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***Pseudomonas* sp. strain 273 degrades fluorinated alkanes**

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12 **Key words:** Fluorinated alkanes, defluorination, degradation

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25 **Abstract**

26 Fluorinated organic compounds have emerged as environmental constituents of concern. We
27 demonstrate that the soil isolate *Pseudomonas* sp. strain 273 utilizes fluorinated alkanes,
28 including 1-fluoroheptane, 1-fluorooctane, 1-fluorononane, 1-fluorodecane (FD), and 1,10-
29 difluorodecane (DFD), as the sole carbon and energy sources in the presence of oxygen. Strain
30 273 degraded FD (5.97 ± 0.22 mM, nominal) and DFD (5.62 ± 0.13 mM, nominal) within 7 days
31 of incubation, and $92.7 \pm 3.8\%$ and $90.1 \pm 1.9\%$ of the theoretical maximum amounts of fluorine
32 were recovered as inorganic fluoride, respectively. With *n*-decane, strain 273 attained $(3.24 \pm$
33 $0.14) \times 10^7$ cells per μmol of carbon consumed, while lower biomass yields of $(2.48 \pm 0.15) \times$
34 10^7 and $(1.62 \pm 0.23) \times 10^7$ cells were measured with FD or DFD as electron donors,
35 respectively. The organism coupled decanol and decanoate oxidation to denitrification, but the
36 utilization of (fluoro)alkanes was strictly oxygen-dependent, presumably because the initial
37 attack on the terminal carbon requires oxygen. Fluorohexanoate was detected as an intermediate
38 in cultures growing with FD or DFD suggesting that the initial attack on the fluoroalkanes can
39 occur on the terminal methyl or fluoromethyl groups. The findings indicate that specialized
40 bacteria such as *Pseudomonas* sp. strain 273 can break carbon-fluorine bonds most likely with
41 oxygenolytic enzyme systems and that terminally monofluorinated alkanes are susceptible to
42 microbial degradation. The findings have implications for the fate of breakdown products of per-
43 and polyfluoroalkyl substances (PFAS).

Introduction

The widespread use of fluorinated organic chemicals as refrigerants, surfactants, lubricants, pharmaceuticals, pesticides, etc. has resulted in the release of these compounds into the environment.¹⁻⁴ Many fluorinated organic chemicals are recalcitrant, and recent studies demonstrated that a specific group of fluorinated organics, the so-called per- and polyfluoroalkyl substances (PFAS), are globally distributed and may impact environmental and human health.⁵⁻⁷ Recalcitrance of fluorinated compounds has been explained by the lack of naturally occurring fluoroorganics and the strength of carbon-fluorine (C-F) bond.⁸ Fluorinated organics have been considered xenobiotics; however, there is clear evidence that geologic⁹⁻¹¹ and biologic systems¹²⁻¹⁵ generate C-F bonds. For example, certain plants and some members of the grampositive *Actinomycetales* order synthesize monofluoroacetate and ω -fluorinated fatty acids.¹⁶ A fluorinase that incorporates fluoride into organic molecules has also been identified and characterized in *Streptomyces cattleya*.¹³ The observation that the C-F bond is not solely anthropogenic implies that fluorinated organics have long been part of the biosphere, and microorganisms have had ample time to evolve strategies for breaking C-F bonds.

The strength of the C-F bond has served as an argument against direct enzymatic attack and microbial degradation of fluorinated compounds. The van der Waals radii of the halogen atoms decrease in the order $I > Br > Cl > F$, whereas the electronegativity follows the opposite order, with fluorine being the most electronegative element in the periodic table. These properties confer a strong polarity to the C-F bond and the C-halogen bond strength increases in the order $C-I < C-Br < C-Cl < C-F$.^{17, 18} Despite the strength of the C-F bond, a number of studies have demonstrated that naturally occurring microorganisms possess enzyme systems that break C-F bonds at neutral pH and at room temperature.¹⁹⁻²¹ Bacteria can catalyze C-F bond cleavage

via oxygenolytic,²¹ hydrolytic,²² reductive²³⁻²⁵ and hydration²⁶ mechanisms. In one instance, evidence for organohalide respiration with monofluoroacetate was obtained,²⁷ suggesting that microbes can conserve energy from breaking C-F bonds via reductive defluorination.²⁸ Under oxic conditions, some bacteria and fungi with specific hydrolytic haloacid dehalogenases degrade monofluoroacetate.^{29, 30} *Pseudomonas* sp. strain D2 was reported to perform defluorination reactions to access the sulfur in certain fluorinated aliphatic sulfonates under oxic, sulfur-limiting growth conditions.³¹ Reductive defluorination of certain PFAS has been demonstrated *in vitro* using reduced corrinoids, which are biomolecules.³²⁻³⁶ Further, co-metabolic defluorination linked to aerobic methanotrophy has been reported.³⁷ *Aureobacterium* sp. strain RHO25 possesses an enzyme system that removes the fluorine substituent from 4-fluorobenzoate to form 4-hydroxybenzoate.³⁸ This organism was unable to grow with 4-chlorobenzoate, 4-bromobenzoate, and 4-iodobenzoate, suggesting that the initial enzyme system releasing the para fluorine substituent specifically evolved to catalyze a defluorination reaction. These observations demonstrate that microbes can catalyze C-F bond cleavage, thereby refuting the perception that bond strength prevents biological systems from breaking these bonds.

Terminally and partially fluorinated alkanes have industrial and medical applications.^{4, 39} Certain plant species, in particular members of the family Dichapetalaceae synthesize mono-fluorinated fatty acids, and 1-fluorodecane (FD) has been detected in the abdominal glands of African weaver ants.^{40, 41} A potentially significant source of partially fluorinated alkanes stems from the transformation of PFAS mediated by aggressive physical-chemical treatments⁴² or biological defluorinating systems.^{43, 44} To date, the environmental fate of alkanes with a low degree of fluorination is unclear.

The soil isolate *Pseudomonas* sp. strain 273 has been shown to grow with C₅ to C₁₂ α,ω -dichloroalkanes under oxic conditions.^{45, 46} During growth, stoichiometric amounts of inorganic chloride were released and preliminary data implicated the involvement of a monooxygenase in dechlorination.⁴⁵ Dechlorinating enzyme systems often also cleave C-Br and C-I bonds, but not C-F bonds.⁴⁷ Unexpectedly, we observed growth of strain 273 with C₇ to C₁₀ fluorinated alkanes as the sole source of carbon and energy under oxic conditions with concomitant fluoride release, indicating that this organism has the ability to break C-F bonds. Experiments with *Pseudomonas* sp. strain 273 demonstrated that fluorinated alkanes are not inert and susceptible to defluorination and degradation under oxic conditions.

Materials and Methods

Chemicals. 1-Fluorodecane (FD) (purity > 97%), 1-fluorononane (> 98%), 1-fluorooctane (> 99%), 1-fluoroheptane (> 99%), and 1-fluorohexane (> 99%) were obtained from SynQuest Labs, Inc., Alachua, FL, USA. 1,10-Difluorodecane (DFD) (> 97%) was custom synthesized by Carbosynth, Newbury, UK. Isocetane (> 98%), sodium decanoate, disodium sebacate, sodium fluoride, sodium acetate, sodium trifluoroacetate (TFA), boron trifluoride, nitrous oxide (N₂O, > 99%) and fatty acid methyl ester (FAME) standards were obtained from Sigma-Aldrich, St. Louis, MO, USA. *n*-Decane (decane, > 99%) and 1,10-dichlorodecane (DCD) (> 98%) were obtained from Acros Organics, Fair Lawn, NJ, USA. *n*-Hexane (hexane, > 99.8%), methanol (> 99.9%) and sodium nitrite were obtained from Fisher Chemical, Pittsburgh, PA, USA. Sodium monofluoroacetate (MFA) was obtained from MP Biomedicals, Solon, OH, USA. Dinitrogen, helium (both ultra-high purity) and acetylene (> 99.99%) were obtained from Airgas, Radnor Township, PA, USA. Bradford 1x dye reagent was obtained from Bio-Rad Laboratories,

113 Hercules, CA, USA. Bovine serum albumin was obtained from Fisher Bioreagents, Pittsburgh,
114 PA, USA. All other chemicals used were of analytical reagent grade or higher.

115 **Cultivation of *Pseudomonas* sp. strain 273.** Strain 273 was isolated from garden soil and
116 taxonomically characterized as a pseudomonad.⁴⁵ Strain 273 was grown in completely synthetic,
117 defined mineral salts medium that comprised (per liter): 6.34 g of Na₂HPO₄, 1.33 g of KH₂PO₄,
118 1.0 g of (NH₄)₂SO₄, 0.2 g of MgSO₄·7H₂O, the Wolin vitamin mix⁴⁸ and a trace metal solution.⁴⁵
119 To avoid precipitate formation, a 10-fold concentrated phosphate buffer stock was autoclaved
120 separately and added to the medium after the solutions had cooled to room temperature. The final
121 medium pH was 7.3. The vitamins were added from a filter-sterilized (0.2 µm polyethersulfone
122 membrane filter, Thermo, Waltham, MA, USA) stock solution. The culture was routinely grown
123 in 160-mL serum bottles containing 50 mL of medium and 110 mL of air headspace. The bottles
124 were closed with black butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ, US) held in place
125 with aluminum crimps (Bellco Glass Inc., Vineland, NJ, USA). Neat hydrocarbons (e.g. decane)
126 were added to autoclaved medium with 5-µL glass microsyringes (Hamilton, Reno, NV, US) to
127 reach initial (nominal) substrate concentrations ranging from 0.5 to 5 mM, which is higher than
128 the maximum aqueous phase solubility of the hydrocarbons tested (Table S1). The headspace of
129 each 160-mL culture vessel was flushed daily with air to ensure oxygen was not limiting
130 hydrocarbon oxidation. Due to immiscibility of the medium-chain hydrocarbons with water, the
131 quantification of C₁₀ hydrocarbons required sacrificial sampling and hexane extraction of the
132 entire incubation vessel. For time-series quantification, triplicate culture vessels (i.e., 20-mL
133 glass vials containing 2 mL of medium) were prepared for each anticipated sampling time point.
134 The vials were closed with Teflon-lined butyl rubber septa held in place with aluminum crimps.
135 To ensure that oxygen availability was not limiting alkane degradation (Table S2), the vials were

pressurized by injecting 18 mL of air (~2 atm). Independent experiments with larger incubation vessels and a decreased liquid-to-headspace volume ratio (i.e., 160-mL serum bottles with 10 mL of medium) verified the results obtained with the 20-mL glass vials. All vessels were shaken at 120 rpm in upright position and incubated at 30°C.

Substrate range. The maximum aqueous phase solubility of the fluorohydrocarbons differed according to chain length (μM values for 25°C in parentheses): FD (15.7), 1-fluorononane (51.9), 1-fluorooctane (172.5), 1-fluoroheptane (573.6), and 1-fluorohexane (1,910.4) (Table S1). To test the range of fluorinated alkanes strain 273 could utilize as growth substrates, cultures received fluoroalkanes dissolved in a water immiscible carrier phase (i.e., isocetane). This procedure avoided potential toxicity of shorter fluoroalkanes due to their higher aqueous phase solubility. Stock solutions of fluoroalkanes (14.48 - 15.97 mg) in isocetane (2 mL) were prepared gravimetrically and fluoroalkane stock concentrations ranged from 49.3 to 69.5 mM (Table S1). Stock solutions of fluoroalkanes dissolved in isocetane (0.5 mL) were transferred to 20-mL glass vials containing 10 mL of sterile medium and inoculated (1%, vol/vol). The aqueous phase concentrations of fluoroalkanes (0.32 - 40.45 μM) at equilibrium were calculated based on published *n*-octanol/water partition coefficient (K_{OW}) values (Table S1). Triplicate cultures were established including control cultures that received only isocetane. The vials were covered with aluminum foil and shaken at 120 rpm at 30°C. Growth occurred in positive control cultures that received FD dissolved in the carrier phase, indicating that 0.5 mL of isocetane did not prevent growth with FD. No growth was observed in cultures that received only isocetane.

To investigate the range of fatty acids strain 273 could utilize as carbon sources, stock solutions (prepared in Milli-Q water) of sodium decanoate, disodium sebacate, MFA or TFA were autoclaved and added to the medium with sterile 1-mL plastic syringes (Becton, Dickinson

and Company, Franklin Lakes, NJ, USA). The utilization of MFA (1 and 2 mM) and TFA (2 mM) was also tested with decane (5 mM, nominal) supplied as primary substrate.

To determine if strain 273 is a complete denitrifier, the organism was grown in anoxic medium (50 mL) with nitrate (2 mM, 100 μ mol), nitrite (2 mM, 100 μ mol) or N₂O (0.42 mM, 100 μ mol) as electron acceptor in 160-mL glass serum bottles. Acetate (10 mM, 500 μ mol) was provided as electron donor. Control cultures received acetylene (11 mL, 10% of headspace) to inhibit N₂O reduction.⁴⁹

To test if oxygen was required for fluoroalkane degradation, strain 273 was grown under anoxic conditions with 2 mM nitrite as electron acceptor and decanoate, decane, or FD (1 mM, nominal) provided as electron donors (four replicate cultures each). To remove oxygen, the headspace of the 160-mL serum bottles was evacuated (–10 psig for 30 sec) and purged with oxygen-free nitrogen (three cycles). Four replicate cultures of strain 273 with FD and an air headspace served as positive controls. Four additional control cultures were maintained under anoxic conditions with 2 mM nitrite and FD for 7 days before the dinitrogen headspace was replaced with air. All cultures were inoculated (1%, vol/vol) from a strain 273 culture grown with decanoate and nitrite under anoxic conditions.

Resting cell assays. Strain 273 was grown with decane (5 mM, nominal) under oxic conditions in two 2-L glass bottles each containing 1.5 L of medium. The bottles were covered with aluminum foil to allow gas exchange and shaken at 120 rpm at 30°C. The cells were harvested after 6 days (OD_{600nm} readings of about 0.1) by centrifugation at 11,325 $\times$ g at 20°C for 20 min. The cell pellets were suspended in 200 mL of sterile mineral salt medium, and 2 mL of cell suspension (0.7 mg protein mL^{–1}, Figure S1) was transferred to three sets of 20-mL glass vials, each set receiving 2 μ L of decane (5.1 mM), FD (4.9 mM), and DFD (4.7 mM, all nominal). The

vials were sealed with Teflon-lined butyl rubber septa held in place with aluminum crimps and pressurized (~ 2 atm) with air. At each sampling event, triplicate vials from each set were sacrificed for liquid extraction and quantification of hydrocarbon remaining.

Growth yields. To compare growth yields with different (halo)carbon substrates, strain 273 cultures received decane (15.5 ± 0.7 μmol , 1.55 ± 0.07 mM), FD (14.6 ± 0.4 μmol , 1.46 ± 0.04 mM), DFD (15.3 ± 0.6 μmol , 1.53 ± 0.06 mM), DCD (14.4 ± 0.7 μmol , 1.44 ± 0.07 mM), acetate (69.6 ± 1.8 μmol , 6.96 ± 0.18 mM), sodium decanoate (12.6 ± 0.2 μmol , 1.26 ± 0.02 mM) or disodium sebacate (12.3 ± 0.4 μmol , 1.23 ± 0.04 mM) in 160-mL serum bottles containing 10 mL of medium and closed with black butyl rubber stoppers. Dedicated 5- μL glass microsyringes were used to add 3 μL of the different hydrocarbons and gravimetric measurements determined the exact amounts of hydrocarbons added. The amount of oxygen in the headspace allowed complete oxidation of the carbon substrates (i.e., electron acceptor present in at least 5-fold excess of the theoretical demand) (Table S2). After a 7-day incubation period, 2 mL of culture suspension samples were collected for liquid extraction to quantify hydrocarbons remaining in the medium. Cell yields were determined with qPCR and the Bradford assay (see SI for details). For qPCR, forward primer (5'-3') F-GCTAGTCTAACCTTCGGGGG ($T_m = 59^\circ\text{C}$) and reverse primer R-TCCCCTACGGCTACCTTGTT ($T_m = 60^\circ\text{C}$) were designed using Primer-BLAST NCBI to amplify a consensus region of the strain 273 five 16S rRNA genes.⁵⁰

Identification of C₁₀ hydrocarbon metabolites. Strain 273 cultures were grown with decane, FD or DFD as the sole carbon source (7 mM, nominal) in closed 160-mL serum bottles containing 50 mL of medium and 110 mL of air headspace, which was replaced daily. Following a 4-day incubation period ($\text{OD}_{600\text{nm}} > 0.08$), 10-mL cell suspension samples were centrifuged at $8,000 \times g$ for 15 min at room temperature and washed once with sterile medium. To identify

potential metabolites generated from C₁₀ hydrocarbons, fatty acids were extracted from cell pellets and derivatized for gas chromatography-high resolution mass spectrometry (GC-HRMS) analysis (see SI for details).

Analytical procedures. Aliphatic hydrocarbons were extracted from culture medium with hexane and quantified with an Agilent 7890A GC-MS system equipped with an autosampler, a DB-624 column and a mass-selective detector (Agilent, Santa Clara, CA, USA) (see SI for details). Fluoride was quantified with an ICS-2100 (Dionex, Sunnyvale, CA, US) ion chromatography system equipped with a 4 mm × 250 mm IonPac AS18 hydroxide-selective anion-exchange column and a conductivity detector. MFA, TFA and acetate were measured with an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA, USA) equipped with an Aminex HPX-87H ion exclusion column (Bio-Rad Lab., Hercules, CA, USA) and a G1365D multiwavelength detector (Agilent, Santa Clara, CA, USA). Decanoate, sebacate were quantified as methyl esters, and FAME were analyzed by GC-HRMS following an established method (see SI for details).⁵¹ Oxygen and N₂O in culture headspace samples were quantified with an Agilent 7890A GC system equipped with an HP-PLOT/Q column (30 m length, 0.32 mm inner diameter, 20.0 µm film thickness) and a micro-electron capture detector. Optical density (OD_{600 nm}) readings were obtained after gentle vortexing 1 mL of culture suspension using a 20D+ spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Statistical analysis. Significant differences between growth yields with different hydrocarbons were examined by Duncan's multiple range test using SPSS Statistics (Version 26, SPSS Inc., Chicago, IL). The calculation of Pearson correlation coefficient between cell number and protein concentration measurements and the linear regression of OD_{600nm} and protein concentration measurements were both performed using SPSS Statistics (Version 26).

Results

Growth of *Pseudomonas* sp. strain 273 with fatty acids, alkanes and fluoroalkanes.

Pseudomonas sp. strain 273 grew with 1-fluorodecane (FD) as the sole source of carbon with concomitant release of inorganic fluoride in the presence of oxygen (Figure 1). Cultures completely consumed 5.97 ± 0.22 mM (11.94 ± 0.44 μ mol) FD within 1 week and produced 5.53 ± 0.23 mM (11.06 ± 0.46 μ mol) fluoride, which accounted for $92.7 \pm 3.8\%$ of the total fluorine contained in the substrate (Figure 1A). Growth and defluorination were also observed in cultures amended with 1-fluorononane, 1-fluorooctane and 1-fluoroheptane (Table 1). No growth occurred in cultures that received 0.5 mM 1-fluorohexane or 1-fluorohexane dissolved in an inert isocetane carrier phase that lowered the aqueous phase concentration of 1-fluorohexane to ~ 40 μ M. Strain 273 grew with the α,ω -fluorinated alkane DFD, and cultures completely consumed 5.62 ± 0.13 mM (11.24 ± 0.26 μ mol) DFD within 1 week with concomitant release of 10.14 ± 0.22 mM (20.28 ± 0.44 μ mol) fluoride, which accounted for $90.1 \pm 1.9\%$ of the total fluorine contained in the initial amount of DFD (Figure 1B). Neither defluorination nor growth occurred in autoclaved control cultures or in vessels without inoculum.

Growth also occurred with 1,10-dichlorodecane (DCD), decane, decanol, decanoate, sebacate, and acetate as the sole carbon and energy sources in the presence of oxygen (Table 1). No growth was observed in cultures amended with monofluoroacetate (MFA) or trifluoroacetate (TFA) as the sole carbon source; however, partial MFA degradation occurred in cultures that received decane as the primary substrate in the presence of oxygen. Over the course of a 17-day incubation period, 0.15 ± 0.03 mM MFA (7.35 ± 1.30 μ mol or $12.4 \pm 2.7\%$ of the initial amount) was consumed with the concomitant release of 0.15 ± 0.03 mM (7.26 ± 1.59 μ mol) fluoride (Figure S2). Defluorination of TFA was not observed.

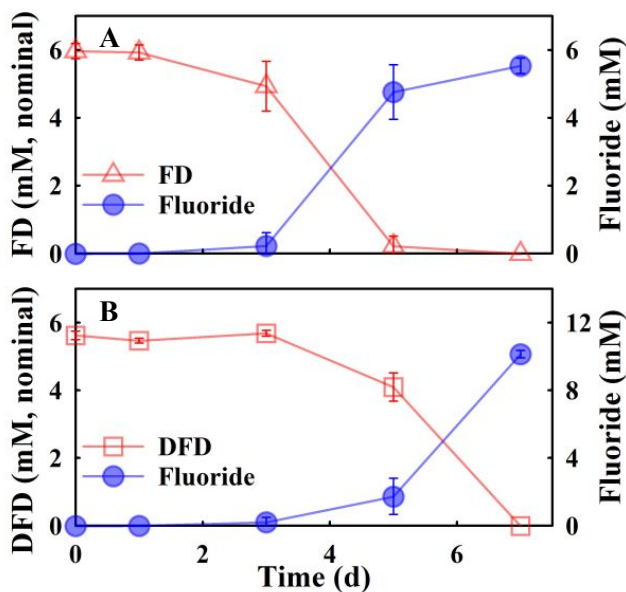


Figure 1. Degradation of C₁₀ fluorodecanes by *Pseudomonas* sp. strain 273 and the concomitant release of inorganic fluoride. Panel A shows degradation of FD and panel B shows DFD degradation. The data represent the averages of triplicate cultures and the error bars represent the standard deviations.

Strain 273 cultures that received decanoate, sebacate or acetate (i.e., fatty acids) as a carbon substrate produced $(10.55 \pm 0.82) \times 10^7$, $(9.71 \pm 1.15) \times 10^7$ and $(5.22 \pm 1.34) \times 10^7$ cells per μmol of carbon consumed, respectively (Table 2). Among all the alkane substrates, the highest growth yield of $(3.24 \pm 0.14) \times 10^7$ cells per μmol of carbon consumed was observed in cultures grown with decane. A slightly lower growth yield of $(2.48 \pm 0.15) \times 10^7$ cells per μmol of carbon consumed was observed with FD. Cultures grown with DFD or DCD reached significantly lower cell densities and $(1.62 \pm 0.23) \times 10^7$ and $(1.61 \pm 0.61) \times 10^7$ cells per μmol of carbon consumed were determined, respectively. Protein quantification was used as an independent measure of growth and corroborated the growth yields determined with qPCR (Table 2). Strain 273 produced 5.35 ± 0.32 , 5.03 ± 0.21 and 2.53 ± 0.14 μg of protein per μmol

of carbon consumed in cultures that were fed decanoate, sebacate or acetate, respectively. With decane, FD, DCD or DFD as a carbon substrate, 2.04 ± 0.31 , 1.89 ± 0.43 , 1.51 ± 0.75 and 1.24 ± 0.58 μg of protein per μmol of carbon consumed were measured, respectively (Table 2). Cell and protein yield measurements showed a significant correlation (Pearson correlation coefficient, $r = 0.95$, $p < 0.01$) (Figure S3), supporting the observation of lower growth yields with halogenated alkanes.

Resting cell experiments. Dense cell suspensions of strain 273 obtained with decane as a carbon source consumed decane, FD and DFD without apparent lag phase (Figure 2). DFD was consumed at the highest rate of 0.89 ± 0.09 $\mu\text{mol h}^{-1} \text{mg}^{-1}$ protein, and 10.30 ± 0.14 μmol of DFD was completely consumed over a 12-hour incubation period (Figure 2). FD and decane were consumed at lower rates of 0.55 ± 0.08 $\mu\text{mol h}^{-1} \text{mg}^{-1}$ protein and 0.63 ± 0.05 $\mu\text{mol h}^{-1} \text{mg}^{-1}$ protein, respectively.

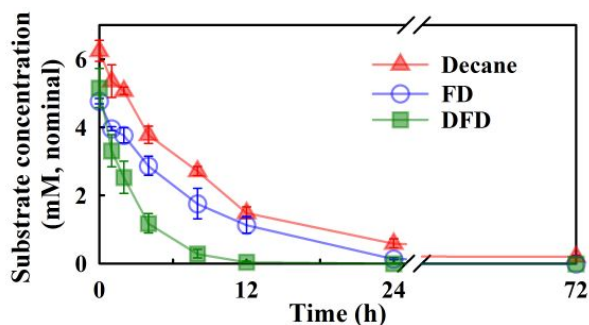


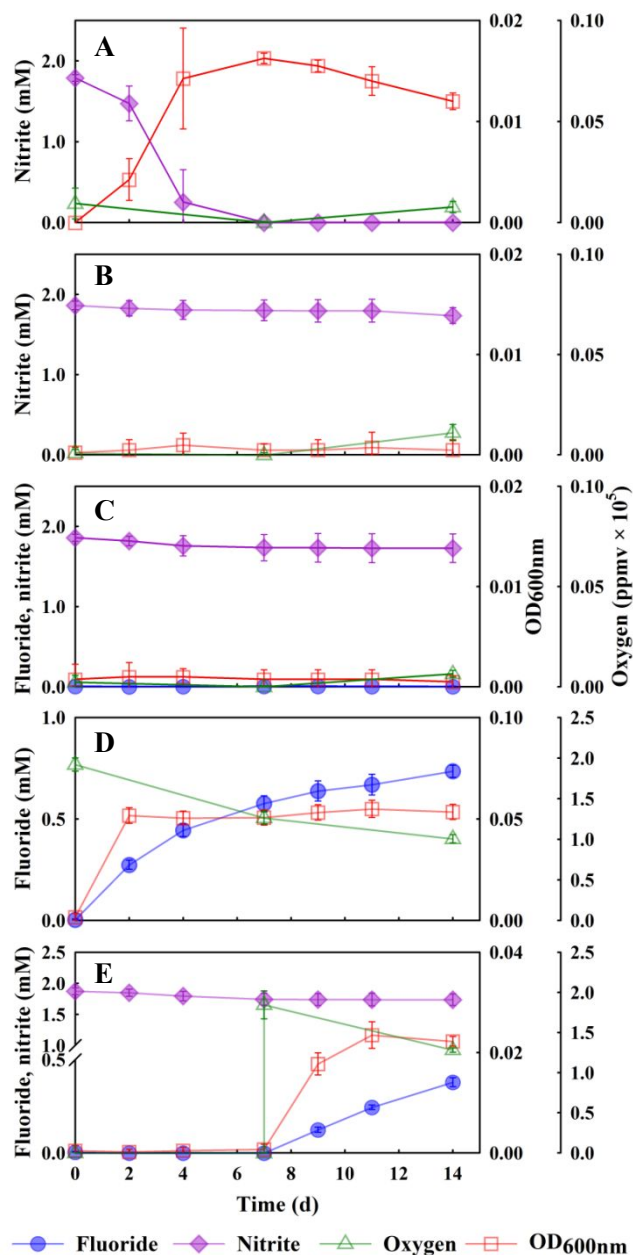
Figure 2. Consumption of decane, FD and DFD in resting cell suspension assays using *Pseudomonas* sp. strain 273 cells grown with decane. The data represent the averages of triplicate incubations and the error bars represent the standard deviations.

Oxygen requirement for (halo)alkane degradation. Growth without oxygen was tested in anoxic medium with nitrate, nitrite or N_2O as electron acceptor and excess acetate as electron

286 donor (Table S3). Complete consumption of $104.2 \pm 4.5 \mu\text{mol}$ ($2.08 \pm 0.09 \text{ mM}$) of nitrite was
287 coupled with the oxidation of $42.7 \pm 1.1 \mu\text{mol}$ ($0.85 \pm 0.02 \text{ mM}$) of acetate. No N_2O was
288 detected in the cultures at the end of the 7-day incubation period. In cultures that received 100.0
289 $\pm 11.6 \mu\text{mol}$ ($0.42 \pm 0.05 \text{ mM}$) of N_2O as electron acceptor, N_2O was completely reduced and
290 $27.8 \pm 4.4 \mu\text{mol}$ ($0.56 \pm 0.09 \text{ mM}$) of acetate were consumed over a 7-day incubation period,
291 indicating that strain 273 possesses a functional *nos* operon responsible for the reduction of N_2O
292 to N_2 . In cultures that received $112.0 \pm 9.2 \mu\text{mol}$ ($2.24 \pm 0.18 \text{ mM}$) of nitrite as electron acceptor
293 and the N_2O reductase inhibitor acetylene, $60.0 \pm 6.2 \mu\text{mol}$ ($0.25 \pm 0.03 \text{ mM}$) of N_2O were
294 measured after a 7-day incubation period indicating stoichiometric conversion of nitrite to N_2O .
295 Electron recoveries calculated for cultures that received nitrite or N_2O as electron acceptors
296 corroborated the involvement of the canonical denitrification pathway (Table S3). In cultures
297 amended with nitrite, nitrite plus acetylene, or N_2O , $91.5 \pm 4.0\%$, $84.8 \pm 7.0\%$ and $89.9 \pm 10.4\%$
298 of the electrons released from acetate oxidation were accounted for in the amount of electron
299 acceptor consumed, respectively. No electron recoveries were calculated for nitrate-amended
300 cultures because phosphate in the medium interfered with nitrate quantification.

301 In addition to acetate, strain 273 utilized decanol and decanoate as electron donors for
302 nitrate and nitrite reduction (Table 1). With decanoate (1 mM , $50 \mu\text{mol}$) as electron donor, 1.79
303 $\pm 0.04 \text{ mM}$ nitrite ($89.5 \pm 2.0 \mu\text{mol}$) was consumed over a 7-day incubation period (Figure 3A).
304 In anoxic cultures amended with nitrite as electron acceptor and decane as electron donor,
305 neither growth, nitrite consumption, nor decane degradation occurred (Figure 3B). Similar
306 observations were made with FD as electron donor, and no inorganic fluoride was released in
307 anoxic incubations with nitrite as electron acceptor (Figure 3C). Conversely, both growth and
308 fluoride release occurred in positive control vessels that received FD with oxygen as electron

309 acceptor (Figure 3D). In anoxic cultures amended with nitrite, the consumption of FD and the
 310 release of inorganic fluoride emerged after the nitrogen headspace had been replaced with air
 311 (Figure 3E).



312
 313 **Figure 3.** Utilization of C₁₀ (fluoro)alkanes and carboxylates by strain 273 under oxic (light red
 314 background) and anoxic (light grey background) conditions. Cultures were supplied with
 315 different electron donor/acceptor combinations including nitrite (2 mM), decanoate (1 mM),

nitrogen headspace (panel A); nitrite (2 mM), decane (1 mM), nitrogen headspace (panel B); nitrite (2 mM), FD (1 mM), nitrogen headspace (panel C); FD (1 mM), air headspace (panel D); FD (1 mM), nitrite (2 mM), nitrogen headspace, which was replaced with air after 7 days (panel E). The data represent the averages of four replicate cultures and the error bars represent the standard deviations.

Detection of (halo)alkane fatty acid metabolites. Microbes metabolize alkanes via β -oxidation to shorter chain fatty acids.⁵² GC-HRMS was used to profile the free fatty acid content of strain 273 grown with decane, FD, or DFD at one time point during active growth (Table S4). Non-fluorinated C₅ to C₁₀ saturated fatty acids were detected in decane-grown cells. Even-chain fluorinated fatty acids, including fluorodecanoate, fluorooctanoate, and fluorohexanoate, were detected in cells grown with DFD and fluorohexanoate was measured in cells grown with FD (Figure 4).

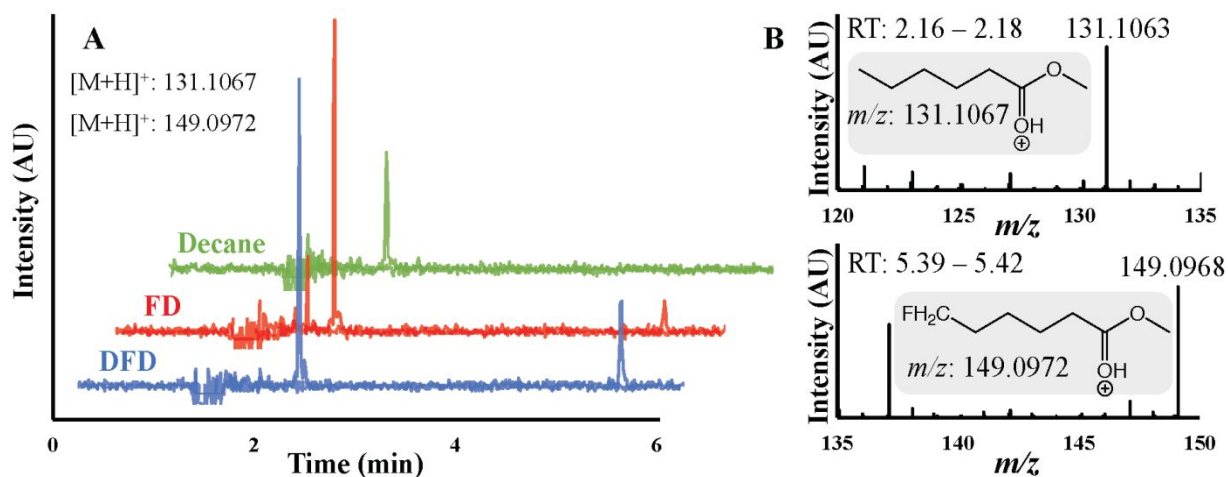


Figure 4. Detection of fluorohexanoate as an intermediate of FD and DFD metabolism by strain 273. (A) Merged extracted ion chromatograms of methyl hexanoate ($[M+H]^+ = 131.1067$ m/z) and methyl fluorohexanoate ($[M+H]^+ = 149.0972$ m/z) in cultures of strain 273 grown with

decane, FD or DFD. (B) GC-HRMS identification of methyl hexanoate and methyl fluoroheptanoate with a mass accuracy tolerance of 3 ppm to the monoisotopic mass of the putative structure illustrated.

Discussion

Only a few organisms have been described that degrade halogenated alkanes, and the substrate range for most of the characterized halogenated alkane degraders has been restricted to chlorinated-, brominated- and iodinated- alkanes.⁵³⁻⁵⁵ In contrast, *Pseudomonas* sp. strain 273, the focus of the current study, utilizes C₇ to C₁₀ monofluoroalkanes and DFD as growth substrates with concomitant release of inorganic fluoride. Apparently, this organism possesses one or more enzyme systems that lead to C-F bond breakage in alkanes with fluorine substitution in one or both terminal methyl groups.

Oxidation of the terminal carbon and degradation pathway of (halo)alkanes in strain 273.

The growth studies demonstrated that alkane degradation in strain 273 only occurred in the presence of oxygen. Presumably, oxygen is a required co-substrate for the initial attack in the terminal carbon atom and conversion to the respective alcohols via a monooxygenase catalyzed reaction.⁵⁶ Prior work implicated the same monooxygenase enzyme system of strain 273 in the initial attack on decane and on DCD.⁴⁵ The lack of an apparent lag phase when dense cell suspensions of decane-grown cells were challenged with FD or DFD (Figure 2) suggested that *de novo* enzyme biosynthesis was not required to initiate FD and DFD degradation. Therefore, it is likely that the same monooxygenase enzyme system is responsible for the initial attack on decane, FD and DFD. An alternate explanation would be the constitutive expression of a monooxygenase that causes C-F bond cleavage, and detailed expression studies under various

growth conditions would be needed to reveal if a single or multiple monooxygenase enzyme systems are involved in (halo)alkane degradation.

In light of the experimental observations, a single pathway is likely utilized for the degradation of (halo)alkanes in strain 273 (Figure 5). The detection of fluorinated fatty acids (e.g., fluorohexanoate) in cells grown with FD and DFD suggests that the initial oxidation can occur at either the terminal methyl ($-\text{CH}_3$) or fluoromethyl ($-\text{CH}_2\text{F}$) carbon. With decane as growth substrate, the initial oxidation product would be the corresponding alcohol (i.e., decanol), which would then be oxidized to a carboxylic acid to undergo β -oxidation or channeled into anabolic pathways. Analogously, the initial attack of a monooxygenase on the terminal fluoromethyl group carbon would generate an unstable geminal fluorohydrin that would spontaneously eliminate fluoride to form the corresponding aldehyde, which would be further oxidized to the carboxylic acid.^{19,57} No monooxygenase enzyme system of strain 273 has been characterized, and it is unknown whether the enzyme(s) has (have) a preference for the terminal methyl group carbon or the fluoromethyl group carbon. For both FD and DFD, β -oxidation could lead to the formation of fluoroacetyl-CoA, which is potentially toxic (Figure 5). Utilization of fluoroacetyl-CoA by the tricarboxylic acid cycle (TCA) cycle would lead to the formation of fluorocitrate, a known inhibitor of aconitase, a key TCA cycle enzyme.⁵⁸⁻⁶⁰ Although MFA did not support growth of strain 273, some fluoride release was observed with decane serving as the primary substrate, suggesting that strain 273 has inherent mechanisms to defluorinate MFA and potentially other ω -fluorofatty acids (or the respective CoA thioesters).

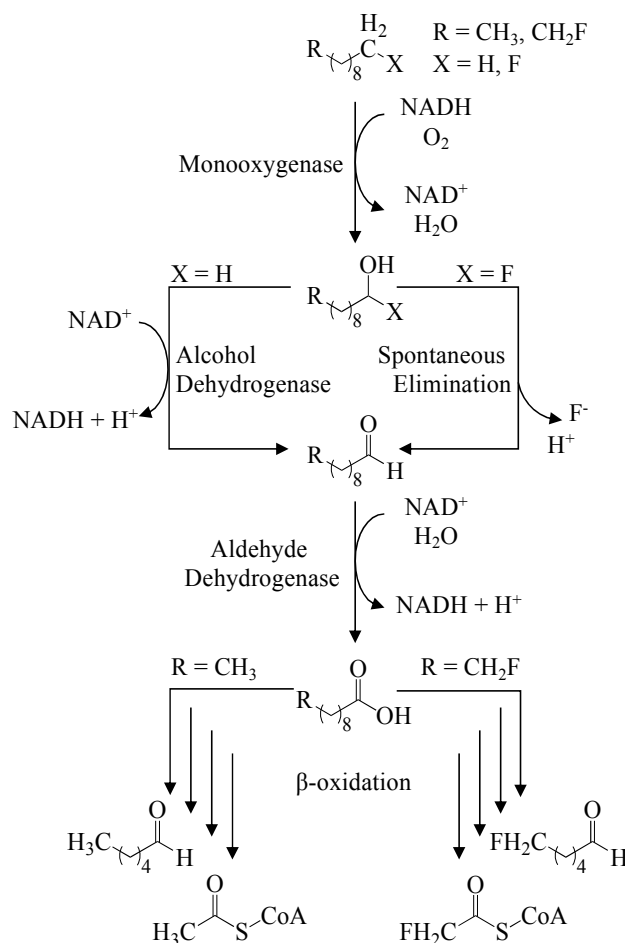


Figure 5. Proposed pathways for decane, FD, and DFD catabolism in *Pseudomonas* sp. strain 273. Fluorohexanoate and hexanoate were identified by GC-HRMS analysis and are shown as intermediates.

Theoretically, both terminal carbon atoms of the alkane could be attacked by the monooxygenase^{19,61}, which would transform FD and DFD to the corresponding dicarboxylates. Such a mechanism would avoid the formation of fluoroacetyl-CoA. A purified monooxygenase from the alkane degrader *Pseudomonas oleovorans* could not only hydroxylate *n*-alkanes to the corresponding primary alcohols but also convert fatty acids to their ω -hydroxy derivatives^{62,63}, suggesting that attacks on both ends of the alkane molecule by the same monooxygenase enzyme

system are feasible. However, the GC-HRMS analysis provided no evidence for the formation of C_4 to C_{10} dicarboxylic acids, suggesting that an attack on both ends of the molecule did not occur (Figure 5).

Metabolic burden of (fluoro)alkane degradation. Strain 273 attained approximately 3-fold more biomass per μmol of carbon utilized with decanoate or sebacate compared to decane as carbon substrate (Table 2). Even lower growth yields were measured in cultures grown with halogenated alkanes, which declined about 50% with DFD and DCD compared to the yield with decane (Table 2). The standard Gibbs free energy changes associated with complete oxidation of decane, halogenated C_{10} alkanes, decanoate and sebacate are in a similar range (Table S5); thus, thermodynamics alone cannot explain the measured growth yield differences. Possible explanations for the lower growth yields with decane and (halo)alkanes compared to the corresponding fatty acids may include the required synthesis of the monooxygenase enzyme system(s) and/or the production of biosurfactant(s) needed for emulsifying the sparingly soluble (halo)alkanes. Strain 273 efficiently solubilizes water-insoluble alkanes with a yet uncharacterized biosurfactant(s).⁴⁵ The production of biosurfactants by prolific oil hydrocarbon-degrading bacteria is a well-studied phenomenon, and several *Pseudomonas* spp. were shown to produce rhamnolipids to emulsify hydrocarbons with a low solubility in water.⁶⁴⁻⁶⁶ Conversely, growth with water-soluble decanoate and sebacate does not burden the cells with the synthesis of the monooxygenase enzyme system and biosurfactant(s). This reasoning cannot explain the lower growth yields with FD and DFD compared to cultures that were grown with decane. The GC-HRMS analysis detected ω -fluorofatty acids as intermediates during growth with both FD and DFD. ω -Fluorofatty acids are precursors of potentially inhibitory fluoroacetyl-CoA. For FD, preferential initial oxidation of the fluoromethyl carbon would avoid the formation of ω -

fluorinated intermediates and fluoroacetyl-CoA. In this case, cultures fed with FD or decane should exhibit similar growth yields; however, the experimental data determined about $23.4 \pm 4.7\%$ lower growth yields with FD. This observation suggests that the monooxygenase may not discriminate between the α and the ω positions of FD and oxidizes both the methyl and the fluoromethyl group carbon atoms. In the case of DFD, the initial attack must occur on one of the two fluoromethyl groups, and the formation of fluorinated metabolites (e.g., ω -fluorofatty acids, fluoroacetyl-CoA) could explain the observed reduction in growth yields with DFD (Table 2). Fluoride toxicity offers another potential explanation for the lower growth yields with FD and DFD, which would only occur during growth with fluorinated electron donors.⁶⁷

Implication for the environmental fate of fluorinated compounds. Knowledge about the presence and the distribution of organofluorines in environmental systems is rapidly evolving, and improved understandings of the processes that affect the fate and longevity of fluorinated compounds is needed. Physical,⁶⁸ chemical,⁶⁹ and biological^{70, 71} approaches, as well as combined treatments⁷² have been shown to transform PFAS and generate partially defluorinated alkyl products. The data herein demonstrate that α,ω -fluorinated alkanes are susceptible to microbial degradation, and organisms with defluorinating oxygenase enzyme systems may contribute to the degradation of partially defluorinated transformation products generated during aggressive remedial treatment of PFAS. Therefore, future efforts should explore whether organisms like *Pseudomonas* sp. strain 273 have value in sequential treatment (i.e., combined remedy) approaches for PFAS.

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437

438 **Supporting information**

439 Detailed experimental procedures including protein measurements, cell enumeration with qPCR,
440 derivatization of decanoate, sebacate and FAME analysis, and supplemental analytical
441 procedures are provided in the Supporting Information. Tables S1, S2 and S5 are provided in a
442 separate Excel file. Table S1: Solubility and *n*-octanol/water partition coefficient data for the
443 carbon substrates tested; Table S2: Oxygen demand for complete oxidation of different carbon
444 substrates; Table S3: Electron recovery in strain 273 cultures grown with nitrite or nitrous oxide
445 as electron acceptors; Table S4: FAME metabolites detected in cultures of strain 273 grown with
446 decane, FD or DFD. Table S5: Gibbs free energy changes associated with the complete oxidation
447 of different carbon substrates. Figure S1: Linear regression of OD_{600nm} readings versus protein
448 concentration measurements; Figure S2: Co-metabolism of MFA with decane as the primary
449 substrate by strain 273; Figure S3: Pearson correlation between cells produced and protein yields
450 per μmol of carbon consumed during growth of *Pseudomonas* sp. strain 273 with different
451 carbon substrates.

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639

640 **Figure legends**

641 **Figure 1.** Degradation of C₁₀ fluorodecanes by *Pseudomonas* sp. strain 273 and the concomitant
642 release of inorganic fluoride. Panel A shows degradation of FD and panel B shows DFD
643 degradation. The data represent the averages of triplicate cultures and the error bars represent the
644 standard deviations.

645
646 **Figure 2.** Consumption of decane, FD and DFD in resting cell suspension assays using
647 *Pseudomonas* sp. strain 273 cells grown with decane. The data represent the averages of
648 triplicate incubations and the error bars represent the standard deviations.

649
650 **Figure 3.** Utilization of C₁₀ (fluoro)alkanes and carboxylates by strain 273 under oxic (light red
651 background) and anoxic (light grey background) conditions. Cultures were supplied with
652 different electron donor/acceptor combinations including nitrite (2 mM), decanoate (1 mM),
653 nitrogen headspace (panel A); nitrite (2 mM), decane (1 mM), nitrogen headspace (panel B);
654 nitrite (2 mM), FD (1 mM), nitrogen headspace (panel C); FD (1 mM), air headspace (panel D);
655 FD (1 mM), nitrite (2 mM), nitrogen headspace, which was replaced with air after 7 days (panel
656 E). The data represent the averages of four replicate cultures and the error bars represent the
657 standard deviations.

658
659 **Figure 4.** Detection of fluorohecanoate as an intermediate of FD and DFD metabolism by strain
660 273. (A) Merged extracted ion chromatograms of methyl hexanoate ($[M+H]^+ = 131.1067\ m/z$)
661 and methyl fluorohecanoate ($[M+H]^+ = 149.0972\ m/z$) in cultures of strain 273 grown with
662 decane, FD or DFD. (B) GC-HRMS identification of methyl hexanoate and methyl

663 fluorohexanoate with a mass accuracy tolerance of 3 ppm to the monoisotopic mass of the
664 putative structure illustrated.

665

666 **Figure 5.** Proposed pathways for decane, FD, and DFD catabolism in *Pseudomonas* sp. strain
667 273. Fluorohexanoate and hexanoate were identified by GC-HRMS analysis and are shown as
668 intermediates.

Table 1. Growth and defluorination observed in cultures of *Pseudomonas* sp. strain 273 with different electron donor/electron acceptor combinations.

Electron donor (mM)	Electron acceptor (mM)	Growth ^a	Defluorination ^b
Decane (1 ^c , 5 ^c)	Oxygen	+	NA
Decane (1 ^c)	Nitrite (2)	-	NA
1-Fluorodecane (0.5 ^c , 1 ^c , 5 ^c , carrier ^d)	Oxygen	+	+
1-Fluorodecane (0.5 ^c , 1 ^c)	Nitrite (2)	-	-
1-Fluorodecane (0.5 ^c)	Nitrous oxide (0.42)	-	-
1,10-Difluorodecane (1 ^c , 5 ^c)	Oxygen	+	+
1-Fluorononane (0.5 ^c , carrier ^d)	Oxygen	+	+
1-Fluorooctane (0.5 ^c , carrier ^d)	Oxygen	+	+
1-Fluoroheptane (0.5, 1 ^c) ^e	Oxygen	+	+
1-Fluorohexane (0.5, carrier ^d)	Oxygen	-	- ^f
1,10-Dichlorodecane (1 ^c)	Oxygen	+	NA
Acetate (2, 5, 10)	Oxygen	+	NA
Acetate (5, 10)	Nitrite (1, 2)	+	NA
Acetate (5, 10)	Nitrate (2)	+	NA
Acetate (5, 10)	Nitrous oxide (0.42)	+	NA
Monofluoroacetate (1, 2)	Oxygen	-	-
Trifluoroacetate (2)	Oxygen	-	-
Monofluoroacetate (1) + decane (5 ^c)	Oxygen	+	+
Trifluoroacetate (2) + decane (5 ^c)	Oxygen	+	-
Decanol (1 ^c)	Oxygen	+	NA
Decanol (1 ^c)	Nitrite (2)	+	NA
Decanoate (1)	Oxygen	+	NA
Decanoate (1)	Nitrite (2)	+	NA
Decanoate (1)	Nitrate (2)	+	NA
Sebacate (1)	Oxygen	+	NA

^a Growth was determined by the increase of culture turbidity and the degradation of substrates

^b Defluorination was determined by the detection of released fluoride in medium

^c Nominal concentration

^d An isocetane carrier phase was used to sustain a low concentration of electron donor in the aqueous phase.

676 ^e Independent experiments demonstrated growth with 0.5 and 1 mM 1-fluoroheptane but not in
677 systems with an isocetane carrier phase.

678 ^f Some fluoride release from 1-fluorohexane was observed in cultures that received decane as a
679 primary substrate.

680 NA: Not applicable

681 **Table 2.** *Pseudomonas* sp. strain 273 growth yields with different carbon substrates.

Carbon substrate (electron donor) ^a	Carbon substrate consumed (mM)	Carbon consumed (μmol)	Growth yield ^b	
			Cell numbers (× 10 ⁷ /μmol carbon)	Protein (μg/μmol carbon)
Acetate	6.96 ± 0.18	139.18 ± 3.54	5.22 ± 1.34 ^B	2.53 ± 0.14 ^B
Decanoate	1.26 ± 0.02	125.86 ± 1.64	10.55 ± 0.82 ^A	5.35 ± 0.32 ^A
Sebacate	1.23 ± 0.04	123.37 ± 4.49	9.71 ± 1.15 ^A	5.03 ± 0.21 ^A
Decane	1.55 ± 0.07	154.60 ± 7.03	3.24 ± 0.14 ^C	2.04 ± 0.31 ^{B,C}
FD	1.46 ± 0.04	145.56 ± 3.60	2.48 ± 0.15 ^{C,D}	1.89 ± 0.43 ^{B,C,D}
DFD	1.53 ± 0.06	153.30 ± 6.48	1.62 ± 0.23 ^D	1.24 ± 0.58 ^D
DCD	1.44 ± 0.07	143.62 ± 7.23	1.61 ± 0.61 ^D	1.51 ± 0.75 ^{C,D}

682 ^a The carbon substrates were completely consumed after a 1-week incubation period.

683 ^b Cell numbers were determined with qPCR and protein amounts were estimated after alkaline
684 cell lysis using the Bradford assay. The yield data represent the net increase of cells and protein
685 per μmol of carbon consumed. For both the qPCR and the protein datasets, the Duncan test (α =
686 0.05) observed significant differences between A, B, C and D.

687 The data represent the averages and the standard deviations of triplicate cultures.

689

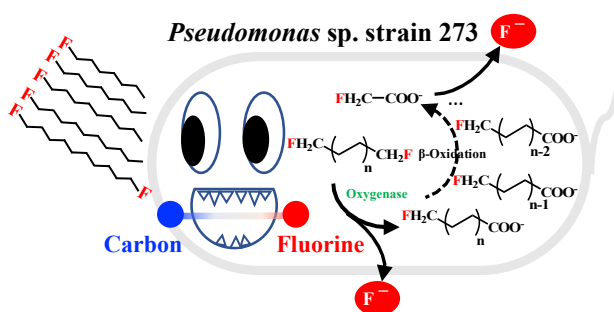


Figure 1

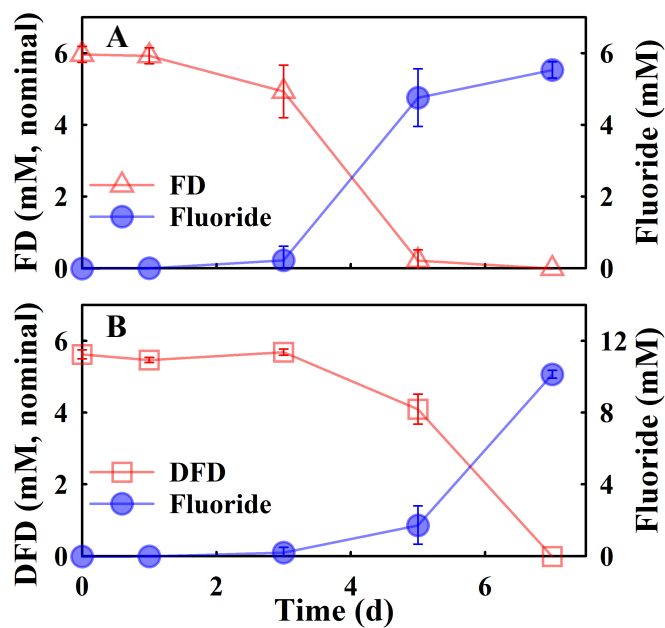


Figure 2

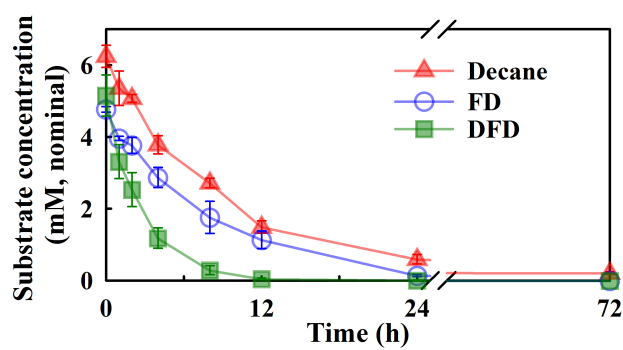


Figure 3

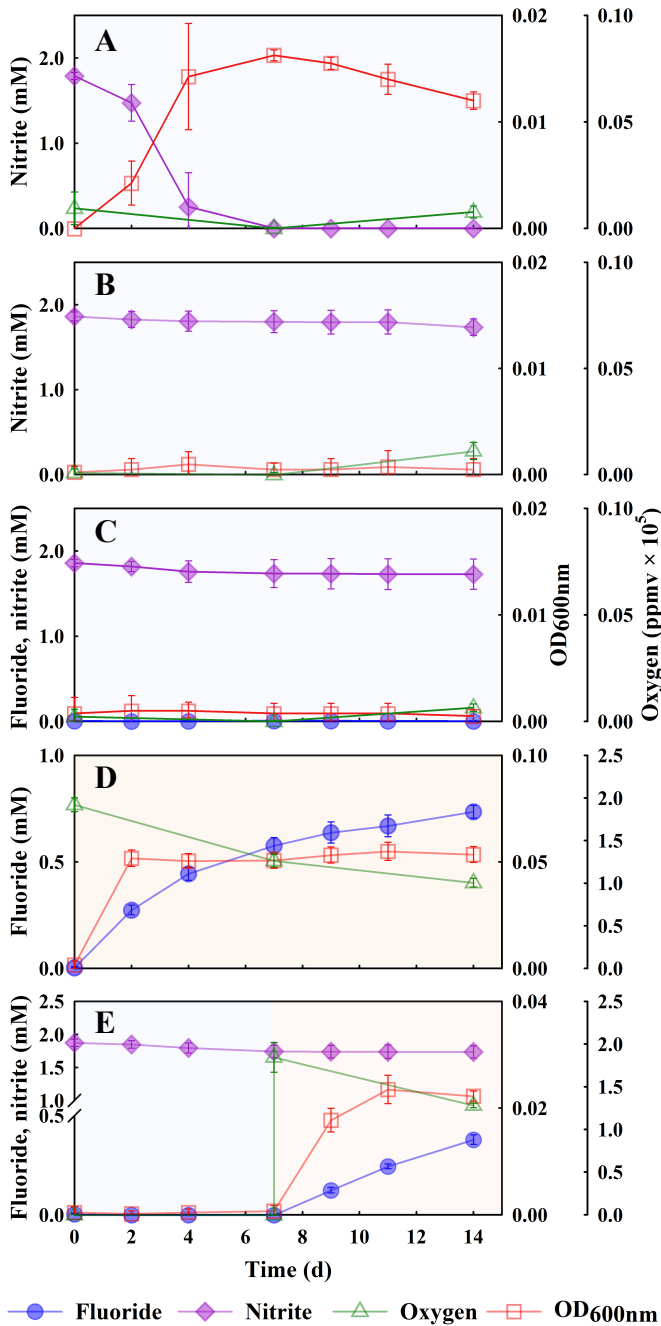


Figure 4

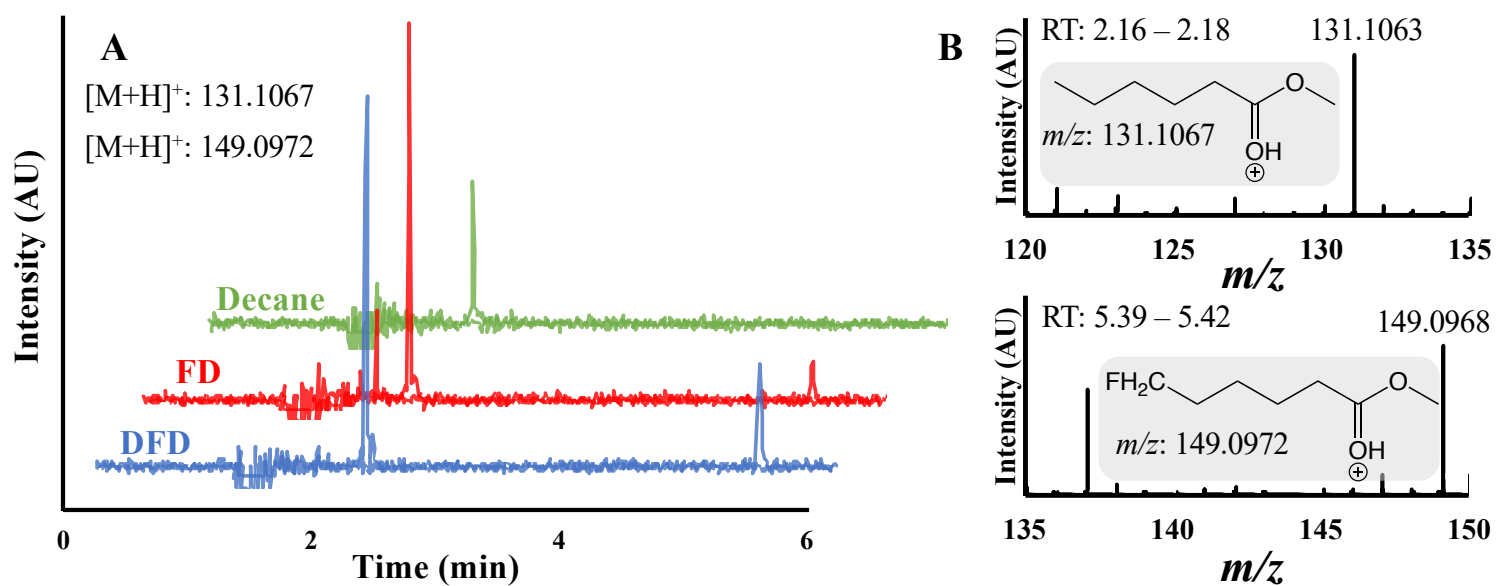


Figure 5

