

Screening of perfluoroalkyl substances and their environmental impact in sequencing batch reactors combined with nature-based solutions

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ABSTRACT

Perfluoroalkyl substances (PFAS) are a growing problem in the environment. The research indicates that they are present in surface water, groundwater, drinking water sources, wastewater treatment plant (WWTP) effluents, and landfill leachates. Additionally, the conventional methods of wastewater treatment are ineffective in their removal.

This study aimed to indicate the concentration of PFAS in wastewater during treatment processes in sequential biological reactors (SBRs), followed by two ponds working in series. Samples were collected after individual stages of treatment, during the beginning of touristic seasonality. The research also determined the environmental impact of the PFAS by determining the ecotoxicity and performing a risk assessment of the analyzed wastewater.

The analyzed wastewater samples were collected from the different stages of the WWTP in Swarzewo, which uses SBRs. In the collected samples, basic parameters such as total suspended solids (TSS), biological oxygen demand (BOD₅), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), as well as identification and concentrations of PFAS were determined. Based on this data, an ecotoxicological assessment and risk assessment of the wastewater was performed.

The research indicated that the basic parameters and Microtox toxicity assay are not sensitive to changes in the PFAS content in wastewater. As the hydrophobicity of the PFAS increases, their solubility in the water decreases. However, these substances may still be present in suspended particles, leading to an increase in their global concentration in the water and, consequently, may pose environmental hazards. The proposed technology of wastewater treatment is an effective PFAS retention system in the sediment (removal of over 90 %). Meteorological conditions affect the PFAS transformation processes taking place in SBRs.

1. Introduction

Perfluoroalkyl substances (PFAS) comprise a large and growing in number class of chemicals that include perfluoroalkyl acids (PFAA), perfluoroalkyl carboxylic acids (PFCA), and perfluoroalkyl sulfonic acids (PFSA) (Dixit et al., 2021).

Since the 1950s, these compounds have been commonly used worldwide in a range of industrial and household consumer products, such as stain-repellants, textile coatings, food paper packaging (e.g., compostable coffee cups and pizza boxes), lubricants, firefighting foams,

insecticides, paints, cosmetic formulations, semiconductors, and many more (Gallen et al., 2018).

Their unique and useful chemical properties, including acting as surfactants, their thermal and acid resistance, and their water and oil repellency, have resulted in their massive use and thus the ubiquitous presence of these compounds in the environment. Unfortunately, these same properties that make PFAS useful also contribute to their persistence and potential accumulation in the environment. This raises concerns about their impact on both ecosystems and human health. The interest of scientists in PFAS has expanded from not only determining

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their increasing levels but also to the growing risks they pose, particularly in the context of drinking water and wastewater contamination as well as looking for effective technologies for their removal to mitigate PFAS migration into the environment (Gobelius et al., 2023; Hu et al., 2016; Pan et al., 2018; Munoz et al., 2023; Nxumalo et al., 2023; Sadia et al., 2023). The primary objective of this study is to evaluate the effectiveness of PFAS removal in a real-world wastewater treatment plant (WWTP) setting by combining sequencing batch reactor (SBR) processes with nature-based solutions (NBS). Additionally, the study aims to determine the impact of environmental conditions on the efficiency of PFAS treatment.

PFAS are persistent pollutants found across various environmental compartments, including surface water, groundwater, drinking water sources, wastewater treatment plant (WWTP) effluents, and landfill leachates. Notably, they have even been detected in areas where products containing PFAS are not manufactured, such as the Gulf of Gdansk (Baltic Sea region) and the mouth of the Vistula, the longest river of Poland (Gałęzowska et al., 2020, 2021). In the Gulf of Gdansk, for instance, 17 PFAS were detected in samples collected near treated wastewater discharges, with concentrations ranging significantly ($\Sigma 17$ PFAS were in the range from 0.004 ng/g dry weight (d.w.) to 614 ng/g d.w.) (Gałęzowska et al., 2021). This evidence strongly suggests that wastewater treatment plants (WWTPs) are significant contributors to PFAS contamination in aquatic environments (Gałęzowska et al., 2021; Zhang et al., 2015).

Given the persistence of PFAS, their removal through conventional wastewater treatment processes—such as biological degradation, oxidation, reduction, and coagulation—has proven largely ineffective (EPA, 2020; Karbassiyazdi et al., 2023; Leung et al., 2022; Yadav et al., 2022). To address these limitations, nature-based solutions (NBS) in the third stage of treatment have been explored as a complementary approach to SBRs. NBS, such as constructed wetlands or, as in this case, free water surfaces—ponds—utilize natural processes involving plants, microbes, and soil to further treat and remove contaminants, including PFAS, from wastewater. This integration leverages the synergies between SBRs' mechanical processes and the NBSs' biological and chemical mechanisms to enhance overall treatment efficiency (Cross et al., 2021; Deshpande, 2024).

Moreover, research specifically addressing PFAS removal in sequencing batch reactors (SBRs) remains limited. One of the few studies, conducted by Gonzalez et al. (2021), investigated the removal of PFAS associated with aqueous film forming foam (AFFF) in an SBR, and reported an up to 70 % reduction in PFAS levels over a 4-day hydraulic retention time (HRT).

Among the most effective technologies currently available for PFAS removal are: adsorption techniques using ion exchange resin or granular activated carbon, with removal efficiencies ranging from 89 to 99 %, as well as and high-pressure membrane filtration, which achieves similar results (EPA, 2020).

In addition to these technologies, natural methods such as treatment wetlands have shown promise. For example, research by Li et al. (2021) demonstrated that 33.59–88.99 % of PFCA and PFSA could be removed via sediment sorption, phytoextraction, and bioaccumulation by microbiota in constructed wetlands.

Another study, by Mei et al. (2021), further explored the processes affecting the bioavailability of PFAS in soil, finding that the PFAS root concentration was not significantly correlated with NBSs' hydrophobic properties, but translocation within the plant was negatively correlated with PFAS hydrophobicity.

Additionally, Arslan and El-Din (2021) provided evidence that processes occurring in wetlands can mineralize the PFAS, highlighting the potential for effective plant-microbe interaction to degrade these compounds.

Despite advances in PFAS removal technologies, residual levels of PFAS often remain in treated wastewater, posing a risk to ecosystems and potentially human health when discharged into the environment.

Consequently, assessing the potential toxicity of such wastewater is crucial. Since the 1990s, various ecotoxicity tests have been employed to evaluate the impacts of wastewater on organisms from different trophic levels, including prokaryotic bacteria, eukaryotic cells, and multicellular organisms such as algae, plants, mussels, crustaceans, and fish (Abbas et al., 2018). These tests not only help to understand the ecological risks, but also inform environmental risk assessments by integrating contamination levels with ecotoxicity data (Sardiña et al., 2019). For example, the hazard quotient (HQ) has been recommended by the EU as a tool for ecological risk assessment (European Commission, 2003). Nevertheless, gaps in knowledge about PFAS, particularly their environmental and health impacts, remain.

This study aimed to address these gaps by: (i) evaluating the effectiveness of PFAS removal from wastewater during treatment in sequencing batch reactors (SBRs) followed by two ponds working in series as a nature-based solution (NBS), (ii) identifying conditions that influence the PFAS removal efficiency, and (iii) assessing the ecotoxicity and environmental risks associated with the treated wastewater.

2. Materials and methods

2.1. Sampling site

The analyzed wastewater samples were collected from the WWTP in Swarzewo (Northern Poland, 54° 46' N, 18° 25' E).

This WWTP treats wastewater from a touristic region, which causes significant irregularity in the inflow (Fig. 1). The flow rate of wastewater in the summer season is 14,000 m³/d, while after the summer, it decreases to one-third and amounts to about 5000 m³/d. The wastewater treatment technology in WWTP Swarzewo is based on three stages of treatment: (i) mechanical, (ii) biological, and (iii) chemical. The wastewater, before being discharged to the receiver, which is the Baltic Sea, is also treated in two ponds working in series and being the fourth stage of treatment, called renaturalization.

The biological process takes place in aeration chambers with a high content of activated sludge using sequential biological reactors (SBRs). In these reactors, the wastewater periodically flows through the installation. In Swarzewo, the SBRs have been designed as three concrete tanks with diameters of 24, 30, and 34 m each, and they work independently. The applied periodical system of the sewage treatment plant is based on portion wastewater treatment. The wastewater treatment is divided into the following stages in WWTP Swarzewo: (i) filling, (ii) treatment, (iii) sedimentation, and (iv) decantation. Initially, the reactor receives a portion of wastewater. Subsequently, the treatment phase commences. The cycle time and duration of individual phases in the SBR are variable. The duration of each phase is regulated automatically based on the concentrations of oxygen and nitrogen forms present in the reactor. That is why this time depends on the load and quality of the effluent as well as the removal efficiency. This causes the duration of individual phases as well as the cycle time to differ not only between winter and summer, but also within these seasons. Following this, the activated sludge undergoes separation from the wastewater during the sedimentation phase, involving both the decantation of treated wastewater and the simultaneous removal of excess sludge.

For the chemical reduction of phosphorus, the WWTP uses iron (II) sulfate (coagulant (commercial name PIX 112), which is added before the SBR. The excess sewage sludge is processed in a digester and then, after dewatering, undergoes the composting for certified compost.

After the SBR, the wastewater flows through an additional secondary tank and two ponds, where further treatment by microorganisms and hydrophyte plants occurs. The tank volume is 21,300 m³. The HRT in the ponds depends on the amount of inflowing wastewater. When the flow is about 5000 m³/d, the HRT is about 102 h. At a flow rate of 20,000 m³/d, the HRT is about 25 h. Sediments from the ponds are removed once every few years (as needed) and are processed together with the sewage sludge.



Fig. 1. Scheme of WWTP in Swarzewo with biological treatment tank (SBR), settling tank, pond 1 and 2 with its influent (raw wastewater) and effluent points.

2.2. Sampling events and basic measurements

Wastewater at various stages of treatment was collected with a scoop equipped with a telescopic handle, except for influent and effluent, which were collected as averaged 24-h samples using an automatic flow-rate sampler. The samples from the ponds and the SBR reactor were collected as grab number one. However, the grab sample was, in fact, an averaged sample (due to the relatively long residence time in the SBR as one chamber without continuous flow and working mode: filling—working—decanting). The samples from the SBR were collected in the treatment phase. In the process of sampling, the HRT (average 102 h in winter and 25 in summer) of the wastewater in the ponds was taken into account. The collection of samples was prepared with two companies in May and June after preliminary sampling from Pond 2 in April. The wastewater samples were collected in those periods because this approach allows us to evaluate the impact of increased load and environmental conditions during the start of the tourist season. Samples were taken from all stages of the WWTP, including raw wastewater, biological treatment, settling tank, pond 1, pond 2, and effluent. A total of 13 samples were collected.

The samples were poured into sterile glass bottles (1 L) with caps and a silicone membrane until full. After replenishing the vessel with water, the bottles were immediately closed with the stopper held during the sampling in the hand through the cap, bottom part down, protecting it from contamination. After collection and immediate transport to the laboratory, the following factors were determined: basic parameters such as total suspended solids (TSS), biological oxygen demand (BOD), chemical oxygen demand (COD), total nitrogen (TN), total phosphorus (TP), as well as identification and concentration of PFAS. The remainders of the samples were stored at a temperature below 10 °C. Their maximum storage time did not exceed 20 days for chromatographic analysis and ecotoxicological tests were performed the following day after natural warming up to room temperature in the morning.

The basic parameters were carried out according to guidelines from the American Public Health Association (APHA, 2005) and Polish Standards (Kotecka et al., 2019). The efficiency of the pollutant removal (RE) in the analyzed samples was calculated according to the formula:

$$RE = \frac{Li - Le}{Li} 100 (\%).$$

where

Li—load of a selected pollutant in influent,

Le—load of a selected pollutant in effluent.

Changes in meteorological conditions were observed during sampling. The samples were collected during spring, the recorded temperature was in the range from 10.4 °C to 18.2 °C with a contrast of about 8 °C between collections. The preliminary sampling was from pond 2 in April (average monthly temperature 5 °C). April and May were the rainy months (18.6 and 29 mm of precipitation, respectively). In June, no significant levels of precipitation were recorded and on average, they amounted to 3.2 mm of water column for the whole month.

2.3. PFAS detection with chromatographic analysis

The following 17 PFAS (13 carboxylic and 4 sulfonic acids) were included in the standard mixture applied: perfluoro-n-butyric acid—PFBA, perfluoro-n-pentanoic acid—PFPeA, perfluoro-n-hexanoic acid—PFHxA, perfluoro-n-heptanoic acid—PFHpA, perfluoro-n-octanoic acid—PFOA, perfluoro-n-nonanoic acid—PFNA, perfluoro-n-decanoic acid—PFDA, perfluoro-n-undecanoic acid—PFUDA, perfluoro-n-dodecanoic acid—PFDoA, perfluoro-n-tridecanoic acid—PFTrDA, perfluoro-n-tetra-decanoic acid—PFTeDA, perfluoro-n-hexadecanoic acid—PFHxDA, perfluoro-n-octadecanoic acid—PFODA, potassium perfluoro-1-butanedisulfonate—PFBS, sodium perfluoro-1-hexanesulfonate—PFHxS, sodium perfluoro-1-octanesulfonate—PFOS, sodium perfluoro-1-decanedisulfonate—PFDS.

As internal standards, the following PFAS isotopes at the level of 10 ng each were applied: sodium perfluoro-1-75-hexane [18O2] sulfonate (MPFHxS), sodium perfluoro-n-[1,2,3,4-13C4]octanoic acids (MPFOA), perfluoro-n-76[13C4]butanoic acids (MPFBA), perfluoro-n-[1,2-13C2]hexanoic acid (MPFHxA), perfluoro-1-[1,2,3,4-77 13C4]octanesulfonate (MPFOS), perfluoro-n-[1.2-13C2]decanoic acid (MPFDA), perfluoro-n-[1.2-13C2]undecanoic acid (MPFUDA), perfluoro-n-[1.2-13C2]dodecanoic acid (MPFDoA). All the standards were purchased from Wellington Laboratories (Canada). Methanol (MeOH) LC-MS grade

and ammonium acetate (NH₄Ac) MS grade were purchased from Merck (Germany). Ultrapure water was produced using a Hydrolab system (Poland).

The PFAS analysis included sample preparation with the SPE technique, and liquid chromatography analysis combined with tandem mass spectrometry according to the method described in ISO21675 “ISO, 2019 Water quality—Determination of perfluoroalkyl and polyfluoroalkyl substances (PFAS) in water—Method using solid phase extraction and liquid chromatography-tandem mass spectrometry (LC-MS / MS)”. The chromatographic method used to perform the analyses was described based on Gałęzowska et al. (2020, 2021).

All liquid samples (500 mL) were subjected to PFAS purification and isolation using the solid phase extraction (SPE) technique using columns dedicated to PFAS isolation (Bond Elut PFAS WAX, 500 mg, 6 mL, Agilent, US) after stabilization of their pH at the value of 3. The cartridges were washed before use in the following sequence: with 4 mL of ammonia/methanol solution, 4 mL of methanol, and finally 4 mL of ultrapure water. Then the extractions were started immediately after conditioning the sorbent packing. The samples were added at a rate of one drop per second (3 mL/min to 6 mL/min). The cartridges were washed with 4 mL of water and 4 mL of acetate buffer solution. Then the target substances were eluted with 4 mL of methanol followed by 4 mL of 0.1 % ammonia/methanol at a rate of one drop per second and collected into a glass tube. The solvent was subsequently evaporated in a nitrogen stream and the dry residue was dissolved in the mobile phase (0.25 mL) used for chromatographic analysis.

The quantitative analysis was performed using the method of adding an isotope internal standard (isotope dilution) for all analyzed PFAS, considering the determined response factors (Gałęzowska et al., 2020, 2021 and Table S1).

The prepared samples were analyzed by ultra-high performance liquid chromatography with a Nexera X2UHPLC-MS/MS (Shimadzu Corp., Japan). The general MS/MS operating parameters were as follows: Interface Temperature = 300 °C, Desolvation Line Temperature = 250 °C, Nebulizing Gas Flow = 3 L/min, Heating Gas Flow = 10 L/min, Heating Block = 350 °C, Drying Gas Flow = 10 L/min and with electrospray ionization in negative ion mode (details in Table S4). The chromatographic analysis of an Acquity UPLC BEH C18, 1.7 μm, 2.1 mm, 100 mm (Waters, USA) analytical column using the mobile phase consisting of methanol and 2.5 mM ammonium acetate in water, at a flow rate of 0.6 mL/min, was performed. The column temperature was 40 °C and the injection volume was 2 μL.

The quantitative analysis was performed using the method of adding an isotope internal standard (isotope dilution) for all analyzed PFAS, considering the determined response factors (Gałęzowska et al., 2020, 2021).

The quality assurance of the measurement results was carried out considering the calibration of the LC-MS/MS system (using a tuning mixture produced by the spectrometer manufacturer), and the determination of selected validation parameters based on calibration curves (Supplementary Table S1 and S4).

2.4. Ecotoxicological assessment of the wastewater

2.4.1. Microtox® test

The measurement is a system based on standard ASTM (American Society for Testing and Materials) methods and the PN-EN ISO 11348-3: 2002 standard. The tests presented in this study were performed according to the test manufacturer's guidelines and the Microtox Model 500 analyzer manual. The basic test method was used with dilutions: Basic Test 81.9 %. A series of dilutions of each sample was prepared (4 dilutions, 1:1, v/v), and then the luminescence of the bacteria *Aliivibrio fischeri* was measured before and after the incubation (15 min and 30 min). Together with each batch of tested samples, the correctness of the test performance and the appropriate sensitivity of the bacteria were regularly assessed. This was carried out by testing the toxicity of the

standard solution, which is ZnSO₄·7H₂O at a concentration of 20 mg / L, and the incubation time was 15 min. The obtained data should be within the range established by the test manufacturer (for more details, see the Supplementary materials).

2.4.2. Thamnotoxkit F test

The applied acute toxicity Thamnotoxkit F test complies with ISO 14380 and is used for routine screening of chemical or environmental samples. The assay is performed in a disposable 24-well plate (6 × 4). Organisms (*Thamnocephalus platyurus*) are activated on demand from their cryptobiotic form (cysts). Each sample is tested for toxicity in triplicate; the wells of the test plate are filled with 1 mL of the test sample. Ten organisms are then transferred to the sample. The test plate is covered with Parafilm and incubated for 24 h in the darkness at 25 °C. Diluted standard freshwater was used as the control and the percentage mortality compared to the control sample was observed and calculated. For toxic samples, EC₅₀ values were determined by preparing a series of standard freshwater dilutions of samples containing from 3.125 % to 100 % of the original sample and testing their toxicity (for more details, see the Supplementary materials).

2.5. Environmental risk assessment

Risk quotient (RQ) values for the selected PFAS (determined in treated sewage discharged into the sea) were calculated by comparing the measured environmental concentration (MEC) in the sea samples to the predicted no-effect concentration (PNEC) according to Eq. (1) (EPA, 2023; Gałęzowska et al., 2021; Kolečka et al., 2020):

$$RQ = \frac{MEC}{PNEC} \quad (1)$$

where:

MEC—measured environmental concentration [ng/L]

PNEC—predicted no effect concentration [ng/L].

The MEC values were the maximum concentration of PFAS determined in the treated wastewater samples. If the substances were not detected in the samples (or they did not exceed the limit of detection), they were not subjected to further analysis.

The available ecotoxicity data using three different species from different trophic levels, which best represent the aquatic ecosystem (algae, crustaceans, and fish) were the basis for determining the PNEC values (Supplementary Table S2). Then, according to the procedure, these values were divided by the assessment factor (AF) if there was at least one short-term exposure (e.g. LC₅₀, EC₅₀) from each of the three evaluated trophic levels available, 100 if one long-term exposure (e.g. NOEC) either for algae, crustaceans or fish was available, 50 if two long-term exposure ecotoxicity data were available for organisms from two different trophic levels and 10 if three long-term exposure values were available for organisms from the three trophic levels (European Commission, 2003). There is a need for additional laboratory experiments to be performed for both acute and chronic ecotoxicity of PFAS, since the data are still lacking. In the case of an absence of toxicity data, the U.S. EPA Ecological Structure Activity Relationships (ECOSAR – Class Program VI.11) (US EPA, 2013) database was used to estimate the parameters.

The environmental risk characterization of perfluorinated compounds in the aquatic environment was performed using ranking criteria (European Commission, 2003):

- HQ < 0.1—no adverse effect, no risk
 - < HQ < 1—low risk
- < HQ < 10—probable risk
- 10 < HQ—high risk

2.6. Statistical analysis

Data analysis was performed with Machine Learning (ML)—Unsupervised Learning; solving cauterization problems. This technique was chosen to the group samples, considering the relationships and patterns in the data. To understand and interpret the “Black Box” of a model, Interpretable AI tools (for instance, SHAP) were used (Doshi-Velez and Kim, 2017). The research was conducted according to the following steps:

- **Data Preparation**—data preparation was conducted with Python 3.9 using the Pandas framework, allowing data preprocessing and feature engineering.
- **Clusterization**—clusterization was performed with Python 3.9 using Scikit Learn (ML framework) and the low-code tool PyCaret (for model selection and batch preparation). Kmeans was performed as the main ML algorithm as it showed the best performance. The selected metrics that were performed were Silhouette and Calinski-Harabaszscore. Data visualization was performed with the 3D TSNE technique.
- **Interpretable AI**—model interpretation was performed with SHAP (Lundberg and Lee, 2017) and interpretation tools within the Scikit Learn framework. The most important features and SHAP values with an additive force layout were described and visualized.
- **Data visualization**—was performed with Python 3.9 using the Plotly tool (Plotly Technologies Inc., 2015).

3. Results

3.1. Efficiency of basic pollutant removal

The WWTP was characterized by very high efficiency of removal of basic parameters (Table 1). For COD, the efficiency of removal was an average of 97.7 %, for BOD 98.9 %, for TSS 95.7 %, for TN 91.0 %, and for TP 97.0 %. Such high removal efficiency, especially in the case of TP, is probably caused by the use of SBR technology. This technology allows for more efficient phosphorus removal compared to typical treatment plants, as it enhances the chemical reactions necessary for phosphorus precipitation. Additionally, the controlled conditions within the SBR, including extended contact time with the coagulant, further contribute to this high efficiency. Thus, the quality of the treated wastewater meets the requirements of Polish standards (Journal of Laws, 2019, item 1311) which comply with the EU's Water Framework Directive (WFD, Directive 2000/60/EC1) and Urban Waste Water Treatment Directive (UWWTD, Council Directive 91/271/EEC6).

3.2. PFAS concentration

Changes in the content of the sum of individual PFAS (at all stages of wastewater treatment) for two consecutive months are shown in Figs. 2 and 3. A significant increase in the total PFAS concentration was observed between May and June in the raw wastewater, where the sum of PFAS increased from 366.67 ng/L to 1254.32 ng/L. This indicates a substantial rise in the PFAS load entering the treatment plant during the warmer month of June, which may be influenced by the considerable

Table 1
Efficiency of basic pollutants removal.

Pollutant	RE [%]		
	April	May	June
COD	97.48	96.67	99.07
BOD ₅	98.75	98.25	99.72
TSS	98.24	96.18	92.59
TN	95	89.64	88.25
TP	98.78	97.87	94.3

increase in average temperature from 10.4 °C in May to 18.2 °C in June. In the case of most substances, an increase in their concentration was noted from May to June. Only the content of PFDA, PFUDA, PFDoA, PFTeDA, and PFOS decreased during this period.

During the biological treatment, a reduction in the total amount of PFAS introduced into the treatment plant was observed. However, an interesting pattern emerged where certain PFAS, such as short-chain PFBA, became more prominent during the treatment process, particularly in May. This suggests that the biological treatment may lead to the breakdown of longer-chain PFAS into shorter-chain forms. The presence of long-chain PFAS such as PFODA in the SBR stages was unexpected, indicating possible desorption or transformation processes occurring within the system.

In the subsequent stages of treatment, an initial increase followed by a decrease in the effluent concentration of total PFAS was observed. This trend indicates the complex dynamics of PFAS behavior in the treatment process, where certain compounds may be transformed or desorbed at different stages before ultimately being removed or reduced by the final treatment steps. The very low rainfall in June (3.2 mm compared to 29 mm in May) likely contributed to the observed concentration changes, as reduced precipitation may have limited the dilution effect and surface runoff, resulting in higher PFAS concentrations in the treated water.

3.3. Ecotoxicity tests

In the case of toxicity tests, high toxicity was observed for the raw sewage, and low for the treated wastewater. The wastewater did not show any toxicity at the consecutive stages of treatment, and hormesis was observed toward *Aliivibrio fischeri*. An increase in the PFAS concentration above 500 ng/L in the settling tank (first SBR stage) was not associated with an increase in toxicity to *Aliivibrio fischeri*, but an increase in toxicity to *Thamnocephalus platyurus* (Table 2). It should be noted that it is not possible to observe hormesis in the Thamnotox test, so it is not known how the samples affected the larvae. Only their death was tested and no other changes in their metabolism or changes in appearance.

3.4. Statistical analysis

The results from the Microtox test were not considered in the statistical analysis, as the results of the data interpretation showed no relationships between the Microtox results and the PFAS concentration.

According to the K-means algorithm, all indexes in the dataset were divided into 4 clusters: Cluster 0, Cluster 1, Cluster 2, and Cluster 3. The impact of the features is presented in Fig. 4 and Fig. 6. The algorithm forming Cluster 0 considered the detected concentration of PFAS as it had the highest impact; likewise, the mean temperature was also considered. An infinitesimal imbalance in the detected concentrations could cause the appearance of this cluster (this is not ideal, but acceptable in work with scientific data). The main feature in the case of Cluster 1's formation was the mean temperature only. Cluster 2 was formed considering rainfalls and seasonality months; the temperature depends on seasonality. However, the contrast in temperature between May and June is not high enough. Cluster 3 was created taking into consideration features such as the Thamnotoxkit F results and the mean temperature represented in the data. Fig. 4 shows the cluster visualization, and the heatmap, which shows how important each feature is to characterize a cluster. Fig. 5 shows the distribution of PFAS and their concentration in individual clusters.

Fig. 6 shows a summary of the SHAP analysis, which explicitly points to a significant contribution in specific clusters of rainfall (cluster 0 about 50 %, and cluster 1 about 30 %) and levels of PFAS concentration (about 10 % in clusters 1 and 3). The temperature and month are of less importance.

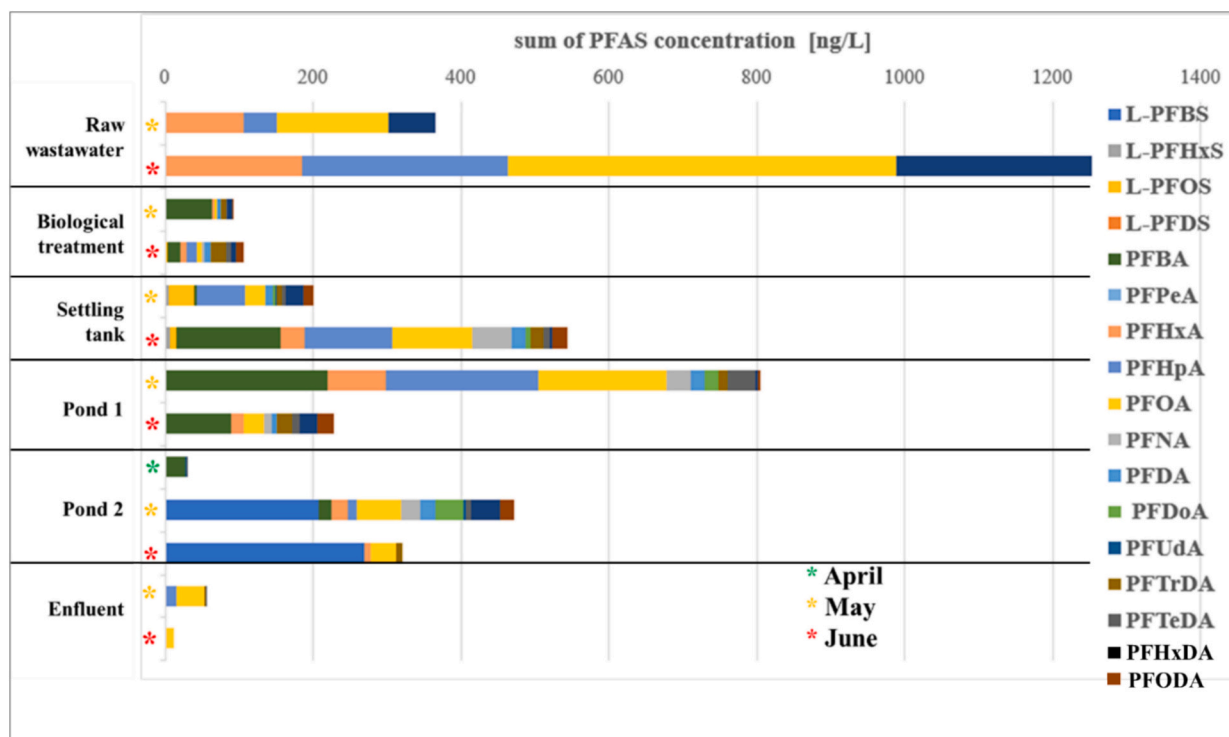


Fig. 2. Sum of PFAS monitored at individual stages of wastewater treatment.

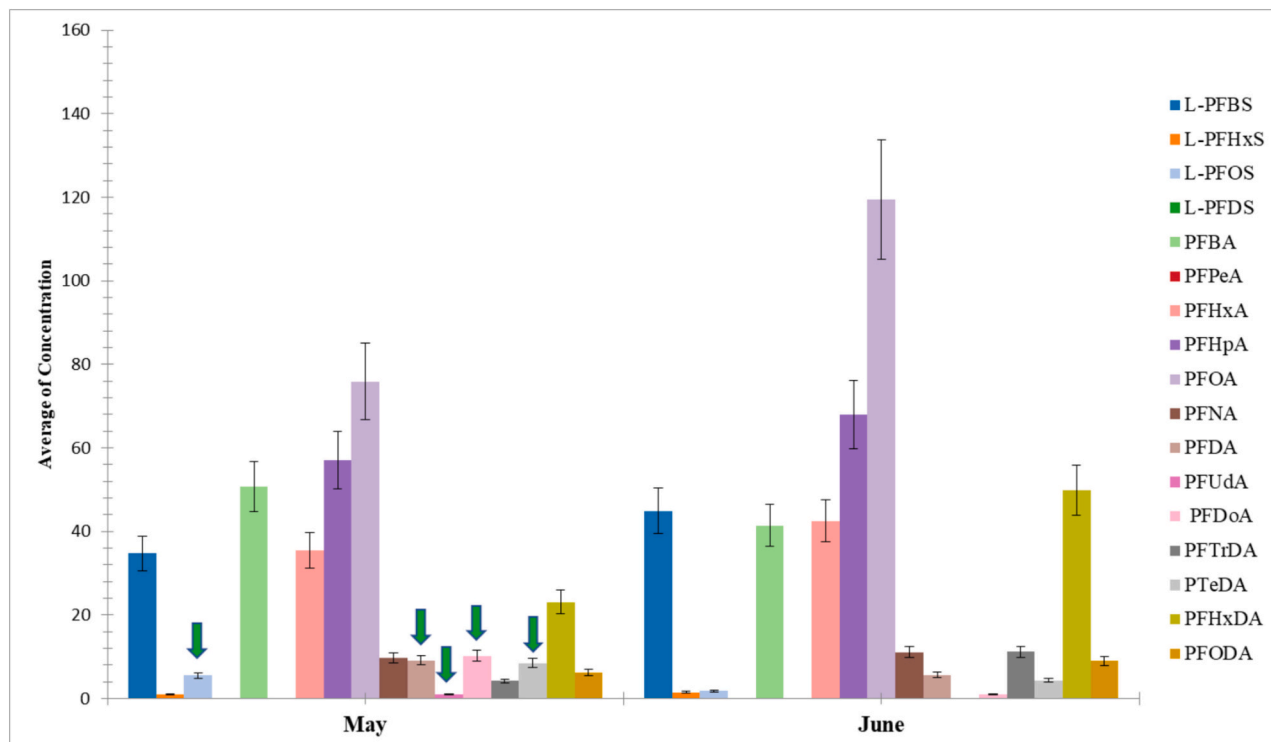


Fig. 3. Sum of PFAS levels monitored in May and June.

3.5. Environmental risk assessment

In the research, data found for the three trophic levels to determine

the PNEC values were used (Table 3). For all PFAS, except for PFOA and PFOS, the data about their ecotoxicity is scarce, which is why this data were obtained from the ECOSAR database. The Hazard Quotients (HQs)

Table 2
Ecotoxicity results at various stages of wastewater treatment, green – not toxic, orange -toxic, red- highly toxic.

Biotox Microtox		Sampling points											
		Raw wastewater		Biological treatment	Settling tank		Pond 1		Pond 2			Effluent	
		May	June	June	May	June	May	June	April	May	June	May	June
Ecotoxicity measurements [%]	15'	55	97	-39	-50	-24	-46	-35	-15	56	17	21	-36
	30'	50	97	-71	-146	-31	-88	-66	-2	39	-6	14	-49
Sample classification		toxic	highly toxic	not toxic	not toxic	not toxic	not toxic	not toxic	not toxic	toxic	not toxic	not toxic	not toxic

Biotox Thamnotoxkit F		Sampling points						
		Raw wastewater	Biological treatment	Settling tank	Pond 1	Pond 2	Effluent	
		June	June	June	June	June	June	
Ecotoxicity measurements [%]		100	37.7	100	13	0	19	
Sample classification		highly toxic	Toxic	highly toxic	not toxic	not toxic	not toxic	

3d TSNE Plot for Clusters

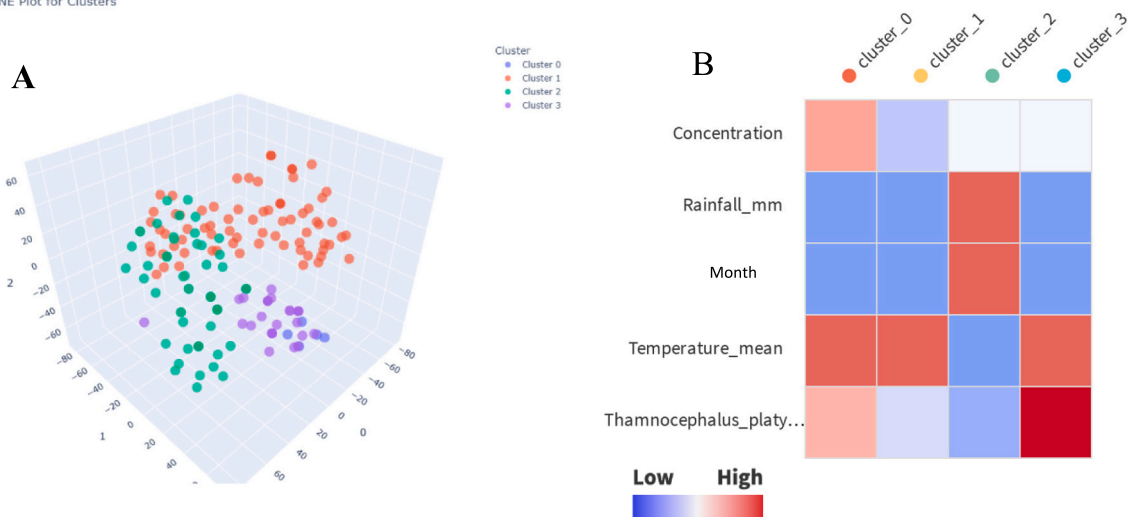


Fig. 4. Visualization of clusters: A—distribution of points in clusters, B—heatmap of dependents in each cluster.

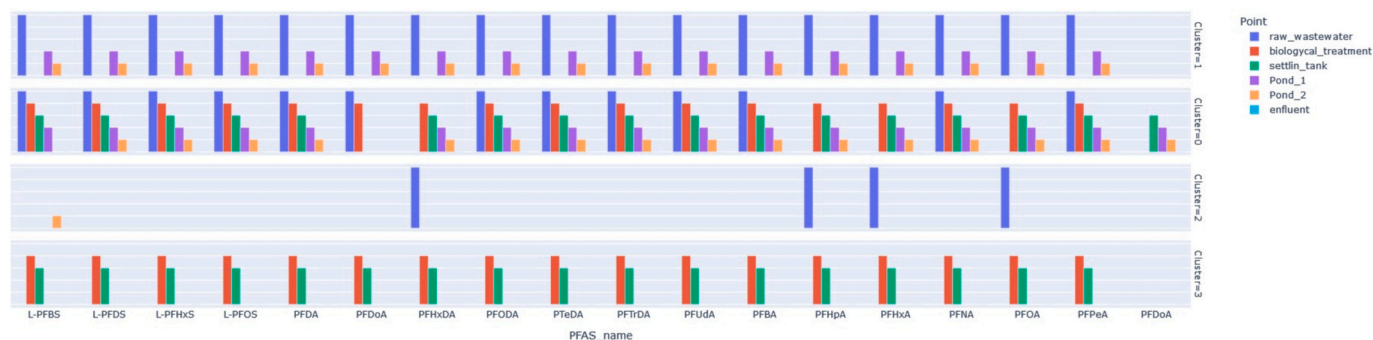


Fig. 5. Distribution of individual PAFS and their concentrations in individual clusters.

(Table 3) for all PAFS determined in the effluent was calculated for their maximum concentration.

The highest HQ values were determined for PFTeDA. However, PFTeDA and all the remaining PAFS determined in the effluent after SBR treatment appeared to show no adverse ecological risk effect. The HQ for the sum of the determined PAFS in the effluent was also at low levels.

4. Discussions

4.1. Basic pollutant removal

Statistical analysis showed no correlation between the determined basic wastewater pollutants and the PAFS content. At the same time, the

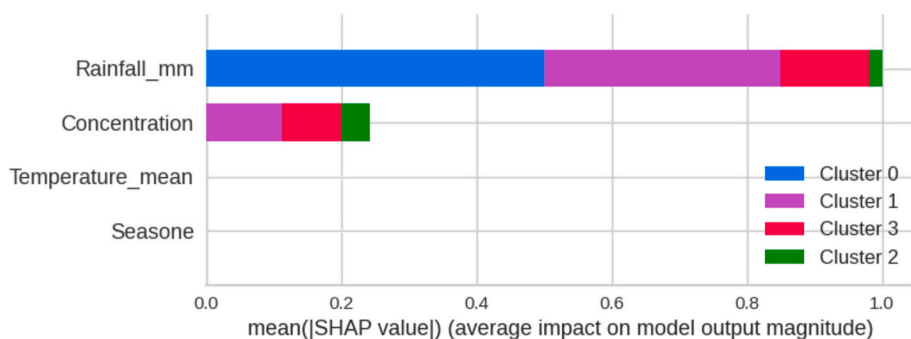


Fig. 6. SHAP summary plot of the average impact on model output magnitude.

Table 3

Hazard Quotients (HQs) values calculated for each compound based on PNEC calculation.

Compound	Ecotoxicological data [mg/L]			AF	PNEC [ug/L]	Reference	HQ
	Green algae	Crustaceans	Fish				
	EC ₅₀ /96 h	LC ₅₀ /48 h	LC ₅₀ /96 h				
PFHxS	220	190	301	1000	190		0.00000053
PFDoA	0.338	0.056	0.059	1000	0.056		0.0014
PFTeDA	0.047	0.0046	0.0043	1000	0.0043		0.020
PFTrDA	0.126	0.016	0.016	1000	0.016	ECOSAR PREDICTION	0.0063
PFHpA	41.4	24.5	35.4	1000	24.5		0.00067
PFOA	16.2	7.44	10.1	1000	7.44		0.0051
						sum of HQ	0.033

assessment of the importance of individual parameters was difficult due to the analysis of individual basic pollutants only in influent and effluent wastewater. Basic data analysis was not carried out at individual stages of wastewater treatment. Moreover, the degree of treatment indicated by the basic parameters is higher than in the case of PFAS removal. It seems that monitoring of basic parameters is insufficient to determine the PFAS content in the wastewater. [Gonzalez et al. \(2021\)](#) also showed no connection between the levels of PFAS before and after the SBR technology and levels of removal efficiency. They showed that COD and TN increases are further supported by slight reductions in nitrification rates in both the first and second aerobic time frames after the addition of the highest concentration of aqueous film forming foam. No change of COD compared to the input PFAS load is observed. However, there is a significant difference between BOD for influent with different levels of PFAS ([Table 1](#) and [Fig. 2](#)).

4.2. PFAS concentration

The concentration of PFAS in raw wastewater varies significantly, likely due to seasonal changes (also related to temperature changes) in the wastewater volume and composition. It should be noted that the WWTP is a small one, designed for a capacity of 5000 m³/d. However, during the summer, this load nearly triples to 14,000 m³/d due to the touristic character of the region, and June is considered the beginning of the summer season. This may explain the significant increase in total PFAS load. This substantial increase in wastewater load corresponds with a notable rise in the total PFAS load, where the sum of PFAS increased about 3 times in the analyzed period. This increase can be attributed not only to the higher volume of wastewater but also to elevated temperatures (average 18.2 °C in June, compared to 10.4 °C in May), which likely enhanced the desorption of PFAS from sediments and promoted transformation processes ([Fredriksson et al., 2022](#); [Lenka et al., 2021](#)). What is more, a 3.5-times increase in the concentration was observed in June (\sum PFAS from 367 to 1254 ng/L, for May and June, respectively). Considering the location of the treatment plant, the specificity of the area, which is a tourist one, and the invariability of the types of PFAS supplied in the sewage, it is probably related to the

increased production of domestic sewage and tourism activity. June is the period when practically every seasonal tourist resort starts its operation for the season. Additionally, global studies have highlighted the widespread presence of PFAS in the influent and effluent of wastewater treatment plants, with concentrations varying from 2.2 to 2156 ng/L in the influent and 1.9 to 4800 ng/L in the effluent across different regions ([Saliu & Saliu and Sauve, 2024](#)). Our observations are consistent with these global data, indicating that the concentrations detected in this study are within the reported global range, underscoring the challenge of PFAS management in wastewater systems. This alignment with global data emphasizes the importance of continued monitoring and development of advanced treatment processes to mitigate PFAS contamination.

This observation aligns with the data presented in the latest publications on PFAS in wastewater sources ([O'Connor et al., 2022](#)).

Besides the standard PFOA, which is commonly present in the environment, short-chain PFAS (PFHxA and PFHpA) in the raw wastewater were determined. This is especially disturbing given the increased number of publications indicating the problem of short-chain compounds ([Göckener et al., 2021](#)).

Changes in the qualitative profile of PFAS in subsequent stages of wastewater treatment may be related to the processes of their transformation as well as sorption and desorption. As pointed out by [Dickman and Aga \(2022\)](#), PFAS can undergo biotic and abiotic transformations. However, this applies to PFAS precursors such as polyfluoroalkyl substances containing carbon-hydrogen, carbon-oxygen, and carbon-nitrogen bonds. These transformations could be oxidation, photolysis, and hydrolysis, depending on meteorological parameters such as temperature and rainfall (higher RE in June compared to May). In our research, the statistical analysis indicated that PFHxA, PFHpA, PFOA, PFHxDA, and PFBS ([Figs. 4 and 5](#)) can enter the wastewater system mainly from wet deposition and surface runoff. This is particularly notable for PFBS, which was only detected in one reactor. Namely, the concentration was higher in Pond 2 during the period of less rain. This suggests that, in addition to rainfall, photolysis processes and increased solar radiation in open ponds may significantly contribute to PFAS dynamics. Further investigation is warranted to gain a comprehensive understanding.

In the case of PFDA, PFuDA, PFDA, PFTeDA, and PFOS, their lower sum of PFAS levels may have been due to the reduction of oxygen in the wastewater (Fig. 3). However, in the settlement tanks, their concentration was higher during the warmer period (June). This suggests faster desorption or release from precursors under warmer conditions. Additionally, there is the possibility of their emission from materials used in the treatment plant installations. The significant reduction in the amount of PFAS observed in ponds 1 and 2 is the main contributor to the overall reduction of PFAS in the water phase. The processes occurring in these ponds, including potential degradation or sorption to sludge, are crucial in mitigating PFAS levels. Certainly, increasing the residence time in subsequent ponds proves beneficial for reducing PFAS concentrations. This could be attributed to their degradation or sorption to sludge. Moreover, it signifies a reduction in the influx of new PFAS into the wastewater system.

In sewage sludge, abiotic processes and the adsorption of PFAS could occur. [Mussabek et al. \(2020\)](#) found that the decomposition of PFAS is strongly related to the mineral content of the sludge (i.e., Fe, Pb, Rb, and As), while the organic carbon content of the sludge had no direct effect on the decomposition of PFAS. In the case of adsorption, mechanisms such as electrostatic, hydrophobic, and ligand exchange interactions can have an impact ([Mukhopadhyay et al., 2021](#)). These phenomena should be investigated further to better understand the potential for PFAS removal through sludge management.

The PFAS concentration in the settling tank was higher than that in the biological treatment in the SBR in May and June, and similarly higher in Pond 1 compared to the settling tank in May. This disparity may be attributed to PFAS emissions from materials utilized in the treatment plant installations. Furthermore, significant rainfall was observed in May, potentially contributing to an elevation in PFAS concentration in the open pond. Additionally, the significant rainfall observed in May might have contributed to elevated PFAS concentrations in the open pond due to increased surface runoff.

The statistical analysis reveals that, in the subsequent stages of wastewater treatment, no seasonality of change over time is visible, except in the raw wastewater. Nevertheless, the amount of PFAS introduced into the Baltic Sea from this point source—the WWTP—averages 214.9 mg/month for the sum of PFAS. This emphasizes the ongoing need for monitoring and improving treatment processes to minimize the environmental impact of PFAS, particularly in regions with sensitive ecosystems, such as the Baltic Sea.

4.3. Ecotoxicity tests

In the Microtox test, the samples were toxic only as raw wastewater. The compounds toxic to *Aliivibrio fischeri* were effectively removed by the biological treatment in the investigated WWTP. The standard acute toxicity test, Microtox, fulfills its role at the stage of determining the degree of environmental hazard of treated wastewater. However, worryingly, it was not sensitive to changes in the level of PFAS in the subsequent stages of wastewater treatment. The concentrations of PFAS were probably too low to have an acute effect on the tested microorganisms. [Lashuk et al. \(2022\)](#), using the Microtox acute toxicity, showed that the EC₂₀ for mixed PFAS had values of 1.53 mg/L and 2.02 mg/L for contact times of 5 min and 15 min, respectively. In our research, the highest monitored sum concentration of the mixture of PFAS was 1.2 mg/L, which was below the EC₂₀ concentrations. Based on the obtained results, we are not able to indicate potency or synergism, because, for the sum, the value is still below EC₂₀.

The preliminary use of the Thamnotox test showed different results than the Microtox test. The Thamnotox test is sensitive to compounds present after biological treatment and settling. The difference may also be because Microtox® is a short acute toxicity test—the organisms had no more than 30 min of contact with the toxic solution, while for the Thamnotoxkit®, it was 24 h of contact. Therefore, the Thamnotox assay may be more sensitive. Additionally, statistical analysis shows that this

could be related to the PFAS content of the samples, especially for clusters 0 and 3. *Thamnocephalus platyurus* crustaceans are freshwater organisms that are extremely sensitive to the presence of ions in the sample. The literature does not indicate the use of this test in assessing the ecotoxicity of PFAS. The mechanism of action of PFAS on these organisms, however, should be thoroughly investigated.

4.4. Environmental risk assessment

The HQ for mixtures can be calculated as the sum of the HQ values of each compound. For the study of the PFAS mixture, there is no risk of adverse effects (HQ < 0.1). In determining the HQ coefficient for the PFAS mixture (using the sum of their individual HQs), an additive toxic effect was assumed, which in many cases is a great simplification. In addition to the additive effect, other effects may take place in the sample, such as potentiation, synergism, or antagonism. Moreover, for different compounds, these interactions may be different, thus several effects may take place simultaneously in one sample. In many cases, synergism is observed, i.e. the enhancement of the effect of the substance in a mixture with other substances. Unfortunately, there is a lack of studies on PFAS interactions in the environment. The indicated threats are their durability in the environment due to the specific chemical structure.

Additionally, the lack of information on new PFAS, their precursors as well as their degradation products, means that the environmental risks assessment of PFAS is still incomplete. Similar conclusions also come from other authors. [Sinclair et al. \(2020\)](#) emphasize that sub-lethal and/or chronic effects should also be taken into account.

Considering the solubility decreases with the increasing hydrophobicity of PFAS in water, their low concentration in wastewater samples does not mean that there are no environmental hazards. In previous studies, [Gałęzowska et al. \(2021\)](#) showed that the accumulation of PFAS in sediments may be a threat. Due to the chemical structure, sorption of PFAS into sludge can be very effective, which also means similar processes in the case of sewage sludge. The risk quotient for sediments in Chinese seas shows toxic effects only for a few selected PFAS ([Zhong et al., 2021](#)).

5. Conclusion

Based on the analyses of the screening, we can assume:

- the basic pollutants' parameters have been efficiently removed in the analyzed configuration of treatment: SBR followed by nature-based solutions;
- biological treatment in an SBR and settling tank followed by two ponds ensure an effective PFAS removal from wastewater of over 90 %, in individual stages: SBR—74–91 %, after settling tank—45–56 %, after ponds—85–99 %;
- the basic parameters and Microtox toxicity assay are not sensitive to changes in PFAS content in wastewater;
- the Thamnotox test could be used as an indicator of toxic levels of PFAS followed by validation of its efficiency with pure PFAS, assessing cross-sensitivity, and addressing other relevant factors;
- meteorological conditions affect the PFAS content in a WWTP.

The practical implications of the research suggest that sequencing batch reactors (SBR) combined with nature-based solutions can enhance PFAS removal. To improve PFAS removal, adjusting operational parameters, such as the hydraulic retention time, and ensuring proper sediment management to maximize PFAS retention are crucial. Additionally, monitoring meteorological conditions can help to understand and improve the efficiency of PFAS transformation processes.

The conducted research shows the following gaps that should be considered in future studies:

- the essential need to comprehensively investigate the distribution of PFAS within a WWTP operating with an SBR followed by NBS technology; this involves considering various polluted matrices at each stage of the process, including liquid phase, solid phase (sewage sludge), and dry and wet precipitation (especially to open systems);
- considering the release of PFAS from materials used in the wastewater treatment plant installations;
- the need to analyze various forms of PFAS, including precursors and short-chain PFAS;
- the impact of PFAS on the nitrification process.

In summary, a comprehensive approach involving the consideration of liquid and solid phases, PFAS precipitation, PFAS release, and monitoring various PFAS types is crucial for a thorough understanding of PFAS' spatial distribution in SBR followed by NBS wastewater treatment plants. The practical insights gained from this study can be applied to optimize existing treatment processes and guide the development of more effective PFAS management strategies in WWTPs globally.

CRediT authorship contribution statement

Grażyna Gałęzowska: Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Conceptualization. **Katarzyna Kolečka:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Conceptualization. **Monika Cieszyńska-Semenowicz:** Writing – original draft, Formal analysis. **Vladysław Redko:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Magdalena Gajewska:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

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