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Evaluation of Inoculum Sources for Aerobic Treatment of 2,3,4-Trifluoroaniline During Start-up and Shock

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Abstract Contamination with fluoroaromatics (FAs), particularly polyfluorinated aniline, is becoming a serious environmental problem worldwide. To shorten the start-up time, and increase the stability of treatment systems, this work focused on the effects of three seeding sources on treatment performances of 2,3,4-trifluoroaniline (2,3,4-TFA) during start-up and shock, as well as the acclimated strategy. After 246–323 days of acclimation in a stepwise feeding according to the inhibition degree, three sequencing batch reactors (SBRs) successfully achieved efficient removal, i.e., 300.00 mg/L of 2,3,4-TFA, with over 95.00% of degradation efficiency and 60.00–80.00% of defluorination rates. The sludge obtained from the fluorizated hydrocarbon wastewater treatment plant(FHS) without prior exposure to fluoroaniline was determined to be optimal, based on the observed shortest start-up time of 246 days, the highest defluorination rate of 70.00–80.00%, the fastest recovery time of 7 days after shock, and the highest microbial diversity with nine dominant bacterial groups. Furthermore, compared with the sludge obtained from pharmaceutical wastewater containing part of municipal wastewater treatment plant(PMS), the seeding source used in treating the comprehensive wastewater in industrial park (CIS) exhibited earlier defluorination reaction, higher defluorination rate and microbial diversity, but lower shock resistance. High-throughput sequencing demonstrated that microbial diversity was dependent on the origin of the inoculum after acclimation. We identified two predominant phyla in PMS, namely, Deinococcus-Thermus (24.43%) and Bacteroidetes (18.44%), whereas these were Acidobacteria and Chloroflexi in FHS and CIS. During the shock of 400 mg/L 2,3,4-TFA, the predominant bacteria norank_f_Blastocatellaceae and norank_f_Methylobacteriaceae disappeared, and the defluorination reaction hardly occurred, indicating that the bacterial genera could contribute to the defluorination reaction.

Keywords Inoculum sources · Acclimation · Shock · Microbioal community · 2,3,4-trifluoroaniline

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1 Introduction

Because of many unique chemical and physical properties of fluorine, fluoroaromatics have been widely used

as pesticides, pharmaceuticals, or starting materials for chemical syntheses over the past few years (Kiel and Engesser 2015). In turn, a growing number of fluoroaromatics have been detected in the environment due to metabolites of drug detoxification or components of industrial wastewaters (Kiel and Engesser 2015). Most fluoroaromatics are manmade, and only less than a dozen organofluorines occur naturally (Kiel and Engesser 2015). Moreover, biological and chemical inertness is often considered in product design, which makes biodegradation of these xenobiotic compounds more difficult than biogenic substrates (Adams and Halden 2010). Therefore, fluoroaromatics are considered potential hard-to-treat environmental contaminants.

Biological treatment is considered an inexpensive and environmental method for removing pollutants from the environment. Despite the fact that fluoroaromatics have low natural biodegradability owing to their molecular structure, there have been some efforts towards their biological treatment. Several studies have shown that some strains exhibit degradation capacity for several fluoroaromatics despite their highly recalcitrant nature, including fluorophenols (Duque et al. 2012; Franco et al. 2014), fluorobenzenes (Strunk and Engesser 2013; Moreira et al. 2012), and fluoroanilines (Song et al. 2014; Wang et al. 2013; Amorim et al. 2013). However, a single culture is usually incapable of degrading polyfluoroaromatics, and to date, there is hardly any reported success. In contrast, due to synergistic interactions occurring in mixed cultures, they usually have stronger degradation ability and can utilize wider ranges of substrates than individual isolates (Cui et al. 2017; Carvalho et al. 2010). Thus, mixed cultures are particularly important when the emphasis is placed on the degradation of highly recalcitrant fluoroaromatics. Unfortunately, information on the biodegradation characteristics of fluoroaromatics in such mixed culture systems is limited (Ramos et al. 2017; Duque et al. 2014; Zhao et al. 2015), those relating to polyfluoroaromatics are scarce.

To date, activated sludge processes have been one of the most common biotechnologies for industrial wastewater treatment. Therefore, the treatment of fluoroaromatics using an activated sludge process in which a mixed culture is in action in the absence of a special growth substrate would be more meaningful, and practical. The pre-adaptation of the activated sludge cultures to the xenobiotic can usually acquire novel metabolic pathways for xenobiotic degradation and referred to as acclimation (Orozco et al. 2013; Movahedyan et al. 2008; Wosman et al. 2016). The duration of acclimation process, when mixed cultures are exposed to new or unusual chemicals, ranges from a few hours to several weeks or months and depends on several factors (Orozco et al. 2013), including type and concentration range of the xenobiotic compounds, as well as the quantity and quality of the inoculum utilized. The initial seeding source is a critical factor in determining the dominant community, and seeding the proper inoculum source can shorten the acclimation period (Orozco et al. 2013; Alexandrino et al. 2018; Moreno and Buitron 2004). Therefore, it is important to understand the effect of seeding sources on xenobiotic compound treatment. However, to the best of our knowledge, no relevant information about fluoroaromatics could be obtained. Additionally, xenobiotic concentrations readily fluctuate in the actual industrial wastewater (Osuna et al. 2008). Under this condition, the operation of the biological treatment facility can be severely disrupted and even lose degradation capability at shock loads, and therefore, the desired effluent quality may not be reached, even when pre-acclimated microbial consortia are used (Movahedyan et al. 2008; Alves et al. 2017). However, how microorganisms respond to fluoroaromatic shock loads remains unclear. Our focus in this paper will investigate the influences of inoculum sources on treatment of fluoroaromatic during start-up and shock, as well as the response of microorganism.

Fluoroanilines (FAs) are a group of typical xenobiotic compounds, belonging to the priority pollutants identified by the US EPA and China (Cui et al. 2017). Therefore, this study was conducted to evaluate the acclimation process by acquiring microorganisms capable of degrading 2,3,4-fluoroaniline (2,3,4-TFA) and assessing microbial responses to 2,3,4-TFA shock loading in three sequencing batch reactors (SBRs) inoculated with three types of activated sludge, namely the sludge used for the treatment of pharmaceutical wastewater containing part of municipal wastewater (PMS), fluorizated hydrocarbon wastewater (FHS), and comprehensive wastewater in industrial park (CIS). The dynamic changes in genera within microbial communities were also monitored using high-throughput DNA sequencing technology. Furthermore, a stepwise acclimation strategy, dependent on the degree of 2,3,4-TFA inhibition, was tested. This study improves our understanding of the biodegradation process and behavior of fluoroanilines during the actual wastewater treatment. Results could also provide the important information on

selecting seed sources for stable operation of treatment systems, as well as the application of an effective acclimation strategy.

2 Materials and Methods

2.1 Chemicals and Seeding Sludge

The reagent grade 2,3,4-trifluoroaniline (2,3,4-TFA, CAS 3862-73-5; > 99.0% purity) was purchased from Saen Chemical Technology Co., Ltd (Shanghai, China). All other reagents used in the present work were commercial products of reagent grade from Shenke Analysis Technology Service Co. Ltd (Quzhou, China).

Three different types of activated sludge were used to seed SBRs. PMS, FHS, and CIS were obtained from a return line of the activated sludge from secondary clarifier of a pharmaceutical wastewater containing portion of the municipal wastewater, the aerobic pool of a fluorizated hydrocarbon wastewater treatment system, and the oxidation ditch of comprehensive wastewater treatment plant in an industrial park, respectively. These were located in Quzhou, Zhejiang, China, and none of them were exposed to any fluoroanilines before acclimation. The properties of the seed sludge are presented in Table 1.

2.2 Influent Components

The synthetic wastewater components were as follows (in mg/L): sucrose 0~500, sodium acetate(NaAc) 0~850, 2,3,4-TFA 25~400, NH_4Cl 237, KH_2PO_4 27.69, $K_2HPO_4.3H_2O$ 46.15, $FeSO_4.7H_2O$ 6.25, $MgCl.7H_2O$ 5.10, $CaCl_2$ 6.00, and 1.00 ml/L of micronutrient solutions which was described in our previous report (Zhao et al. 2015). The initial pH was adjusted to 7.20 ± 0.20 with 1 M HCl or NaOH.

2.3 Inhibition Assays

The activated sludge respirometric test is considered a direct method for evaluating the toxic effect of chemicals on sludge activity and the potential effect on its microbial population (Lepik and Tenno 2012; Vasiliadou et al. 2018). To select the suitable concentration ranges of 2,3,4-TFA during start-up and shock, the respirometric test was performed according to the standard method. Briefly, the unacclimated activated sludge (MLSS = 2320 mg/L, VSS/SS = 0.78) was obtained from a municipal sewage treatment plant in Quzhou, and concentrated to 4,000 mg/L after washing three times with phosphate buffer saline (PBS). The activated sludge was exposed to the synthetic wastewater (16.00 g peptone, 11.00 g beef extract, 3.00 g urea, 0.40 g NaCl, 2.80 g K_2HPO_4, 0.20 g $CaCl_2{\cdot}2H_2O$, and 0.20 g $MgSO_4{\cdot}7H_2O$ in 1000 mL H_2O) together with different concentrations of 2,3,4-TFA (25 mg/L, 50 mg/L, 100 mg/L, 200 mg/L, and 400 mg/L), and aerated for 3 h at a temperature of 20 °C ± 1 °C. After that, the activated sludge was transferred into the respirometer with 500 mL of working volume reactor and was continuously stirred with a magnetic stir-bar at a constant rotation speed to ensure homogeneity. As the dissolved oxygen (DO) concentration reached saturation, aeration was stopped, and DO was monitored for 10 min using a portable dissolved oxygen meter (JPBJ-608, Rex, Shanghai, China). The activated sludge only exposed to the synthetic wastewater was used as the control. 3,5-Dichlorophenol (3,5-DCP), a reference toxicant, was included in the test as a kill control to ensure that the test works properly. At least three replicates were conducted for every sample, and the mean values were calculated and utilized as the rate of inhibition.

Table 1 The properties of three inoculum sources used in the study

Parameters index*	PMS (R1)	FHS (R2)	CIS (R3)
MLSS (mg/L)	6566 ± 144	10179 ± 1114	6901 ± 738
MLVSS (mg/L)	2929 ± 80	5860 ± 627	3088 ± 706
SVI (mL/g)	120 ± 6	59 ± 9	81 ± 12
VSS/SS	0.45 ± 0.013	0.58 ± 0.003	0.44 ± 0.061

**MLSS*, mixed liquor suspended solids; *MLVSS*, mixed liquor suspended solids; *SVI*, sludge volume index (30 min)

Oxygen uptake rates (OURs) were obtained from the slope of DO decline over time using linear regression. Biomass activity was measured in terms of specific exogenous oxygen uptake rate (SOUR), and the inhibition ratio was determined according to the following formula (Eq. (1)) (Polo et al. 2011):

$$\text{Inhibition}(\%) = \left(1 - \frac{\text{SOUR}_r}{\text{SOUR}_c}\right) * 100\% \quad (1)$$

where SOUR_r represents the OUR for the synthetic wastewater in the presence of a given concentration of

2,3,4-TFA (mg O_2 /(g VSS h)); and $SOUR_c$ is the OUR of the control sample (mg O_2/(g VSS h)).

In addition, inhibition was assessed in terms of the median lethal concentration (EC_{50}), causing a 50% reduction in SOUR. The parameter γ is defined in Eq. (2) following the ISO 11348-3 (ISO 1998) standard. The relationship between γ and the toxicant concentration, C, can be expressed in Eq. (3).The linear fit between the logarithm of parameter γ and the logarithm of 2,3,4-TFA concentration are used to calculate EC_{50}, which is expressed in Eq. (4) as follows:

$$\gamma = \left(\frac{SOUR_c}{SOUR_r} - 1\right) * 100\% \quad (2)$$

$$\gamma = KC^p \quad (3)$$

$$\log\gamma = p\log C + \log K \quad (4)$$

where γ is the inhibition effect of 2,3,4-TFA on SOUR, in percent; and p is the value of the slope of the described line; log$K$ is the value of the intercept of the described line.

Thus, when the 2,3,4-TFA causes a 50% reduction of the SOUR, γ takes the value of 1, and EC_{50} can be obtained in Eq. (5) as follows:

$$EC_{50} = 10^{-\left(\frac{\log K}{p}\right)} \quad (5)$$

2.4 SBR and Operation Procedure

The study was conducted in three SBRs with a working volume of 2 L (named R1, R2, and R3). These were cylindrical in shape and made of polymethyl methacrylate. R1, R2, and R3 were seeded with predetermined amounts of PMS, FHS, and CIS, respectively, with the initial mixed liquor volatile suspended solids (MLVSS) at a concentration of approximately 1500 mg/L. During the whole acclimation period, cycle time, feeding, aerobic reaction, settling, and effluent withdrawal were kept constant at 24 h, 5 min, 23 h, 45 min, and 10 min, respectively. Three reactors operated at a 70% volumetric exchange ratio, and a stable reactor temperature was maintained at 28 °C ± 2 °C using a thermostat-controlled electric heating rod. Additionally, the oxygen concentration was maintained at over 3.00 mg O_2/L at aerobic phase in three SBRs.

Whole acclimation included two stages. At the first stage, the initial 2,3,4-TFA concentration was 25.00 mg/L, using sucrose and sodium acetate as the supplementary substrates; 2,3,4-TFA concentration then gradually increased to 200.00 mg/L based on the inhibition degree of 2,3,4-TFA on biomass, and decreased the supplementary substrates content until zero (Wosman et al. 2016). During the second stage, the 2,3,4-TFA concentration was further increased from 200.00 to 300.00 mg/L, and acclimation was performed when the degradation efficiency and defluorination rate reached over 85.00%, and about 70.00%, respectively. During whole acclimation, samples were periodically collected to determine 2,3,4-TFA, and fluoride ion (F^{-1}) concentrations

2.5 Shock Tests

Even when the activated sludge is successfully acclimated to a xenobiotic compound and achieved a steady state at a certain concentration, insufficient degradation capability is also prevalent after the xenobiotic compound concentration is increased enough to inhibit for a length of time (Chong and Chen 2007). Thus, three SBRs were subjected to shock tests when these successfully achieved a steady state at 300.00 mg/L of 2,3,4-TFA. Shocks were administered using a sudden increase in 2,3,4-TFA concentration from 300.00 mg/L to above EC_{50} value according to the inhibition test for a certain time, then reverted back to 300.00 mg/L.

2.6 Molecular Analysis

2.6.1 DNA Extraction and Illumina MiSeq Sequencing

Based on the results of above experiments, a total of eight sludge samples were collected, including seed sludge (R1seed, R2seed, and R3seed), three types of acclimation sludge (SFR1, SFR2, and SFR3), and two shock samples obtained from R2 and R3 (SFR2shock and SFR3shock), to analyze microbial community composition. Genomic DNA extraction was performed using a Fast DNeasy® PowerSoil® Kit for soil (Carlsbad, CA, USA) following the manufacturer's protocol. The V3-V4 fragment of the *16S rRNA* gene was amplified using primer set 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) in an Applied Biosystems 9700 PCR System (ABI GeneAmp® 9700). The PCR conditions were as follows: an initial

denaturation of DNA for 3 min at 95 °C; followed by 27 cycles of denaturation 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. Finally, the DNA library was sequenced using the Illumina Miseq PE300 platform (Illumina, USA) in Majorbio Co., Ltd (Shanghai, China). Raw sequencing data obtained from the seeding sludge and other sludge in this study has been deposited in Sequence Read Archive (SRA) database with accession number PRJNA531184.

2.6.2 Biodiversity Analysis and Phylogenetic Classification

Corrected sequences were processed using the Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso et al. 2010) and were clustered into operational taxonomic units (OTUs: to easily analyze the phylogenetics or population genetics, the same marker is artificially assigned to a taxon (strain, species, genus, and grouping)) using the Usearch algorithm (version 7.0; http://drive5.com/uparse/) on the basis of 97.00 % nucleotide similarity (Edgar 2010). The same and unique OTUs in multiple samples were expressed by Venn analysis (displaying overlapping areas of element sets). The coverage of each sample was generated by MOTHUR. Good's coverage values were greater than 99.80% in all samples, indicating a satisfactory number of sequences for characterization of microbial communities (Choi et al. 2018). Based on these clusters, Alpha diversity indices, including Shannon index (indicating community diversity) and Chao 1 (indicating community richness), were calculated. The diversity index is a quantitative measure that reflects how many different types there are in a dataset, and simultaneously takes into account how evenly the basic entities are distributed among those types. The value of a diversity index increases both when the number of types increases and when the evenness increases. Reads, which are sequences derived from high-throughput sequencing, were classified using the Classifier tool from the Ribosomal Database Project (version 2.2 http://sourceforge.net/projects/rdp-classifier/).

2.7 Analytical Methods

Liquid samples were periodically withdrawn from the influent and effluent of three SBRs to determine the concentrations of 2,3,4-TFA and F^-. F^- was analyzed using the ion-selective electrode method (composed of PF-1-01 fluorine ion-selective electrode and 232-01 calomel electrode). 2,3,4-TFA was analyzed by Waters' high-performance liquid chromatography (HPLC) coupled with UV absorbance detector at a wavelength of 230 nm, with C18 reversed-phase column, according to the procedure of Zhao et al. (2015). The determination procedures of MLSS, MLVSS, and SVI were detailed in the Standard Methods (APHA 2005).

3 Results and Discussion

3.1 Inhibitory Effect and Determination of EC_{50}

To prevent the bacteria from being severely inhibited during acclimation period, it is crucial to evaluate the toxicity effect of 2,3,4-TFA on activated sludge. Figure 1 shows the inhibition percentage vs. log C and the corresponding linear plot of log γ vs. log C. We observed a general trend towards inhibition ratio, which enlarged with increasing 2,3,4-TFA concentrations. At 25–400 mg/L of 2,3,4-TFA, the inhibition ratios were within the range of 21.20–51.80%. Additionally, there was a good agreement between log γ and log C, and EC_{50} value for the 2,3,4-TFA obtained from the linear method was 345.00 mg/L. Although no data on the inhibition of 2,3,4-TFA on activated sludge respiration were obtained, it apparently has high ecotoxicity compared with those of the same species recognized as highly toxic, such as aniline, phenol, and chlorobenzene (Cai et al. 2010). The luminescent microbial test with the microorganism *Photobacterium phosphoreum* has also shown that 2,3,4-TFA has high ecotoxicity (Yang et al. 1997).

3.2 Treatment Performances of 2,3,4-TFA During Start-up

Generally, with the concurrent presence of the xenobiotic and biogenic substrates, the provisions for growth and energy can be obtained from the readily utilized substrates, providing better resistance against toxicity, and also can enhance certain heterogeneous microbial communities' capacity of degrading xenobiotics (Chong and Chen 2007). Furthermore, considering the toxicity of high xenobiotic compound concentration, the stepwise acclimation procedure that consisted of gradually increasing the proportion of xenobiotic compounds relative to biogenic substrates when the removal rate was >

85.00% is often applied (Orozco et al. 2013; Vasquez and Nakasaki 2016). In this study, based on the results of activated sludge respirometric tests, the initial 2,3,4-TFA concentration was set to 25.00 mg/L, and then gradually increased to 50.00 mg/L, 100.00 mg/L, 150.00 mg/L, 200.00 mg/L, and 300.00 mg/L during start-up, and the degradation performances of 2,3,4-TFA in each bioreactor are shown in Fig. 2.

During 2,3,4-TFA degradation, fluoride release serves as an indicator for the biodegradation and mineralization, but the defluorination reaction hardly occurred with acclimation of 110 days at 25.00 mg/L 2,3,4-TFA (Fig. 2). Chong and Chen (2007) and Rezouga et al. (2009) reported the existence of two types of microorganisms, including degraders and non-degraders of the xenobiotic compound, and the selective pressure for the degraders was proportional to the xenobiotic concentration as a mixed culture (such as activated sludge) was in contact with a xenobiotic compound. The microbes may preferentially degrade biogenic substrates and thus fail to develop the activity towards the toxic substances due to the low selective pressure imposed on the microbes during acclimation (Chong and Chen 2007). Based on these considerations, it can be deduced that the higher the xenobiotic concentration, the bigger the selective pressure, and the microbial populations carrying functional genes exhibit a higher growth rate (Chen et al. 2016). Thus, in the following days, a new acclimation

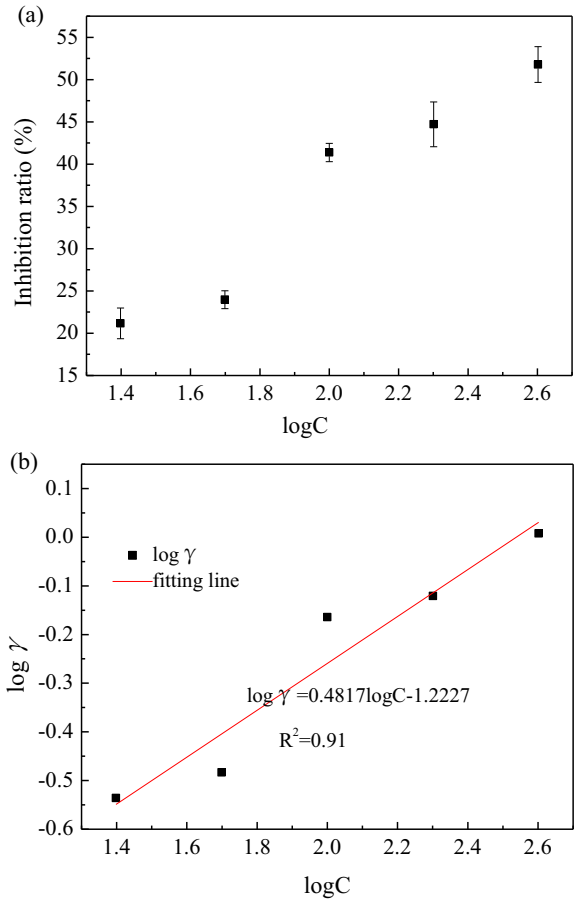


Fig. 1 **a** Respirometric inhibition curve of 2,3,4-TFA and, **b** its linear fitting equation

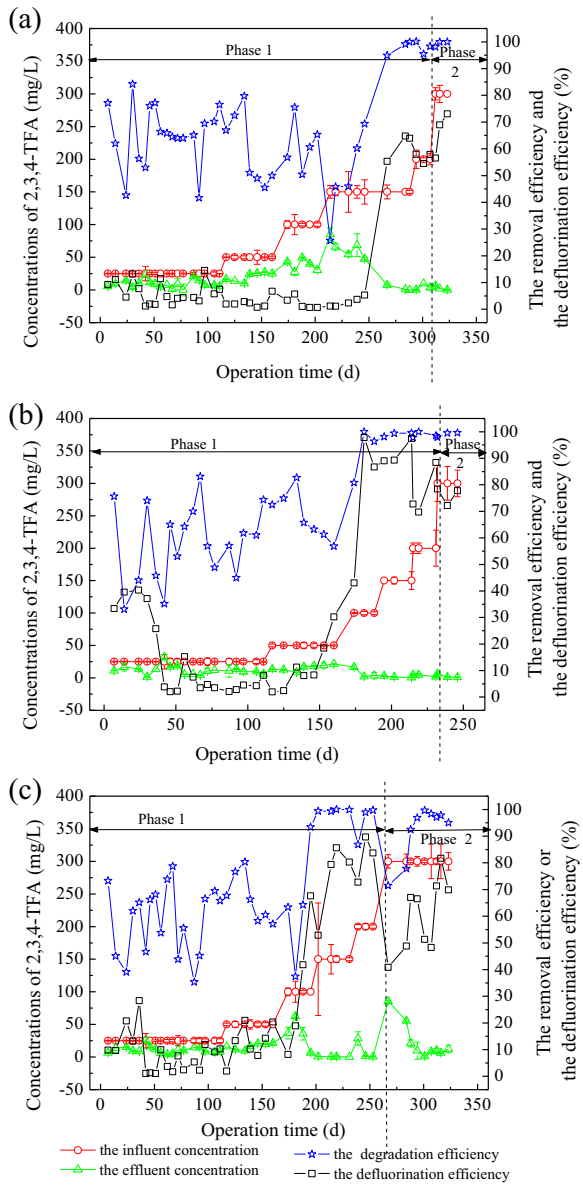


Fig. 2 Evolution of 2,3,4-TFA removal performances for **a** R1, **b** R2, and **c** R3 during start-up. Sucrose and sodium acetate were used as supplementary substrates in phase 1, whereas no supplementary substrates were employed in phase 2

strategy was tested, namely, the influent 2,3,4-TFA concentration was further increased as long as no obvious inhibition was observed or the biomass adaptation was obtained during start-up.

Figure 2 shows that the 2,3,4-TFA degradation reaction was initialized, and the performance was stabilized in the three bioreactors after the long-term operation. For R1, defluorination (55.31%) occurred on 267 days at 150.00 mg/L. After 267 days, even when the concentration was further increased to 200 mg/L and even without any biogenic substrate at 300 mg/L, the removal rates of 2,3,4-TFA (95.00–100.00%) and defluorination efficiency (54.00–73.00%) were maintained until the end of operation (Fig. 2a). Figure 2 c shows a similar trend for R3, the defluorination reaction (41.80%) was initiated on 188 days at 100.00 mg/L and was maintained at steady state (70.00–80.00%) during the subsequent days. When the concentrations were further increased to 200.00 mg/L and 300.00 mg/L, a slight inhibition occurred at the early stage, with the removal rate of 2,3,4-TFA and defluorination efficiency decreasing, although gradual adaptation was observed, and 2,3,4-TFA was almost completely removed at the end of the assay. These findings could be attributed to the fact that a longer time is required to synthesize essential enzymes for degrading the higher concentrations of 2,3,4-TFA for R3. However, compared with R1 and R3, a shorter enrichment time was observed for R2, the defluorination reaction was initiated on 174 days at 100.00 mg/L and drastically increased to over 85.00% on 181 days. When the concentrations were further increased, the removal rates and defluorination efficiency remained > 95.00% and 70.00%, respectively.

Comparing the sources of inoculum, a shorter acclimated time was obtained with the sludge coming from the fluorizated hydrocarbon wastewater treatment plant, followed by the comprehensive and the pharmaceutical one. These results suggested that the 2,3,4-TFA degrading biomass could be induced by three different inocula, although the origin of the inoculum also influenced the acclimation process. The observed differences in behavior among the three inocula could be explained by microbial population diversity, which would be discussed later.

In addition, the above results confirmed the feasible acclimation strategy wherein an increase in xenobiotic concentration is dependent on the degree of inhibition, yet independent of the removal degree. In our previous study, the concentrations further increased only as degradation rate was > 85.00% during start-up. Here, the reaction time was extended to achieve that efficiency, and the whole start-up period lasted 350 days (Zhao et al. 2015). Although the required total start-up time was similar between two acclimation strategies, it is important to note that the degraders only adapt to 100.00 mg/L and not 300.00 mg/L of 2,3,4-TFA, and a long lag period was required at 100.00 mg/L in the previous study. Thus, compared with the regular acclimation mode, the acclimation way based on inhibitory effect has an obvious advantage, which can be attributed to the different seeding sources, but the high selection pressure for microorganisms is also an important factor. In this study, only feasibility aspects have been clarified, some detailed information remains unclear, and further studies would be performed, especially on how much time is required for different selection pressures.

3.3 Treatment Performances of 2,3,4-TFA During Shock

According to the result of inhibition tests, high concentrations of 2,3,4-TFA may be toxic to biomass and can adversely affect the performance of biological wastewater treatment processes. To investigate the effect of three inocula on the resistance capacity of 2,3,4-TFA treatment systems to shock loading, the concentration of 2,3,4-TFA was increased to 400.00 mg/L (higher than EC_{50}). In the aerobic activated sludge treatment system, 7–9 days are often used as the all mean cell residence time (Chong and Chen 2007). Therefore, the treatment systems were successively administered with shock load for 8 days and 40 days, and the results are summarized in Fig. 3.

When the concentration of 2,3,4-TFA increased from 300 to 400 mg/L, the performances of three treatment systems were disrupted (Fig. 3). In the last (eighth) day of the shock, the degradation efficiency of 2,3,4-TFA in R1, R2, and R3 decreased to 14.60%, 38.40%, and 12.00%, and the defluorination rates only reached 2.90%, 10.60%, and 1.20%, respectively. As normal operation conditions (300.00 mg/L of 2,3,4-TFA) were re-established, recovery possible in about 20 days and 7 days for R1 and R2, respectively. However, R3 only slightly contributed to the improvement in system performance, and the overall 2,3,4-TFA removal efficiency was not higher than 55.00%. In particular,

defluorination hardly occurred during 20 days of the recovery period, indicating that an irreversible inhibition of 2,3,4-TFA could occur in these microorganisms, and re-acclimation was required to regain the previous steady state under such 2,3,4-TFA shock load. The results suggested that the resistance capacity of the three inocula to 2,3,4-TFA shock loading was in the order of FHS > PMS > CIS.

To further investigate the influence of shock time, reactor R2 was selected for the second shock test. When a second shock of 400.00 mg/L 2,3,4-TFA was applied, we observed similar results as those of the first shock on 8th day. On the 16th day, an adaptation of the biomass in R2 to 400.00 mg/L 2,3,4-TFA shock was detected, with 2,3,4-TFA removal increasing from 35.14 to 65.40%, indicating an increase in tolerance of the degraders in R2 to such 2,3,4-TFA load. However, after 40 days of shocks, no complete recovery was observed after 42 days, especially when defluorination rate was only within the range of 8.00–12.00%. This suggested that 2,3,4-TFA toxicity might have limited microbial activity under prolonged exposure of the microorganisms to such 2,3,4-TFA levels, and re-acclimation is required, as previously reported using phenol shock (Alves et al. 2017).

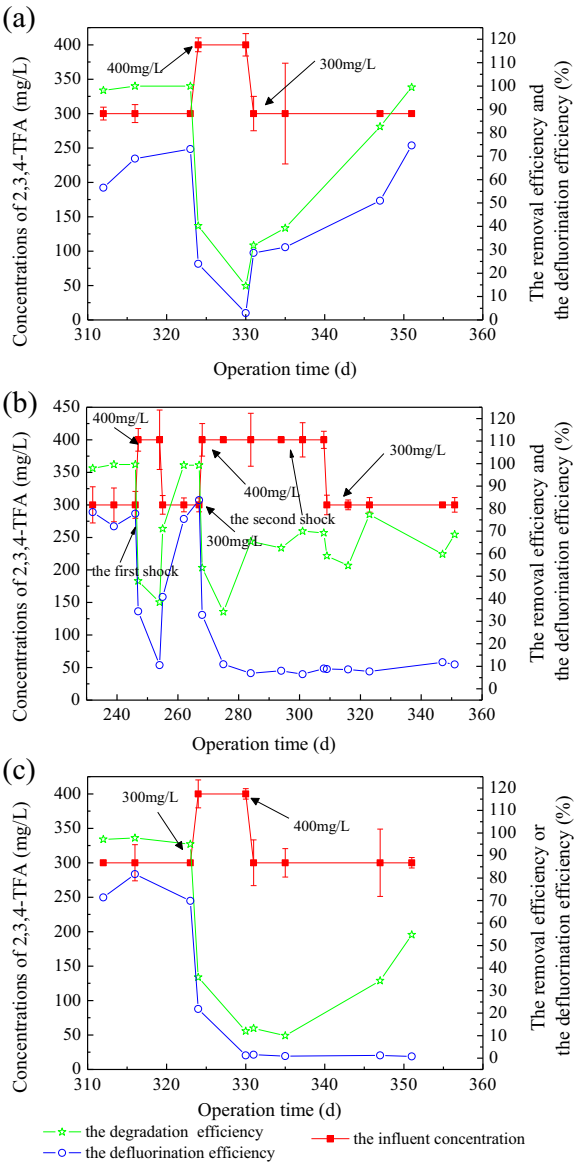


Fig. 3 Evolution of 2,3,4-TFA removal performances for **a** R1, **b** R2, and **c** R3 during shock

The above results indicate that the resistance capacity of the systems largely depends on the inocula. Furthermore, the longer the inhibition time, the more serious the inhibition. Thus, the toxicity of the influent stream should be evaluated using the activated sludge respirometric test before it is treated using the activated sludge system. With the awareness that the influent concentration over EC_{50} is enough to cause the system to fail its treatment, wastewater should be diluted before it is directed to the bioreactor, or a post-treatment step can be implemented in biological treatment processes. However, unlike the results of the start-up tests, PMS showed higher resistance to shock load than CIS, which can be explained by the microbial population found in the two inocula. Compared with the comprehensive wastewater in the industrial park, the pharmaceutical wastewater has or could have higher levels of pollution involving toxic organic chemicals. Thus, selection of a specific strain or species present in the inoculum with the capability to resist toxic compounds possibly occurred.

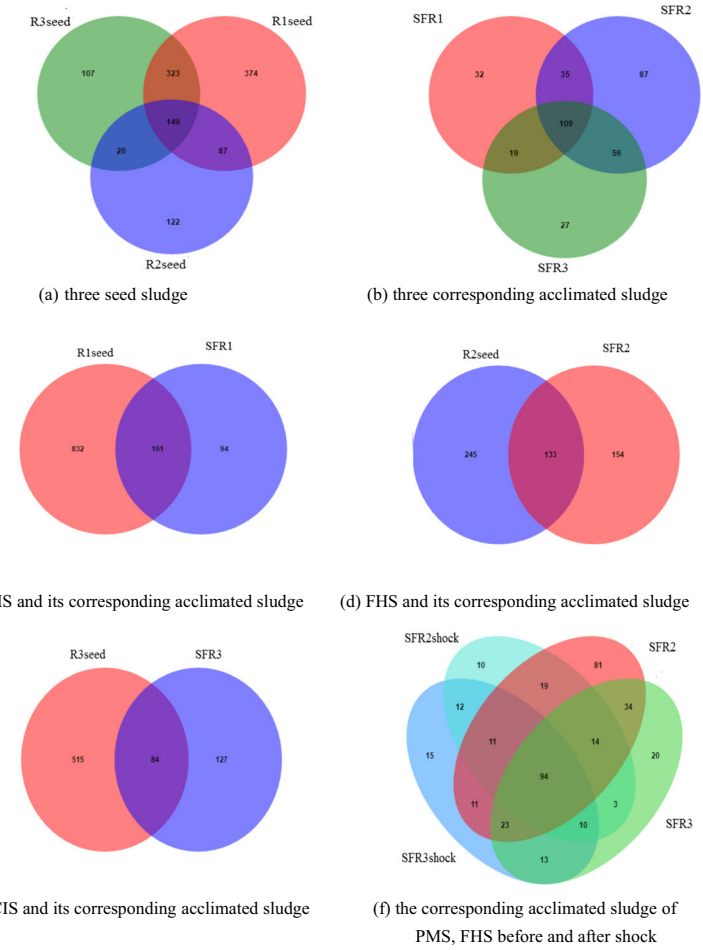
3.4 Bacterial Community Composition

To provide further insights into the microbial compositions for 2,3,4-TFA biodegradation and assess the effect of 2,3,4-TFA shock load on the bacterial community composition in three SBRs, eight samples were analyzed using the Illumina MiSeq sequencing technology. In total, 46,500, 45,300, 33,600, 45,536, 45,383, 33,986, 46,757, and 39,354 sequences were obtained for activated sludge samples of R1seed, R2seed, R3seed, SFR1, SFR2, SFR3, R2shock, and R3shock, respectively.

3.4.1 Shifts in Bacterial Community Composition During Start-up

Figure 4 a shows that 933, 378, and 599 OTUs were determined for the seeding activated sludge samples of R1–R3, respectively. Among the total OTUs, 149 OTUs were shared, accounting for 15.97%, 39.42%, and 24.87% in the seed sludge of R1–R3, respectively, which indicated that the bacterial community composition in three seed sludge was significantly different. Compared with the OTUs in the seed sludge of R1–R3, bacterial diversity decreased after acclimation (Fig. 4b), which might be due to non-degrader endogenous decay. In addition, the bacterial community composition in the three acclimation sludge samples showed a higher degree of similarity than that of three seed sludge types, which may be due to the same operation conditions during long-term enrichment. Additionally, Venn analysis further showed that only 9.83% (101 out of 1,027 OTUs), 25.00% (133 out of 532 OTUs), and 11.57% (84 out of 726 OTUs) species shared by seed sludge and corresponding acclimation sludge in R1–R3 (Fig. 4c–e). These findings suggest that the bacterial community composition in seed sludge and corresponding acclimation sludge was significantly different, and the microorganisms in the seed sludge of R2 showed the greatest contribution to acclimation, leading to the shortest acclimation time.

Table 2 shows that the Chao1 and Shannon indices of the acclimation sludge in R2 were the biggest, followed by the acclimation sludge in R3. In addition, in the three samples of R1–R3, Venn analysis further showed that 195, 287, and 211 OTUs were determined at 97.00% similarity, respectively. These results indicated that the acclimation sludge of R2 has the higher biodiversity than the other two, followed by the acclimation sludge in R3. Among total OTUs, 109 OTUs were shared among the total



(a) three seed sludge

(b) three corresponding acclimated sludge

(c) PMS and its corresponding acclimated sludge

(d) FHS and its corresponding acclimated sludge

(e) CIS and its corresponding acclimated sludge

(f) the corresponding acclimated sludge of PMS, FHS before and after shock

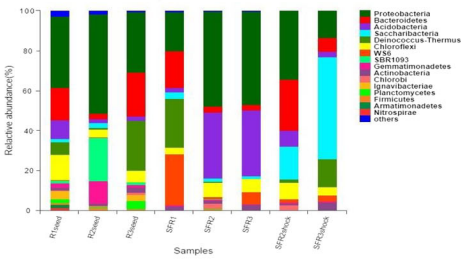
Fig. 4 Shifts of microbial community structure during start-up and shock in R1, R2, and R3 as revealed by Venn analysis. **a** Three seed sludge. **b** Three corresponding acclimated sludge. **c** PMS and its corresponding acclimated sludge. **d** FHS and its corresponding acclimated sludge. **e** CIS and its corresponding acclimated sludge. **f** The corresponding acclimated sludge of PMS and FHS before and after shock

Table 2 The microbial data statistics for acclimation samples and shock samples

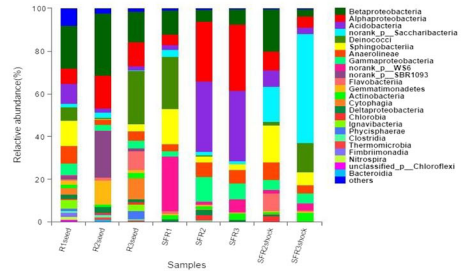
Sample\estimators	No. of high-quality reads	Shannon index	Chao1	Coverage
R1seed	46500	5.28	1014.49	0.998
R2seed	45300	3.16	419.76	0.999
R3seed	33600	4.31	774.22	0.995
SFR1	45536	2.97	228.16	0.999
SFR2	45383	3.16	376.38	0.999
SFR3	33986	2.95	246.29	0.999
R2shock	46757	3.06	210.06	0.999
R3shock	39354	2.53	273.00	0.999

OTUs, these shared OTUs accounted for 55.90%, 37.98%, and 51.66% in R1–R3, respectively (Fig. 4b), suggesting that the three acclimation sludges of R1–R3 harbored distinct microorganisms by seeding three different sources, as reported in previous investigations (Cortes-Tolalpa et al. 2016; Song et al. 2015b). These results, to a certain extent, agree with the acclimation time required in the order of PMS > CIS > FHS.

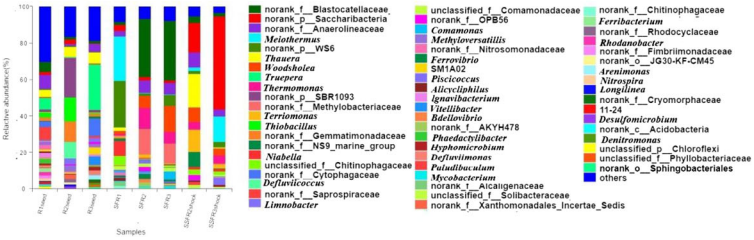
Compared with the sequencing results of the initial raw sludge (Fig. 5a), clear shifts in bacterial community composition occurred after incubation in the presence of



(a)



(b)



(c)

Fig. 5 **a** Phylum-level, **b** class-level, and **c** genus-level relative abundance of microbial communities in different samples

Table 3 Shifts of dominant bacterial genera in R1 during start-up

R1seed		SFR1	
Bacterial genus	Relative abundance	Bacterial genus	Relative abundance
norank_f_Anaerolineaceae	7.69%	norank_p_WS6	25.43%
norank_f_Saprospiraceae	7.13%	*Meiothermus*	24.40%
Truepera	6.22%	*Niabella*	8.26%
norank_f_Blastocatellaceae	5.67%	unclassified_f_Chitinophagaceae	5.26%
Thauera	3.93%	norank_p_Saccharibacteria	3.43%
norank_f_Cytophagaceae	2.86%	norank_f_Anaerolineaceae	3.12%
Ignavibacterium	2.63%	unclassified_f_Comamonadaceae	2.16%
Phaeodactylibacter	2.70%	norank_f_Blastocatellaceae	1.55%
norank_f_Nitrosmonadaceae	2.49%	*Thauera*	1.88%
norank_f_Fimbriimonadaceae	1.85%	*Woodsholea*	1.83%
Ferribacterium	1.85%	*Alicycliphilus*	1.38%
Rhodanobacter	1.78%	norank_f_Chitinophagaceae	1.23%
Nitrospira	1.46%	*Commonas*	1.15%

2,3,4-TFA. The three dominant phyla in the raw sludge of R1 were Proteobacteria (35.08%), Bacteroidetes (14.95%), and Chloroflexi (14.11%); in the raw sludge of R2 were Proteobacteria (54.16%), SBR1093 (17.73%), and Gemmatimonadetes (12.82%); in the raw sludge of R3 were Proteobacteria (34.44%), Deinococcus-Thermus (20.35%), and Bacteroidetes (17.04%). After acclimation, the microbial community showed that Deinococcus-Thermus (24.43%), Proteobacteria (19.65%), and Bacteroidetes (18.44%) were the three predominant phyla in sludge of R1, and these were Proteobacteria, Acidobacteria, and Chloroflexi in the acclimation sludge of R2 and R3. At the class level (Fig. 5b), the top five predominant classes in the raw sludge of R1 were Betaproteobacteria (20.05%), Sphingobacteriia (11.99%), Acidobacteria (9.35%), Alphaproteobacteria (7.04%), and Anaerolineae (8.12%). In the raw sludge of R2, these were Betaproteobacteria (29.01%), SBR1093 (21.48%), Alphaproteobacteria (15.39%), Gemmatimonadetes (11.32%), and Anaerolineae (2.58%). In the raw sludge of R3, these were Deinococci (25.02%), Betaproteobacteria (14.44%), Alphaproteobacteria (11.29%), Cytophagia (10.04%), and Anaerolineae (4.47%). The shift in microbial communities after incubation in the presence of 2,3,4-TFA led to a relative dominance of Deinococci (24.43), Sphingobacteriia (16.52%), Betaproteobacteria (11.04%), Alphaproteobacteria (5.08%), and Saccharibacteria (3.43%) in R1. The five classes were predominant in the acclimation sludge of R2, including Acidobacteria (33.11%), Alphaproteobacteria (27.98%), Gammaproteobacteria (11.64%), Anaerolineae (6.93%), and Betaproteobacteria (5.46%). The same five classes were predominant in the acclimation sludge of R3. The results showed that Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, and Sphingobacteriia were present in the raw sludge, and also in the acclimation sludge. The five classes have been found in degradation systems of aniline (Jiang et al. 2019) and trichlorophenol (Song et al. 2018). Thus, it can be inferred that the four classes in the raw sludge are very important and useful for the treatment of 2,3,4-TFA in SBRs.

At the genus level, shifts in the predominant genera (relative abundances > 1.00%) in the seed sludge and acclimated sludge in the three SBRs were further investigated (Fig. 5c). Tables 3, 4, and 5 show that the predominant genera in seed sludge of R1–R3, for example, *Truepera*, *Thauera*, *Defluviicoccus*, *Thiobacillus*, and unclassified affiliated to genera including norank_f_Saprospiraceae, norank_f_Cytophagaceae, and norank_f_Gemmatimonadaceae, decreased or even disappeared during start-up, suggesting that these taxonomies likely the predominant genera in the seed sludge showed minimal contributions to 2,3,4-TFA degradation. However, in the acclimation sludge, the abundance of norank_f_Blastocatellaceae and

Table 4 Shifts of dominant bacterial genera in R2 during start-up and shock

R2seed		SFR2		SFR2shock	
Bacterial genus	Relative abundance	Bacterial genus	Relative abundance	Bacterial genus	Relative abundance
Thiobacillus	14.70%	norank_f_Blastocatellaceae	32.10%	*Thauera*	18.35%
norank_f_Gemmatimonadaceae	12.82%	norank_f_Methylobacteriaceae	14.06%	norank_p_Saccharibacteria	16.36%
Defluviicoccus	12.22%	*Thermomonas*	11.30%	norank_f_Anaerolineaceae	8.20%
Thauera	5.53%	norank_f_Anaerolineaceae	6.93%	*Terrimonas*	12.10%
Methyloversatilis	3.40%	*Woodsholea*	6.51%	*Woodsholea*	8.12%
Limnobacter	3.30%	*Ferrovibrio*	2.74%	norank_f_Blastocatellaceae	3.56%
norank_p_Saccharibacteria	2.12%	unclassified_f_Comamonadaceae	2.14%	unclassified_f_Chitinophagaceae	2.76%
norank_f_Rhodocyclaceae	1.78%	*Bdellovibrio*	1.90%	*Meiothermus*	1.79%
norank_f_Blastocatellaceae	1.01%	*Terrimonas*	1.80%	unclassified_f_Solibacteraceae	1.57%
norank_f_Anaerolineaceae	0.98%	*Limnobacter*	1.77%	*Niabella*	1.40%
norank_f__Xanthomonadales_Incertae_Sedis	2.09%	norank_p_Saccharibacteria	1.74%	norank_f__NS9_marine_group	8.12%
		Comamonas	1.13%		

norank_f_Methylobacteriaceae significantly increased, and some minor genera (relative abundance < 1.00%) in the seed sludge became the predominant genera, whereas some novel predominant genera appeared. For the acclimation sludge of R1, the predominant genera were *Meiothermus* (24.40%), *Niabella* (8.26%), *Thauera* (1.88%), *Woodsholea* (1.83%), *Alicycliphilus* (1.38%), *Commonas* (1.15%), and unclassified genera including unclassified_f_Chitinophagaceae (5.26%), norank_p_Saccharibacteria (3.43%), norank_f_Anaerolineaceae (3.12%), unclassified_f_Comamonadaceae (2.16%), norank_f_ Alcaligenaceae (2.07%), norank_f_Blastocatellaceae (1.55%), and norank_f_Chitinophagaceae (1.23%). In R2, the main genera included *Thermomonas* (11.30%), *Woodsholea* (6.51%), *Ferrovibrio* (2.74%), *Bdellovibrio* (1.89%), *Terrimonas* (1.80%), *Commonas* (1.14%), and unclassified Blastocatellaceae (32.10%), Methylobacteriaceae (14.06%), Anaerolineaceae (6.93%), and Saccharibacteria (1.74%). In R3, these included *Thermomonas* (6.43%), *Commonas* (4.32%), *Mycobacterium* (1.38%), *Woodsholea* (14.28%), and unclassified norank_f_Blastocatellaceae (32.92%), norank_f_Methylobacteriaceae (10.82%), norank_f_Anaerolineaceae (6.45%), unclassified_f_Comamonadaceae (2.05%), norank_p_Saccharibacteria (1.23%), and norank_f_Chitinophagaceae(1.22%).

The Blastocatellaceae species is known to degrade ethyl *tert*-butyl ether (van der Waals et al. 2019). The Methylobacteriaceae has been reported in polyhydroxyalkanoates degradation system (Dionisi et al. 2005). Several reports have shown that *Thermomonas* can degrade polycyclic aromatic hydrocarbons (Nzila et al. 2018) and aniline (Hou et al. 2018). The bacteria Anaerolineaceae can also play a very important role in the biodegradation of tetrabromobisphenol A (Chen et al. 2019) and aniline (Sun et al. 2015). Several literatures have reported that Saccharibacteria, Alcaligenaceae, and Comamonadaceae can play major roles in the biodegradation of chlorophenol (Sun et al. 2018; Zhao et al. 2018; Zilouei et al. 2006). Additionally, *Mycobacterium* and *Niabella* have been reported in degradation systems of polycyclic aromatic hydrocarbons (Wang et al. 2018) and phenanthrene (Jiao et al. 2016), respectively. *Ferrovibrio* has been shown to be capable of degrading cyhalothrin (Song et al. 2015a), Chitinophagaceae has the capacity of degrading polycyclic aromatic hydrocarbons (Blanco-Enriquez et al. 2018), and *Alicycliphilus* can degrade

Table 5 Shifts of dominant bacterial genera in R3 during start-up and shock

R3seed		SFR3		SFR3shock	
Bacterial genus	Relative abundance	Bacterial genus	Relative abundance	Bacterial genus	Relative abundance
Truepera	20.35%	norank_f_Blastocatellaceae	32.94%	norank_p_Saccharibacteria	51.08%
Thauera	6.84%	*Woodsholea*	14.28%	*Meiothermus*	13.92%
norank_f_Anaerolineaceae	6.30%	norank_f_Methylobacteriaceae	10.82%	*Thermomonas*	4.46%
norank_f_Cytophagaceae	4.71%	norank_f_Anaerolineaceae	6.45%	*Woodsholea*	3.99%
Limnobacter	3.64%	*Thermomonas*	6.43%	norank_f_Anaerolineaceae	3.96%
Vitellibacter	3.97%	*Comamonas*	4.32%	*Piscicoccus*	3.69%
norank_f_Blastocatellaceae	2.48%	unclassified_f_Comamonadaceae	2.05%	*Terrimonas*	2.18%
Arenimonas	2.63%	*Mycobacterium*	1.38%	norank_f_Blastocatellaceae	2.03%
Hyphomicrobium	1.88%	norank_p_Saccharibacteria	1.23%	norank_f_Chitinophagaceae	1.76%
Defluviimonas	1.79%	norank_f_Chitinophagaceae	1.22%	*Niabella*	1.24%
norank_f_Cryomorphaceae	1.68%	*Ferrovibrio*	1.03%	unclassified_f_Comamonadaceae	1.08%

aromatic hydrocarbon such as phthalate polyester polyurethanes (Perez-Lara et al. 2016) and nonylphenol (Bai et al. 2017). Thus, it can be inferred that the presence of these genera is very important and useful for 2,3,4-TFA degradation. Unfortunately, the genera with the highest abundance, including *Meiothermus*, *Woodsholea*, and *Commonas*, were the first to be reported in aromatic hydrocarbon degradation systems, although their capacity of degrading 2,3,4-TFA remains unclear.

3.4.2 Shifts in Bacterial Community Composition After Shock

In the shock sludge samples of R2 and R3, only 173 and 189 OTUs were determined to have 97% similarity, respectively, suggesting that the bacterial diversity decreased after shock (Fig. 4f). Approximately 138 OTUs and 140 OTUs were shared with the corresponding acclimation sludge, accounting for 79.77%, and 74.07% in the shock sludge samples, of R2 and R3, respectively (Fig. 4f), which was consistent with the above results that R2 had the stronger resistance to 2,3,4-TFA shock load than that of R3. These results indicated that a part of microorganisms disappeared, whereas some new distinctive ones appeared to adapt to the high concentration of 2,3,4-TFA.

At the phyla level of R2 and R3, clear shifts in the relative abundance of every phylum were observed, especially for the obvious decrease of Proteobacteria and Acidobacteria and a significant increase in Bacteroidetes and Saccharibacteria. Table 4 shows that the predominant genera in the acclimation sludge of R2, *Thermomonas*, *Bdellovibrio*, *Comamonas*, *Ferrovibrio*, and unclassified genera including norank_f_Blastocatellaceae and norank_f_Methylobacteriaceae, decreased or even disappeared after shock. Interestingly, in the shock sludge, *Thauera* was the most abundant predominant genus (18.35%) and is known to degrade phenol (Gui et al. 2019). Its emergence might be related to the high concentration of 2,3,4-TFA used in the shock experiment. In addition, the abundance of *Saccharibacteria* and *Terrimonas* remarkably increased from 1.74% and 1.80% in the acclimation sludge to 16.36% and 12.10% in shock sludge, respectively. However, for R3, the abundance of *Woodsholea*, *Thermomonas*, *Comamonas*, *Ferrovibrio*, norank_f_Blastocatellaceae, norank_f_Methylobacteriaceae, and norank_f_Anaerolineaceae decreased or even disappeared after shock. *Meiothermus* (13.92%), *Piscicoccus* (3.69%), and *Terrimonas* (2.18%) became the predominant bacterial genera, and the abundance of norank_p_Saccharibacteria remarkably increased from 1.23% in the acclimation sludge to 51.08% in the shock sludge. *Terrimonas* can effectively degrade polycyclic aromatic hydrocarbon (Singleton et al. 2016). Our results suggest that changes in the composition of new predominant bacterial genera occurred to adapt the adverse operation conditions in R2 and R3, and norank_p_Saccharibacteria and *Terrimonas* could have the stronger resistance to 2,3,4-TFA shock load than the other predominant bacterial genus. Additionally, based on the fact that the defluorination hardly occurred

during recovery period, it can be inferred that norank_f_Blastocatellaceae and norank_f_Methylobacteriaceae largely contributed to defluorination during 2,3,4-TFA degradation.

The functional microbial community potentially involved in 2,3,4-TFA biodegradation in the acclimation sludge of the three SBRs shows different abundances and diversity after exposure to 2,3,4-TFA, indicating an activated sludge condition-dependent response of the functional activated sludge bacteria to 2,3,4-TFA contamination. Additionally, the responses of the microbial community in the acclimation sludge of the three SBRs to 2,3,4-TFA shock load varied. Taken together, we conclude that the inoculum influences the treatment performance of 2,3,4-TFA in SBR, as well as shock resistance of the reactor.

4 Conclusions

This study investigated the influence of three different seeding sources, including PMS, FHS, and CIS, on 2,3,4-TFA treatment during start-up and shock. Acclimation of the activated sludge for the biodegradation of 2,3,4-TFA was achieved using the stepwise feeding of 2,3,4-TFA according to the inhibition situation. Among the three seeding sources, FHS exhibited better treatment efficiency, shorter acclimation time, and faster recovery than the other two types of sludge. Compared with PMS, CIS showed earlier defluorination reaction, but had lower resistance to 400 mg/L of 2,3,4-TFA shock load. 16S rDNA sequence analysis revealed that the bacterial genera *Blastocatellaceae* and *Methylobacteriaceae* could play critical roles in degrading 2,3,4-TFA. These results clearly suggest that the type of seeding source is a significant factor influencing the treatment performances of 2,3,4-TFA, but these effects may vary during start-up and shock. FHS was optimal for the treatment of 2,3,4-TFA. In the absence of FHS, CIS could be employed as an alternative for the treatment of the low-concentration 2,3,4-TFA due to short start-up duration of the reactor, but PMS instead of CIS as inoculum could increase shock resistance.

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