aromáticos, respetivamente), incluindo: (1) a interação entre concentração de flúor e defluorinação, (2) co-metabolismo e (3) uma combinação de nanofotocatálise e degradação bacteriana. A sensibilidade ao flúor das estirpes MFA5 e F11, bem como de uma estirpe não defluorinadora, E. coli Seattle 1946, foi inicialmente determinada através da exposição a diferentes concentrações de fluoreto de sódio (NaF) sob diferentes condições tróficas e de pH. A disponibilidade de nutrientes desempenhou um grande papel na resposta bacteriana ao flúor (mais do que o pH), dado que foram observadas inibições mais severas em condições oligotróficas. As estirpes foram então expostas ao limiar tóxico de NaF determinado (0,4 mM) em condições oligotróficas e os seus desempenhos de defluorinação foram avaliados contra os seus substratos fluorados preferenciais. A eficácia de defluorinação foi inibida pela exposição ao flúor, diminuindo de degradação completa em condições isentas de flúor para eficiências de defluorinação de 63,3% e 7,6% para o fluoroacetato (*D. acidovorans* MFA5) e fluorobenzeno (*L. portucalensis* F11), respetivamente. Estes resultados sugerem que o flúor afeta indiretamente a defluorinação, prejudicando o crescimento bacteriano, apesar de ser um produto final inevitável destas reações. Para os testes co-metabólicos, o desempenho de defluorinação das estirpes MFA5 e F11 foi testado para o ácido perfluorohexanóico (PFHxA) e mefentrifluconazol (MFZ), seguindo uma estratégia co-metabólica, com o uso de ácido hexanóico e tolueno como co-substratos. Esta abordagem não teve um efeito positivo na defluorinação, uma vez que as estirpes testadas foram igualmente incapazes de defluorinar eficazmente qualquer poluente na presença ou ausência dos co-substratos selecionados. Os resultados sugerem assim que as vias de degradação do ácido

hexanóico e do tolueno não estão envolvidas na degradação do PFHxA e do MFZ, respetivamente. Por fim, uma abordagem de tratamento híbrido, combinando nanofotocatálise e degradação bacteriana, foi testada como uma possível estratégia para melhorar a defluorinação da trifloxistrobina. Apesar de terem sido construídos diferentes consórcios bacterianos para defluorinar água contaminada com trifloxistrobina, pré-tratada por nanofotocatálise, nenhum destes consórcio exibiu sinais de defluorinação. A falta de crescimento bacteriano sugere que as nanopartículas libertadas durante o pré-tratamento exerceram efeitos tóxicos nos diferentes consórcios, impedindo ainda mais a sua eficiência catabólica. No geral, os resultados recolhidos neste trabalho apresentam limitações e ajudam a identificar possíveis otimizações e futuras prioridades de investigação no sentido de melhorar os processos de defluorinação bacteriana capazes de combater a poluição causada por compostos fluorados persistentes.

Palavras-chave: Flúor, Compostos fluoroogânicos, Sensibilidade a fluor, Defluorinação, Biorremediação, Mefentrifluconazol, Ácido perfluorohexanoíco, Trifloxistrobina, Co-metabolismo, Nano-fotocatálise,

Abstract

Fluorinated organic compounds are persistent pollutants that are widespread across most ecosystems and that can resist most treatment technologies due to the stability of their C-F bonds. Still, bacterial degradation remains one of the few approaches with potential to substantially degrade and mineralize these pollutants, as some bacteria can catalyse defluorination reactions. However, this catabolic process is rare and tends to be highly inefficient. Finding new ways to improve the productivity of bacterial defluorination has the potential to open new doors for the bioremediation of these critical environmental pollutants. As such, this work aimed to explore the influence of different strategies and bottlenecks on the bacterial defluorination of different polyfluorinated compounds by two model defluorinating bacterial strains (*Delftia acidovorans* MFA5 and *Labrys portucalensis* F11, known degraders of aliphatic and aromatic organofluorines, respectively), including: (1) the interplay between fluoride sensitivity and defluorination, (2) structure-driven co-metabolism and (3) a combination of nanophotocatalysis and bacterial degradation. The fluoride sensitivity of strains MFA5 and F11, as well as non-defluorinating strain *E. coli* Seattle 1946, was initially determined, by exposure to increasing concentrations of sodium fluoride (NaF) under different trophic and pH conditions. Nutrient availability played a large role in the bacterial response to fluoride (more than pH), as more severe inhibitions being observed in oligotrophic conditions. Strains were then exposed to the determined NaF toxic threshold (0.4 mM) under oligotrophic conditions and their defluorination performances were evaluated against their preferred fluorinated substrates. Defluorination performances were impaired by fluoride exposure, decreasing from complete degradation under fluoride-free conditions to defluorination efficiencies of 63.3% and 7.6% for fluoroacetate (*D. acidovorans* MFA5) and fluorobenzene (*L. portucalensis* F11), respectively. These results suggest fluoride indirectly affects defluorination by impairing bacterial growth, despite being an inevitable end-product of these reactions. For the co-metabolic tests, the defluorination performance of strains MFA5 and F11 was tested for perfluorohexanoic acid (PFHxA) and mefentrifluconazole (MFZ), following a structure-driven co-metabolic strategy using hexanoic acid and toluene as co-substrates. This approach did not have a positive effect on defluorination, as the tested strains were equally unable to effectively defluorinate either pollutant in the presence or absence of the selected co-substrates. Results thus suggest that hexanoic acid and toluene degrading pathways are not involved in the degradation of PFHxA and MFZ, respectively. Finally, a hybrid treatment approach combining nanophotocatalysis and bacterial degradation was tested as a possible strategy to improve the defluorination of trifloxystrobin. While multiple bacterial consortia were assembled to

defluorinate trifloxystrobin contaminated water pre-treated by nanophotocatalysis, no consortium exhibited signs of defluorination. Lack of bacterial growth suggests that released nanoparticles during pre-treatment exerted toxic effects on the different consortia, further preventing their catabolic efficiency. Overall, results collected across this work present limitations and help identify possible optimizations and future research priorities towards improved bacterial defluorination processes capable of tackling pollution caused by persistent fluorinated compounds.

Keywords: Fluorine, Fluoorganic compounds, Fluoride sensitivity, Defluorination, Bioremediation, Mefentrifluconazole, Perfluorohexanoic acic, Trifloxystrobin, Co-metabolism, Nano-photocatalysis,

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List of Abbreviations

FCUP	FACULTY OF SCIENCES OF THE UNIVERSITY OF PORTO
UP	UNIVERSITY OF PORTO
ICBAS	SCHOOL OF MEDICINE AND BIOMEDICAL SCIENCES
CIIMAR	INTERDISCIPLINARY CENTRE OF MARINE AND ENVIRONMENTAL RESEARCH
PFAS	PER- AND POLYFLUOROALKYL SUBSTANCES
PFOA	PERFLUOROOCTANOIC ACID
PFHXS	PERFLUOROHEXANESULFONIC ACID
PFOS	PERFLUOROOCTANE SULFONATE
PFHXA	PERFLUOROHEXANOIC ACID
EPA	ENVIRONMENTAL PROTECTION AGENCY
PPARY	PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR
HPF	HOURS POST FERTILIZATION
SVHC	SUBSTANCE OF VERY HIGH CONCERN
PPB	PARTS PER BILLION
GSH	GLUTATHIONE
ECHA	EUROPEAN CHEMICALS AGENCY
MFZ	MEFENTRIFLUCONAZOLE
DT_{90}	PERIOD REQUIRED FOR 90% DISSIPATION
DT_{50}	HALF-LIFE
LC_{50}	LETHAL CONCENTRATION 50%
EFSA	EUROPEAN FOOD SAFETY AUTHORITY
TRF	TRIFLOXYSTROBIN
TFSA	TRIFLOXYSTROBIN ACID
PFMEUPA	4-(TRIFLUOROMETHYL) HEXAFLUOROPENT-2-ENOIC ACID
TIO_2	TITANIUM DIOXIDE
ZNO	ZINC OXIDE
NB	NUTRIENT BROTH
MSM	MINIMAL SALTS MEDIUM
NAF	SODIUM FLUORIDE
OD	OPTICAL DENSITY
FA	FLUOROACETATE
FB	FLUOROBENZENE
TISABIII	TOTAL IONIC STRENGTH ADJUSTMENT BUFFER

Introduction

1. Fluoorganic compounds

Elemental fluorine was originally isolated on June 1886 by Henry Moissan by electrolysis of potassium fluoride using anhydrous hydrofluoric acid, however fluoride only saw wide application in multiple fields in the 20th century (Tressaud, 2018; Wisniak, 2002). Fluorine is an element belonging to the Halogens group, with an atomic mass of 19. It is also the most electronegative element in the periodic table, due to its small atomic radius, causing fluorine to have a stronger attraction of shared electrons and, thus, higher reactivity with non-fluorine elements, particularly carbon. In organic molecules, carbon-fluorine (C-F) bonds are highly polarized, with a high electrostatic attraction between fluorine and carbon. Furthermore, the C-F bond has a short length (1.35 Å), being comparable to the C-O (1.43 Å) and C-H (1.09 Å) bonds, improving the overall stability of this already highly polarized bond (O'Hagan, 2008; Tressaud, 2018). Furthermore, the dissociation energy of C-F has been reported as the highest among common carbon covalent bonds (105.4 Kcal mol^{-1}), couple with a strong dipole moment (1.41 D), further increasing the stability of this bond (Gillis et al., 2015; O'Hagan, 2008). The junction of these properties makes organic fluorinated compounds extremely stable molecules. Due to its electronegativity, when bonded with carbon, fluoride draws electron density towards itself, adopting a noble gas-like configuration, impeding both nucleophilic and electrophilic attacks (Alexandrino, Almeida, et al., 2022; Gillis et al., 2015; Thiehoff et al., 2017) which further increases the stability of fluorinated compounds.

Most fluorinated organic compounds are man-made molecules as the natural occurrence of these is a rarity, with only ~30 of the existing thousands of organohalogens containing fluorine (Richardson, 2021). Furthermore, all of the known natural occurring fluorinated organic compounds are monofluorinated (Walker & Chang, 2014), which contrasts with the thousands of synthetic polyfluorinated organic compounds that are known (Lewandowski et al., 2006). Synthetic fluoroorganic compounds have seen ample uses in many fields, including refrigeration with the invention of Freon®, multiple fluoropolymers (Teflon®), fluorinated plastics and surfactants use for fabrics and kitchen appliances, as well as pesticides and pharmaceuticals (Chambers, 2004; Cui et al., 2020; Dolbier, 2005; Gaines, 2023; Gluge et al., 2020; Tressaud, 2018). These compounds represent a class of molecules whose origin dates back to the mid-19th century, although their wide dissemination came in the 1930s, with the introduction of chlorofluorocarbons (CFCs) in the refrigeration industry (Adams & Halden, 2010; Okazoe, 2009; Sicard & Baker, 2020). Since then, fluoroorganic compounds have steadily grown in representation across most

economic sectors, as fluoroorganic chemistry has seen a massive expansion in the 21st century due to more efficient synthesis processes (Tressaud, 2018).

The physical-chemical properties that have led to the successful expansion of fluorinated compounds in organic chemistry to produce industrial and commercial products, are also responsible for the environmental relevance of these compounds as xenobiotics and pollutants. Their large-scale production and widespread use, combined with the typical high thermal and chemical stability of these compounds, have caused fluoroorganic compounds to become ubiquitous in the environment. Conventional treatment processes used in wastewater treatment plants, such as secondary biological treatment, have shown to be largely ineffective in the removal of fluoroorganic compounds, with reported cases of only partial transformation into shorter, more mobile metabolites (Arvaniti & Stasinakis, 2015; Coggan et al., 2019; Hepburn et al., 2019). Furthermore, as these compounds are almost exclusively synthetic, biological degradation is not common, which further contributes to the environmental persistence of fluoorganics (Alexandrino, Mucha, et al., 2022; Wackett Lawrence, 2021).

In the environment, fluoroorganic compounds show accumulation in the atmosphere, water, wildlife and in humans (Lind et al., 2022; Nilsson et al., 2022; Zhang et al., 2012). In soils, samples collected from paddy fields in the industrialized area of Shifang City, China found perfluorooctanoic acid (PFOA) concentrations reaching a maximum of 0.905 ng g^{-1} in topsoil, with PFOA and other per- and polyfluoroalkyl substances (PFAS) being also shown to migrate towards lower levels of the soil column, in some cases being detected at depths of 80-100 cm with a maximum concentration of 0.356 ng g^{-1}, (Gan et al., 2022). In China, in downstream regions of the Changshu fluorine-chemical industrial park, maximum total PFAS concentrations for agricultural soil and drinking water were 65.7 ngg^{-1} and 369.9 ng L^{-1}, respectively, with PFOA accounting for 60.2% of PFAS load in all irrigation water samples (Li et al., 2019). In a test population of 799 firefighters from the Airservices Australia organisation, in blood serum samples the PFAS PFOA, perfluorohexanesulfonic acid (PFHxS) and perfluorooctane sulfonate (PFOS) were present in all samples at mean concentrations of 1.5, 6.5 and 14 ng mL^{-1}, respectively (Nilsson et al., 2022). This widespread environmental circulation coupled with the high toxicity exerted by some fluoorganics, turns them into concerning pollutants. Many organofluorines have been found to be hazardous for both environmental and human health (Murphy et al., 2012).For instance, the fluorinated pesticide epoxiconazole is responsible for endocrine disruption in rats exposed to either 15 or 50 mg/ kg of the pesticide. High doses of epoxiconazole have shown to cause increased gestational periods, dead fetuses and postanatal deaths as well

as a sevenfold increase in progesterone and a twofold increase in testosterone levels (Taxvig et al., 2007). Ciprofloxacin, a fluoroquinolone antibiotic, has shown to act as an inhibitor for the photosynthetic process in *Arabidopsis thaliana* and *Spinacia oleracea*, when occurring as an environmental pollutant at concentrations higher than 10 µM (Evans-Roberts et al., 2016) (Aristilde et al., 2010). Similarly, PFOS exert immunotoxic activity on both animals and humans (Liang et al., 2022). In adult male mice, PFOS induced the apoptosis of thymocites for mice exposed (Zhang et al., 2013). In humans PFOS was found to alter lymphocyte function, by altering the expression of cell differentiation genes as well as affecting cell signalling pathways, leading to the apoptosis of immune cells, such as phagocytes and leukocytes (R. Li et al., 2020).

2. Per- and polyfluoroalkyl substances (PFAS)

Among the class of fluoorganic compounds, PFAS present themselves as extremely concerning pollutants as they showcase the improved physical-chemical attributes induced by fluorination in much higher extent, as a result of having multiple C-F bonds in their molecular structure. Indeed, PFAS are characterized by their unique properties, including high thermal, chemical and biological stability as well as simultaneous hydrophobic and lipophobic behaviour (Buck et al., 2011; Cui et al., 2020; McCarthy et al., 2017).

PFAS were originally separated into two groups, long-chain, and short-chain PFAS. According to Buck et al. (2011), long-chain PFAS included both perfluoroalkyl sulfonic acids with at least 6 carbon atoms and perfluoroalkyl carboxylic acids with at least 7 carbon atoms and their respective anions, with the rest being classified as short-chain PFAS. This definition has since been revised for a more consistent and coherent one focused on their chemical structure. PFAS are now characterized as " fluorinated substances that contain at least one fully fluorinated methyl or methylene carbon atom…" (Wang et al., 2021). This new classification is comparatively broader, including previously excluded fluorinated compounds.

2.1. Perfluorohexanoic acid

Perfluorohexanoic acid (PFHxA) is considered a traditional PFAS, being a short-chain perfluorinated compound composed by eleven C-F bonds. Being a short-chain PFAS, it is introduced into the environment via the non defluorinating degradation of long-chain PFAS, in addition to its direct environmental contamination via production, use and waste of

PFHxA-containing products (Dasu et al., 2022; Dauchy et al., 2012; F. Li et al., 2020; Smorada et al., 2024). The discharge of PFHxA, due to this non defluorinating degradation, from industrial wastewater treatment plants (WWTPs), has been already observed in Australia (Coggan et al., 2019; Hepburn et al., 2019). According to Coggan et al. (2019), the concentration of PFHxA, among other PFAS, was found to increase between the influent and the final effluent of WWTPs, likely because of microbial degradation processes of other fluorinated compounds, such as known precursor 6:2 fluorotelomer sulfonate(6.2FTS)(Hamid et al., 2020). In this study, PFHxA was detected in over 90% of samples, with the highest mean concentration of 16 ngL^{-1} in aqueous samples from 19 different WWTPs (Coggan et al., 2019). PFHxA has been found in municipal landfill leachates, as well (Eschauzier et al., 2013). In Hepburn et al. (2019), groundwater samples were collected from seven legacy landfills with PFHxA being detected in 85% of the samples collected, with concentrations ranging from <0.2 to 46 ngL^{-1}.

After entering the environment, PFHxA is known to be environmentally persistent due to the carbon-fluoride bonds present, with the ability to bioaccumulate in marine animals (Kreychman et al., 2024). In European carp, traces of PFHxA was found in the brain, gallbladder, gonad, kidney and liver tissues in concentrations up to 1.2, 2.51, 5.34, 1.55 and 37.5 ngg^{-1} respectively, after an exposure period of 14 weeks and 3 weeks of depurination (Petre et al., 2023). In whole fish homogenates from samples collected from the Missouri, Mississippi and Ohio Rivers, PFHxA was present in the samples from all three rivers , with the lowest concentration of <2 ng/g, a maximum concentration of 18.4 ng/g and a median concentration of 3.71 ng/g across all rivers (Ye et al., 2008).

An estimation of the elimination kinetics of PFHxA in humans, using multiple blood serum samples collected from eight ski wax technicians, indicated that this compound has a human mean half-life ($DT_{50)}$ of 32 days (ranging from 14 to 49 days). Comparisons conducted with previous studies on mice, rats and monkey suggested a proportional relationship between mammalian body-weight and DT_{50} for PFHxA (Russell et al., 2013).

According to the United States Environmental Protection Agency (EPA), once in the environment PFHxA exposure is likely responsible for endocrine (decreased total and free thyroxine, decreased triiodothyronine, increased thyroid epithelial cell hypertrophy and thyroid weight), hematopoietic (decreased blood proteins, hematocrit, hemoglobin, red blood cells and increased reticulocytes), developmental (decreased fetal body weight and increased perinatal mortality) and hepatic effects (increased hepatocellular hypertrophy, relative liver weight and hepatocellular necrosis) in humans (EPA, 2023). Regarding hepatoxicity, PFHxA was found to affect mice through the upregulation of the peroxisome

proliferator-activated receptor (PPARγ) signalling pathway, responsible for the regulation of the metabolism of fatty acids, promoting hepatic steatosis (Jiang et al., 2021). Furthermore, in this same study, PFHxA was found to affect both glutathione (GSH) metabolism, superoxide dismutase (SOD) activity and purine synthesis, generating oxidative stress (Jiang et al., 2021). Using zebrafish as a model organism, PFHxA was found to induce significant morphological changes at 72 hours post fertilization (hpf) in exposed embryos, affected fish were found to have a decrease in body length, head length and width at the concentrations of 40 and 400 ppb PFHxA (Wasel et al., 2022). Due to its persistence, increasing environmental presence and confirmed toxic potential, PFHxA has recently been classified as a substance of very high concern (SVHC) by the European Commission following a recommendation by Germany (ECHA, 2018). The reasoning for this stance relies on the fact that PFHxA is described as “extremely persistent, mobile in the aquatic environment, can be distributed easily within and between environmental compartments by aqueous media, has a long-range transport potential and the potential to enrich in plants.”. Currently, the European Commission is in the process of restricting PFHxA and its’ related substances (compounds with a possibility to be degraded or transformed into PFHxA) . During the REACH committee meeting on 28 February 2024, the EU member states voted in favour of PFHxA limits previously proposed by Germany in 2020, with the support of the European Chemicals Agency (ECHA) in 2022, of 25 parts per billion (ppb) for the aggregate of PFHxA and its salts, and the limit of 1000 ppb for the sum of all PFHxA related substances.

2.2. Mefentrifluconazole

Mefentrifluconazole (MFZ) is a chiral fluorinated triazole fungicide that was originally discovered by Badische Anilin-und-Soda-Fabrik (BASF), and is a member of the isopropanol azoles, a sub-group of triazole fungicides (He et al., 2023; Tesh et al., 2019). Harbouring a trifluoromethyl functional group in its chemical structure, MFZ is also classified as PFAS according to the revised PFAS definition.

Azole fungicides are mainly introduced into the environment via domestic sewage, entering wastewater treatment plants and ultimately released into the environment (Chen & Ying, 2015; Chen et al., 2014). These compounds have been detected previously in water streams, such as estuaries. In the U.K, clotrimazole, another azole fungicide, was found to be present in multiple samples of various estuaries, with concentrations reaching up to 22 ngL^{-1} in the Tees estuary (Thomas & Hilton, 2004). MFZ has been found to be a soil contaminant, described by the European Food Safety Authority (EFSA) as exhibiting “high

to very high persistence in soil" with a required period for 90% dissipations (DT_{90}) ranging from 616 to 1000 days, and expecting accumulation in fields treated yearly with this pesticide (European Food Safety Authority et al., 2023). The DT_{50} of MFZ is dependent on crop type, temperature, humidity, soil pH conditions and organic matter content, with alkaline soils and rich organic matter favouring the dissipation of MFZ (Liu et al., 2021; Zhang et al., 2023). In watermelon cultivars, MFZ dissipation was found to be enantioselective, with higher temperature and humidity conditions, originating from a subtropical climate, being responsible for the increase of the DT_{50} of MFZ in these plants, reaching a maximum 6.6 ± 0.04 days for R-mefentrifluconazole (Liu et al., 2021). In mango, MFZ has a reported DT_{50} of 5.6 to 11.8 days, with the lowest observed value being attributed to the temperature, precipitation and sunlight conditions of the area the mango samples were collected from (Wang et al., 2023). In brown rice, the DT_{50} of MFZ was reported to range from 2.8 to 9.0 days, from samples collected from the Ezhou and Shenyan regions (country?) respectively, with slower dissipation possibly being a result of lower average temperatures present in the Shenyang region (Zhang et al., 2023).

Triazole fungicides perform their antifungal properties by blocking the synthesis of ergosterol, interfering with the formation of the fungal cell membrane. This is achieved by the inhibition of the enzyme sterol 14α-demethylase, belonging to the superfamily of the heme-containing cytochrome P450 enzymes (Zarn et al., 2003). However, this process is also responsible for the inhibition of other sterol enzymes, such as aromatase, a cytochrome P450 enzyme that plays a vital role in converting both androstenedione and testosterone into, respectively, estrone and estradiol, leading to endocrine disruption effects in mammals (Zarn et al., 2003). In mammals, MFZ has also been linked to multiple acute (skin sensitization, neurotoxicity) and chronic (liver cell hypertrophy and necrosis) effects (Tesh et al., 2019). Additional concerns have arisen due to other toxicological effects of MFZ, which seem to be enantioselective. On earthworm, the acute toxicity of the enantiomer S-mefentrifluconazole was the highest, with skin exposure being considered the main route for MFZ, using the filter paper contact test for earthworms (OECD, 1984) a Lethal Concentration 50 (LC50) at 48 hours of 4.1 μgcm^2 (Xu et al., 2022). In zebrafish, S-mefentrifluconazole also exerted higher toxicity when compared to R-mefentrifluconazole, consistently presenting lower LC_{50} values across all life stages of the tested zebrafish after a period of 96 hours. Here, both enantiomer were found to exert teratogenic effects on zebrafish, such as yolk sac and pericardial edema in embryos, pericardial and spinal curvature in larvae, and heartbeat inhibition in both larvae and embryos (Cui et al., 2022).

2.3. Trifloxystrobin

Trifloxystrobin (TRF) is a fluorinated fungicide introduced in 1998, belonging to the group of the strobilurin pesticides (Bartlett et al., 2002; Morton & Staub, 2008; Sharma et al., 2019), being the first strobilurin pesticide featuring a oximether side chain responsible for a broad efficacy against multiple fungal pests (Banerjee et al., 2006; Zhu et al., 2015).

Strobilurin pesticides, including TRF, act by inhibiting the mitochondrial respiration activity in fungal cells, by binding to the Q_0 site of cytochrome b, stopping electron transfer from cytochrome b to cytochrome c, leading to inhibition of the generation of adenosine triphosphate in fungi (Balba, 2007; Bartlett et al., 2002; Liu et al., 2020; Sharma et al., 2019). Concerns about the toxicological effects of TRF on non-target organisms have arisen. In zebrafish embryos, TRF exposure resulted in development abnormalities, such as growth retardation, pericardial and yolk sac edemas and pigmentation defects, acting in a dose-dependent manner (Li et al., 2018). TRF has also shown to significantly lower the predation rate of different tadpole species by eel *S. marmoratus* in cases for exposure in both predator and prey as well as cases of only predator exposure, explained by a reduction in prey detection by the predator species (Junges et al., 2012). Earthworm *Eisenia fetida* suffers from oxidative stress when exposed to 10 mg kg^{-1} of TRF in soil, with protein synthesis and intracellular homeostasis also showing to be impaired (Liu et al., 2020).

In the environment, TRF has a recorded DT_{50} ranging from 1.8 to 6.7 days, described to have a “very low to moderate persistence “in soils (European Food Safety et al., 2017). In soils collected from four different location of the Himalayan region of India, TRF was found to have a $DT_{50}$of 0.54 to 8.8 days, with a decrease in dissipation time for the locations with higher sunlight exposure, indicating that photolysis acts as a dissipation pathway for TRF. (Wang et al., 2014). TRF is also degraded via aquatic hydrolysis with a $DT_{50}$of 0.7 to 1.3 days, however this process results in the metabolite trifloxystrobin acid (TFSA), which is known to persist in neutral of weak acid conditions allowing for its persistence in these environmental conditions for up to several years (Banerjee et al., 2006; Luo et al., 2020). According to EFSA, TFSA presents “moderate to very high persistence” in soil, with DT_{50} values ranging between 35 to 755 days (European Food Safety Authority et al., 2017). TFSA also shows higher mobility in groundwater than TRF, while exerting the same toxicological effects than its parental compound, which combined with its larger persistence turns this metabolite into a very concerning pollutant for soils and water systems (Luo et al., 2020) (European Food Safety et al., 2017) (Liu et al., 2020).

3. Degradation and remediation of fluorinated compounds: physical-chemical and biological approaches

As previously established, fluoroorganic compounds present themselves as an environmental hazard, mainly due to their high stability, as well as the toxic effects these compounds might exert. For the complete degradation of these compounds, the cleavage of the C-F bonds is a vital step as it reduces the stability of these pollutants, facilitating any posterior steps in the degradation process and their eventual mineralization (Alexandrino, Mucha, et al., 2022). However, not many treatment strategies lead to defluorination. Multiple propositions for the effective environmental removal of these compounds have come forth over the years, including electrochemistry, advanced oxidation/reduction processes and incineration, all of which have shown inefficient of removing fluoride from these persistent pollutants (Arvaniti & Stasinakis, 2015; F. Li et al., 2020; Winchell et al., 2021). In addition, the application of these processes presents significant drawbacks. Incineration presents the danger of releasing toxic hydrogen fluoride, alongside harmful atmospheric emissions (Winchell et al., 2021). Regarding electrochemistry and advanced oxidation/reduction processes, these tend to be expensive and non-specific processes that have a possibility of creating toxic compounds as sub-products (Yu et al., 2020).

Bioremediation, namely through bacterial degradation processes, has been less contemplated for the environmental remediation of fluoroorganic pollutants, including PFAS. Bioremediation is defined as the process whereby organic pollutants are biologically degraded under controlled conditions to safer sub-products, or removed to levels below concentration limits established by regulatory authorities (Mueller et al., 1996). This can be achieved using naturally occurring organisms for the degradation and detoxification of pollutants that are hazardous to the environment or to human health, which may be native to the contaminated area or previously isolated and introduced into the contaminated site (Kensa, 2011). Several bacteria have demonstrated the ability to metabolically achieve the cleavage of the C-F bonds in multiple fluorinated pollutants, a process that is not frequently observed during the treatment of fluoroorganic pollution following physical or chemical remediation approaches (Alexandrino, Mucha, et al., 2022). Still, bacterial defluorination of these compounds is a rare process due not only to the high stability of C-F bonds, but also due to scarcity of natural fluorinated organic compounds, contrasting with the large diversity of man-made fluoroorganic compounds that are present at relatively low concentrations in the environment, which does not favour the selection and evolution of defluorination phenotypes in environmental bacteria (Alexandrino, Mucha, et al., 2022; Wackett Lawrence, 2021).

Nevertheless, bacterial defluorination can be a promising venture for this issue, as the existence of bacteria encoding for enzymes capable of defluorination, such as reductive or hydrolytic dehalogenases, could imply a cost-effective and environmentally safe means for the effective removal of fluorinated persistent pollutants from affected environments (Ang et al., 2018). Recent studies have highlighted the role that bacterial defluorination can play on mitigating the persistence of fluorinated pesticides and antibiotics, which are both recalcitrant and highly toxic pollutants. For instance, epoxiconazole and fludioxonil, two persistent fluorinated pesticides, have been found to be completely defluorinated after a period of 9 and 12 days respectively by cultures derived from agricultural soil of a small traditional farm (Alexandrino et al., 2020). Norfloxacin, enrofloxacin and moxifloxacin, three fluoroquinolone antibiotics with extensive use in human and veterinary applications (Murphy, 2016), were found to be defluorinated into metabolites with less antibacterial activity and potentially further degraded and mineralized by different environmental bacteria (Alexandrino et al., 2017; Carvalho et al., 2016; Harrabi et al., 2019; Kim et al., 2011).

4. Potential strategies to improve bacterial defluorination

4.1. Improving fluoride homeostasis in degrading bacteria

An important factor for the effective bacterial remediation of organofluorines is the toxicity exerted by the released fluoride ions into the intracellular media (Alexandrino, Mucha, et al., 2022). Historically some bacteria have shown to present specific modes of resistance to fluoride toxicity, notably Fluc channels, highly specific channels responsible for transporting fluoride from the intracellular to the extracellular media (Baker et al., 2012; Ji et al., 2014; Macdonald & Stockbridge, 2017). These resistance systems depend on fluoride riboswitches for the detection of fluoride, and subsequent expression of said systems for fluoride cellular export (Breaker, 2012). Furthermore, in slightly acidic environments, anionic fluoride quickly converts to hydrofluoric acid, a membrane-permeant acid with a pKa of 3.4, that can flood the intracellular environment (Breaker, 2012; Ji et al., 2014; Marquis et al., 2003). This influx can exacerbate fluoride stress through a mechanism known as the weak acid accumulation effect. Fluoride is a known enzymatic inhibitor in bacteria, notably the metalloenzyme enolase, responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate during the glycolysis process, affecting cell division and RNA processing (Curran et al., 1994; Krucinska et al., 2019). Furthermore, fluoride toxicity has shown to be an enabler of the catabolic defluorination process (Wackett, 2024). *Acetobacterium* strains with capacity to enzymatically defluorinate 4-(Trifluoromethyl)hexafluoropent-2-enoic acid (PFMeUPA) was only able to achieve this in

the presence of functioning fluoride export systems, as the same strains with no functional export system showed no defluorinating activity (Yu et al., 2024). So, for a bacterial strain to be considered an apt candidate for use in bioremediation ventures, it needs not only to be able to effectively catabolize the cleavage of C-F bonds, but also to have the ability to endure the toxic effects that result from the release of fluoride into the intracellular media.

4.2. Co-metabolism

Co-metabolism is a biodegradation process typically employed to degrade recalcitrant compounds, in which the target pollutant represents a substrate uncapable of sustaining microbial growth. To meet this gap, co-metabolic approaches make use of secondary substrates that act as easily accessible carbon sources, thus driving central metabolism and biomass multiplication in the presence of the pollutant (Brandt et al., 2003; Wang et al., 2019). According to Kiel & Engesser (2015), co-metabolic approaches can fall into two categories: structure-driven (induction of enzymes for the degradation of the target pollutant by using growth substrates similar to the pollutant or its functional groups) or energy-driven (a surplus of an easily accessible carbon and energy source is available to increase microbial growth and improve the chances of the target pollutant being biodegraded as a result). The first recorded case of co-metabolism was provided by Leadbetter and Foster (1958), with the discovery of the oxidation of ethane and propane by *Pseudomonas methanica,* exclusively in the presence of methane as a co-substrate. Since then, co-metabolism has been consolidated as a viable strategy for the removal of otherwise recalcitrant compounds. For example, *Pseudomonas oleovorans* DT4 showed a higher efficiency in benzene degradation when grown with either tetrahydrofuran, pyruvate or ethanol (Zhou et al., 2011). When using ethanol as co-substrate, complete degradation of benzene was 6 hours faster when compared to using benzene as a sole carbon source. Furthermore, the same study showed that, while strain DT4 was unable to degrade either xylene or ethylbenzene, the presence of tetrahydrofuran was enough to trigger the bacterial degradation of both these compounds (Zhou et al., 2011). *L. portucalensis* F11, a bacterial strain known to be able to degrade aromatic fluorinated organic compounds (Moreira et al., 2012b; Moreira et al., 2014), is unable to degrade chlorobenzene when supplied as a sole carbon source, but when FB is co-supplemented the complete degradation of both compounds is observed after 10 days with resulting halogen release (Moreira et al., 2012a). Both *Rhodococcus rhodochrous* and *Aspergillus niger* were unable to grow when exposed to five different pharmaceuticals *(*carbamazepine, sulfamethizole, sulfamethoxazole, trimethoprim and metroprolol), however the presence of glucose as co-substrate led to significant degradation of both carbamazepine and sulfamethoxazole, with the

accumulation of stable, less likely to biotransform metabolites in both cases (Gauthier et al., 2010).

Furthermore, the use of co-metabolic approaches to improve the degradation and defluorination of fluorinated organic compounds has also been observed. Strain *L. portucalensis* F11 is able to degrade racemic fluoxetine as a sole carbon source, but the addition of acetate as a secondary carbon source allowed for a higher transformation rate of fluoxetine and achieve its complete biodegradation (Moreira et al., 2014). In addition, the strain was shown to have an increase in biodegradation of 450 µg/L of racemic ofloxacin when continuously supplied with acetate, with a removal of 96.0% for both (R)-ofloxacin and (S)-ofloxacin, a significant increase when compared to sole carbon source tests, with maximum removal of 34.6% ,for (S)-ofloxacin (Maia et al., 2018). Similarly, strain *Ralstonia sp.* FD-1 has been found to yield near to no degradation of multiple 4-fluoroaniline (4-FA) isomers and homologs [2-fluoroaniline(2-FA); 2,4-difluoroaniline(2,4-DFA), 3,4-difluoroaniline(3,4-DFA), and 2,3,4-trifluoroaniline (2,3,4-TFA)] when used as the sole carbon and nitrogen source, however the strain is able to completely defluorinate 4-FA. The use of 500 mg/L of 4-FA as a primary substrate in a co-metabolic test, with 72 hours of incubation and different target substrate concentrations (50; 100; 200 and 400 mg/L) was responsible for the increase of defluorination efficiency for all previously mentioned fluoroanililes, with a maximum yield of 100% observed for 3,4-DFA with a concentration of 50 mg/L (Cao et al., 2015). 3,3,3-trifluoropropionic acid (C3a) was found to be nearly fully degraded by activated sludge samples collected from the aeration tank of a local wastewater treatment plant in a co-metabolic process using either propionate or glucose as the growth substrate. Comparatively, the use of C3a as the sole carbon source for the activated sludge resulted in only the removal of ~10%, or ~0.3 mM of C3a (Che et al., 2021).

4.3. Combination of photocatalysis and biodegradation

Photocatalysis is an advanced oxidation process that relies on the use of photosensitive semiconductors that catalyse the chemical oxidation of the target pollutants when irradiated by natural or synthetic radiation (Beydoun et al., 1999; Enesca & Andronic, 2020; Tong et al., 2012). This process is dependent on multiple factors, including the properties of the catalyst used, the medium in which the process occurs, the light source and oxygen concentration (Zhang et al., 2019). Regarding the use of natural sunlight in photocatalysis, this has been widely used for the treatment of wastewater effluents, where it presents itself as a low cost, sustainable, efficient option for the removal of environmental pollutants (Bernabeu et al., 2011; Beydoun et al., 1999; Gutierrez-Mata et al., 2017). Photocatalysis

using UV radiation has shown to be very effective for the treatment of textile wastewater, namely the degradation of organic and highly water-soluble dyes that would otherwise not be degraded by other wastewater treatment processes (Al-Mamun et al., 2019). More specifically, the use of titanium dioxide (TiO_2) nanoparticles, irradiated by UV-light has been found to be very effective for the removal of these dyes. TiO_2 nanoparticles completely degraded the dye Acid Orange 7 after an irradiation time of 20 minutes under UV-light. Comparatively, photocatalysis with direct sunlight caused degradation efficiencies below 5% (Juang et al., 2010). Reactive Red 195 has been found to be completely degraded by photocatalysis, using TiO_2 as the catalyst irradiated by UV-light at 542 nm wavelength for 20 minutes in aqueous solution (Belessi et al., 2009).

More recently, nanophotocatalysis, consisting of the junction of nanotechnology and photocatalysis, has emerged as an updated photocatalytic strategy efficient towards more persistent pollutants. The use of nanoparticles (such as zinc oxide (ZnO) and TiO_2) as photocatalysts has shown to be effective on the removal of heavy metals, as well as organic pollutants such as dyes and pharmaceutical active compounds (Enesca & Andronic, 2020; Tahir et al., 2019). Fluorinated antibiotic ciprofloxacin is degraded by the action of Au/TiO_2 nanoparticles, with the highest recorded degradation efficiency of ≈90%, however the highest nanoparticles concentration of 1.3 gL^{-1} caused a decrease in efficiency, attributed to water turbidity (Martins et al., 2020). Ciprofloxacin and ofloxacin were also shown to be degraded with an efficiency of 73.6 and 71.5%, respectively, using NCQDs-BNiO catalysts after an irradiation period of 180 minutes, using a 400 W halogen lamp simulating sunlight (Mohammadi et al., 2023). The defluorination of PFOA has been tested using TiO_2 nanoparticles and a nanocomposite of TiO_2 and zeolite with efficiencies of 48.8 and 54.5% respectively, after a reaction period of 8 hours in UV light (Halder et al., 2024). These findings indicate that nanophotocatalysis is a promising prospect for the effective breakdown of fluorinated organic compounds from contaminated sites, which can possibly yield fluorinated sub-products that can be more easily defluorinated when coupled with the process of bacterial defluorination.

Aim of this thesis

Organofluorines are extremely persistent contaminants, due to the characteristics gifted by C-F bonds, posing unique obstacles and requiring degradation strategies tailored to improved defluorination performances. As already described, fluoride is an expected sub-product from biological defluorination, but it is also a known antibacterial agent, so prospective bacterial degraders must be tested not only on their ability to defluorinate, but

also on their sensitivity to fluoride stress resulting from the cleavage of C-F bonds. On the other hand, co-metabolism poses itself as a promising strategy to promote the defluorination yield of persistent organofluorines, but there is a lack of studies showing how strategic co-substrates can facilitate bacterial defluorination. Finally, for extreme cases of environmental persistence, namely when concerning highly fluorinated PFAS, additional measures are likely to be required to streamline their productive degradation, namely the combination of different remediation strategies.

In light of this, the work presented in this dissertation has the following objectives:

1. To investigate the role of fluoride toxicity as a modulator for the catabolic defluorination process.
2. Exploring co-metabolism as a possible bioremediation strategy for the removal of the fluorinated recalcitrant compounds PFHxA and MFZ.
3. To combine the photocatalytic process with conventional bacterial remediation for the removal of TRF and identify optimization possibilities.

Materials and Methods

1. Tested strains

In this work, two bacterial strains were selected based on their ability to defluorinate fluorinated substrates: *Labrys portucalensis* F11, a fluoroaromatics-degrading strain originally isolated from an industrially contaminated sediment in northern Portugal (Carvalho et al., 2008; Carvalho et al., 2005a) and *Delftia acidovorans* MFA5, a bacterial strain known to be able to effectively degrade aliphatic fluorinated compounds, such as monofluoroacetate (Alexandrino et al., 2018). Whenever mentioned, the *Escherichia coli* strain Seattle 1946 (ATCC® 25922) was used as a non-defluorinating control. All strains were routinely maintained in the lab in Plate-Count Agar (PCA) at room temperature and routinely streaked onto fresh agar plates every 2 weeks.

2. Fluoride Sensitivity Assays

In these assays, the sensitivity of the strains *E. coli* Seattle 1946, *D. acidovorans* MFA5 and *L. portucalensis* F11 to different fluoride concentrations (0 to 0.6 mM NaF) was tested in oligotrophic [Minimal Salts Medium (MSM)] and mesotrophic conditions [Nutrient Broth

(NB)], using bacterial growth against the control condition (0 mM NaF) as an indicator of inhibition.

Fluoride sensitivity assays were conducted in Nutrient Broth and Minimal Salts Medium supplied with 1 gL^{-1} of sodium acetate, for the concentrations of 0, 0.01, 0.05, 0.1, 0.2, 0.4 and 0.6 mM of sodium fluoride (NaF), which served as a source of fluoride in these assays. For each assay, NaF concentrations were prepared to their final concentrations in 10 mL of the respective culture media in sterile Falcon tubes. In parallel, separate inocula of *L. portucalensis* F11*, D. acidivorans* MFA5 and *E. coli* Seattle 1946 were created by resuspending their biomass in 2 mL of sterile culture media from PCA plates grown for up to 4 days at 28 ºC. Bacterial strains were then individually inoculated in the culture media supplemented with different NaF concentrations to an optical density at 600 nm (OD_{600}) of 0.1. Finally, 0.2 mL of each strain/NaF combination was transferred to a sterile 96-well plate in triplicates and allowed to incubate for 48 hours at 28 ºC.

Bacterial growth was determined by measuring the OD_{600} at 0 and 48 hours, using a 96-well plate reader and the impact of fluoride on the tested strains was estimated by comparing their growth at different NaF concentrations with that of the control condition (0 mM NaF). To experimentally confirm the tested NaF concentrations in each condition, as well as to rule out any potential mechanism of fluoride removal by the tested strains, the remaining solutions of each strain/NaF combination were incubated at 28ºC for 48 hours in a rotary shaker (130 rpm) after which their fluoride contents were analysed by potentiometry.

To test for the possibility of the weak acid accumulation effect affecting the tested bacterial strains, acidification of NB and MSM was done by addition of sulfuric acid to both media. Verification of the final pH of each medium was done using a pH sensitive electrode, with a pH of 5,25 and 5,40, for NB and MSM, respectively. Following the previously mentioned conditions, in this assay only the NaF concentrations of 0, 0.01, 0.1 and 0.6 were tested.

3. Defluorination assays under fluoride stress

Considering the fluoride sensitivity thresholds of *L. portucalensis* F11 and *D. acidovorans* MFA5 previously determined, the defluorination performance of these strains was evaluated under fluoride stress and compared to fluoride-free conditions. Defluorination assays were conducted in 30 mL glass serum vials containing 15 mL of MSM with either 0 (non-stressed condition) or 0.4 mM NaF (stressed condition), inoculated axenically with each bacterial strain to an OD_{600} of 0.1 (confirmed spectrophotometrically), and supplemented with 0.5 mM of their preferred fluorinated substrates: fluoroacetate (FA) for *D. acidovorans* MFA5

cultures and fluorobenzene (FB) for *L. portucalensis* F11 cultures. *E. coli* Seattle 1946 was used as the non-defluorinating control, being exposed to each fluorinated substrate separately. After their preparations, serum vials were sealed with a rubber septum and the cultures were incubated for two weeks at 28 ºC in a rotary shaker (130 rpm). OD_{600} values and fluoride concentrations were monitored at 0 hours and after 2 weeks of incubation by spectrophotometry and potentiometry, respectively.

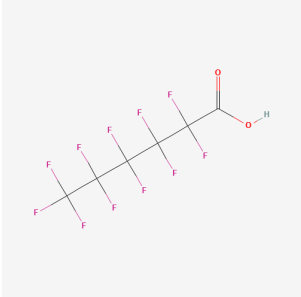
4. Co-metabolic defluorination of PFHxA and MFZ

L. portucalensis F11 and *D. acidovorans* MFA5 were tested on their ability to effectively defluorinate PFHxA and MFZ under structure-driven co-metabolic conditions. Prospective co-substrates were first selected from a list of different compounds with structural analogy to PFHxA, MZF or some of their functional groups (Table 1), based on the capacity of strains F11 and MFA5 to use them as growth substrates. To test this, *L. portucalensis* F11 and *D. acidovorans* MFA5 were inoculated into 15 mL of MSM (in 30 mL serum vials) individually supplemented with 100 mgL^{-1} of hexanoic acid, toluene or 1,2,4-triazole to a final OD_{600} of 0.1. Serum vials using 100mgL^{-1} of acetate as the sole carbon source were used as the control for this experiment. Triplicate cultures were established for each experimental condition and all cultures were incubated for up to 6 days at 28 ºC in a rotary shaker (130 rpm). Bacterial growth was used as the endpoint to determine co-substrate suitability, which was analysed by determining OD_{600} values after 48, 72 hours post-inoculation. For the case of hexanoic acid, the incubation period was extended, with an additional analysis of OD_{600} after 144 hours.

Table 1: Target compounds for co-metabolism assay with respective growth substrate candidates

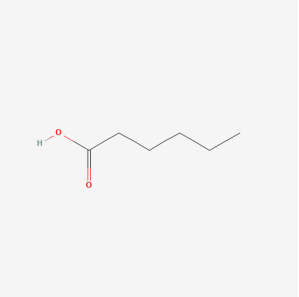
Target compound	Candidate co-substrate

PFHxA



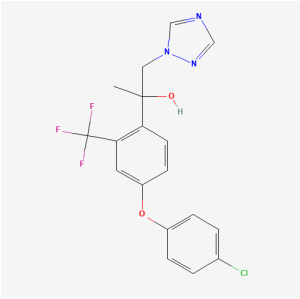
PubChem Identifier: ***CID 67542***
URL: https://pubchem.ncbi.nlm.nih.gov/compound/Perfluorohexanoic-acid#section=2D-Structure

Hexanoic Acid



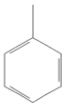
PubChem Identifier: ***CID 8892***
URL: https://pubchem.ncbi.nlm.nih.gov/compound/Caproic-Acid#section=2D-Structure

MFZ



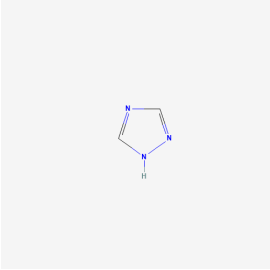
PubChem Identifier: ***CID 71230671***
URL: https://pubchem.ncbi.nlm.nih.gov/compound/Mefentrifluconazole#section=2D-Structure

Toluene



PubChem Identifier: ***CID 1140***
URL: https://pubchem.ncbi.nlm.nih.gov/compound/Toluene#section=2D-Structure

1,2,4-Triazole



PubChem Identifier: ***CID 9257***
URL: https://pubchem.ncbi.nlm.nih.gov/compound/1_2_4-Triazole#section=2D-Structure

After selecting the most suitable co-substrates, co-metabolic assays were established for PFHxA and MFZ in 30 mL serum vials with 15 mL of MSM supplemented with 100 mgL^{-1} of

hexanoic acid (for PFHxA) or toluene (for MFZ), 1 mgL^{-1} of each target pollutant and inoculated with *L. portucalensis* F11 or *D. acidovorans* MFA5 to an initial OD_{600} of 0.1. Control cultures were established in parallel, consisting of each strain inoculated in 15 mL of MSM supplemented with each co-substrate, but not with the target pollutants. In parallel, these strains were also cultivated in MSM with 1 mgL^{-1} of PFHxA or MFZ as sole carbon sources, to determine the efficacy of the co-metabolic process. All cultures were established in triplicate and incubated for 14 days at 28 ºC in a rotary shaker (130 rpm). During the incubation period, co-metabolism cultures were resupplied with 100 mgL^{-1} of each co-substrate weekly, except for the cultures *D. acidovorans* MFA5 supplemented with PFHxA and hexanoic acid which were re-supplied with hexanoic acid every 3 days. In addition, all cultures were force aerated with filter-sterilized atmospheric air, to ensure appropriate aerobic conditions. OD_{600} values of all cultures were monitored weekly in a spectrophotometer and defluorination of PFHxA and MFZ was estimated by measuring fluoride ion release after the 14-day incubation period by potentiometry.

5. Defluorination of TRF after a pre-treatment with nanophotocatalysis

A combination of nanophotocatalysis and bacterial degradation was tested to investigate if the overall defluorination of a persistent fungicide, TRF, could be significantly improved following this 2-step hybrid approach. For the nanophotocatalytic stage, 1 L of deionized water supplemented with 1 mgL^{-1} of TRF was combined with 1 gL^{-1} of ZnO nanoparticles, acting as nanocatalysts, and exposed to UV-A radiation for a period of 9 hours. Radiation exposure was conducted in a photoreactor with vertical irradiation at a distance of 14 cm by 8 lamps (Philips 8W) with a maximum wavelength of 365nm. This nanophotocatalytic treatment was conducted at the Electroactive Smart Materials laboratory from the Centre of Physics of University of Minho, in the scope of an ongoing research collaboration.

After this stage, pre-treated water samples were organized in five different batches (corresponding to each bacterial consortium and a non-inoculated control condition) of quadruplicate 30 mL serum vials consisting of 15 mL of each pre-treated water, which were then inoculated with their corresponding bacterial consortium. Each bacterial consortium was prepared by creating suspensions of biomass from each bacterial isolate (Table 2), obtained from 4-days old PCA plates, in 2 mL of sterile MSM. After the mixture, the OD_{600} of each suspension was determined and the appropriate calculations were performed to determine the inoculation volume of each batch of pre-treated water sample so that a OD_{600}

of 0.5 was obtained at the onset of the incubations. After this, quadruplicate vials of all pre-treated water batches were inoculated with the corresponding bacterial consortium and supplemented with a 10-fold concentrated solution of MSM salts, to ensure appropriate oligotrophic conditions for bacterial metabolism. The non-inoculate control condition solely consisted of pre-treated water and MSM salts. Defluorination was estimated via fluoride ion released, with samples taken before and immediately after nanophotocatalysis treatment, as well as after an incubation period of 21 days at 28ºC, in an orbital incubator (130 rpm).

Table 2: Composition of each bacterial consortia used in hybrid degradation assay

Consortium	Composition	References
1	*Labrys portucalensis* F11 *Delftia acidovorans* MFA5 *Hydrogenophaga eletricum* 3EE *Leadbeterella* sp. DPS8	(Carvalho et al., 2005a)) (Alexandrino et al., 2018)
2	*Hydrogenophaga eletricum* 3EE *Pseudomonas stutzeri* 5EE *Acinetobacter* sp. 7EE	Alexandrino et al. (2020)
3	*Pseudomonas xanthomarina* 1EF *Pseudomonas* sp. 2EF *Ochrobactrum anthropic* 3EF *Hydrogenophaga eletricum* 8EF	
4	*Comamonas testosteroni* MFA1 *Stenotrophomonas maltophili* MFA2 *Herbaspirillum frisingense* MFA4 *Pseudomonas* sp. MFA9	(Alexandrino et al., 2018)

Variovorax paradoxus MFA10
Chryseobacterium taeanense MFA25
Comamonas testosteroni MFA35

6. Analytical methods

Fluoride release was determined by potentiometry, by measurement of fluoride ion concentration using a fluoride-sensitive electrode (Crison 9655C, Crison Instruments, S.A., Barcelona, Spain. Calibration curves were constructed prior to samples analysis, using NaF standards (ranging from 0.001 to 1 mM) in MSM. Total ionic strength adjustment buffer (TISABIII) was added to the samples in a ratio of 1:10, to reduce possible interference during analysis.

Microbial growth was observed via optical density of culture samples, using a V-1200 spectrophotometer (VWR International, LLC, Pennsylvania, USA), at a wavelength of 600 nm.

Results and Discussion

Fluoride Sensitivity Assays

Under mesotrophic conditions, *D. acidovorans* MFA5 showed similar growth across all NaF concentrations tested to the one observed under fluoride free conditions, except for the 0.2 mM NaF concentration where the strain showed a positive ΔOD_{600} of 0.219 ± 0.234 (Fig 1A). Comparatively, *L. portucalensis* F11 presented a higher ΔOD_{600} than *D. acidovorans* MFA5 for the lowest NaF concentrations, with ΔOD_{600} values of 0.051 ± 0.023 and 0.052 ± 0.021 for 0.01 and 0.05 mM NaF, respectively (Fig. 1B) but increasing concentrations of NaF resulted in progressively higher bacterial growth inhibitions (Fig. 1B). *E. coli* Seattle 1946 showed higher growth inhibition for the concentrations of 0.1, 0.4 and 0.6 mM NaF, but for the remaining NaF concentrations tested (0.01, 0.05 and 0.2 mM NaF) the impacts on bacterial growth were less noticeable (Fig. 1C).

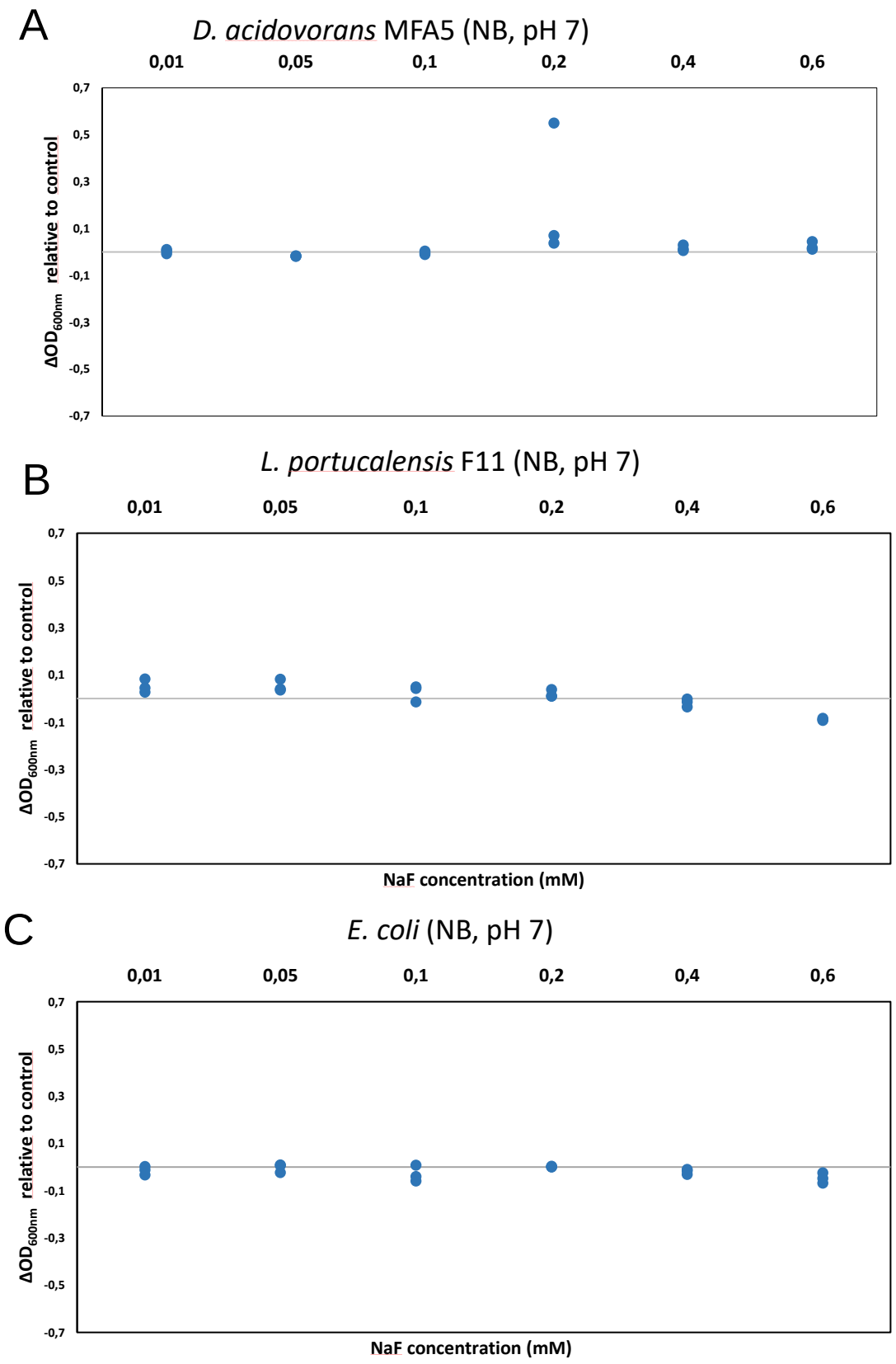


Fig 1: ΔOD_{600} values relative to control for *D. acidovorans* MFA5 (A), *L. portucalensis* F11 (B) and *E. coli* Seattle 1946 *(C)* in NB (pH 7) for the tested NaF concentrations.

In oligotrophic conditions, all strains showed a harsher growth inhibition as a result of fluoride exposure, compared to mesotrophic conditions, with D. *acidovorans* MFA5 and *L. portucalensis* F11 remaining the least and most sensitive strains to fluoride exposure, respectively. *D. acidovorans* MFA5 showed higher or similar growth in all concentrations tested than the control condition, expect for 0.2 and 0.4 mM NaF where negative ΔOD_{600} values were determined (Fig. 2A). As for *L. portucalensis* F11, growth yields higher than the control condition were never observed under these conditions, with clear growth inhibitions having been observed for the 0.01, 0.2 and 0.4 mM NaF concentrations (Fig. 2B). Similarly, *E. coli* Seattle 1946 showed sharper growth declines for the 0.1, 0.2 and 0.4 mM NaF concentrations under these conditions, with the remaining NaF concentrations not resulting in clear differences in growth against the control condition (Fig. 2C).

Based on these results, nutrient availability clearly played a large role in the cellular tolerance to fluoride, as all strains saw more severe cases of growth inhibition when exposed to fluoride under oligotrophic conditions compared to mesotrophic media (Figs 1, and 2). Fluoride compounds, including NaF (van Loveren et al., 2008), are known inhibitors of the enzyme enolase (Krucinska et al., 2019), a metalloenzyme that participates in the glycolysis, responsible for the conversion of 2-phosphoglycerate to phosphoenolpyruvate, cell wall biosynthesis, for cell division, and RNA processing in bacteria (Krucinska et al., 2019). Fluoride has been found to be a “quasi-irreversible” inhibitor of enolase in permeabilized cells of *Streptococcus mutans* GS-5, however high levels of either the metabolite phosphoenolpyruvate or the glycolysis substrate 2-phosphoglycerate were responsible for the re-activation of enolase (Curran et al., 1994). Considering the observed results, it is possible that the abundance of substrate to act as a carbon source in mesotrophic media alleviated the inhibitory effects of fluoride on enolase, allowing for glycolysis, as well as cell wall biosynthesis for cell replication. Surprisingly, there were instances where *E. coli* Seattle 1946 revealed higher tolerance to fluoride than the defluorinating strains. Given that the genome of the *E. coli* strain used in this study is completely mapped, it was possible to confirm the presence of gene operons coding for the highly specific Fluc fluoride channels. Fluc channels belong to the crcB family of transporters and export fluoride ions from the intracellular media to the extracellular one as a specific resistance mechanism for known intracellular fluoride ion toxicity (Baker et al., 2012; Barbier et al., 2010). As such, their genomic presence likely contributed to the tolerance demonstrated by *E. coli* Seattle 1946.

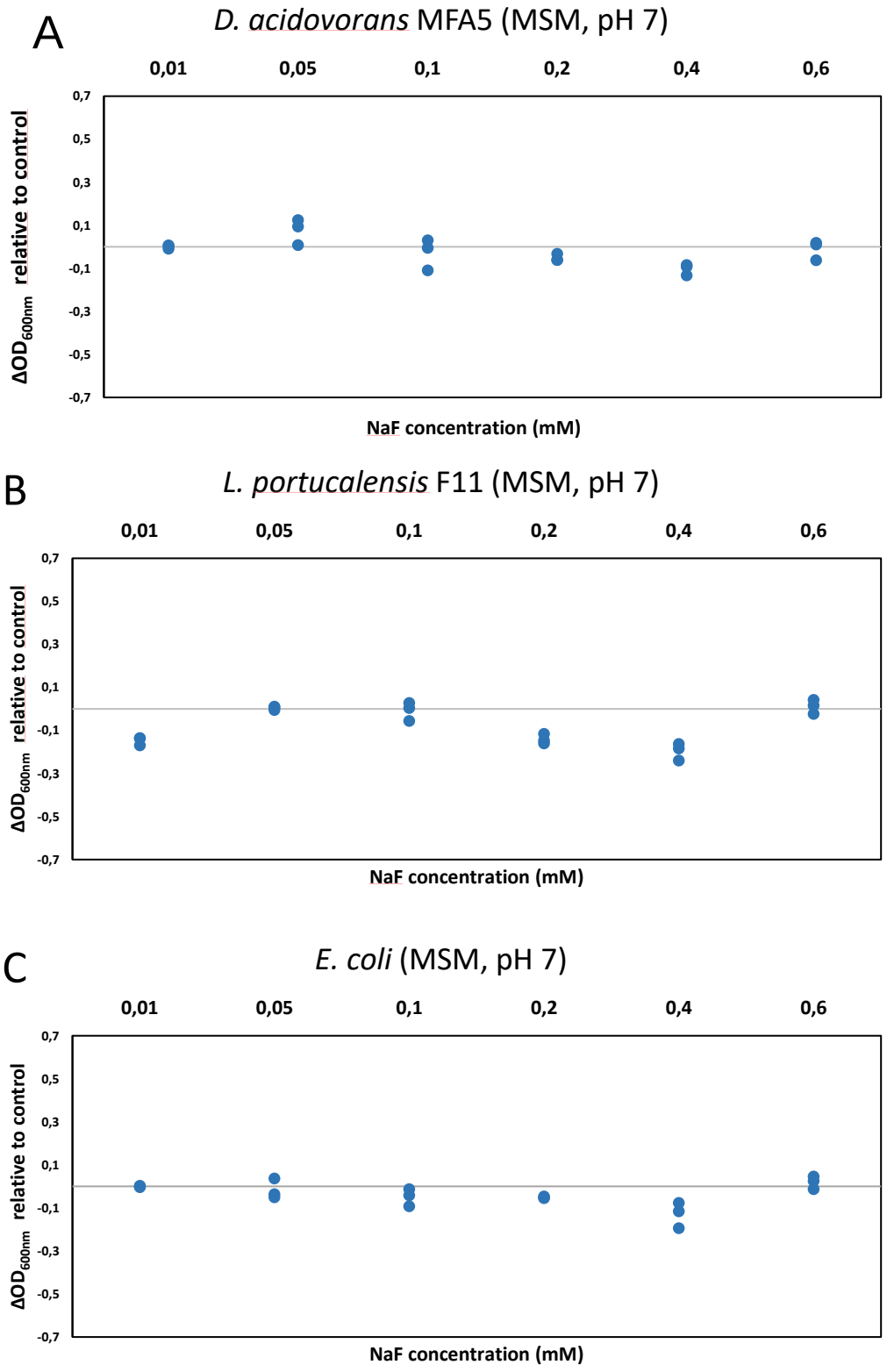


Fig 2: ΔOD_{600} values relative to control for *D. acidovorans* MFA5 (A), *L. portucalensis* F11 (B) and *E. coli* Seattle 1946 *(C)* in MSM (pH 7) for the tested NaF concentrations.

Another critical factor in regulating bacterial homeostasis to intracellular fluoride is pH, particularly related with what is known as the weak acid accumulation effect, responsible for an increased fluoride toxicity in bacteria when exposed at a slightly acidic pH due to conversion of the fluoride anion into hydrofluoric acid (Breaker, 2012; Ji et al., 2014; Wilks & Slonczewski, 2007). As such, a new assay was conducted to observe the correlated impacts of slightly acidic mesotrophic and oligotrophic media in fluoride tolerance of the tested strains.

In mesotrophic conditions at pH~5, *E. coli* Seattle 1946 showed slightly positive ΔOD_{600} of 0.032 ± 0.025 at 0.01 mM NaF, but growth differences were not observed for the remaining NaF concentrations (Fig. 3C). At physiological pH (pH~7) and in the same medium, *E. coli* Seattle 1956 showed higher bacterial growth when exposed to 0.01 mM NaF but relatively similar growth patterns than the control conditions for the remaining concentrations tested. *D. acidovorans* MFA5 showed positive ΔOD_{600} values for the concentrations of 0.01 and 0.6 mM NaF, but similar growth than the control condition for the intermediate NaF concentrations tested. As for *L. portucalensis* F11, the strain saw increase in density for concentrations 0.01 and 0.1, corresponding to ΔOD_{600} of 0.037 ± 0.035 and 0.024 ± 0.005 respectively, but for 0.6 mM NaF no differences were observed compared to the control condition (Fig 3B). When compared to the mesotrophic conditions at physiological pH, both these strains were shown to either be unaffected by the difference in pH or to present higher OD_{600} values. This is further confirmed as *D. acidovorans* MFA5 presented the two highest OD_{600} values at slightly acidic conditions, corresponding to 0.230 ± 0.294 and 0.175 ± 0.043 for 0.01 and 0.6 mM NaF, respectively. *L. portucalensis* F11 presented OD_{600} values closer to the fluoride free control, most notable when comparing 0.6 mM conditions in both pH cases. At a pH~7 ΔOD_{600} was valued at -0.089 ± 0.004, however for the same NaF concentration at a pH~5 growth was more similar to the control conditions, with a value of -0.020 ± 0.026.

In oligotrophic conditions at pH~5, *E. coli* Seattle 1946 showed progressively higher growth inhibitions with increasing NaF concentrations (Fig. 4C), clearly contrasting the results observed for the same media at physiological pH. On the other hand, *D. acidovorans* MFA5 showed growth dynamics similar to the control condition across all NaF concentrations tested, showing once again to be the more robust to fluoride stress (Fig. 4A). *L. portucalensis* F11 only revealed different growth dynamics at 0.6 mM NaF, with a negative ΔOD_{600} of -0.175 ± 0.043, while showing similar growth yields for the other NaF concentrations tested compared to the control condition (Fig. 4B).

The pH~5 results showed different behaviour to the initial results. *E. coli* Seattle 1946 was the most sensitive to fluoride specifically under the acidic conditions tested, however using all the data collected, *L. portucalensis* F11 was the most sensitive across all conditions tested. Contrastingly, *D. acidovorans* MFA5 was consistently the most tolerant strain to fluoride in all tested conditions. Nutrient availability was a large factor in the response to fluoride stress, as bacterial strains were more severely affected when exposed under oligotrophic conditions. This was particularly noticeable for *E. coli* Seattle 1946 and *L. portucalensis* F11, which showed sharper growth declines in oligotrophic media. Furthermore, results were mostly consistent when comparing the same NaF concentrations at different pH levels, with the clearest difference between pH levels for *L. portucalensis* F11 in oligotrophic media with a NaF concentration of 0.01 mM. Other results indicated a similar trend in the bacterial sensitivity to fluoride.

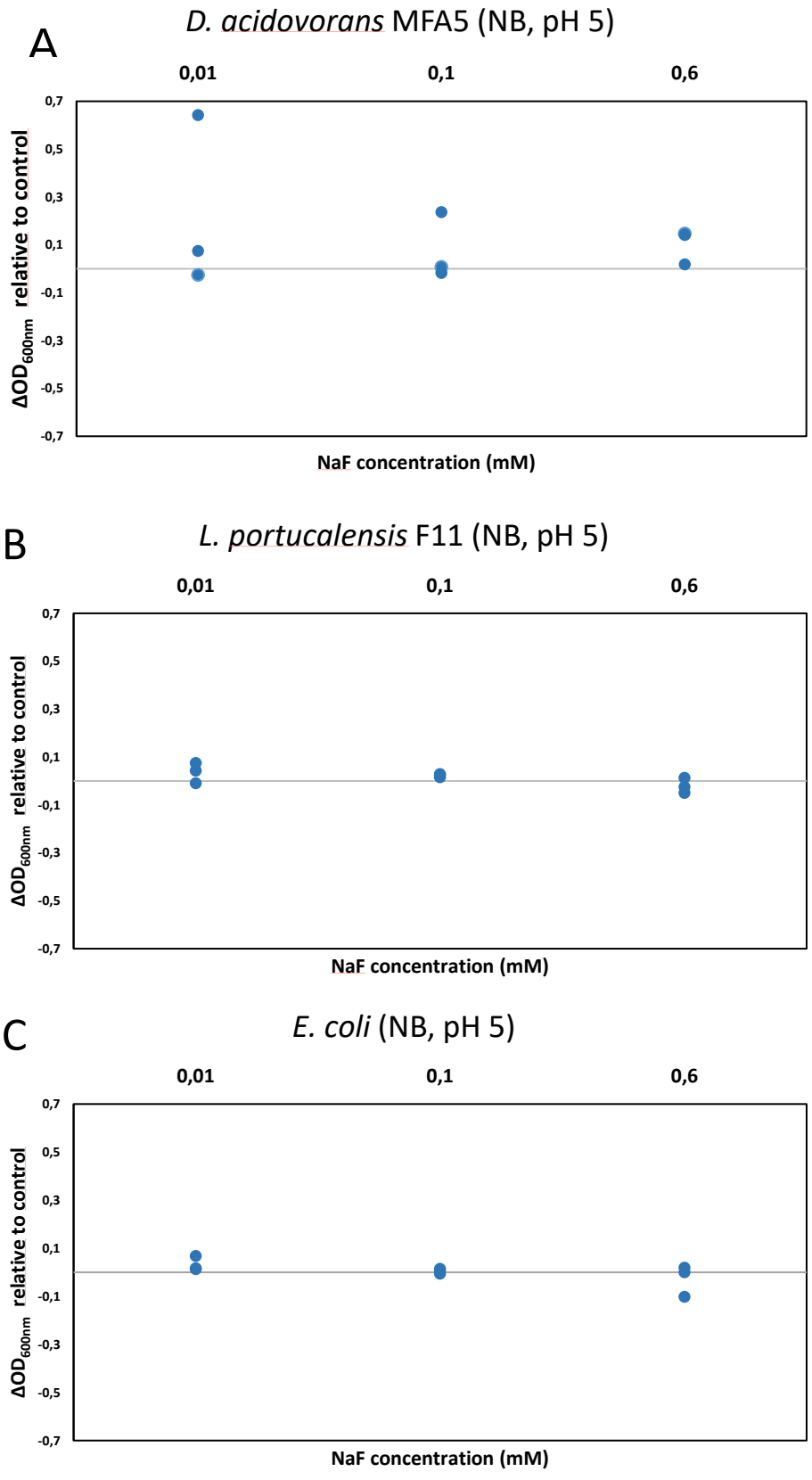


Fig 3: ΔOD_{600} values relative to control for *D. acidovorans* MFA5 (A), *L. portucalensis* F11 (B) and *E. coli* Seattle 1946 *(C)* in NB (pH 5) for the tested NaF concentrations.

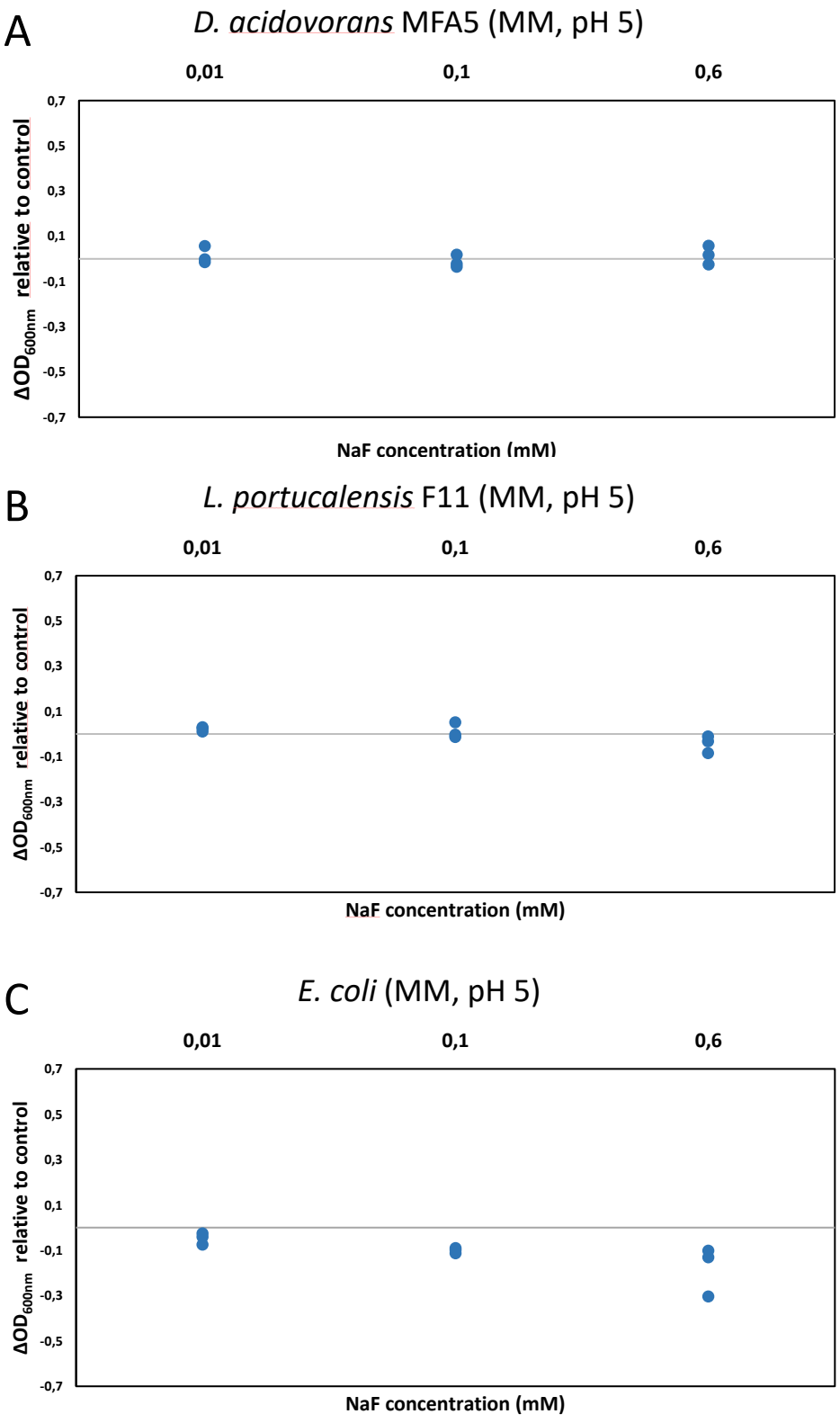


Fig 4: ΔOD_{600} values relative to control for *D. acidovorans* MFA5 (A), *L. portucalensis* F11 (B) and *E. coli* Seattle 1946 *(C)* in MSM (pH 5) for the tested NaF concentrations.

Defluorination under fluoride stress assay

In this assay, the ability of *D. acidovorans* MFA5 and *L. portucalensis* F11 to defluorinate their preferred fluorinated substrates (FA and FB, respectively) under fluoride stress was tested. To induce fluoride stress, the concentration of 0.4 mM NaF was selected, as previous assays showed this concentration to be the most impactful for bacterial growth across all strains and conditions tested. Defluorination under fluoride stress was analysed by measuring the defluorination efficiency of the fluorinated substrates at 0.4 mM NaF, which were then compared to the defluorination efficiencies obtained at fluoride-free conditions. *E. coli* Seattle 1946 was also used as a non-defluorinating control.

In the absence of fluoride, *D. acidovorans* MFA5 and *L. portucalensis* F11 were able to effectively defluorinate 0.5 mM FA or FB after two weeks, as expected (Fig 5). However, when co-supplemented with 0.4 mM NaF, strains *D. acidovorans* MFA5 and *L. portucalensis* F11 only yielded a defluorination efficiency of 62.3 ± 1.34% and 7.6 ± 1.16% (Fig. 5), respectively, indicating that exposure to 0.4 mM of NaF negatively affected the catabolic defluorination process in both test strains. The non-defluorinating bacterial strain *E. coli* Seattle 1946 showed no defluorination activity in any tested condition.

Concerning bacterial growth, all test strains showed a consistent decrease in OD_{600} after the incubation period of 2 weeks, regardless of the defluorination efficiencies demonstrated. Values for the growth of all the bacterial strains tested are presented in Table 3. *L. portucalensis* F11 saw the largest decline in OD_{600} while using fluorobenzene as a sole carbon source under fluoride-free conditions, with an initial value of 0.091 that lowered to 0.054 after the two-week incubation period. A similar growth development was observed for the fluoride stress conditions, where OD_{600} of strain F11 decreased from an initial density value of 0.101 to 0.063 (Table 3). *D. acidovorans* MFA5 also showed decreased OD_{600} after the incubation in both conditions tested, despite the recorded degradation efficiencies. While the collected OD_{600} data does not allow a robust inference of the growth dynamics for both defluorinating strains, it is possible that their negative growth fluctuations were due to the limited availability of a suitable energy and growth-supporting substrate. Under fluoride free conditions, both *L. portucalensis* F11 and *D. acidovorans* MFA5 consumed the available fluorinated substrates before the end of the incubation period, which likely led to an abrupt decrease on cellular density after that threshold. Under fluoride stress, growth was likely limited by a combination of NaF toxicity and due to deficient catabolic utilization of the supplemented substrates. Indeed, fluoride is known to interfere with different enzymes, such as phosphatases or enolases, mostly by inhibiting their catalytic function

when present in concentrations at the mM level (Barbier et al., 2010). In some cases, these enzymes are known to be involved in defluorination pathways, as is the case of ATPases, which also inhibits this important catabolic process (Wackett Lawrence, 2021). In this experiment, strains were exposed to 0.4 mM of fluoride (as NaF), the concentration previously established to most severely affect their growth in either oligotrophic or mesotrophic conditions, which may have inhibited the function of the enzymes involved in both defluorinating and central metabolic pathways in *L. portucalensis* F11 and *D. acidovorans* MFA5. While multiple defluorinating enzymes are currently known (Alexandrino, Mucha, et al., 2022; Bygd Madison et al., 2021; Yu et al., 2020), the metabolic process by which test strain *D. acidovorans* MFA5 is able to effectively defluorinate its respective substrates is not yet known, so a genomic/proteomic based study would be advantageous to elucidate the defluorination pathways for *D. acidovorans* MFA5 and to identify potential targets of inhibition caused by fluoride stress. *L. portucalensis* F11 performs the defluorination process of FB by the partial conversion of FB to 4-fluorocathecol and catechol, followed by the degradation of these metabolites using the *ortho*-cleavage pathway enzymes catechol 1,2-dioxygenase, muconase cycloisomerase, maleylacetate reductase and 3-oxoadipate:succinyl-coenzyme A transferase (Carvalho Maria et al., 2006). Furthermore, this process has been seen to be non-specific, with strain *L. portucalensis* F11 being able to metabolize other fluorinated aromatic compounds, namely 1,3- and 1,4-difluorobenzene (Moreira et al., 2012b). Previously, *L. portucalensis* F11 has shown to be negatively affected by FB concentrations above 0.4 mM, with severity increasing with concentration (Carvalho et al., 2005b). In this assay total fluoride concentration (FB + NaF) was calculated to be 0.9 mM, consistent with what was previously reported, and it is possible that fluoride exposure at such inhibitory levels negatively affected the functioning of enzymes responsible for the metabolism of fluorinated compounds present in this strain.

Likewise for the non-defluorinating control, the OD_{600} decrease observed for *E. coli* Seattle 1946 was also likely due to a combination of NaF toxicity and limited availability of a suitable growth substrate, as the strain was uncapable of degrading FA or FB. Still, *E. coli* Seattle 1946 showed milder reductions of OD_{600} values than *L. portucalensis* F11, despite not having a compatible growth and energy substrate, which is indicative of strain F11's large sensitivity to fluoride stress and the capacity of this *E. coli* strain to endure intracellular fluoride accumulation, despite not being a defluorinating strain.

Table 3: OD_{600} and ΔOD_{600} values for the tested strains during the incubation period of 14 days with FB or FA under fluoride-free and fluoride stress conditions.

Condition	**Bacterial strain**	**Initial OD_{600}**	**Final OD_{600}**
Substrate: Fluorobenzene			
No fluoride stress	*E. coli* Seattle 1946	0.070	0.048
	L. portucalensis F11	0.091	0.054
With fluoride stress	*E. coli* Seattle 1946	0.121	0.088
	L. portucalensis F11	0.101	0.063
Substrate: Fluoroacetate			
No fluoride stress	*E. coli* Seattle 1946	0.100	0.071
	D. acidovorans MFA5	0.092	0.079
With fluoride stress	*E. coli* Seattle 1946	0.105	0.074
	D. acidovorans MFA5	0.094	0.075

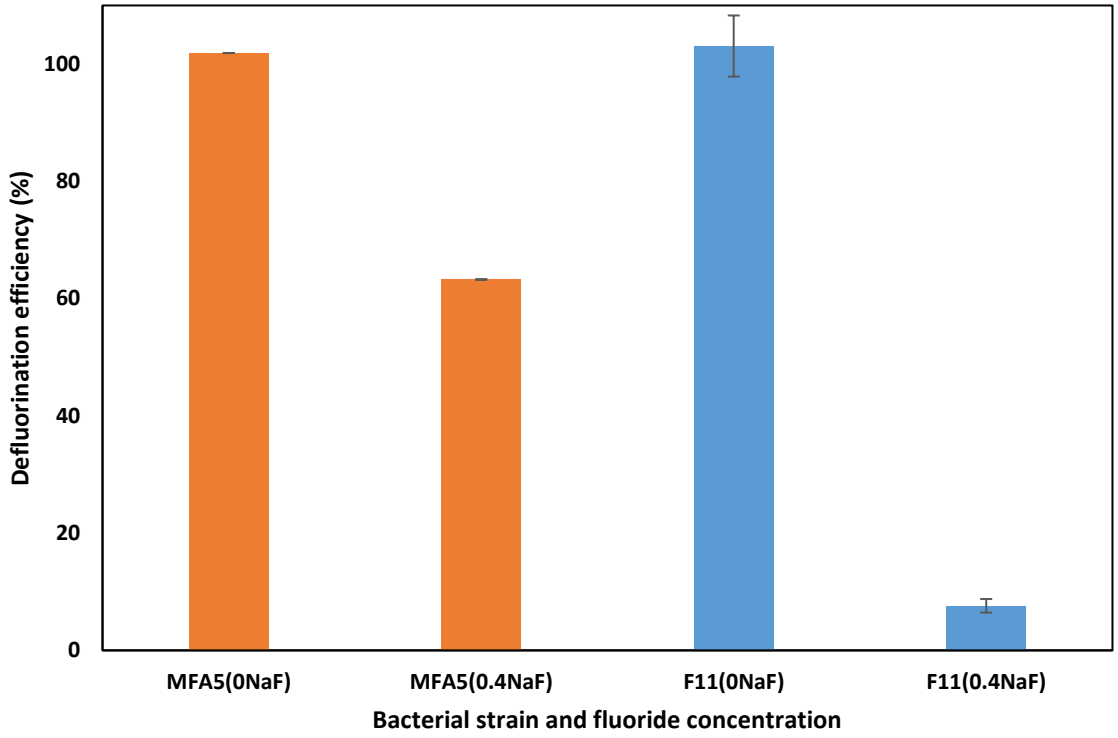


Fig 5: Average defluorination performances of *D. acidovorans* MFA5 and *L. portucalensis* F11 using their preferred substrates FA and FB, respectively, under fluoride free and fluoride stress conditions (with 0.4 mM NaF). Error bars indicate standard deviation of three replicates.

Co-metabolic defluorination of PFHxA and MFZ

In order to evaluate the co-metabolic defluorination of PFHxA and MFZ, there was the need to first select the most suitable co-substrates for *D. acidovorans* MFA5 and *L. portucalensis* F11, among a pre-selection of different compounds with structural analogy to the target pollutants, based on the capacity of the strains using them as growth substrates.

Hexanoic acid, a potential co-metabolite for the degradation of PFHxA, was found to be an effective growth substrate for *D. acidovorans* MFA5, as OD_{600} values were consistently higher than the acetate control along the screening period (Fig. 6). After 48 hours of incubation, strain MFA5 doubled its cellular density when supplemented with this substrate, reaching almost double the OD_{600} observed in the acetate control. Cellular densities of *D. acidovorans* MFA5 remained stable until 72 hours of incubation after which they started to decline (Fig. 6), indicating the complete consumption of both substrates. In the case of *L. portucalensis* F11 using hexanoic acid as growth substrate, a consistent increase in OD_{600} was observed during the whole incubation period, peaking at 0.198 ± 0.009 after 6 days of incubation, suggesting that this strain had a slower consumption rate of hexanoic acid than that observed for *D. acidovorans* MFA5 (Fig. 7). Another difference from *D. acidovorans*

MFA5 was that, when compared to acetate controls, growth of *L. portucalensis* F11 was consistently lower until the 6th day of incubation (Fig. 7), when OD_{600} values exceed those seen in acetate controls with values of 0.198 ± 0.009 and 0.187 ± 0.048, respectively. Nevertheless, given that hexanoic acid could sustain the growth of strains MFA5 and F11, although with different rates, this substrate was validated for further use in the co-metabolic assays with PFHxA.

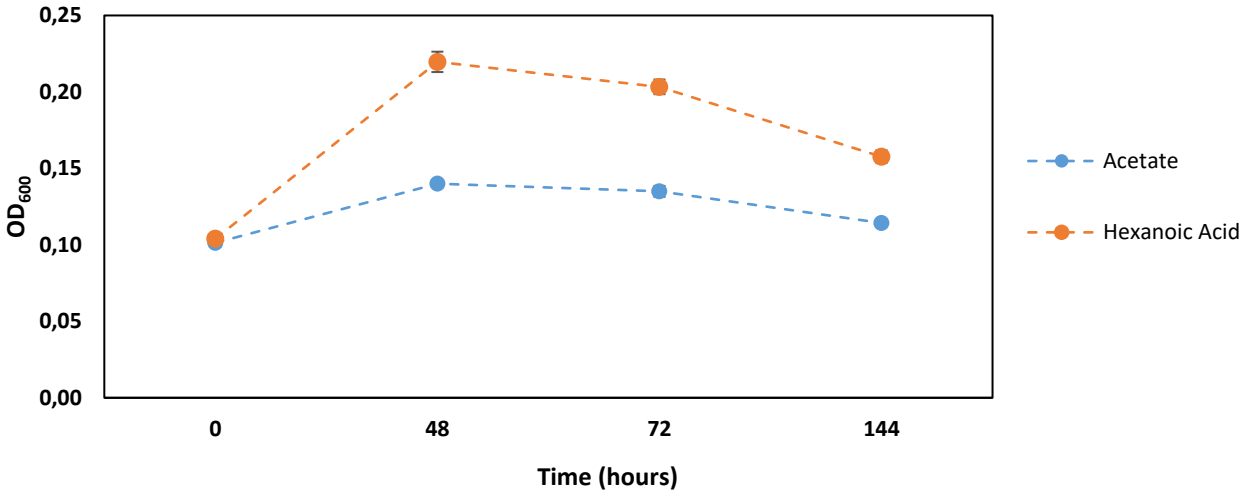


Fig 6: Growth of *D. acidovorans* MFA5 (expressed as OD_{600} values) in 100 mgL^{-1} of hexanoic acid or acetate supplemented as sole carbon sources, over a period of 6 days. Errors bars express standard deviation and lines connecting the data points are just for guiding purposes.

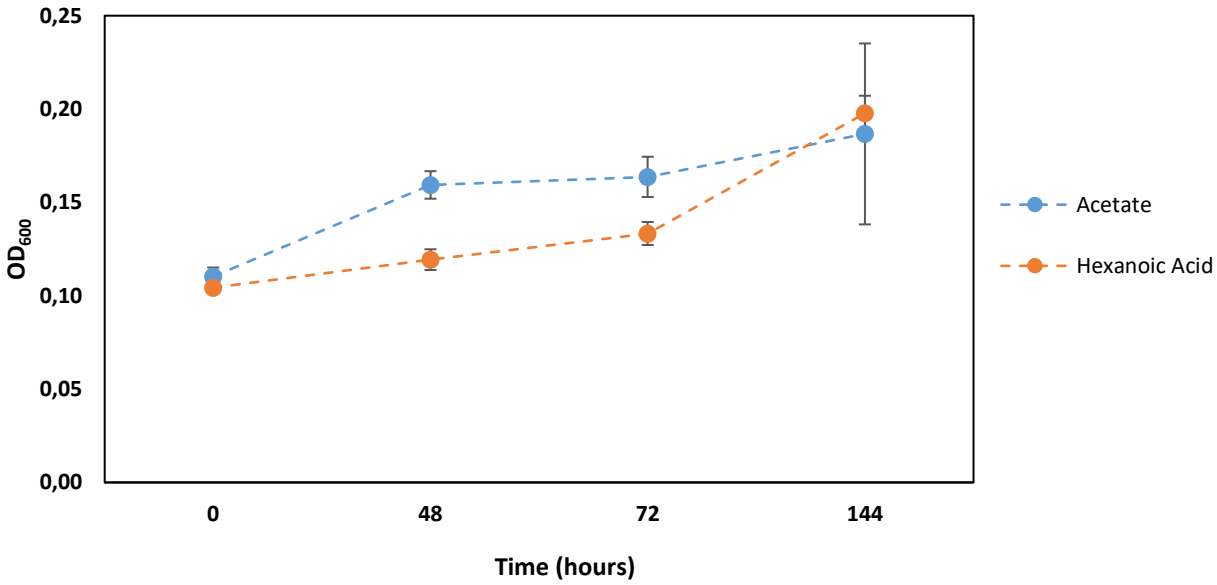


Fig 7: Growth of *L. portucalensis* F11 (expressed as OD_{600} values) in 100 mgL^{-1} hexanoic acid or acetate supplemented as sole carbon sources, over a period of 6 days. Errors bars express standard deviation and lines connecting the data points are just for guiding purposes.

As for toluene and 1,2,4-triazole, potential co-metabolites for the degradation of MFZ, growth assays using these substrates were conducted simultaneously for strains MFA5 and F11, while using acetate as control. *D. acidovorans* MFA5 and *L. portucalensis* F11 showed similar growth dynamics on both these substrates, consistently exhibiting higher OD_{600} values in the acetate controls in relation to toluene or 1,2,4-triazole, for which stable OD_{600} values were observed throughout the 72-hours screening period (Fig. 8; 9). Considering these results, both toluene and 1,2,4-triazole cannot support productive growth of *D. acidovorans* MFA5 and *L. portucalensis* F11. These results indicate that both substrates wouldn't be apt for an energy driven co-metabolic strategy. Structure driven co-metabolism calls for structural similarities of the co-substrate with the target compound (Kiel & Engesser, 2015). Due to this, one of these co-substrates was still selected for this work, to test the possible activation of the catalytic defluorination process. While toluene and 1,2,4-triazole share structural similarities to different functional groups of MFZ, ultimately toluene was selected for the co-metabolic defluorination of MFZ in this study due to being the non-fluorinated equivalent of the fluorinated aromatic ring in MFZ, increasing the likelihood of this co-substrate leading to the enzymatic activation of defluorinating pathways for MFZ (Table 1).

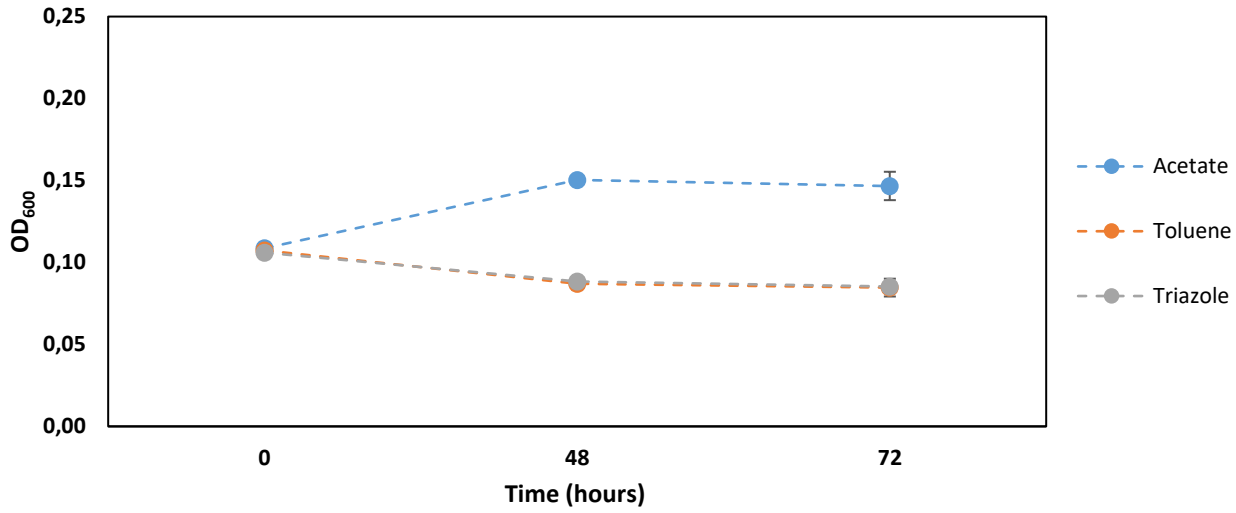


Fig 8: Growth of *D. acidovorans* MFA5 (expressed as OD_{600} values) in 100 mgL^{-1} of toluene, triazole or acetate supplemented as sole carbon sources, over a period of 6 days. Errors bars express standard deviation and lines connecting the data points are just for guiding purposes.

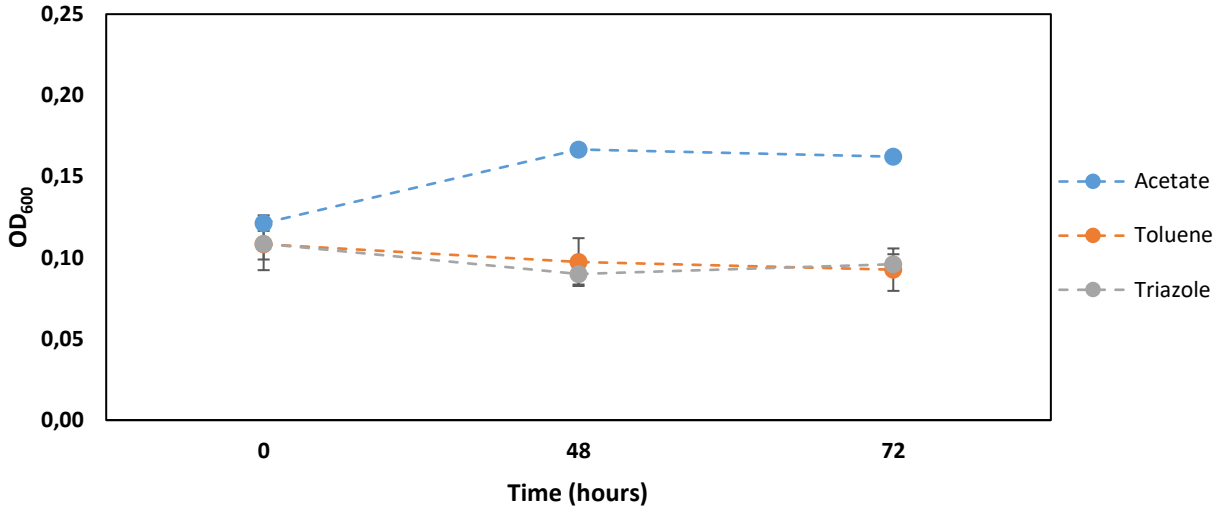


Fig 9: Growth of *L. portucalensis* F11 (expressed as OD_{600} values) in 100 mgL^{-1} of toluene, triazole or acetate supplemented as sole carbon sources, over a period of 6 days. Errors bars express standard deviation and lines connecting the data points are just for guiding purposes.

Using all the obtained data, the experimental approach and the co-substrate/strain combinations adopted were the ones summarized in Table 4.

Table 4: Frequency of supplementation of each co-substrate, depending on the target fluorinated pollutant and degrading bacterial strain

Condition	**Bacterial strain**	**Frequency of supplementation (days)**
Substrate: Perfluorohexanoic acid		
Co-substrate: Hexanoic acid	*D. acidovorans* MFA5	3
	L. portucalensis F11	7
Substrate: Mefentrifluconazole		
Co-substrate: Toluene	*D. acidovorans* MFA5	7
	L. portucalensis F11	7

To better assess the impact of the co-metabolic strategy laid out in Table 4 on the defluorination of PFHxA or MFZ, degradation assays where the target pollutants were the sole carbon sources were conducted in parallel, to elucidate the defluorination efficiency of the test strains under non-cometabolic conditions. *D. acidovorans* MFA5 and *L. portucalensis* F11 were unable to defluorinate the target pollutants as sole carbon sources, as fluoride concentrations determined in the culture media after the incubation period were below the limit of detection (Table 5). However, neither strain was able to co-metabolically defluorinate MFZ or PFHxA as well, as substantial fluoride release was not observed in either combination of target compound and co-substrate (Table 5). As such, comparing these results to the ones obtained with PFHxA or MFZ acting as the sole carbon source for both tested bacterial strains, it is clear that hexanoic acid and toluene do not serve as effective co-substrates for the biotransformation of these fluorinated pollutants. The different toluene degradation pathways all begin with an initial oxidation of the substrate, catalysed by multiple enzymes. For instance, *Burkholderia cepacia* G4 utilizes toluene 2-monooxygenase, oxidizing the aromatic ring forming o-cresol as a product that is then oxidized to form 3-methylcatechol (Shields et al., 1989), while *Pseudomonas putida* F1 uses toluene dioxygenase to produce cis-1-2dihydrotoluene-1,2-diol from toluene, which is then converted into 3-methylcatechol and further degraded by meta ring fission (Jindrová et al., 2002; Parales Rebecca et al., 2000). The biodegradation of toluene by bacterial strain *Pseudomonas putida* PaW15 primarily targets the methyl group present in toluene, leading to the production of benzoate which is then converted to catechol and assimilated into central metabolism via the meta cleavage pathway (Williams Peter & Murray, 1974). As for hexanoic acid, this aliphatic molecule is degraded via the universal β-Oxidation pathway for fatty acids (Yuan et al., 2022), where acetyl-CoA is terminally produced to trigger the tricarboxylic acid cycle (Usman et al., 2022). As neither of the tested co-substrates were able to trigger the defluorination process in *D. acidovorans* MFA5 and *L. portucalensis* F11, it is possible that the previously described enzymatic pathways are not directly involved in the defluorination of MFZ and PFHxA.

Table 5: Combinations of target compound and growth substrate and the defluorination efficiencies for each inoculate of Delftia acidovorans MFA5 and Labrys portucalensis F11 for each combination tested.

Condition	**Bacterial strain**	Defluorination efficiency (%)
Substrate: Perfluorohexanoic acid		
No growth substrate	*D. acidovorans* MFA5	0
	L. portucalensis F11	0.026 ± 0.03
Growth substrate: Hexanoic acid	*D. acidovorans* MFA5	0
	L. portucalensis F11	0
Substrate: Mefentrifluconazole		
No growth substrate	*D. acidovorans* MFA5	0.01 ± 0.009
	L. portucalensis F11	0.025 ± 0.036
Growth substrate: Toluene	*D. acidovorans* MFA5	0
	L. portucalensis F11	0

OD_{600} results obtained during the co-metabolism assays were much higher than those obtained during the selection process for the growth substrates. After a period of 7 days, *D. acidovorans* MFA5 and *L. portucalensis* F11 reached OD_{600} values of 0.307 ± 0.01 and 0.312 ± 0.046, respectively, when fed with PFHxA and hexanoic acid, and after 14 days of incubation cellular densities reached 0.485 ± 0.021 and 0.460 ± 0.018, respectively (Fig 10). Comparatively, after a total of 6 days of incubation with a single supplementation of hexanoic acid as the sole carbon source, *D. acidovorans* MFA5 and *L. portucalensis* F11 had only reached OD_{600} of 0.158 ± 0.004 and 0.198 ± 0.009 respectively (Fig 6;7). Similar results were seen in the conditions of MFZ with toluene as a co-substrate. After a period of 7 days, *D. acidovorans* MFA5 and *L. portucalensis* F11 reached OD_{600} values of 0.259 ± 0.048 and 0.383 ± 0.160, respectively, which decreased to 0.073 ± 0.004 and 0.097 ± 0.013

after 14 days of incubation (Fig 11). During the selection of the co-substrate for MFZ, the cellular densities of toluene-fed cultures reached a maximum of 0.085 ± 0.005 and 0.093 ± 0.01 for *D. acidovorans* MFA5 and *L. portucalensis* F11, respectively, after 3 days of incubation (Figs. 8 and 9). This difference in OD_{600} between the selection of growth substrates and the co-metabolic defluorination assays is likely due to the consistent supplementation of a productive co-substrate to the culture medium, compared to the single dosage used at the beginning of the co-substrate selection process. However, the possible non-defluorinating degradation of the target compounds PFHxA and MFZ by the tested bacterial strains, due to possible promotion of non-defluorinating catabolic pathways caused by the growth substrates cannot be excluded.

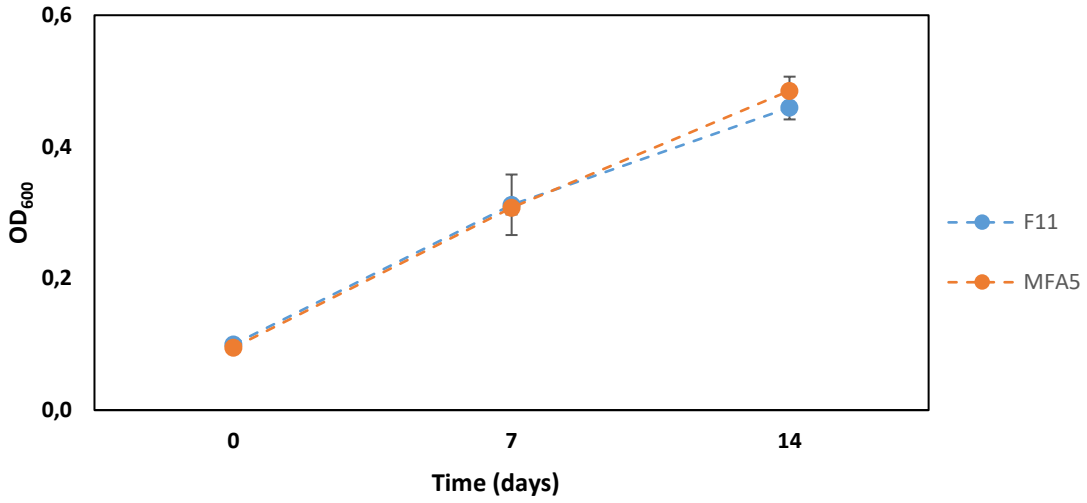


Fig. 10: Average OD_{600} for *L. portucalensis* F11 (blue) and *D. acidovorans* MFA5 (Orange) in MSM with 1 mgL^{-1} of PFHxA and 100 mgL-1 of Hexanoic Acid for an incubation period of 14 days. Error bars express standard deviation, and lines connecting the data points are just for guiding purposes.

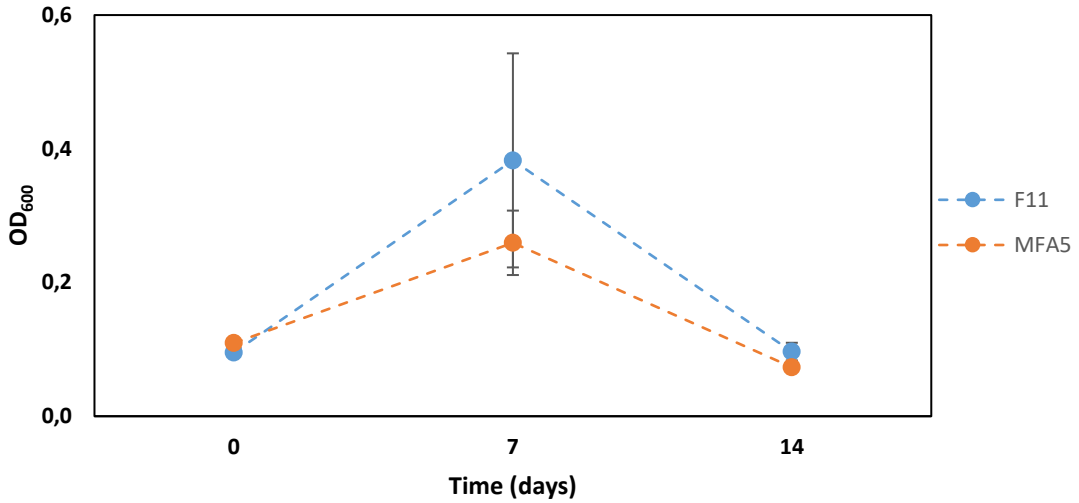


Fig. 11: Average OD_{600} for *L. portucalensis* F11 (blue) and *D. acidovorans* MFA5 (Orange) in MSM with 1 mgL^{-1} of MFZ and 100 mgL^{-1} of toluene for an incubation period of 14 days. Error bars express standard deviation, and lines connecting the data points are just for guiding purposes.

Defluorination of TRF by a nanophotocatalysis-biodegradation hydrid process

Biodegradation and defluorination of TRF, a persistent foliar fungicide, was investigated by combining a nanophotocatalytic pre-treatment with biodegradation by different bacterial consortia. This assay was conducted in the scope of an ongoing R&D collaborative work, which has previously confirmed that TRF could not be biodegraded or defluorinated solely via biodegradation by each of the bacterial consortia tested in this experiment.

After an exposure period of 9 hours under UV-A radiation with ZnO nanoparticles, the nanophotocatalytic process yielded a TRF defluorination efficiency of 64.96%, corresponding to a release of 0.0047 mM of fluoride to the treated water. After this stage, each bacterial consortia were inoculated into the pre-treated water and incubated for an additional period of 21 days. After this treatment window, no additional fluoride release was observed by any of the tested consortia. No defluorination was observed as well in the non-inoculated controls. As such, the tested bacterial consortia were unable to catalyze the catabolic defluorination of the remaining TRF or of any possible fluorinated subproduct resulting from the nanophotocatalytic degradation. However, the absence of defluorination activity by any of the tested bacterial consortia may also be related to some toxic effects caused by ZnO nanoparticles. Even though these nanoparticles were extracted from the

pre-treated water by centrifugation, it is possible that a residual amount kept in solution and negatively impacted the catabolic activity of the bacterial consortia, as previous studies have shown that ZnO can act as a antibacterial agent for both Gram-positive and Gram-negative bacteria, as well as eukaryotic cells (Emami-Karvani & Chehrazi, 2011; Sirelkhatim et al., 2015). Previous results of this collaborative work have also showed that minor leaching of zinc ions (Zn^{2+}) from ZnO nanoparticles can occur, in some cases leading to a final concentration of ca. 10 mgL^{-1} of Zn^{2+} in solution. The release of Zn^{2+} ions in to the medium may cause the inhibition of active transport and the disruption of enzyme systems in bacteria (Sirelkhatim et al., 2015).

Conclusion

L. portucalensis F11 and *D. acidovorans* MFA5 are strains known to be able to effectively metabolize and use FB and FA as sole carbon source, respectively (Alexandrino et al., 2018; Carvalho et al., 2008). Thus, linking the OD and defluorination performance data, under no pressure from background levels of fluoride, both these strains completely degraded their respective substrates in the two-week period of this assay, as expected. The complete degradation of the substrates was likely the main factor in the decrease in OD observed, due to the extinction of an available carbon source capable of sustaining bacterial growth. In contrast, the exposure to fluoride affected the catabolic defluorination in both strains, causing a clear decrease in catabolic defluorination performance. In future work, multiple OD measurements would be beneficial to be able to more accurately monitor the development of the test strains. No co-metabolic defluorination of either MFZ of PFHxA was observed for the strains *D. acidovorans* MFA5 and *L. portucalensis* F11, while bacterial growth was observed, suggesting that other biotransformations beyond defluorination could have allowed some carbon assimilation from MFZ by the degrading strains. Possibly, further studies using fluorinated co-substrates could be conducted, as these would necessitate the activation of the defluorinating pathways to act as an energy source for the degrading bacteria. The junction of nanophotocalysis and bacterial degradation of TRF did not result in a more effective defluorination of fluorinated compounds by the tested bacterial consortia, possibly due to known toxic effects of ZnO nanoparticles on bacteria that may have inhibited their growth and therefore the degradation of the target compounds. To account for the effects of ZnO on the tested bacterial consortia, further tests are planned, using a ZnO specific membrane to effectively remove the nanocatalyst from treated water samples, prior to a bacterial defluorination treatment.

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