# Biodegradation of mono-, di- and trifluoroacetate by microbial cultures with different origins

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A B S T R A C T

This work focused on the biodegradation of three structurally related fluoroacetates (FAs), mono- (MFA), di-(DFA) and trifluoroacetate (TFA), using as microbial inocula samples collected from a site with a long history of industrial contamination and activated sludge obtained from a municipal wastewater treatment plant. Biodegradation experiments were carried out under different modes of substrate supplementation, which included (i) FAs fed as sole carbon sources; (ii) FAs (only for DFA and TFA) fed in co-metabolism with sodium acetate; and (iii) mixtures of MFA with DFA or TFA. Biodegradation of the target compounds was assessed through fluoride ion release. Defluorination was obtained in the cultures fed with MFA, while DFA and TFA were recalcitrant in all tested conditions. When present in mixture, DFA was shown to inhibit biodegradation of MFA, while TFA had no effect. A total of 13 bacterial isolates obtained from MFA degrading cultures were found to degrade 20 $mgL^{-1}$ of this compound, as single strains, when supplemented as a sole carbon source. Sequencing of the 16S rRNA gene indicated that among these degrading bacteria only *Delftia acidovorans* had been previously reported to be able to degrade MFA. This work shows that, despite their similar chemical structures, biode-gradation of the three tested FAs is very distinct and draws attention to the unknown impacts that the accu-mulation of DFA and TFA may have in the environment as a result of their high recalcitrance.

*Keywords:* Biodegradation Co-metabolism Defluorination Monofluoroacetate Difluoroacetate Trifluoroacetate

## 1. Introduction

Due to the valuable properties that fluorine confers on organic molecules, the use of synthetic organofluorines in industrial, medical and agricultural applications has increased significantly in recent decades [1]. As a result of their wide applications, fluoroorganic molecules are becoming pollutants in several environmental compartments, where they may persist for long periods due to the recalcitrant nature of many of these molecules [2,3]. The degradation of organofluorine compounds constitutes a challenge to microorganisms, not only because the environmental pollution originated by these compounds is a relatively recent problem, causing microorganisms to be exposed to compounds so far unknown, but also because the C-F bond of organofluorines has one of the highest known energies [4].

Fluoroacetates (FAs) are a family of carboxylic aliphatic organofluorines composed of mono- (MFA), di- (DFA) and trifluoroacetate (TFA), that are non-volatile and highly soluble in water. They are important building blocks and intermediary reagents for the chemical synthesis of various fluorinated compounds with different applications [5–11], and also result from the abiotic breakdown of fluorinated polymers [12–15]. MFA is also used in some countries as a pesticide to control mammalian pests and is known to be naturally produced by a number of plants and a few *Streptomyces* species [16–22]. In addition, TFA is a derivative of the tropospheric degradation of several hydrochlorofluorocarbons (HCFCs) and hydrofluorocarbons (HFCs) [23].

Due to the different applications of FAs and various entry routes in the environment, pollution caused by these compounds may be either diffuse or point source. FAs have been reported to occur in several environmental compartments, with the aquatic media being their major

environmental sink [24]. TFA has been detected in seasonal wetlands, marine environments, rainwater and lotic environments, in concentrations ranging from 30 to 600 $ngL^{-1}$ [25–29]. The environmental occurrence of MFA is mainly linked with its application, aerially or in baits, for pest control, though release through discharges from chemical industries may also occur [30], being likely to reach groundwater streams and even surface waters due to its mobility in the environment. The environmental dynamics of DFA are poorly explored in the literature but its structural similarity to the other FAs, namely regarding its physicochemical properties, suggests a similar environmental behaviour.

MFA was found to be biodegraded by a number of soil microorganisms [31]. Kelly et al. [32] first reported the bacterial degradation of MFA, and since then other MFA-degrading bacteria have been isolated from different environmental sources, including bovine rumen, MFA-accumulating plants, anaerobic sludge and aquatic sediments [21,33,34]. Microbial degradation of this compound is mediated by fluoroacetate dehalogenase, which catalyses the cleavage of the C-F bond, yielding glycolate [35–37]. More recently, MFA has been also found to be defluorinated by L-2-haloacid dehalogenases [38]. Biodegradation of TFA has been reported to occur under anaerobic conditions by microbial communities from freshwater and saltmarsh sediments, although its aerobic conversion to fluoroform by the same communities was also described [13,39]. DFA has been identified as a metabolite resultant from the anaerobic biodegradation of TFA, being further converted into MFA and then acetate by a reductive dehalogenation mechanism [13]. Although the data suggest degradation of DFA under anaerobic conditions, no studies on its aerobic biodegradation are available in the literature (as far as we are aware). Moreover, due to their wide industrial applications, FAs may occur simultaneously in the environment and thus it is important to understand how the biodegradation of each compound is affected by the presence of its analogues. In this context, the work reported here aimed to investigate the aerobic biodegradation of MFA, DFA and TFA as sole carbon sources, as well as in mixtures of two FAs which to the best of our knowledge have not been studied previously. In addition, co-metabolic degradation of DFA and TFA in the presence of their non-fluorinated analogue, acetate, was also explored. Biodegradation was investigated using microbial inocula from different origins.

## 2. Materials and methods

### 2.1. Reagents

All chemicals used were of the highest purity grade available (Sigma- Aldrich Chemie, Steinheim, Germany; Merck, Darmstadt, Germany). DFA (97%), TFA (98%) and acetate (99%) were bought to Sigma-Aldrich and MFA (> 95%) was obtained from Supelco (Bellefonte, USA).

### 2.2. Microbial inocula

Sediment samples and rhizosphere of *Phragmites australis* (Cav.) Trin. ex Steud., were collected from a site in Estarreja (Portugal) with a long history of industrial chemical contamination [40], and used as an environmental source of microorganisms. For each sample, approximately 100 g were collected in triplicate and then pooled and homogenised in sterile zip bags. An activated sludge (AS) consortium obtained from a municipal wastewater treatment plant (Gondomar, Porto) was also used as inoculum for this study. AS samples (100 mL) were collected in triplicate from the aeration tank and pooled and homogenised in a sterile flask. Sediment, rhizosphere and AS samples were transported to the laboratory at room temperature and used in the same day of sampling. AS inoculum was obtained by centrifuging 40 mL of AS (5000 rpm for 15 min at 4 °C), washing twice the resultant pellet with a minimal salts medium (MM) and resuspending it in the same medium to one tenth of its original volume.

### 2.3. Biodegradation experiments

Biodegradation experiments were performed in batch mode in 250 mL flasks with 70 mL of sterile MM. MM contained (per litre of ultra-pure water): $Na_2HPO_4{\cdot}2H_2O$ 2.7 g, $KH_2PO_4$ 1.4 g, $(NH_4)_2SO_4$ 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.2 g and 10 mL of a trace elements solution with the following composition, per litre: $Na_2EDTA{\cdot}2H_2O$ 12.0 g, NaOH 2.0 g, $MnSO_4{\cdot}4H_2O$ 0.4 g, $ZnSO_4{\cdot}7H_2O$ 0.4 g, $H_2SO_4$ 0.5 mL, $Na_2SO_4$ 10.0 g, $Na_2MoO_4{\cdot}2H_2O$ 0.1 g, $FeSO_4{\cdot}7H_2O$ 2.0 g, $CuSO_4{\cdot}5H_2O$ 0.1 g and $CaCl_2$ 1.0 g. Flasks were inoculated with 5 g of fresh sediment or rhizosphere samples and for the AS inoculum, flasks were inoculated in order to have an initial optical density (OD) at 600 nm of 0.1. Cultures were fed with FAs individually, in mixtures of two FAs and, for DFA and TFA, in co-metabolism with acetate. When fed individually, FAs were supplemented at a concentration of 20 $mgL^{-1}$ (0.20, 0.17 and 0.15 mM for MFA, DFA and TFA, respectively), while in the binary mixtures of FAs, each compound was fed at the concentration of 10 $mgL^{-1}$ (0.10, 0.085 and 0.074 mM for MFA, DFA and TFA, respectively). Cultures in co-metabolism with acetate were supplemented with DFA or TFA at the concentration of 5 $mgL^{-1}$ (0.042 and 0.037 mM for DFA and TFA, respectively) and fed three times a week with 500 $mgL^{-1}$ of sodium acetate. In this latter condition, cultures were weekly transferred to new sterilised flasks in order to ensure sufficient oxygen for the aerobic degradation of the target compounds. Biodegradation of FAs was followed during a three week period, after which half of the cultures were transferred to new flasks containing the same proportion of MM and refed with the respective carbon sources. Culture flasks were maintained closed throughout the experiments and incubated in a rotary shaker (130 rpm) at 25 °C in the dark. Abiotic controls consisting in MM supplemented individually with each of the FAs (5 $mgL^{-1}$) and incubated under the same conditions were also included. Experiments were conducted in duplicate. FAs biodegradation was followed by measuring bacterial growth and fluoride ion release at the beginning and at the end of each three week period.

### 2.4. Microbial characterization of MFA-degrading cultures

At the end of the biodegradation experiments, the microbial diversity of MFA degrading cultures was analysed by spreading several tenfold dilutions of culture samples onto minimal salts agar plates supplemented with MFA (20 $mgL^{-1}$) as sole carbon source and Plate-Count Agar (PCA). The plates were incubated at 25 °C until growth was detected. Bacterial diversity was analysed by visual inspection and morphologically distinct colonies were purified by streaking the different colonies in new agar plates.

### 2.5. Biodegradation capacity of microbial isolates obtained from MFA-degrading cultures

The capacity of the different isolates retrieved from the MFA-degrading cultures to degrade this compound in axenic cultures was investigated by inoculating single strains into 30 mL sterile flasks, filled to two thirds of their volume with MM and supplemented with MFA at 20 $mgL^{-1}$. The initial OD (600 nm) of the cultures was 0.1. Flasks were incubated in a rotary shaker (130 rpm at 25 °C), in the dark. Biodegradation was followed along a three week period by monitoring bacterial growth and fluoride ion release. A microbial culture consisting of a mixture of all MFA-degrading isolates was also created and used as inoculum for investigating its capacity to degrade DFA and TFA fed individually as sole carbon source (20 $mgL^{-1}$) and co-metabolism with MFA (20 $mgL^{-1}$ of MFA and 5 $mgL^{-1}$ of DFA or TFA).The experiment was conducted under the same conditions described above.

### 2.6. Identification of MFA-degrading isolates

All the isolates capable of degrading MFA as single strains were identified through 16S rRNA gene sequence analysis. DNA was extracted from colonies grown on minimal salts agar plates supplemented with MFA or PCA plates following a standard phenol-chloroform extraction method, as described elsewhere [41]. Extracted DNA was amplified by Polymerase Chain Reaction (PCR) using the universal primers 27F and 1492R [42]. PCRs were prepared in a total volume of 10 µL as follows: 5 µL of Multiplex PCR Master Mix (Qiagen, Valencia, CA), 1 µL of primer mix (2 µM of each primer) and a 2–4 µL of template DNA sample. Negative controls were included in all reactions. The PCR amplification conditions included an initial denaturation at 95 °C for 15 min, followed by 30 cycles at 94 °C for 30 s, 48 °C for 90 s and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Amplification products were separated by electrophoresis in a 1.5% agarose gel containing SYBR® Safe (ThermoFisher Scientific, Massachusetts, USA). DNA fragments were visualised under UV light in a BioRad Molecular Image® Gel Doc™ XR+ with Image Lab™ Software, and those showing amplification bands with a suitable size (~1500 bp) were submitted for Sanger sequencing in a commercial provider. The obtained 16S rRNA gene sequences were aligned using the *Geneious* v4.8.2 software and the resulting consensus sequences were compared to those present in the nucleotide collection database of the National Center for Biotechnology Information (NCBI) and in the EZTaxon database (http://www.ezbiocloud.net/eztaxon/database). The 16S rRNA gene sequences of all MFA-degrading strains have been deposited in GenBank® (NCBI, Maryland, USA) with the accession numbers indicated in Table 1.

### 2.7. Analytical methods

Fluoride release was analysed by potentiometry, through the measurement of the concentration of fluoride ions in the supernatant of culture samples, using a fluoride-selective electrode (Crison 9655C, Crison Instruments, S.A., Barcelona, Spain). Prior to sample analysis, a calibration curve was constructed using standards of sodium fluoride (0.001 to 1 mM) prepared in MM. In order to remove possible interference in the potentiometric analysis, a total ionic strength adjustment buffer (TISAB III) was supplemented to the samples and standards in a 1:10 ratio. Microbial growth was monitored through the measurement in a spectrophotometer of the OD at 600 nm of culture samples (Model V-1200, VWR International, LLC, Pennsylvania, USA).

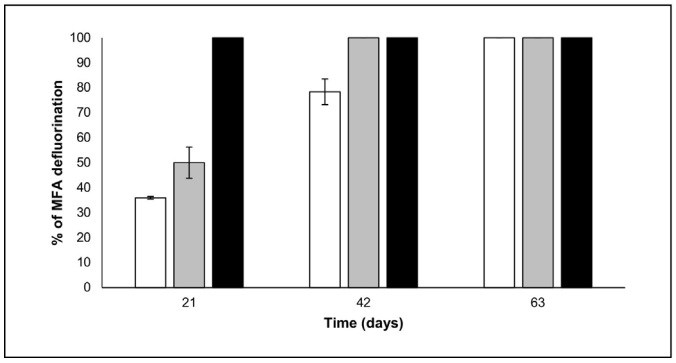


**Fig. 1.** Biodegradation performances, based on fluoride release, of monofluoroacetate (MFA) supplemented as a sole carbon source along a two months period. White bars represent rhizosphere inoculum, grey bars, sediment inoculum and black bars, activated sludge consortia. Days 21, 42 and 63 correspond to the end of the 1st, 2nd and 3rd MFA feeding periods, respectively. The results represent the mean of duplicates and error bars show standard deviation.

## 3. Results

### 3.1. Biodegradation of FAs by the different microbial inocula

The microbial capacity to degrade three structurally related FAs, MFA, DFA and TFA, as sole carbon sources, in mixtures of two FAs and in co-metabolism with acetate, was investigated using microbial inocula with distinct origins. Fluoride release was used as a key biodegradation indicator, since the main obstacle to the microbial degradation of these compounds lies in the presence of this atom in their molecular structures. Acetate was chosen as a co-metabolite since it is a growth supporting substrate that can be easily metabolised by most microorganisms and is a non-fluorinated analogue of the target FAs.

When supplemented as a sole carbon source, only MFA was degraded by the tested microbial inocula. The AS consortium already showed complete defluorination of MFA by the end of the first feeding period, whereas the treatments inoculated with sediment or rhizosphere samples revealed a gradual increase in MFA degradation performance (Fig. 1). In these latter cultures, complete defluorination was also achieved. For cultures inoculated with sediment samples, complete defluorination was obtained when fed a second time with MFA, while for rhizosphere cultures this was observed in the following feeding period (Fig. 1). Total defluorination of MFA was maintained in further MFA feedings for an additional period of 2 months. None of the tested

**Table 1**
Microbial isolates obtained from the different monofluoroacetate-degrading consortia and taxonomic identification of the microbial isolates capable of degrading monofluoroacetate as sole carbon source.

| Inoculum | Carbon source supplemented to the medium | Number of microbial isolates recovered | Number of isolates with capacity to degrade MFA | Identification of MFA degrading microorganisms | GenBank accession numbers |
|---|---|---|---|---|---|
| Activated sludge | MFA | 5 | 3 | Comamonas testosteroni strain MFA1 | KX400799 |
| | | | | Stenotrophomonas maltophili strain MFA2 | KX400881 |
| | | | | Herbaspirillum frisingense strain MFA4 | KX756676 |
| | MFA and TFA | 7 | 3 | Delftia acidovorans strain MFA5 | KX400852 |
| | | | | Pseudomonas putida strain MFA15 | KX400880 |
| | | | | Achromobacter anxifer strain MFA16 | KX398363 |
| Rhizosphere | MFA | 8 | 1 | Pseudomonas sp. strain MFA9 | KX404994 |
| | MFA and TFA | 7 | 2 | Variovorax paradoxus strain MFA10 | KX400967 |
| | | | | Arthrobacter humicola strain MFA12 | KX400776 |
| Sediment | MFA | 8 | 1 | Chryseobacterium taeanense strain MFA25 | KX400798 |
| | MFA and TFA | 8 | 3 | Achromobacter anxifer strain MFA31 | KX400775 |
| | | | | Pseudomonas sp. strain MFA32 | KX756677 |
| | | | | Comamonas testosteroni stran MFA35 | KX400851 |

MFA, monofluoroacetate; TFA, trifluoroacetate.

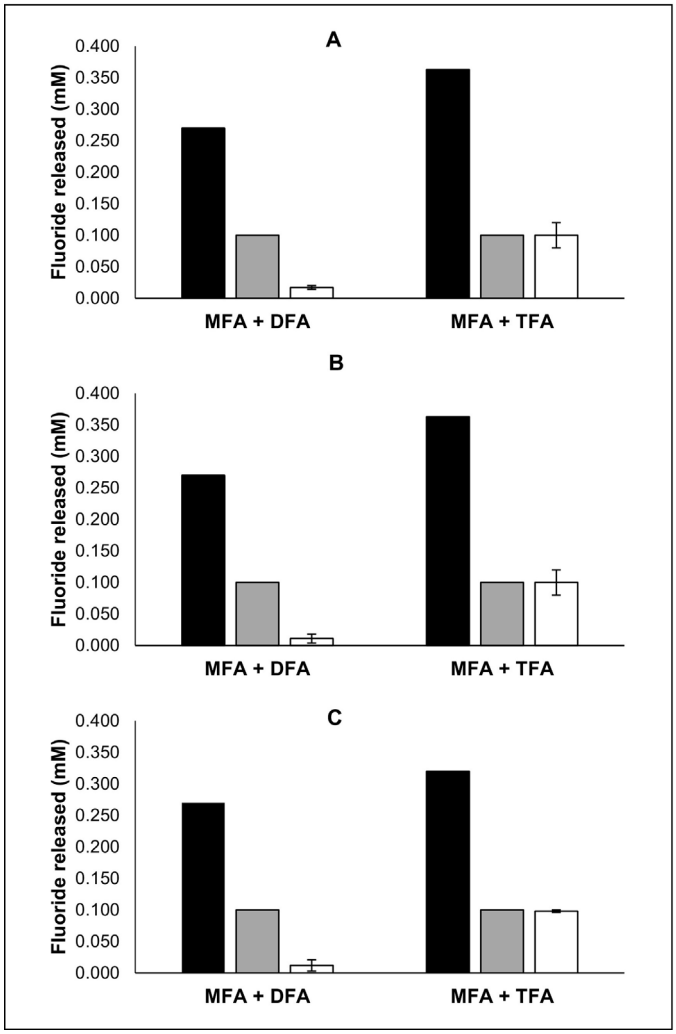


**Fig. 2.** Defluorination performance of the tested microbial cultures when supplemented with mixtures of fluoroacetates (FAs) after a two months period. A – activated sludge consortia; B – rhizosphere inoculum; C – sediment inoculum. Black bars show expected fluoride concentrations considering complete defluorination of both FAs in the mixture, grey bars show expected fluoride concentration considering total defluorination of only monofluoroacetate and white bars show the concentration of fluoride ion released to the culture medium. Results represent the mean of duplicates and error bars are relative to standard deviation. MFA, monofluoroacetate; DFA, difluoroacetate; TFA, trifluoroacetate.

microbial inocula were capable of defluorinating DFA or TFA, either when supplemented as sole carbon sources or in co-metabolism with acetate.

Biodegradation of MFA in mixture with DFA or TFA was also investigated. A mixture of DFA and TFA was not tested considering that no biodegradation has been obtained when these compounds were supplemented individually. When MFA was supplemented with DFA, only a small fraction of fluoride was detected in the culture medium of the different tested microbial consortia (Fig. 2). The low concentration of fluoride released in these cultures indicates that the simultaneous presence of the two FAs did not stimulate the biodegradation of DFA, but also produced a negative effect in the biodegradation of MFA, as the fluoride concentration obtained in the culture medium was not proportional to the complete defluorination of this compound (Fig. 2). In the cultures supplemented with a mixture of MFA and TFA, the concentration of fluoride ion analysed in the culture medium was higher than that obtained in the cultures fed with MFA and DFA, and the extent of fluoride released suggests that MFA was fully degraded, being in agreement with its stoichiometric defluorination (Fig. 2). This result suggests that, unlike DFA, the presence of TFA in the mixture does not interfere with MFA biodegradation and that, similarly to what happened with DFA, the addition of MFA does not stimulate biodegradation of TFA.

In the cultures fed with MFA, both as sole carbon source and in mixture with TFA, a slight OD increase (about 0.01) was observed, while a residual growth (about 0.002) was obtained in the cultures fed with MFA and DFA. No detectable growth was observed in the cultures fed with DFA or TFA as sole carbon sources, but when these compounds were fed in co-metabolism with acetate an OD increment of ca. 0.8 was obtained, indicating that apparently these compounds are not toxic to the cultures. For the cultures inoculated with sediment or rhizosphere samples, the OD parameter could not however be analysed along the first three feeding periods due to the interference of the inocula in this analysis. Abiotic controls were also established in parallel with the biodegradation experiments, revealing no fluoride release in any of the flasks under the tested experimental conditions.

### *3.2. Characterization of MFA-degrading microbial consortia and biodegradation capacity of the isolated strains*

All the cultures degrading MFA (individually or in mixture with TFA) were analysed in terms of their microbial diversity. A total of 43 microbial isolates were obtained from the degrading cultures: 12 strains were recovered from activated sludge, 15 strains from cultures inoculated with rhizosphere samples and 16 strains from cultures inoculated with sediment samples (Table 1). All these isolates were tested individually for their capacity to degrade MFA when supplemented as a sole carbon source, revealing that out of the 43 isolates recovered, 13 were capable of completely defluorinating MFA (Table 1). Cultures of degrading isolates revealed OD increments between 0.01 and 0.02. The highest number of MFA-degrading isolates was obtained from activated sludge consortia.

A mixed culture composed by all MFA-degrading isolates was also created and tested for its capacity to degrade DFA and TFA as sole carbon sources and in co-metabolism with MFA. Based on fluoride release, no biodegradation of DFA and TFA fed individually was observed with this consortium and no growth was obtained. When these compounds were supplemented in co-metabolism with MFA, the results obtained were very similar to those previously observed with the mixtures of two FAs, i.e. the concentration of fluoride ion analysed in the culture medium when MFA was fed with TFA correlated with the total defluorination of MFA, suggesting that this defluorination pattern is attributed solely to the degradation of MFA, but when DFA was present in the mixture, biodegradation of MFA was inhibited and only ca. 10% of this compound was defluorinated (Fig. 3). Microbial growth

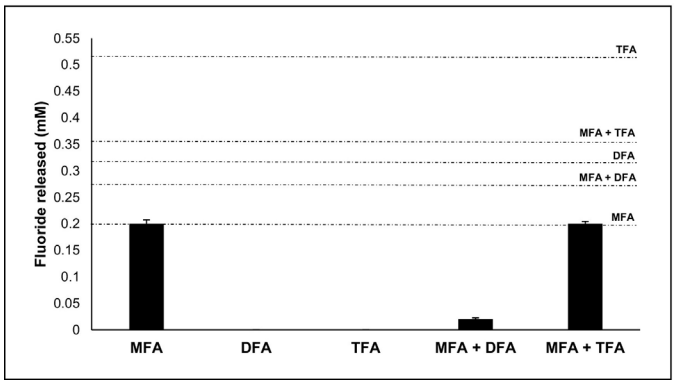


**Fig. 3.** Biodegradation, based on fluoride release, of fluoroacetates supplemented as sole carbon sources and in co-metabolism with monofluoroacetate (MFA), by a mix of the 13 MFA-degrading microbial isolates. Dotted lines indicate theoretical fluoride concentration corresponding to complete defluorination of the tested compounds. Results represent the mean of duplicates and error bars show standard deviation. MFA, monofluoroacetate; DFA, difluoroacetate; TFA, trifluoroacetat.

obtained in these cultures was also consistent with defluorination patterns, revealing an OD increment of ca. 0.01 in the consortium fed with MFA and TFA and a residual growth (0.001) in the consortium fed with MFA and DFA. These results suggest that, as with the enriched cultures, the culture consisting of the mixture of all MFA-degrading isolates was unable to metabolise DFA and TFA, being capable of defluorinating MFA in the presence of TFA, but not with DFA.

### 3.3. Identification of the MFA-degrading microbial isolates

Microbial isolates capable of degrading MFA as sole carbon source were identified through 16S rRNA gene sequence analysis. The isolates were identified as 9 distinct species, belonging to different genera, mainly assigned to the *Proteobacteria* phylum (Table 1). Activated sludge comprised 6 genera of MFA-degrading isolates: *Stenotrophomonas*, *Herbaspirillum*, *Delftia*, *Pseudomonas*, *Comamonas* and *Achromobacter*. A *Pseudomonas* species, as well as the species *Comamonas testosteroni* and *Achromobacter anxifer* were present in both activated sludge and cultures inoculated with sediment samples. An isolate identified as *Chryseobacterium taeanense* was also obtained from these latter cultures. In the cultures inoculated with rhizosphere samples, isolates capable of degrading MFA as single strains were found to belong to *Variovorax*, *Arthrobacter* and *Pseudomonas* genera (Table 1).

## 4. Discussion

Aliphatic organofluorines represent an important class of pollutants that are released to the environment, mainly as a consequence of the wide industrial applications of fluorinated compounds [43]. The critical step in the biodegradation of these compounds is the removal of fluoride ion [1]. Complete defluorination of FAs was reported to yield easily degradable compounds that may be readily dissipated from the environment and have no potential for ecosystems damage, such as glycolate, a known metabolic product of the biodegradation of MFA, or acetate, which is thought to result from the anaerobic biodegradation of TFA [13,37]. In the present study, microbial potential to aerobically biodegrade MFA, DFA and TFA was investigated with the target compounds being fed both as sole carbon sources and in mixture. Sediment and rhizosphere samples from an industrially contaminated site and activated sludge from a municipal WWTP were used as inocula, as the first two samples constitute a good source of microorganisms used to the presence of xenobiotic compounds and WWTP commonly receive and treat effluents with a myriad of pollutants. However, no information about the potential presence of FAs in these samples was available.

Complete defluorination of MFA supplemented as a sole carbon source was achieved with all tested inocula. While activated sludge cultures defluorinated the supplemented MFA during the first feeding period (i.e. in the first 21 days), a longer period was required for the other two microbial consortia to degrade the target compound. This period may be necessary because activated sludge microbial cells might be in a more active metabolic condition than the other two microbial consortia, since these communities are typically subjected to high organic loads, having an easy access to growth substrates and to the presence of a wide range of organic molecules in wastewaters. Nonetheless, the fact that the other tested microbial inocula also degraded MFA, indicates that microorganisms capable of metabolising this compound were originally present in these microbial consortia, though they needed a longer lag phase in order to prevail in the communities. Due to the fact that no fluoride release was obtained in abiotic controls containing MFA, defluorination observed in the tested cultures can be solely attributed to the biological action of microorganisms in these cultures. Biodegradation of MFA has been reported previously, either when supplemented as sole carbon source or in the presence of a secondary carbon source, such as D-ribose and yeast extract [20,21,31,33,44]. Some of the described MFA-degrading microorganisms originated from environments where MFA was known to be present, such as soils in the neighbourhood of MFA-producing plants or soils adjacent to baits impregnated with this compound [31,33]. However, isolation of MFA-degrading microorganisms has also been reported from samples not contaminated with this compound, indicating that the microbial capacity to metabolise MFA is widespread in the environment and among microorganisms [20,21,44]. This is in agreement with our results, as the obtained MFA-degrading isolates originated from environmental samples where MFA is not expected to be present.

DFA and TFA were not defluorinated by any of the tested cultures along an enrichment period of ca. 4 months. The absence of TFA defluorination under aerobic conditions is in agreement with the results reported by other authors, while DFA biodegradation has never been investigated to the best of our knowledge [13,45]. Visscher et al. [13] reported the accumulation of a dead-end metabolite, identified as fluoroform, resultant from the aerobic biodegradation of TFA. This metabolite still bears a trifluoromethyl group in its structure, and is more toxic than the parent compound. Benesch et al. [45] found no aerobic biodegradation of TFA along a three month period by microbial communities from vernal pool soils. Contrastingly, complete defluorination of TFA under anaerobic conditions has been reported by Visscher et al. [13] and Kim et al. [39], with TFA (in concentrations ranging from 0.2 to 51 $mgL^{-1}$) being reductively dehalogenated under methanogenic conditions to DFA, MFA and acetate. Co-supplementation of the microbial cultures with acetate, a compound structurally similar to FAs and a common microbial substrate, did not produce any effect in defluorination of DFA or TFA. The co-feeding of substrates with chemical structures similar to their halogenated counterparts has been shown in some cases to have a positive effect in their biodegradation, through the induction of metabolic enzymes capable of acting on their metabolism. For example, *Burkholderia* sp. was capable of metabolising a group of mono- and dichlorophenols in the presence of phenol as a growth-supporting substrate, though for highly substituted chlorophenols, such as trichlorophenols and pentachlorophenol, this strategy was inefficient [46]. This result may be due to the more significant molecular steric and biochemical properties that are associated with the increasing halogenation of organic compounds, which may cause differences in enzyme recognition and, consequently, substrate interaction. The results obtained in our study indicate that the enzymatic mechanisms involved in the degradation of acetate are not efficient in the biodegradation of DFA or TFA. On the other hand, the addition of acetate to the cultures fed with DFA or TFA could also have benefited the biodegradation of these compounds by stimulating microbial growth, as reported for other organofluorines [47,48], but this was also not verified in the present study.

In order to understand how mixtures of FAs influence their biodegradation, MFA was supplemented to microbial inocula in a mixture with DFA or TFA. The biodegradation of mixtures of these compounds has not been investigated previously and is relevant due to the high likelihood of these FAs coexisting in the environment. MFA defluorination was found to be negatively affected by the presence of DFA in the culture medium, while TFA did not seem to exert any effect on the biodegradation of this compound. This negative influence of DFA in MFA defluorination may be associated with an enzymatic inhibition, as MFA and DFA share a greater stereochemical similarity than MFA and TFA. This could allow DFA to bind to the active site of the enzyme that metabolises MFA, preventing the binding of the latter compound to the enzymatic system, blocking its action and causing defluorination inhibition. As the trifluoromethyl moiety of TFA has a higher steric bulk than DFA, the MFA degrading enzyme may have a higher capacity to discriminate between these two compounds, and so inhibition does not occur. However, it should be noted that this metabolic inhibition may not be verified for lower concentrations, which are usually more representative of these compounds in the environment. Nevertheless, and to the best of our knowledge, the inhibitory effect of DFA in the metabolism of MFA has never been reported before. The results obtained

with MFA fed in mixture with DFA or TFA also suggest that the metabolic enzymes responsible for the biodegradation of MFA are selective for this compound and, thus unable to attack DFA or TFA. This selective MFA catabolism has been reported earlier by Donnelly and Murphy [49]. These authors isolated a fluoroacetate dehalogenase from a *Pseudomonas fluorescens* strain and found that the enzyme was highly selective for MFA and not capable of metabolising DFA and TFA. This finding is a clear example of the impact that the degree of fluorination may have in the microbial metabolism of fluorinated compounds.

A total of 13 bacterial strains with the capacity to degrade MFA as sole carbon source was isolated from the different MFA-degrading cultures. Taxonomic identification of these strains revealed several microbial species not previously associated with the biodegradation of MFA, such as *Herbaspirillum frinsingense,* a nitrogen-fixing bacterial strain which has never been linked with the biodegradation of environmental contaminants, including MFA. On the other hand, some of the species identified here were found to belong to the *Pseudomonas* genus, which is known to include a number of MFA-degrading strains [35,49,50]. The bacterial isolates identified as *Comamonas testosteroni*, *Variovorax paradoxus* and *Delftia acidovorans*, all belonging to the *Comamonadaceae* family, were also capable of degrading MFA as sole carbon source, being isolated from the different MFA-degrading cultures. *D. acidovorans* (formerly *Moraxella* sp.) is the only microbial species obtained in this study that has been demonstrated before to degrade MFA [36,51,52]. *C. testosteroni* and *V. paradoxus* have never been associated with the biodegradation of MFA, but their capacity to degrade other recalcitrant compounds, including several chlorinated aromatics, has been described before [53–56].

According to the literature, defluorination of MFA is commonly catalysed by fluoroacetate dehalogenase [35–37]. Since this enzyme generally occurs at the plasmidic level, it is possible that horizontal transfer of this genotype may have occurred in the MFA-degrading bacterial communities, which may have contributed to the significant number of bacterial strains capable of degrading this compound obtained in our study [36,51,52,57]. The combination of all MFA-degrading isolates proved to be ineffective in the metabolism of DFA and TFA, namely concerning defluorination of these compounds, reinforcing the conclusion that the enzyme responsible for the defluorination of MFA is unable to act on its di or tri-fluorinated counterparts. In addition, this result also supports our theory that fluoride release obtained in the cultures fed with mixtures of FAs was due to defluorination of MFA and not of DFA or TFA.

Overall, the results obtained in this study call attention to the recalcitrant nature of DFA and TFA, as well as to the potentially deleterious effects that their continuous release into the environment may have. Although the literature shows that TFA causes no or only slight toxic effects in the environment, increasing environmental release is expected to cause its accumulation, especially in aqueous resources, which may have unknown consequences. The effects of the environmental accumulation of DFA are not yet known, but its resistance to biodegradation together with the fact that it may interfere in the degradation mechanisms of defluorinating enzymes, deserve further attention. The inhibition of MFA defluorination, verified in our experiments, caused by the addition of DFA must be taken into consideration regarding the biological removal of mixtures of structurally related fluorinated compounds.

## 5. Conclusion

This work shows that MFA can be aerobically metabolised by several bacterial strains from different environmental sources, and that the mechanisms responsible for its catabolism do not apply in the biodegradation of its di- and trifluorinated counterparts. Most of the MFA-degrading isolates obtained have not previously been associated with the biodegradation of MFA, expanding the range of microbial species capable of metabolising this fluoroaliphatic compound. Under aerobic conditions, DFA and TFA were recalcitrant to microorganisms and co-supplementation with the structurally related and more easily degradable substrates, acetate and MFA, had no effect in their biodegradation. These results indicate that the degree of fluorination of fluoroaliphatic compounds significantly influences their biological degradation. When present in mixtures, DFA inhibited MFA defluorination, while TFA did not produce any effect, a result that, to our knowledge, had not been reported previously. Such interactions should be taken into account when considering the biodegradation of mixtures of structurally similar fluorinated compounds. Overall, this work emphasises the recalcitrant nature of DFA and TFA under aerobic conditions, as well as the potentially negative interactions induced by mixtures of fluoroorganics. The persistence and accumulation of FAs in the environment is a relevant issue that may lead to disturbance in ecosystems.

## Author's contributions

Diogo A. M. Alexandrino

This author was responsible for all the experimental work, as well as for the treatment of the resulting data and for writing this manuscript.

Inês Ribeiro, Luís M. Pinto and Rafael Cambra

These authors provided assistance on the experimental work, including samplings, maintenance of microbial cultures and the analytical methods.

Rui S. Oliveira

This author was involved in the experimental design and the scientific supervision of this manuscript.

Filipe Pereira

This author provided support on the taxonomical identification of the degrading isolates obtained in this study.

Maria F. Carvalho

This author was responsible for all the conducted work, supervising it throughout the entire experimental period, being involved in the experimental design, discussion of the obtained results and in the scientific supervision of this manuscript.

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