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Study of Inhibitory Effects on Aerobic Biomass: Interaction Between Per-/Polyfluoroalkyl Substances (PFAS) and Traditional Toxic Compounds

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Abstract: This work aims to investigate the inhibitory effects of PFAS on activated sludge biomass and compare them to the impact of conventional toxic substances that may be found in wastewater, such as phenol, trichlorophenol, and copper sulphate. Respirometric assays, i.e., batch and continuous oxygen uptake (OUR) tests, were used to evaluate the response of aerobic biomass to various aqueous wastes containing PFAS and traditional inhibitory compounds. The study is divided into many phases, assessing the inhibitory effects of tested pollutants over different time scales: short-term exposure (10 min contact time in batch tests) and medium-term exposure (several hours in continuous tests). The results highlight that while PFAS did not cause acute or medium-term chronic toxicity on biomass (OUR values between 6 and 8 mgDO $(gSSV{\cdot}h)^{-1}$), copper sulphate (at a concentration of 166.7 mg L^{-1}) involved irreversible inhibition beyond critical exposure time. Furthermore, the biodegradability of the studied substrates was impacted by the interaction between PFAS and conventional toxic substances, with certain mixtures showing the capacity to lessen inhibitory effects (OUR values between 5 and 20 mgDO $(gSSV{\cdot}h)^{-1}$). This study provides new knowledge on the potential inhibitory mechanisms of PFAS and underlines the importance of considering the combined effects of these pollutants with other contaminants. The findings support the development of more effective treatment approaches for PFAS-contaminated wastewater and help in improving the operational strategies of wastewater treatment plants.

Keywords: PFAS; PFOA; PFOS; aqueous waste; phenols; inhibition; wastewater; aerobic biomass

Academic Editors: Florentina Laura Chiriac and Stefania Gheorghe

Received: 17 March 2025
Revised: 17 April 2025
Accepted: 23 April 2025
Published: 25 April 2025

Citation: Collivignarelli, M.C.; Bellazzi, S.; Calabria, L.M.R.; Baldi, M.; Abbà, A. Study of Inhibitory Effects on Aerobic Biomass: Interaction Between Per-/Polyfluoroalkyl Substances (PFAS) and Traditional Toxic Compounds. *Environments* **2025**, *12*, 139. https://doi.org/10.3390/environments12050139

1. Introduction

Per- and polyfluoroalkyl substances (PFAS) comprise over 4700 synthetic chemical compounds, characterized by extremely stable carbon–fluorine bonds [1]. The acronym PFAS refers to "perfluoroalkyl and polyfluoroalkyl substances", which are highly fluorinated aliphatic compounds containing at least one carbon atom where all hydrogen substituents have been replaced by fluorine atoms [2].

Since the mid-20th century, PFAS have been employed in a wide range of industrial and consumer products due to their unique properties, including resistance to heat, oil, stains,

grease, and water. Their hydrophobic and oleophobic nature makes them ideal for various applications, such as fabric protectors, non-stick cookware, adhesives, food packaging, as well as pesticides and herbicides, lubricants, and flame retardants [3]. The most well-known PFAS are perfluorooctanoic acid (PFOA, also known as C_8) and perfluorooctane sulfonic acid (PFOS), which, due to their high molecular weight and complex structure, are highly resistant substances and pose a threat to human and animal health [4]. Furthermore, PFAS are very mobile in the environment because of their high solubility in water; they contaminate surface water, groundwater, and surrounding soil, and they can bioaccumulate in humans and wildlife [5]. Toxicological concerns include increasing cancer risk, negative impacts on growth [6], development [7], reproduction, and thyroid function, as well as impairments to the immune liver and metabolic systems [8].

PFAS are characterized by high stability against both biological and chemical degradation and through the sewage network, PFOS and PFOA enter wastewater treatment plants (WWTPs) [3].

To assess the possible inhibitory effect that perfluoroalkyl and polyfluoroalkyl substances have on biological treatments, respirometry assays have been utilized. Respirometry measures and interprets the oxygen consumption in a biological system to degrade and remove a substrate [9]. It is used to assess the biodegradability of wastewater, measure the kinetic constants, determine the oxygen demand for biomass, and verify any inhibitory effects that wastewater may have [10]. Oxygen uptake rate (OUR) tests were conducted to assess the respiration rate of biomass over time. Variations in the specific oxygen uptake rates (OURs), which is the ratio between dissolved oxygen consumption and the concentration of volatile suspended solids (VSS), can indicate that biomass is being inhibited by any toxic compounds. Specifically, an exogenous OURs value (measured after the contact of the tested substrate with biomass) lower than the endogenous one (measured with the presence of biomass only) can serve as a warning signal of biomass inhibition due to the presence of toxic substances in tested substrate.

To assess the degree of inhibition of perfluoroalkyl substances (PFAS) on aerobic-activated sludge biomass, "conventional" toxic substances (phenol, trichlorophenol, and copper sulphate) are used. These compounds can be found in industrial wastewater and can be treated by the "aqueous waste" platform, which gave us substrates rich in perfluoroalkyl substances.

It is known that phenol (C_6H_5OH) is an aromatic organic compound, composed of a benzene ring attached to a hydroxyl group (-OH). Phenol and its derivatives are hazardous and carcinogenic compounds that are ubiquitous in industrial wastewater generated by the steel industry, coal conversion, coking plants, and oil refineries [11]. The toxicity of phenol strongly reduces bacterial cell activity [12]. Recent studies have found a negative impact on COD removal (over 50% inhibition for phenol concentrations above 80 mg L^{-1}) and on nitrification (over 60% inhibition for phenol concentrations above 250 mg L^{-1}) [13].

Trichlorophenols ($C_6H_3Cl_3O$)—TCP—are a class of organic compounds derived from phenol, in which three chlorine atoms replace hydrogen atoms on the benzene ring. It inhibits many microorganisms, including nitrifiers bacteria. In the study by Shasha Zou et al., 2020 [14] removal rates with 2 mM of TCP resulted in ammonium removal rates of 0.35 mM h^{-1} and 0.15 mM h^{-1}.

Finally, copper sulphate ($CuSO_4$) inactivates microorganisms by blocking their growth and enzymatic functions. Notably, increasing the concentration of this substance leads to a higher inhibition rate. For example, at a concentration of 200 mg L^{-1}, an inhibition of 83% was observed. This effect, however, is not strictly linear across all concentrations. At lower doses, the inhibition increases more gradually, likely due to the microbial community's

buffering and resistance capacity. As concentrations rise beyond a certain threshold, the toxic effects intensify more rapidly, suggesting a non-linear dose–response relationship [15].

This study aims to evaluate the potential toxic effects of aqueous waste containing PFAS on mesophilic activated sludge biomass in biological treatment processes and to analyze the same behaviour with conventional toxic substances. Despite several studies that have investigated the persistence and environmental risk of PFAS, research focusing specifically on their impact on biological treatment systems, particularly on the activity and performance of biological processes, are limited. Prior works have typically examined either the chemical degradation pathways of PFAS or their removal efficiency in advanced treatment processes such as adsorption or oxidation [16,17]. However, few studies have directly compared the inhibitory effects of PFAS with other toxic compounds within the biological processes. This investigation addresses that gap by systematically assessing the biodegradability of substrates in the presence of PFAS and benchmark toxic compounds, thereby offering new insights into how PFAS may disrupt biological treatment and informing more effective management strategies for wastewater systems facing contamination by emerging pollutants.

2. Materials and Methods

The methodological approach followed in this work was developed in two steps:

1. Evaluation of the Potential Inhibitory Effects of Aqueous Waste with High PFAS Concentrations Compared to Traditional Inhibitors;
2. Evaluation of the Potential Inhibitory Effects of Substrates Combining Aqueous Waste with High PFAS Concentrations and Traditional Inhibitors:
   (a) Effects at short term
   (b) Effects at medium-long term

2.1. Equipment Used for the OUR Test

The OUR (Oxygen Uptake Rate) respirometric tests was carried out with the use of a multi-component system (Figure 1). Since multi-OUR tests measure an entire respirogram consisting of multiple successive OUR tests, the need for consecutive aeration periods requires an automated system [10]. The dissolved oxygen (DO) concentration was measured using the portable multiparametric meter WTW® Multi-Line Multi 3510 IDS, with optical sensors for dissolved oxygen WTW® IDS FDO 925 (Xylem Analytics Germany Sales GmbH & Co, Mainz, Germany) (referred to as "DO probe"). The measured DO concentration was transferred to a laptop via USB connection and recorded using the MultiLab Importer add-in for Excel® (Version 2503).

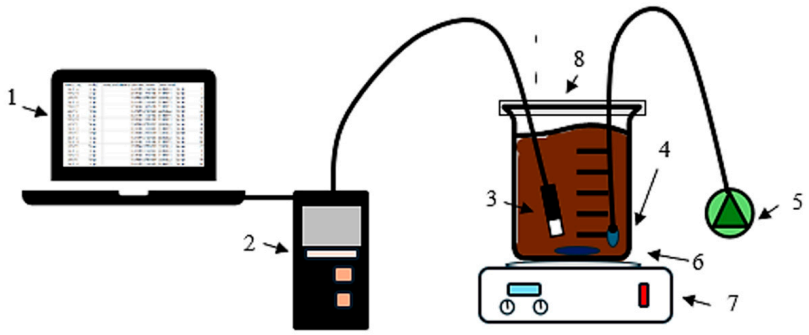

Figure 1. Schematic representation of the laboratory equipment for OUR respirometric tests. Legend: 1: Laptop: data transfer; 2: Oximeter; 3: DO probe; 4: Porous stone; 5: Air compressor; 6: Magnet; 7: Magnetic and thermostatic stirrer; 8: Parafilm.

2.2. Execution and Processing of OUR Tests

The methodology adopted for the batch OUR tests (also called single-track) provided that a known quantity of biomass was transferred into a conical flask, which was placed on a magnetic stirrer to keep the sample well-mixed during the tests. The biomass sample was then aerated until the DO saturation. For exogenous OUR measurements, after stopping aeration, the tested substrate (with a volume equal to the biomass one) was added. To prevent air exchange with the external environment, the flask was sealed with parafilm. Dissolved oxygen concentration data (mgDO L^{-1}), recorded at 5 s intervals by the probe, were transferred to a laptop and plotted in a scatter graph.

OUR was calculated as the slope of the linear regression line interpolating the measured data [10] divided by the VSS concentration in the batch reactor. The OURs (mgDO $gVSS^{-1}$ h^{-1}) indicate the oxygen consumption rate of the biomass relative to the degradation of organic matter in the substrate alone [18].

If necessary, the non-specific OUR value was normalized to a temperature (T) of 20 °C using the following formula:

$$\text{OUR (20 °C)} = \text{OUR} \cdot \theta^{(20-T)}$$

where θ is equal to 1.05 if T < 20 °C and 1.07 if T > 20 °C.

Each measured value of oxygen uptake rate (OUR) was calculated by fitting a linear regression line to the oxygen consumption data over time, with all regressions showing a coefficient of determination (R^2) greater than 0.98, ensuring the reliability of the fit. To improve the robustness of the results, each type of test was replicated four times under identical experimental conditions. The average value of the replicated measurements was then used to represent each experimental condition. Furthermore, for each set of replicates, the standard deviation and the 95% confidence interval were calculated to assess the variability of the data and to quantify the associated measurement, uncertainty. To assess possible medium-long term inhibitory effects and pollutant removal efficiency, continuous OUR tests were carried out only once. These tests were performed with the same instrumentation used for the single-run tests; the main operational differences were the duration and aeration. In this case, the duration of the test was similar to the hydraulic retention time (HRT) of the full-scale bioreactor; however, for the aeration, the compressor was set to switch off at 5 mg L^{-1} (close to the saturation value of DO) and switch on again at 2 mg L^{-1}, to produce a full respirogram.

2.3. Tested Biomass

In this study, activated sludge biomass was collected from a wastewater treatment plant (WWTP) located in Northern Italy. Specifically, the biomass was sampled from the conventional activated sludge (CAS) process of a municipal WWTP (referred to as WWTP-1). This plant treats domestic sewage only and has a capacity of 100,000 Population Equivalents (PE). The selected biomass represents a typical mesophilic microbial community commonly found in municipal wastewater treatment systems. The optimal working temperature of these processes is approximately 20 °C. In the oxidation tank, the VSS concentration was between 3 and 4 gVSS L^{-1}.

2.4. Tested Substrates

The tested substrates are three different industrial wastewater samples containing PFAS, designated as R1, R2, and R3, with their main characteristics listed in Table 1. The three wastewater samples, referred to as R1, R2, and R3, are actual landfill leachates collected from different treatment plants located in Northern Italy. The samples were collected directly from the leachate storage tanks using appropriate sampling procedures to ensure

their representativeness and preservation prior to analysis. The values reported in Table 1 (COD, BOD_5, pH, PFBS, PFOA, PFOS) were quantified by specialized analysis laboratories.

Table 1. Main characteristics of R1, R2, and R3.

Parameter	U.M.	R1	R2	R3
COD	mg L^{-1}	2333	3300	1350
BOD_5	mg L^{-1}	400	1730	460
pH	-	8.00	7.80	8.09
PFBS	µg L^{-1}	21.908	37.570	15.868
PFOA	µg L^{-1}	2870.060	24.142	2.340
PFOS	µg L^{-1}	148.380	0.788	0.084
Sum of PFAS	µg L^{-1}	3267.641	78.703	26.381

Both batch and continuous OUR tests were conducted on these samples to evaluate the behaviour of biomass with each specific wastewater. Additionally, tests were performed on three different pure conventional inhibitors, found in the aqueous waste treated by the platform: phenol (PH), trichlorophenol (TCP), and copper sulphate ($CuSO_4$).

Subsequently, tests were also carried out on substrates obtained by combining the waste (R1, R2, and R3) with these conventional inhibitors.

2.5. Experimental Tests

Following the methodological approach, the initial batch OUR tests were conducted to assess the wastewater samples containing PFAS (R1, R2, and R3) and the three conventional inhibitors (PH: phenol, TCP: 2,4,6-trichlorophenol, and $CuSO_4$: copper sulphate).

In the batch tests, the concentrations of tested toxic pollutants were defined to create an increase ranging from 50 mg L^{-1} to 100 mg L^{-1}, up to a maximum concentration of 200 mg L^{-1}. For practical reasons, during laboratory tests, a minimum (47.62 mg L^{-1}) and a maximum (200 mg L^{-1}) concentration was reached. Subsequently, noticing the strong inhibitory effect of copper sulphate, an additional batch test was conducted to observe its effect at a copper sulphate concentration of 166.7 mg L^{-1}.

Once the initial results were obtained, batch OUR tests and continuous OUR tests were carried out by combining waste and toxic substances to determine whether exceeding the slow threshold of conventional inhibitors created significant toxicity or allowed possible biomass acclimatization. For continuous OUR tests, the amount of inhibitors was dosed to reach a concentration of 200 mg L^{-1}. Table 2 shows all the tests carried out, the substrates tested, and their concentrations in the 2 steps. The concentrations of toxic pollutants used in the batch tests were selected to reflect realistic contamination levels potentially found in industrial liquid wastes while also allowing for the assessment of microbial inhibition dynamics across a meaningful gradient. The concentration range—approximately between 50 mg L^{-1} and 200 mg L^{-1}—was chosen based on values reported in the literature and technical guidelines as representative of heavily contaminated effluents, particularly from industries such as petrochemical, pesticide manufacturing, metal plating, and electronics [19].

Table 2. OUR tests carried out, the substrates tested and their concentrations.

Test Type	Tested Substrate	Concentration of Pollutants				Step
		Sum of PFAS [μg L^{-1}]	PH [mg L^{-1}]	TCP [mg L^{-1}]	$CuSO_4$ [mg L^{-1}]	
Batch	R1	3267.641				1—Comparison between PFAS and traditional inhibitors
	R2	78.703				
	R3	26.381				
	PH		47.62			
			90.9			
			200			
	TCP			47.62		
				90.9		
				200		
	$CuSO_4$				47.62	
					90.9	
					166.7	
					200	
Batch	R1 + PH	3267.641				2a—Combination of PFAS and traditional inhibitors: effects at short term
		2189.319	66.67			
		1633.821	100			
		1110.998	133.34			
	R1 + TCP	3267.641				
		2189.319		66.67		
		1633.821		100		
		1110.998		133.34		
	R1 + $CuSO_4$	3267.641				
		2189.319			66.67	
		1633.821			100	
		1110.998			133.34	
	R2 + PH	78.703				
		52.731	66.67			
		39.352	100			
		26.759	133.34			
	R2 + TCP	78.703				
		52.731		66.67		
		39.352		100		
		26.759		133.34		
	R2 + $CuSO_4$	78.703				
		52.731			66.67	
		39.352			100	
		26.759			133.34	
	R3 + PH	26.381				
		17.675	66.67			
		13.191	100			
		8.97	133.34			

Table 2. *Cont.*

Test Type	Tested Substrate	Concentration of Pollutants				Step
		Sum of PFAS [μg L^{-1}]	PH [mg L^{-1}]	TCP [mg L^{-1}]	$CuSO_4$ [mg L^{-1}]	
Batch	R3 + TCP	26.381				2a—Combination of PFAS and traditional inhibitors: effects at short term
		17.675		66.67		
		13.191		100		
		8.97		133.34		
	R3 + $CuSO_4$	26.381				
		17.675			66.67	
		13.191			100	
		8.97			133.34	
Continuous	R1 + PH	3267.641	200			2b—Combination of PFAS and traditional inhibitors: effects at medium-long term
	R1 + TCP	3267.641		200		
	R1 + $CuSO_4$	3267.641			200	
	R2 + PH	78.703	200			
	R2 + TCP	78.703		200		
	R2 + $CuSO_4$	78.703			200	
	R3 + PH	26.381	200			
	R3 + TCP	26.381		200		
	R3 + $CuSO_4$	26.381			200	

Phenol and chlorinated phenols like 2,4,6-trichlorophenol are frequently detected in industrial wastewaters, with concentrations ranging from a few mg L^{-1} up to over 200 mg L^{-1} depending on the source and treatment efficiency [20]. Similarly, copper sulfate is commonly found in electroplating and mining effluents, where copper concentrations can reach or exceed 100 mg L^{-1} [21]. The maximum tested concentration of 200 mg L^{-1} was selected to simulate worst-case scenarios, while the additional test at 66.7 mg L^{-1} was introduced after observing the high inhibitory effect of copper sulfate, allowing for a more detailed understanding of the inhibition threshold.

These concentrations are, therefore, consistent with those found in real-case liquid waste streams and provide a valid basis for evaluating the performance and resilience of biological treatment systems under stress from toxic compounds.

3. Results and Discussion

3.1. Evaluation of the Potential Inhibitory Effects of Aqueous Waste with High PFAS Concentrations Compared to Traditional Inhibitors

In the first phase of the experimental activity, the OUR tests were performed by dosing waste with a high PFAS content (R1, R2, R3) as is (not diluted). The aim of this analysis was to verify a possible inhibitory effect on the respiration of the activated sludge biomass placed in contact with the raw liquid waste.

OUR tests performed on activated sludge biomass in the presence of different liquid wastes rich of PFAS (R1, R2, R3), reported in Figure 2, show that all exogenous single-OUR values are higher than the endogenous one. This indicates the absence of short-term acute toxicity effects, since the biomass continues to consume oxygen in the presence of the fed substrate.

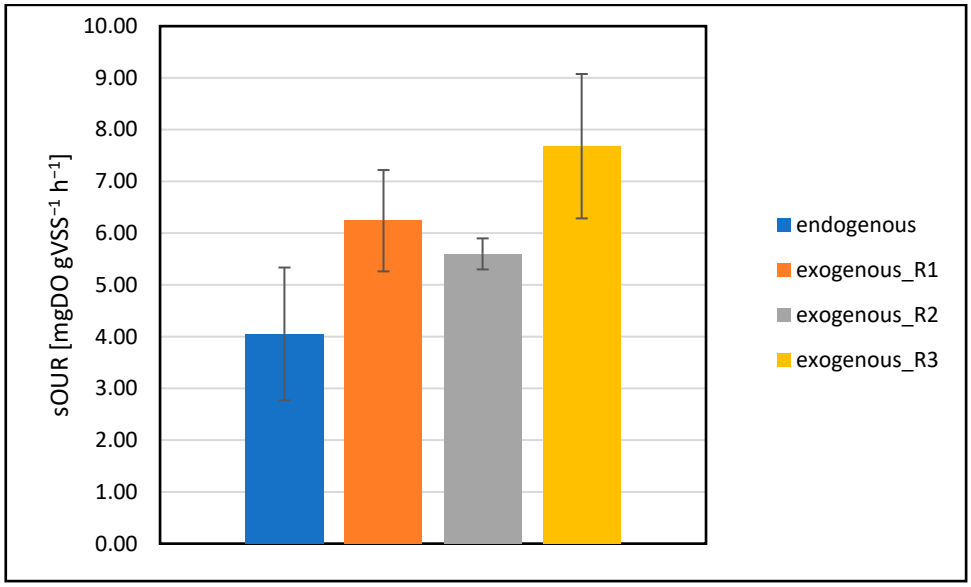

Figure 2. Results of OUR batch tests with dosing of R1, R2, and R3 waste containing PFAS, compared with the endogenous respiration of the biomass used.

The trend of oxygen consumption is strictly related to the biochemical oxygen demand (BOD) load introduced with the different wastes. In particular, the addition of R1, R2, and R3 determines an increase in the rate of oxygen consumption, since the high concentration of BOD present in these wastes contributes to increasing the overall BOD of the final mixture.

However, analyzing the results in relation to the total sum of PFAS present in the different wastes, no direct correlation is observed between the content of these compounds and biomass respiration. In fact, although the three wastes present very different PFAS concentrations, even with differences in orders of magnitude, these variations are not reflected in the measured respiration values. A clear example is the case of R1, which determines a higher respiration than R2, despite the differences in PFAS concentration. This suggests that the presence of PFAS, at least in the experimental conditions analyzed, does not significantly influence the respiratory kinetics of the biomass.

To evaluate a possible inhibitory effect due to the presence of waste containing PFAS, the respiration rates of the activated sludge biomass after exposure to "traditional" inhibitors were calculated. These inhibitors could be present in industrial wastewater treated by the platform that provided us with the PFAS-containing substrates. Phenol, trichlorophenol (TCP), and copper sulphate were tested, each at different concentrations as reported in Table 2. The results obtained are illustrated in Figure 3.

The data analysis showed the specific OUR values obtained by dosing the inhibitory substances and comparing the values with the endogenous respiration value (basal biomass respiration) [22]. The results show that both copper sulphate, mainly, and trichlorophenol induce a partial inhibition of the biomass starting from the first concentration tested (about 50 mg L^{-1}) [14]. This indicates that these compounds interfere with the metabolic activity of the microorganisms present in the activated sludge, reducing their respiratory capacity. Copper sulphate at a concentration of 200 mg/L completely inhibits biomass respiration with an OUR value of 0.62 mgDO $gVSS^{-1}$ h^{-1} (15% of the initial respiration rate value) [23]. The inhibitory effect of copper sulphate on biomass respiration is mainly due to the toxic action of free Cu^{2+} ions. These ions can penetrate microbial cells and interact with key enzymatic systems involved in cellular respiration, particularly by binding to thiol (-SH) groups of enzymes, thereby altering their structure and functionality. In addition, copper can induce oxidative stress by promoting the formation of reactive oxygen species (ROS), which further damages cellular components and reduces microbial viability [24].

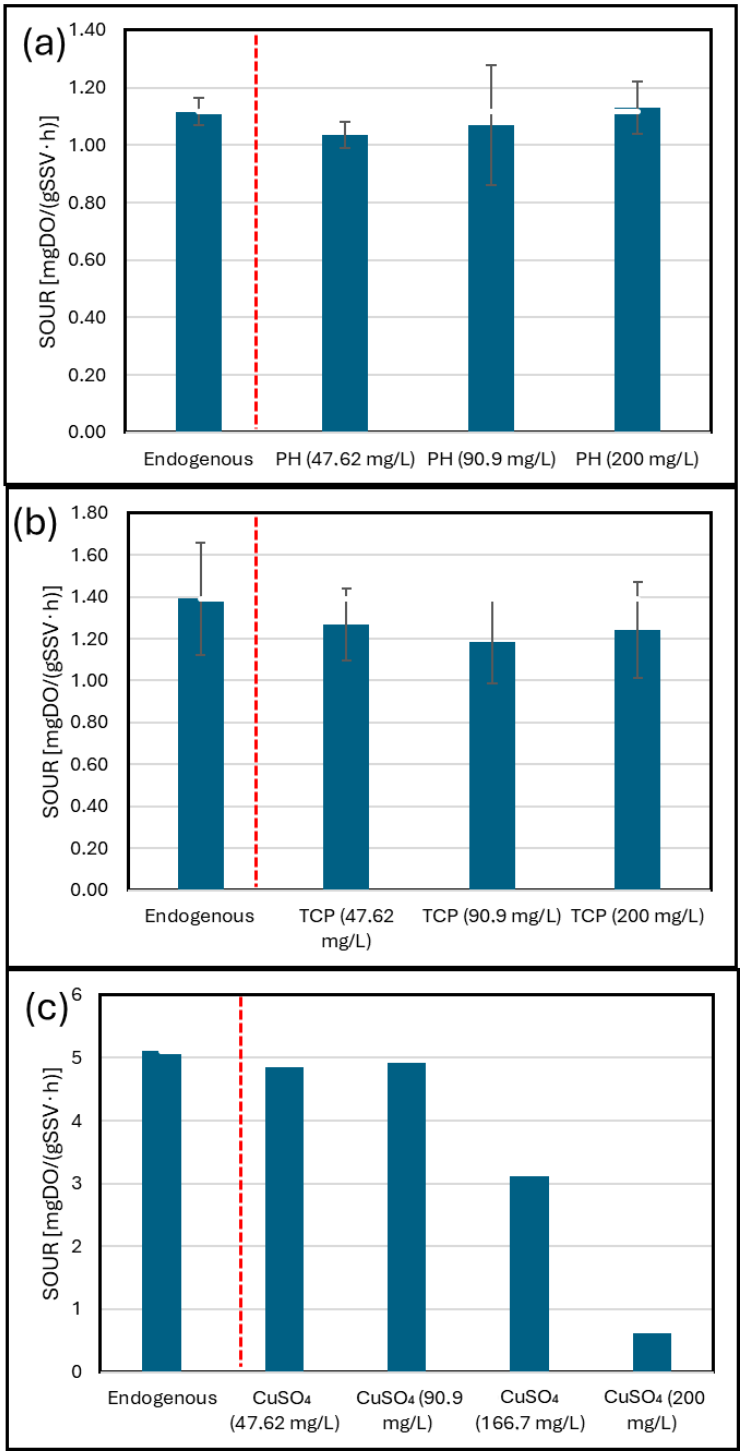

Figure 3. Results of OUR batch tests with dosage of toxic substances compared with the endogenous respiration of the biomass used. (**a**) Effect of phenol; (**b**) Effect of trichlorophenol; (**c**) Effect of copper sulphate.

On the contrary, phenol does not seem to determine a significant inhibitory effect in the short term (10 min of contact) probably due to its strong tendency to volatilize or be physically removed from the aqueous phase under agitation conditions, thereby reducing its effective concentration and contact time with the target organisms [25]. In all the experimental conditions tested, the biomass respiration values in the presence of phenol are comparable to endogenous respiration, suggesting that, at least in the concentrations

and exposure times considered, this compound does not immediately compromise the biological activity of the biomass.

3.2. Evaluation of the Potential Inhibitory Effects of Substrates Combining Aqueous Waste with High PFAS Concentrations and Traditional Inhibitors

In the second phase, batch and continuous OUR tests were used to assess the short- and long-term effects of high-concentration inhibitors (200 mg L^{-1}) mixed with aqueous waste (R1, R2, R3) on biomass activity, providing insight into immediate toxicity and adaptation under complex contamination conditions [9].

3.2.1. Effects at Short Term

The results reported in Figure 4 illustrate the respiration values of activated sludge biomass exposed to different mixtures containing aqueous waste characterized by the presence of PFAS and phenol. The data analysis highlights how the presence of waste containing PFAS and, therefore, the contribution of BOD_5 to the biomass resulting from their dosage (as reported in Table 2) determines a significant increase in respiration. This increase exceeds the levels of endogenous respiration, indicating a good bioavailability of the substrate and confirming that the biomass is able to metabolize the organic compounds present in the waste [26].

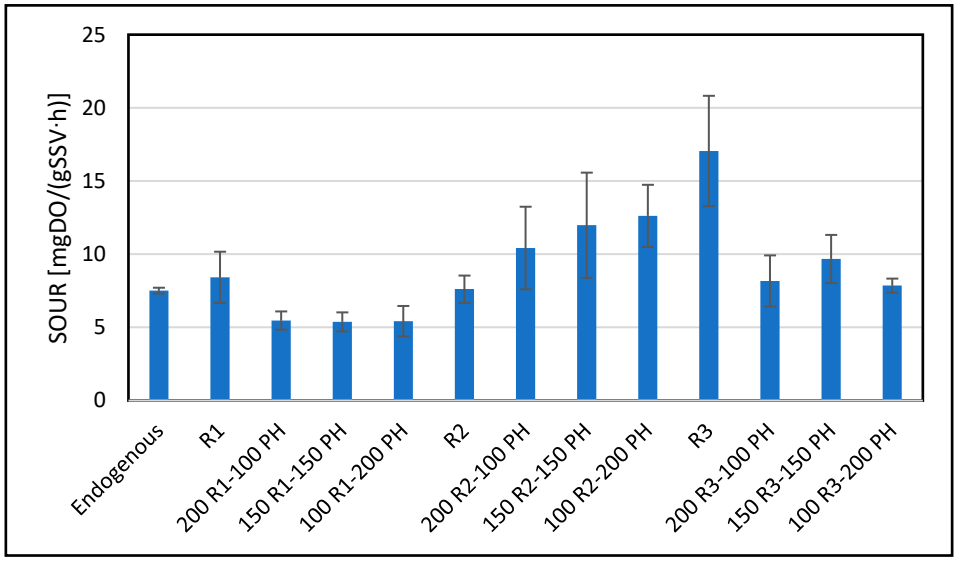

Figure 4. Results of OUR batch tests analyzing the effect of waste containing PFAS (R1, R2, R3) appropriately mixed with phenol in order to evaluate a comparison with a known inhibitor.

The presence of PH does not appear to have an inhibitory impact on tested biomass. However, respiration values similar to the endogenous respiration of biomass are noted when PH is specifically combined with R1. This suggests that, in this specific condition, the addition of phenol does not contribute to a significant increase in respiratory activity, unlike what occurs with the addition of PH to the other two liquid wastes in which the contribution of organic matter still shows palatability for biomass. This suggests a possible interaction between the characteristics of R1 and the availability of phenol as a carbon source for biomass. Additionally, when PH is added to R3, a reduction in respiration is observed, although it remains above endogenous level.

The effect of PFAS substrates mixed with TCP is illustrated in Figure 5. Similarly to what was observed in the previous tests, in which PH was present, the contribution of organic matter deriving from the dosage of the liquid waste containing PFAS involves an increase in the biomass respiration values. However, unlike PH, which in the previous step

had not cause any inhibitory effect on the biomass even when dosed in different concentrations, TCP completely loses the inhibitory capacity previously highlighted. This suggests that, in the context of short-term tests, the biomass is able to select the molecules most suitable for its metabolism, excluding those potentially toxic or inhibitory. A particularly relevant aspect is that liquid waste containing PFAS, despite the high concentrations of these compounds, does not determine any inhibitory effect on the biomass. On the contrary, they seem to enhance respiration activity, probably thanks to the contribution of BOD_5 present in the substrate [27]. This result suggests that, at least in the short term, biomass is able to exploit the organic fraction available in liquid waste containing PFAS, mitigating any toxic effects and transforming the substrate into a metabolizable resource.

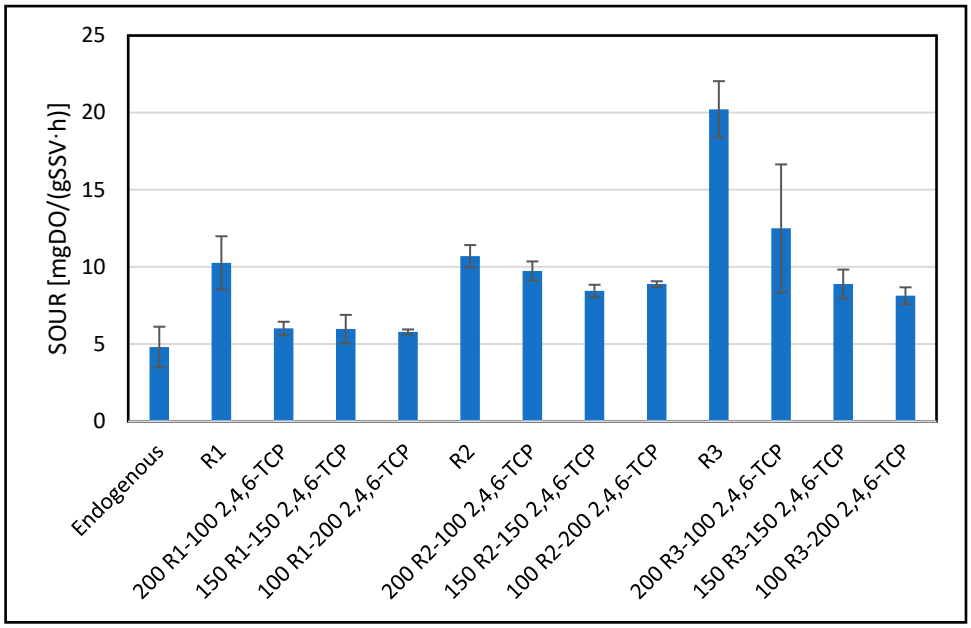

Figure 5. Results of OUR batch tests analyzing the effect of waste containing PFAS (R1, R2, R3) appropriately mixed with trichlorophenol in order to evaluate a comparison with a known inhibitor.

The results reported in Figure 6 illustrate the effect of PFAS substrates mixed with copper sulphate on biomass respiration. In this case, the behaviour observed is even more marked with respect to the presence of TCP. In fact, contrary to what one might expect, as the amount of $CuSO_4$ dosed increases, an increase in biomass respiration values is recorded. This phenomenon could be attributed to a biomass lysis process [28]. As highlighted in the previous step, copper sulphate has an acute toxic effect on the biomass, which could lead to the destruction of some cells. This lysis process can release a significant amount of organic matter from the disintegrated cells into the mixture, making it available for metabolism by the surviving biomass. As a result, the uninhibited fraction of the biomass can utilize this secondary substrate, leading to an apparent increase in respiratory activity. However, in the case of R3, the addition of copper sulphate does not lead to an increase in oxygen uptake rate (OUR), unlike in R1 and R2. Another important observation is that, even in the presence of high concentrations of PFAS, respiration values never fall below the endogenous respiration level, regardless of the mixture composition. This finding confirms that waste containing PFAS, despite its high concentrations, does not exert a significant inhibitory effect on the biomass. Instead, it suggests a certain adaptive capacity of the microbial consortium under the tested experimental conditions.

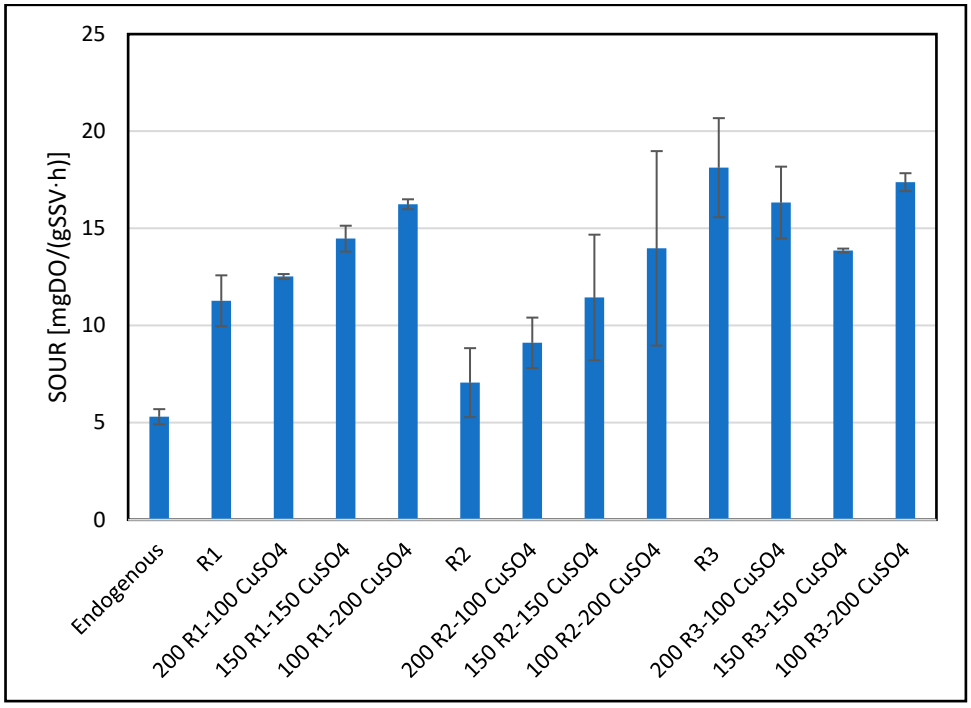

Figure 6. Results of OUR batch tests analyzing the effect of waste containing PFAS (R1, R2, R3) appropriately mixed with copper sulphate in order to evaluate a comparison with a known inhibitor.

The observed effects of copper in the system can be primarily attributed to its role as a heavy metal known to interfere with microbial enzymatic functions and cellular processes, leading to reduced respiration activity. Although copper is the main toxic agent, it is impossible to ignore the presence of the sulfate anion (from copper sulfate) since it may influence microbial dynamics by contributing to ionic strength and osmotic stress [29]. The particular combination of pollutants present may be the cause of the unique behaviour of liquid waste R3, which most likely resulted in synergistic interactions affecting biomass activity. Given the complexity of the system, including the simultaneous presence of PFAS and traditional pollutants, it is plausible that mutual interactions between these compounds mitigated toxicity in unpredictable ways. These findings highlight the importance of considering the combined effects of co-contaminants when evaluating biological responses in engineered treatment systems.

3.2.2. Effects at Medium-Long Term

The results presented in this paragraph illustrate the respiratory behaviour of activated sludge biomasses exposed to different mixtures of aqueous waste containing PFAS and previously studied inhibitory substances (PH, TCP, $CuSO_4$). The aim of the analysis is to evaluate a possible medium-long term inhibition, considering a contact time of 6 h, comparable to the hydraulic retention times of municipal wastewater treatment plants [30]. The tests were conducted in two phases: initially a waste with a specific concentration of PFAS was dosed, followed, after 2 h of contact, by the addition of the pure inhibitory substance, in order to reach the same concentration previously studied for short-term inhibition. This strategy allowed to compare the respiration values before and after the dosages, allowing to separately evaluate the possible inhibitory effect of the waste and, subsequently, that of the addition of the traditional inhibitor.

The results reported in Figure 7 illustrate the behaviour of the activated sludge biomass in contact with three wastes with high concentrations of PFAS (R1, R2, R3), followed by the addition of solid PH up to a final concentration of 200 mg L^{-1}. As highlighted in the

graphs, the dosage of the three wastes determined an increase in the OUR value, confirming the results already observed in the single-track tests described in the previous paragraph. However, the subsequent dosage of PH did not induce any inhibition of the biomass, not even in the medium-long term. The oxygen consumption values relating to the tests reported in Figure 6 are summarized in Table 3, highlighting how the three tested wastes present different levels of biodegradability, measured through the total consumption of dissolved oxygen during the experiment [31]. Furthermore, the peak OUR values do not always appear immediately following the dosage of the substrate containing organic matter: in the case of liquid waste R3, for example, the biomass showed an acclimatization time before reaching the maximum OUR value [32].

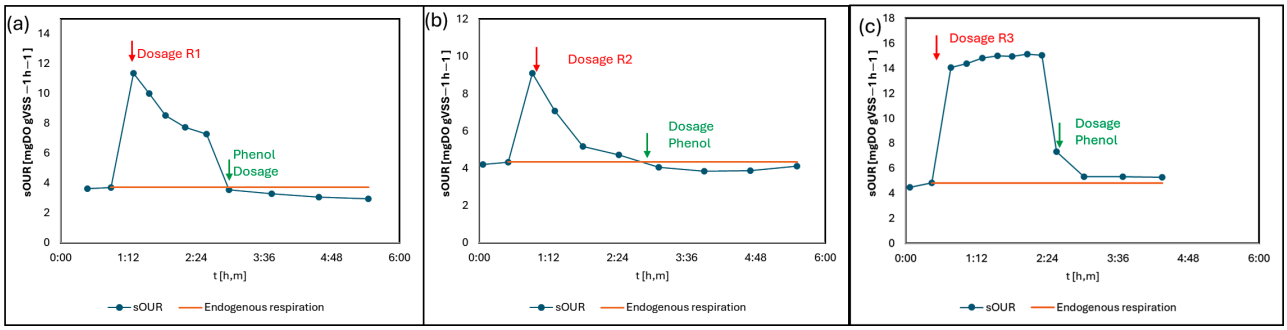

Figure 7. Results of continuous OUR tests analyzing the effect of waste containing PFAS ((**a**) R1, (**b**) R2, (**c**) R3) appropriately mixed with phenol in order to evaluate a comparison with a known inhibitor.

Table 3. Continuous OUR test results: oxygen consumption values during the entire test and maximum respiration recorded after substrate dosing (PH tests).

Substrate	Oxygen Consumption [mgDO gVSS^{-1}]	Maximum Value [mgDO gVSS^{-1} h^{-1}]
R1 + PH	22.93	11.38
R2 + PH	22.17	9.10
R3 + PH	35.95	15.12

The results reported in Figure 8 and Table 4 confirm what was observed in the previous tests, using TCP as an inhibitory substance. Unlike what emerged in the batch tests, the data obtained are substantially similar to those recorded with the phenol dosage, highlighting the absence of medium-long term toxicity phenomena. This behaviour is most likely caused by TCP volatilization, which results in a quick drop in concentration due to effects associated with system movement rather than biological abatement, suggesting a predominantly chemical-physical process [33]. With regard to the treatability of waste containing PFAS, the results confirm the previous observations, highlighting that R2 represents the liquid waste with the most limited treatability, as demonstrated both by the peak value of respiration and by oxygen consumption.

Table 4. Continuous OUR test results: oxygen consumption values during the entire test and maximum respiration recorded after substrate dosing (TCP tests).

Substrate	Oxygen Consumption [mgDO gVSS^{-1}]	Maximum Value [mgDO gVSS^{-1} h^{-1}]
R1 + TCP	35.54	12.3694
R2 + TCP	13.88	4.3411
R3 + TCP	35.95	14.4589

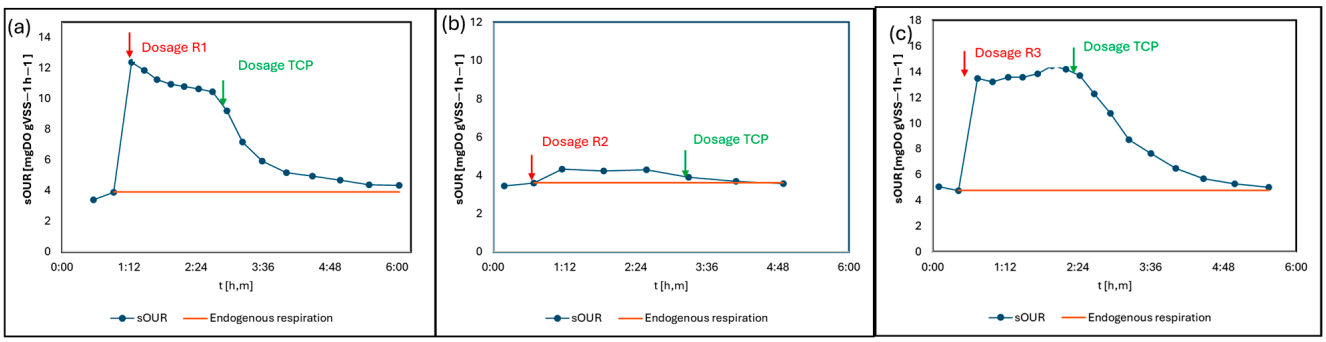

Figure 8. Results of continuous OUR tests analyzing the effect of waste containing PFAS ((**a**) R1, (**b**) R2, (**c**) R3) appropriately mixed with trichlorophenol in order to evaluate a comparison with a known inhibitor.

The results reported in Figure 9, relating to the use of copper sulphate as an inhibitory substance, show a significantly different behaviour compared to that observed with the other two substances (PH and TCP). In particular, the addition of $CuSO_4$ determined a chronic inhibition of the biomass [34], both in the test mixed with R1 (Figure 8a) and in the one with R2 (Figure 8b), leading to a toxic effect, with the complete reduction in respiration capacity of the activated sludge biomass. The comparison between the respiration values relating to the inhibition process and the batch OUR tests highlights that, in the single-track tests, the contact time between the inhibitory substance and the biomass was not sufficient to observe a significant effect. In fact, after 10 min of exposure, the biomass enters an irreversible inhibition process, which leads to the complete loss of the respiratory capacity and, consequently, to the impossibility of biodegrading the organic substance. On the contrary, the results obtained with the dosage of liquid waste R3 confirm what was observed in the batch tests, showing that mixing $CuSO_4$ with a highly biodegradable substance allows the biomass to select the most favourable substrate, reducing the toxic effect of the inhibitor. These trends are further confirmed by the data reported in Table 5, which highlight how the total consumption of dissolved oxygen and the maximum respiration value underline the good biodegradability of the three liquid wastes containing PFAS [35]. Among these, waste R2 is confirmed to be the least biodegradable, while waste R3 is the one most easily assimilated by biomass, confirming that there is no correlation between the presence of an inhibitory effect on biomass respiration and the amount of PFAS contained in the treated substrate.

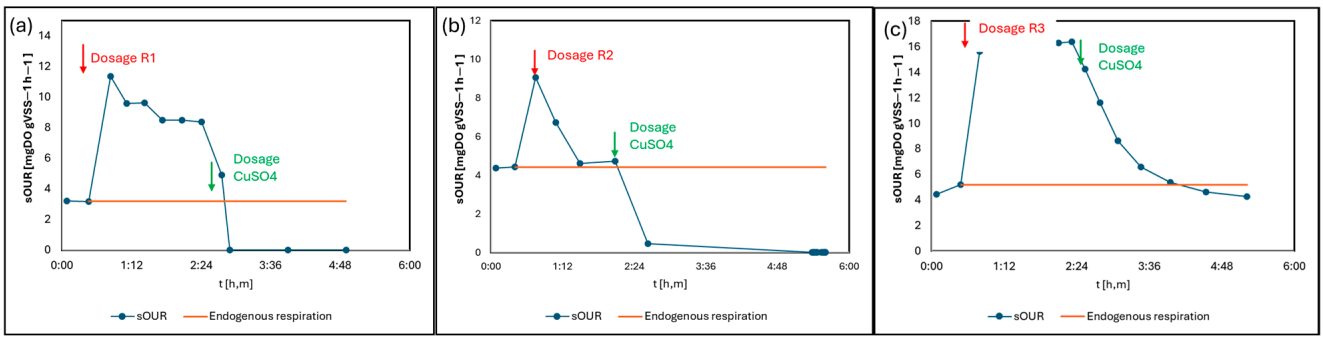

Figure 9. Results of continuous OUR tests analyzing the effect of waste containing PFAS ((**a**) R1, (**b**) R2, (**c**) R3) appropriately mixed with copper sulphate in order to evaluate a comparison with a known inhibitor.

Table 5. Continuous OUR test results: oxygen consumption values during the entire test and maximum respiration recorded after substrate dosing ($CuSO_4$ tests).

Substrate	Oxygen Consumption [mgDO $gVSS^{-1}$]	Maximum Value [mgDO $gVSS^{-1}$ h^{-1}]
R1 + $CuSO_4$	15.41	11.3749
R2 + $CuSO_4$	20.03	9.0753
R3 + $CuSO_4$	47.1	16.4071

4. Conclusions

This study investigated the potential inhibitory effects of PFAS on activated sludge biomass, comparing their impacts with well-known toxic substances (such as phenol, trichlorophenol, and copper sulphate) ones. Respirometric tests, conducted using oxygen uptake rate (OUR) measurements, provided insights into both short-term (batch tests) and long-term (continuous tests) inhibitory effects. The findings indicate that aqueous waste with high content of PFAS did not induce acute or chronic toxicity in the tested biomass. However, copper sulphate produced irreversible inhibition, suggesting that conventional inhibitors may pose a more immediate threat to biological wastewater treatment processes. Additionally, the biodegradability of the studied substrates was impacted by the interaction between PFAS and traditional toxic substances, showing that certain mixtures could mitigate inhibitory effects. From an operational perspective, these findings highlight how crucial it is to comprehend inhibitory mechanisms in wastewater treatment plants (WWTPs) in order to optimize process control. However, the absence of an inhibitory effect from PFAS in liquid waste should not be interpreted as a positive outcome. This study highlights the need for further research on the long-term effects of PFAS exposure, as well as the potential role of biomass acclimatization also to the slight toxic compounds. Furthermore, to ensure more effective wastewater management and prevent the uncontrolled release of PFAS into the environment, it is essential to develop advanced detection and treatment technologies specifically targeting these persistent compounds. Simultaneously, the creation of a centralized database collecting PFAS concentration data from wastewater streams at individual treatment plants could serve as a valuable tool. In addition to supporting risk assessment and regulatory monitoring, such a database would assist in identify critical hotspots, directing focused remediation efforts, and inform the design of more efficient treatment strategies based on real, site-specific contamination profiles.

Author Contributions: Conceptualization, M.C.C. and S.B.; methodology, S.B.; software, S.B.; validation, A.A., S.B. and M.C.C.; formal analysis, A.A.; investigation, L.M.R.C.; resources, M.C.C.; data curation, S.B.; writing—original draft preparation, L.M.R.C. and S.B.; writing—review and editing, S.B. and A.A.; visualization, S.B. and M.B.; supervision, A.A. and M.B.; project administration, M.C.C.; funding acquisition, M.C.C. All authors have read and agreed to the published version of the manuscript.

Funding: The authors received financial support for the research from Asmia S.r.l.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. (Data are not publicly available due to privacy restrictions).

Acknowledgments: The authors sincerely thank the Department of Excellence of the DORIAN Project, Department of Civil Engineering and Architecture, University of Pavia for its support and within which the research work was carried out.

Conflicts of Interest: The authors declare no conflict of interest.

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