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Systematic evaluation of fluoride quantification methods identifies an appropriate measurement for studying fungal defluorination of per- and Polyfluoroalkyl substances

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ABSTRACT

The large-scale manufacturing and disposal of Per- and Polyfluoroalkyl substances (PFASs) are causing global concerns, and investigations are needed to understand their ecological impacts. Saprotrophic fungi are dominant nutrient recyclers in ecosystems, while their roles in transforming PFAS remain largely untapped. Particularly, there is a lack of appropriate means to rapidly measure fluoride anions released by fungal cultures to understand their defluorination (deF) capacities and mechanisms. In this research, we systematically evaluated three prevalent means for quantifying free fluoride anions under cultural conditions of wood decay fungal species. Two spectrophotometric measurings, including one based on the fluoride-catalyzed kinetochromic reaction of Xylenol Orange-Zirconium (XO-Zr^{4+}) and another one based on Alizarin-Lanthanum-Fluoride (Al–La^{3+}-F^-) ternary complexone reaction, were successfully adapted to a high-throughput micro-well setup for detecting micromolar-level fluoride, but essential fungal media components pose significant interference to both assays. The third method, relying on the potentiometer and fluoride ion-selective electrode (F-ISE), demonstrated a strong capacity for anti-interferences of fungal nutrients, metabolites, and PFAS, leaving it a most appropriate method to study fungal deF. With F-ISE, we further demonstrated a deF test using a model PFAS, 4,4,4-trifluoro-3-(trifluoromethyl) crotonic acid, and a wood rot fungus *Trametes versicolor*. Together, our research identifies a reliable fluoride quantification method that can allow assessing fungal cultures for their PFAS-defluorinating phenotypes.

1. Introduction

The large-scale environmental disposal of fluorinated carbon chemicals, such as per- and poly-fluoroalkyl substances (PFAS), is causing emerging concerns of the US EPA (United States Environmental Protection Agency) and the general public (Abunada et al., 2020). PFASs are the structural chemicals of widely used consumable products and fire-fighting foams, and their inert 'F-C' bonds provide them with non-biodegradable features and make them a growing source of pollution of concern (Bentel et al., 2019; Buck et al., 2011; Wang and Liu, 2020). Traditional management approaches are facing difficulties in treating these chemicals (Coggan et al., 2019; Lang et al., 2017; Liou et al., 2010), and as entering into the environment through landfill disposals and run-offs (Anderson et al., 2016; Brusseau et al., 2020; Lang et al., 2017), these chemicals are inevitably causing impacts on the ecosystem and organismal processes therein.

Saprotrophic wood decay fungi are crucial nutrient recyclers in the terrestrial ecosystem, and they use inherent degradative systems to deconstruct the polymeric structure of woody biomass to utilize the embedded nutrient elements that include primarily carbon but also nitrogen, minerals, and others (Andlar et al., 2018; Navarro et al., 2021). Given that the hydrolytic and oxidative enzymes they employ have broad substrate specificity, wood decay fungi are also found capable of degrading xenobiotic pollutants, such as plastics, organic dyes, and halogenated pharmaceuticals (Zhuo and Fan, 2021). In regard to PFAS, recent studies suggested that wood decay fungi may also hold the metabolic potential to degrade the inert fluorinated carbon structures. The relevant research was mainly conducted by Dr. Shaily Mahendra's group (Merino et al., 2018, 2023; Tseng et al., 2014), which studied the degradation of 6:2 fluorotelomer alcohol [6:2 FTOH; F$(CF_2)_6CH_2CH_2OH$] by several wood decay fungal species, using LC/MS/MS to identify the transformation products. Their research suggested that wood decay fungi can employ the yet-to-be-known biochemical pathways to transform 6:2 FTOH into a mixture of shorter

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chain poly- and perfluoroalkyl carboxylic acids (PFCA) [e.g., perfluorooctanoic (PFPeA) and perfluorooctanoic (PFHxA) acids] and other poly-fluoroalkyl analogs/conjugates. However, in these research, free fluorides were not detected; thus, it remains unclear if wood decay fungi can defluorinate PFAS. As the cleavage of the inert F-C bond, namely defluorination (deF), is recognized as a crucial step towards breaking down PFAS (Kucharzyk et al., 2017), being capable of quantifying the fluoride anions in fungal cultures would be crucial to further confirming the fungal potentials to degrade PFAS and to exploring fungal roles in responding to the emerging PFASs.

Establishing an appropriate fluoride anion-detecting method is key to studying the fungal deF. To date, a number of methods for the detection of fluoride have been explored to evaluate bacterial cultures for deF capacity, which include colorimetric methods such as that based on zirconyl xylenol orange (Cabello-Tomas and West, 1969; Harwood and Huyser, 1968; Macejunas, 1969) or alizarin-lanthanum complexone (Belcher and West, 1961a, 1961b; Bygd et al., 2022), as well as the methods using potentiometer [e.g., Fluoride Ion Selective Electrode (F-ISE)] and ion-chromatography (IC) (Huang and Jaffé, 2019). The modified colorimetric approaches in micro-well plates have been adopted as a higher throughput means to screen large numbers of bacterial cultures for deF, even though they are sometimes susceptible to chemicals in the culture media and to reaction conditions. However, no fluoride-detecting tools have been made available for fungi that often require distinct nutrients and cultural conditions to grow.

This work, therefore, seeks to systematically evaluate and compare different fluoride detection methods and to adapt the most appropriate one to screen fungal cultures for their capacities to deF PFASs. Our results indicated that while the colorimetric approaches are both highly sensitive with detection limits comparable to that of F-ISE and compatible with 96-micro-well assays, the essential fungal media components pose significant interference to these methods. F-ISE stood out as the most reliable method for detecting fungal deF. The resilience of F-ISE in measuring fluoride produced by fungal cultures was further proved by monitoring the deF of a model PFAS, 4,4,4-trifluoro-3-(trifluoromethyl) crotonic acid, that has been proved as a defluorinable PFAS in many microbial organisms due to the electron-attracting alkenyl group (C=C) provokes the cleavage of adjacent C-F bond (Che et al., 2021).

2. Materials and Methods

PFAS and fluoride chemicals PFASs and sodium fluoride were purchased from Sigma Aldrich [PFOA, CAS# 335-67-1, 98 % purity; PFOS, 1763-23-1, 98 % purity; NaF, 7681-49-4, 99.99 % purity] and Matrix Scientific [4,4,4-trifluoro-3-(trifluoromethyl) crotonic acid, 1763-28-6, 97 % purity]. Stock solutions at the concentration of 10 mM were prepared aseptically by dissolving PFAS or sodium fluoride in sterilized deionized water and were stored at room temperature under constant agitation at 150 rpm until use. Polypropylene bottles containing the stock solutions were sonicated for 20 min and mixed prior to the spiking of fungal cultures.

Xylenol Orange-Zirconyl reaction The kinetochromic reaction of Xylenol Orange–Zirconyl (XO-Zr^{4+}) catalyzed by fluoride ion was adapted from Cabello-Tomas and West (1969) and was further developed with a microwell system to improve the throughput of F^- measurements. To enable the reaction, a 10 mM zirconium oxychloride octahydrate (CAS#13520-92-8) stock solution was prepared in 12.5 mM hydrochloric acid (HCl) and was aged at 4 °C overnight prior to making the 500 μM Zirconyl working solution by diluting the stock solution in 60 mM HCl. The zirconyl solutions should be aged at 4 °C overnight prior to use and must be discarded after 5 days of storage or if it becomes warm. The 500 μM Xylenol Orange (XO) solution was prepared by dissolving xylenol orange tetrasodium salt (CAS#3618-43-7) in DDI water and was adjusted to the pH of 4.8 with HCl. XO solution should be stable within 72 h. F^- standards were prepared by serial dilution of a NaF stock solution prepared in DDI or media background dissolving the required amount of sodium fluoride in DDI water or in the background solutions of different nutrition required for fungal cultivation, and all fluoride solutions should be stored in polypropylene plastic (silicon-free) bottles to avoid concentration loss caused by the container.

A microwell XO-Zr^{4+} reaction procedure was optimized to determine the F^- concentration, and the proper sequence of reagent addition was essential to this measurement. Specifically, to 100 μL fluoride-containing sample, 12.5 μL XO working solution was added, followed by 17.5 μL of 5 M HCl. After a thorough mix, 25 μL zirconyl working solution was then added as uniformly quickly as possible, which was followed by a rapid dilution with 95 uL cold DDI water and a thorough mix using a multichannel pipette. While setting up the reaction, all solutions should be stored on ice, and the reaction should be kept at a constant temperature (15–20 °C). After a 90-min reaction, the absorbance of the solution was measured at 550 nM with a VarioSkan Lux 40 microplate reader (Thermo Fisher Scientific). Resulting absorbance values were plotted against F^- concentrations for regression analysis, testing the adequacy of the above method for fluoride determination. Spectral scanning from the wavelength of 350–620 nm was conducted to understand the reaction mechanisms. Notably, the reaction under current conditions occurs rapidly and is temperature sensitive. No impact was observed when measuring [F-] in DDI, but a red orange precipitate was rapidly formed when using HMM media that prevented accurate fluoride quantification.

Alizarin-Lanthanum-Fluoride reaction A second colorimetric fluoride-determining method relying on the chelate reaction of Alizarin complexone, Lanthanum (III), and Fluoride ions (Al–La^{3+}-F) was adapted from Belcher and West (1961b) and tested in a microwell setup. This method applies a principle similar to that of the commercial Spectroquant® Fluoride test kit and is analogous to EPA 340.3 and APHA 4500-F-E. To prepare the reaction, the 500 μM alizarin working solution was made by firstly dissolving alizarin-3-methyliminodiacetic acid (CAS# 3952-78-1) in DDI water aided by adding 2 N sodium hydroxide to ensure the complete solution of alizarin, and then by adjusting the final pH to about 5–6 with HCl, a process accompanied by the color change from violet to red. The 500 μM lanthanum solution was prepared by dissolving Lanthanum (III) nitrate hexahydrate (CAS# 10277-43-7) in DDI water. Acetate buffer (pH 5.2) was prepared by dissolving 100 g of sodium acetate trihydrate in DDI water, followed by the addition of 11 mL glacial acetic acid and a final volume fixed to 1 L. F^- standards in the background DDI water and medium components were prepared as above.

The Al–La^{3+} working solution was freshly prepared prior to each test and used within 3 h, which was made by first mixing the 500 μM alizarin solution with acetate buffer, followed by addition of the 500 μM lanthanum solution, thus reaching the final concentrations of 50 μM for alizarin and lanthanum and 84 μM for the acetate buffer, respectively. To setup the microwell assay, the 100 μL fluoride-containing sample was mixed with 50 μL Al–La^{3+} working solution, and then was diluted with 50 μL of acetone. The reaction reached its maximum sensitivity after 90 min when the absorbance values were read at 620 nm and 530 nm with the VarioSkan Lux 40 microplate reader (Thermo Fisher Scientific), and a standard curve was created by plotting the 620/530 nm ratio against fluoride concentration. As for the XO-Zr^{4+} reaction, absorbance values of the Al–La^{3+}-F complex were monitored by scanning a spectrum ranging from 360 to 700 nm to understand the reaction mechanisms.

Fluoride Ion Selective Electrode (F-ISE) measurement The potentiometric method for measuring fluoride ion was optimized using the Orion Star™ A214 pH/ISE benchtop meter (Thermo Fisher Scientific) and the Orion™ Fluoride IonPlus Sure-Flow Solid State Combination Electrode (CAS# 9609BNWP). Two Total Ionic Strength Adjuster Buffers (TISAB) were tested for their effects in eliminating the interference of fungal medium components. The Low-level TISAB was prepared by dissolving 57 mL glacial acetic acid and 58 g of NaCl in DDI water, adjusting the pH with 5 M NaOH to 5.0–5.5, and finally fixing the total

volume to 1 L with DDI water. TISAB II, with the CDTA and a stronger buffering capacity (CAS# 940909), was purchased from Thermo Fisher Scientific.

To measure the F^- in DDI water or in the context of different medium components, TISAB was mixed 1:1 with the F^- solution and incubated for 15 min at room temperature (20–25 °C) before analysis. F-ISE measurement was done according to the manufacturer's instructions. After calibration in storage solution for 5 min, the probe was immersed in F^- solution for recording the millivolt (mV) reading in 5 min or until the reading was stable. The probe was rinsed and allowed to rest in a storage solution between different measurements. In this procedure, all solutions must be kept at the same temperature and pH (5–5.5), given that the electrode potentials are affected by the changes in these two factors. The mV readings were plotted logarithmically against F- standard concentrations for calculating the calibration curve and for determining the F- concentrations in samples.

Fungal medium Highley's minimum medium (HMM) (Highley, 1973) was used as the fungal medium to test its components' influences on F^- quantification, which contains 1 g/L glucose and 10 g/L aspen wood sawdust and the following basal salts additions (per liter): Essential nutrients mix I: 2 g NH_4NO_3, 2 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.1 g $CaC1_2{\cdot}2H_2O$; Trace metal mix II: 0.57 mg H_3BO_4, 0.036 mg $MnC1_2{\cdot}4H_2O$, 0.31 mg $ZnSO_4{\cdot}7H_2O$, 0.039 mg $CuSO_4{\cdot}5H_2O$, 0.018 mg $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.015 mg $FeSO_4{\cdot}7H_2O$; and 0.001 g thiamine hydrochloride.

Fungal strain and cultural conditions Wood decomposer fungal strain *Trametes versicolor* A1-ATF was obtained from Forest Mycology Collection at the University of Minnesota, and it was stored on YMG (Yeast extract 4 g/L, Malt extract 10 g/L, Glucose 10 g/L) agar slants at 4 °C until being used for evaluating fluoride-quantifying methods and monitoring PFAS degradation. Fungal mycelia were activated on YMG agar plates at 28 °C for 6 days prior to testing their defluorination capacities with liquid culturing conditions.

Liquid cultures were conducted in replicates of five in 125 ml Erlenmeyer flasks containing 50 ml of HMM medium spiked to 100 μM of 4,4,4-trifluoro-3-(trifluoromethyl) crotonic acid to test fungal defluorination. HMM was supplemented with 1 g/L glucose and 10 g/L aspen wood sawdust as carbon sources. Ten 5-mm activated mycelial plugs were used to inoculate each flask and cultured at 25 °C and 110 rpm. Two milliliters of sample per flask were aliquoted and were centrifuged at 10, 000 rpm and 4 °C for collecting the supernatant for F^- quantification. The inoculated flasks without spiking PFAS or with killed fungus were prepared as the controls.

Fluoride detection in fungal cultures Fungal extracts collected from liquid cultures, as above, were measured for fluoride concentrations using F-ISE. The TISABII buffer w/CDTA was mixed 1:1 with the extract in a 15 ml Falcon tube on the styrofoam holder and incubated for 15 min at room temperature prior to analysis with a fluoride electrode, as described above.

Statistical analysis At least three bioreplicates were used in this work to allow the statistical analysis. Mean values and standard deviations were presented. Two-tailed paired t-tests were used to establish significant differences when required, given unequal variances among the various treatments. One-way ANOVA and multiple comparisons were used for analyses of the XO-Zr^{4+} and Al–La^{3+}-F methods for investigating the interferences of fungal medium components. Linear and logarithmic regression modeling were used for calculating the F^- calibration curves of colorimetric and ISE measurements, respectively.

3. Results and Discussion

Many spectrophotometric methods for determining fluoride contents in community water have been developed since the 1960s (Cabello-Tomas and West, 1969; Harwood and Huyser, 1968; Macejunas, 1969; Zolgharnein et al., 2009), and these methods are often based on the chelate reaction between the complexometric indicator dye and metal cation to form the complexone whose colors are influenced by the presence of more or less fluoride anions (F^-). Among these, the Xylenol Orange-Zirconium (XO-Zr^{4+}) and Alizarin-Lanthanum (Al–La^{3+}) methods represent the well-studied ones. Here, we adapted these two methods in a micro-well setup for measuring F^- in a high throughput manner and then evaluated if they are suitable for fungal deF studies by assessing the potential interference effects of fungal nutrients and PFASs.

Assessment of the Xylenol Orange-Zirconium (XO-Zr^{4+}) complexone for the micro-well detection of fluorides. The XO-Zr^{4+} method is based on a kinetochromic reaction that involves the catalytic effect of F^- on the complexation between Zirconium (IV) oxychloride octahydrate ($Cl_2OZr{\cdot}8H_2O$) and the dye compound XO ($C_{31}H_{28}N_2Na_4O_{13}S$) (Cabello-Tomas and West, 1969; Davis et al., 2011) (Fig. 1A). The consistent absorbance spectra of the XO-Zr^{4+} products in the absence and presence of F^- reiterated the role of F^- as a catalyst, as previously reported (Cabello-Tomas and West, 1969), rather than as a reactant for ternary product's formation (Fig. 1B). With an optimized micro-well reaction, our results showed a hyperchromic effect of the red-color XO-Zr^{4+} complexone along with the increasing amounts of added F^- (Fig. 1B), and this was then used on a quantitative basis for determining F^- concentrations. This adapted method proved to be reliable and allowed the detection limitation of as low as 1 μM F^- in the context of DDI (Distilled Deionized) water, while it was significantly interfered with by the components of essential fungal nutrients mix (I) provided in the Highley's medium (HMM) and other fungal media (Gruber et al., 1990; Zhang et al., 2012) (Fig. C and D). Further diagnosis revealed that KH_2PO_4, $CaCl_2$, $MgSO_4$, and wood sawdust are the major sources of interference. Among these, the high concentration of KH_2PO_4 (15 mM) used in the current medium recipe significantly bleached out the color by preventing the formation of XO-Zr^{4+} complexone, and this is consistent with other's findings (Cabello-Tomas and West, 1969). On the other hand, the 600 μM $CaCl_2$ and 2 mM $MgSO_4$ in the medium exhibited catalytic effects due to the presence of anions Cl^- and SO_4^{2-}, respectively, like that of fluoride. Wood sawdust also showed the catalytic effect, which may be because the extractives of plant material, such as the phenolic compounds, could enhance the formation of XO-Zr^{4+} complexone. Despite the non-identified catalytic mechanism, wood substrate, as the main nutrient source of wood decay fungi, can pose significant interference to the XO-Zr^{4+} method for F^- determination.

Assessment of the Alizarin-Lanthanum (Al–La^{3+}) method for micro-well quantification of fluorides. Different than the XO-Zr^{4+} method, the Al–La^{3+} chelate can further react with F^- to form the blue-purple ternary complex (Al–La^{3+}-F) that possesses a marked bathochromic shift of the absorption wavelengths relative to the La^{3+}-Alizarin complex reactant (Fig. 2A and B). Along with the increasing amount of added F^-, the wavelength of maximum absorption of the formed ternary complex will also show a hyperchromic change (Fig. 2B), offering the basis for quantifying F^- in solutions. With this Al–La^{3+} method, as little as 1 μM F^- was accurately detected in an adapted micro-well setup in the solutions of DDI water (Fig. 2C), as indicated by the linear calibration curves over the range of 1–48 μM F^-, which is similar to that of the XO-Zr^{4+} method. Notably, both methods obeyed the Lambert–Beer law at the tested F^- range, although the calibration line extension did not pass through the origin point on the optical density axis.

The interference tests showed that the Al–La^{3+} method is less likely influenced by cationic and ionic nutrients provided in the fungal medium than the XO-Zr^{4+} method, except KH_2PO_4 and wood substrate that were found as the major interferences suppressing the reaction of F^- with Al–La^{3+} chelate (Fig. 2D). KH_2PO_4 at the tested concentration of 15 mM, i.e., 300- to 1500-fold excesses of added F^-, completely bleached out the color reaction likely due to its competitive complexation with La^{3+}, which is in line with the previous findings (Belcher and West, 1961a, 1961b) for a similar method based on $Cerium^{III}$-Alizarin complexan-Fluoride reaction. Similarly, phenolic extracts from wood

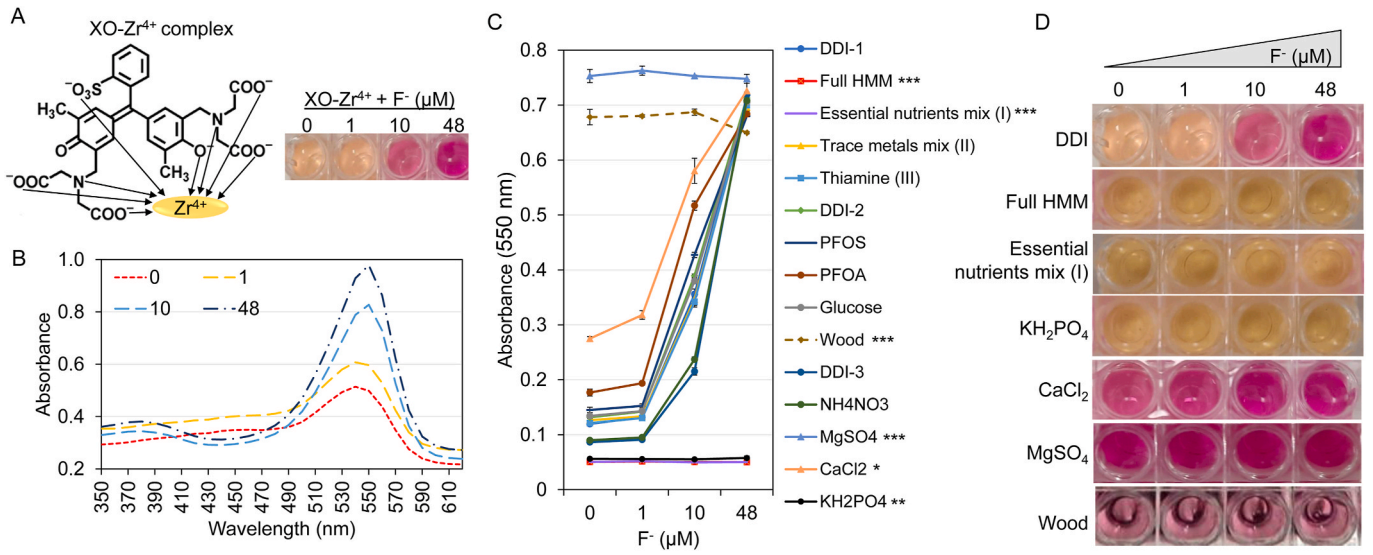

Fig. 1. Assessment of the XO-Zr^{4+} method for measuring fluoride anions of fungal cultures. (A) XO-Zr^{4+} complexation schematic and the colorimetric reactions in the presence of different concentrations of F^-. (B) XO-Zr^{4+} absorbance spectrum showing the hyperchromic effect of the kinetochromic reaction catalyzed by F^-. (C) The absorbance of XO-Zr^{4+} complexan at 550 nm plotted against F^- concentrations in the context of fungal medium components. Significant differences were calculated for each chemical or medium component against DDI water using one-way ANOVA analysis (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). Mean values ± standard deviations of three replicates were shown. (D) Micro-well plates highlighting the interfering sources of fungal cultures to the XO-Zr^{4+} reaction.

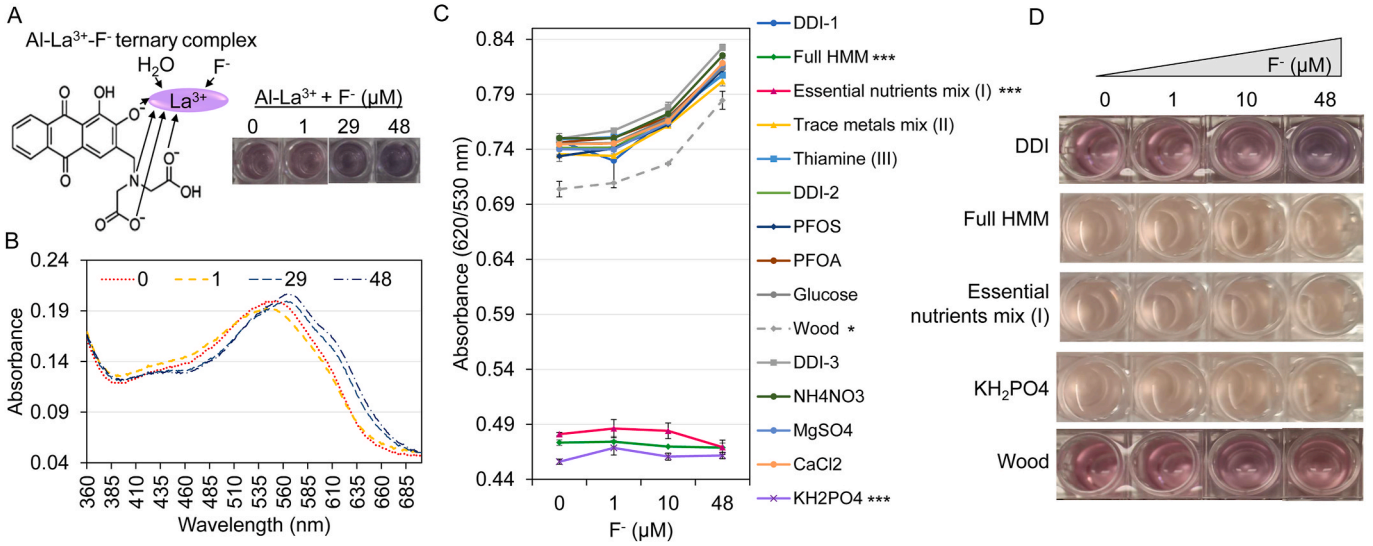

Fig. 2. Assessment of the Al–La^{3+}-F^- reaction for quantifying fluoride anions. (A) The schematic of Al–La^{3+} complex reacting with F^-, and the colorimetric reactions leading the blue ternary complexation products. (B) The absorbance spectrum of the ternary complex showing a hyperchromic effect along with the increasing amounts of added F^-. (C) Absorbance ratios of 620/530 nm of the ternary products plotted against F^- concentrations in the context of fungal medium and PFAS components. Significant differences were calculated for each chemical or medium component against DDI water using one-way ANOVA analysis (*, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$). Mean values ± standard deviations of three replicates were shown. (D) Micro-well plates highlighting the interfering sources of fungal cultures (e.g., KH_2PO_4 and wood substrate) to the Al–La^{3+}-F^- colorimetric reaction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

may outcompete Alizarin to chelate La^{3+}, thereby negatively influencing the absorption values. However, more investigations are still required to understand the inhibiting mechanisms.

At this point, there might be two ways (or in combination) to circumvent the above-mentioned interferences: (1) Avoid the interference nutrients and re-formulate a growth medium that is compatible with colorimetric fluoride measurements while still sustaining fungal proliferation and metabolism for defluorination. (2) Develop a masking agent to chelate and remove cationic interferences or an ion-exchange separation to eliminate interference anions, as a cleaning step prior to applying the colorimetric measuring. Although both can opt to satisfy the needs of the colorimetric determination of F^- in microbial cultures such as bacteria (Bygd et al., 2022), these additional treatments would inevitably lower the throughput of the measuring. Additionally, both KH_2PO_4 and wood substrate have been shown to be essential for the proper growth of the wood decay fungi evaluated here, and for inducing the PFAS degradative capacities (Merino et al., 2023), respectively. Thus, changing the culturing method may not be realistic for maintaining fungal deF capacities. In this regard, we decided to move on to evaluate the third fluoride-measuring method, which is based on the potentiometer equipped with a Fluoride Ion Selective Electrode (F-ISE), as discussed in the following section.

The optimized potentiometric method meets the needs of quantifying fluoride anions in fungal cultures. Besides colorimetric methods, the potentiometer is also recommended for measuring F^- in aqueous samples with the Fluoride Ion Selective Electrode (F-ISE), as seen in the SW-846 Test Method 9214 of US-EPA (US EPA, 2015). However, it is still unknown if the F-ISE measurement would be impacted by fungal cultural nutrients as for the colorimetric methods.

Maintaining ionic stability is the main requirement while using F-ISE to quantify F^- in aqueous solutions, with both protons and polyvalent cations such as Fe(III) can significantly vibrate potentiometer readings. For instance, the pH of the samples must fall into the range of 5.0–5.5 to allow the optimal performance of ISE, as higher proton concentrations at pH < 5 will facilitate the formation of HF that cannot be detected, and on the other hand, hydroxide ion will interfere when pH > 7. To overcome these issues, we mixed a Total Ionic Strength Adjustment Buffer (TISAB; see methods for more details) with samples at a 1:1 ratio to mask cationic interferences and maintain pH constant. The following measuring then showed that the default TISAB formulation (i.e., low-level TISAB; Fig. 3A) was unable to equilibrate the ionic strength of fungal cultures and control the pH to 5.0–5.5, with significant influences evidenced for the Highley's minimal media (HMM) containing variable carbon nutrients, while a stronger TISABII (Carey and Coleman, 2014) buffer containing trace CDTA [1,2-cyclohexylenedinitrilo)-tetraacetic Acid] can efficiently mitigate these interferences (Fig. 3B). With this stronger buffer, a constant pH of about 5.3 was obtained across samples from wood decay fungal cultures, despite that it is known that fungal metabolisms tend to alter environmental pH in different ways (Mali et al., 2017; Shimada et al., 1997). Furthermore, using this optimized buffering system allowed us to extend the detection limit to as low as 1 μM F^- in the presence of fungal medium components, as indicated by the logarithmic calibration curve in Fig. 3C.

Together, the F-ISE method evaluated here is free of interferences of cultural conditions required to support fungal growth and PFAS degradation and offers facile measurement of F^- down to 1 μM, a comparable level to that of the colorimetric methods. Thus, although the periodic calibration and the fixed time for measuring samples in a "one-by-one" manner can pose an obstacle for large-scale screening, the reliability and robustness of F-ISE still leave it the most appropriate method to study fungal deF.

Using the F-ISE method for deF detection in fungal cultures Further tests confirmed that the F-ISE measuring is free of interferences of the fungal metabolites produced by active growth of the white rot wood decay fungus *T. versicolor* in HMM liquid medium and of the context chemical of a short-carbon-chain PFCA 4,4,4-trifluoro-3-(trifluoromethyl) crotonic acid (referred as "crotonic PFCA") (Fig. 4). Crotonic PFCA has been proved as a defluorinable PFAS in many microbial organisms (Che et al., 2021), including wood decay fungi

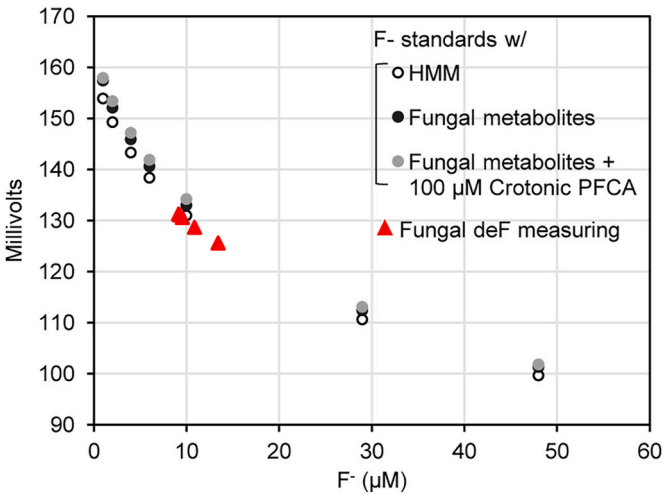

Fig. 4. Defluorination tests with the wood decay fungus *Trametes versicolor*. F-standards ranging from the concentrations of 1–48 μM were measured in different context solutions, and no clear interfering effects were evidenced for HMM medium, fungal metabolites, and crotonic PFCA. Triangles indicate the measuring of free fluorides released after culturing *T. versicolor* on 100 μM crotonic PFCA for 48 h in HMM liquid medium supplemented with 1 % aspen wood sawdust. Five bioreplicates were involved in the experiments.

evidenced in our own research; thus, it is recognized as an ideal model chemical for studying fungal deF mechanisms. While this crotonic PFCA may not be an ideal surrogate to reflect the degradation process of legacy PFASs (e.g., PFOA and PFOS) that are often the focus of PFAS degradation studies, it represents a great model PFAS structure for exploring the relevant biodegradation pathways involved in fungal deF. Also, it is representative of short-carbon-chain carboxylic acid PFASs, which are an important PFAS subgroup derived from legacy PFAS degradation and identified as environmentally concerning pollutants (Brendel et al., 2018; Li et al., 2020). Relevant to this work, the use of crotonic PFCA is expected to facilitate the detection of fungal deF with the optimized F-ISE method. Using the F-ISE method, we then monitored the capacity of *T. versicolor* in defluorinating crotonic PFCA in the HMM liquid medium supplemented with aspen wood sawdust as the natural substrate, which detected 10.43 ± 1.61 μM free F^- (n = 5) after 48 h of culture (Fig. 4). Thus, our work demonstrated that F-ISE is resilient to the changes of reaction conditions, including medium nutrients necessary for fungal growth, fungal metabolites, and PFASs, and it can be used for screening fungal deF capacities.

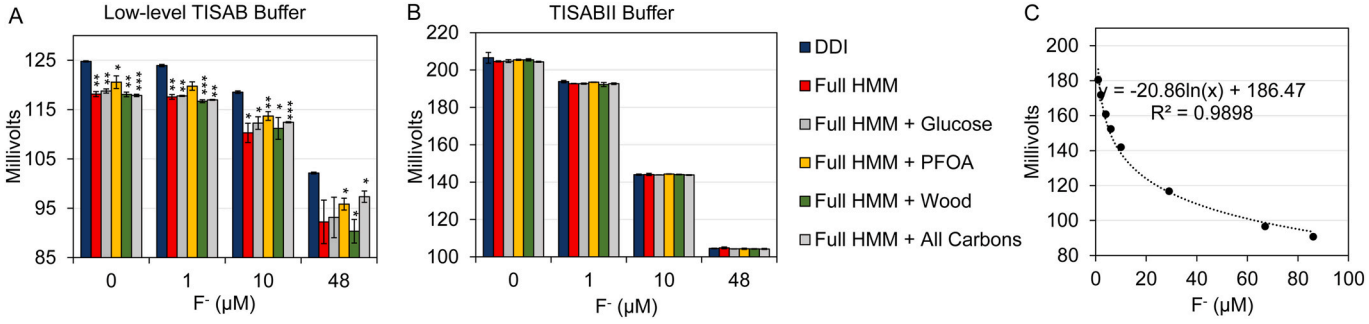

Fig. 3. Quantification of fluoride in the context of fungal cultural conditions using an F-ISE potentiometer. ISE readings in Millivolt units were plotted against F-standards at different concentrations and were measured using either low-level TISAB buffer (A) or TISABII w/CDTA (B) to stabilize the ionic strengths of samples. (C) The logarithmic calibration curve of F^- standards prepared in the full-strength Highley's minimal media (HMM) containing wood substrate, PFOA, and glucose as the carbon sources to support the metabolisms of wood decay fungi. Significant differences were calculated for each medium condition against DDI water using one-way ANOVA analysis (*, p < 0.05, **, p < 0.01, and ***, p < 0.001). Mean values ± standard deviations of three replicates were shown.

4. Conclusions

Wood decay fungi are crucial players in nutrient recycles on terrestrial land, while their roles in responding to and recycling the emerging PFAS pollutants are still understudied. Particularly, their defluorination capacities remain largely unknown due to the lack of a reliable, cheap fluoride anion-quantifying method that can allow a large-scale investigation of PFAS-defluorinating phenotypes in fungal cultures. In this research, we systematically evaluated three F^--quantifying methods, including two that employ spectrophotometric measuring (i.e., XO-Zr^{4+} and Al–La^{3+}) and one that relies upon a fluoride potentiometer (F-ISE), for their appropriateness to quantify free fluoride generated as fungal cultures degrade PFAS. Our results showed that both colorimetric methods can be adapted to the micro-well setup for large-scale assays, while they are significantly interfered with by nutritional elements (e.g., KH_2PO_4) required to support fungal growth and by the wood substrate that lures the production of fungal degradative enzyme systems (Luo et al., 2018; Merino et al., 2023; Steffens et al., 2023). The F-ISE, however, can circumvent these interferences by using an optimized Total Ionic Strength Adjustment Buffering system, although this may need to sacrifice sample processing throughput relative to micro-well-based colorimetric measuring. With the optimized F-ISE, we successfully demonstrated a defluorination test by using a white rot wood decay fungus, *T. versicolor*, to degrade the crotonic PFCA, which is a known defluorinable PFAS structure that carries the electron-attracting alkenyl group (C=C) adjacent to C-F bond.

While the use of crotonic PFCA allowed us to detect fungal deF, we are willing to highlight that the observed deF of this compound does not suggest that the structurally different longer-chain perfluoroalkyl acids (PFAAs), which are of greater environmental concern, would be similarly degraded. Crotonic PFCA is a five-carbon branched PFAS that bears only certain levels of similarity to linear long-chain legacy PFAS such as PFOA and PFOS, and it is among those thousands of PFASs with large variability in structural and chemical properties. Even though, this will not hurt the use of crotonic PFCA to validate the F-ISE method tested here, and it opens new avenues for continued research to identify other PFAS structures susceptible to deF. Taken together, our research provided a reliable fluoride anion-quantifying approach that would facilitate further investigations of the deF mechanisms and eco-roles of the fungal organisms in PFAS-impacted environments.

CRediT Authorship contribution statement

Charles Ayers: Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Jiwei Zhang:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no competing financial interests.

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