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Effect of Newly Synthesized Salts and Three Common Micropollutants on the Biochemical Activity of Nitrifiers

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Abstract: Often, different types of contaminants in wastewater are suspected of adversely affecting the treatment efficiency of a wastewater treatment plant (WWTP). Therefore, it is essential to study the effects of newly synthesized substances on the activity of activated sludge microorganisms. The aim of this study was to determine the effect of innovative biosurfactants, i.e., sophorolipids quaternary ammonium salts (SQAS), and three common micropollutants (MPs), i.e., diclofenac (DCP), 17α-ethynylestradiol (EE2), and 4-nonylenol (4-NP), on the biochemical activity of activated sludge microorganisms. The effect of all tested substances was more significant on nitrite-oxidizing bacteria (NOB) than on ammonia-oxidizing bacteria (AOB), and least on the respiratory activity of heterotrophic organisms (HET). SQAS inhibited nitrification even at the lowest concentration tested (5 mg L^{-1}) and the inhibition degree was in the range of 37% to 78%; at the highest concentration of SQAS studied (160 mg L^{-1}), it was about 45–96%. In most cases, the degree of inhibition increased when the SQAS concentration approached 80–160 mg L^{-1}. MPs influenced the activity of nitrifiers to a lower extent than SQAS. The inhibition degree varied from 25% to 75%, depending on the micropollutant tested and its concentration.

Keywords: MPs; nitrifying bacterial activities; nitrification inhibition; wastewater treatment; SQAS

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1. Introduction

The term "micropollutants" is commonly used to describe organic and inorganic compounds present in water and wastewater in concentrations of μg L^{-1} or ng L^{-1}. Pharmaceutical micropollutants belong to the group of emerging organic micropollutants that include, e.g., human and veterinary drugs, steroid and thyroid hormones, phytoestrogens, and endocrine disruptors [1,2]. These compounds and their metabolites enter into wastewater treatment plants (WWTPs), where some of them may not be completely eliminated or transformed during the wastewater treatment process, leading to their presence in surface waters [3]. The removal of micropollutants from wastewater is an increasingly adopted goal for every conventional wastewater treatment plant. Usually WWTPs, constructed for the efficient elimination of organic nutrients (g L^{-1}) by biological degradation or coagulation, are ineffective in removing these substances [4,5].

Nitrification is a microbial process through which reduced nitrogen compounds (primarily ammonia) are sequentially oxidized to nitrite and nitrate. In the first step of nitrification, ammonia-oxidizing bacteria (AOB) oxidize ammonia to nitrite; *Nitrosomonas* is the most frequently identified genus associated with this step. In the second step of the

process, nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate; *Nitrobacter* is the most frequently identified genus associated with this second step [6–9]. The two nitrification steps occur simultaneously, but the process can be disconnected and investigated individually using inhibitors of one of the two steps. Inhibition is the result of the blockage or inactivation of the normal catalytic cycle of the enzyme responsible for a specific function, i.e., nitritation or nitratation [10,11].

The inhibition of nitrification in wastewater treatment plants is most often caused by components in industrial wastewater from many different industries. Nitrification inhibition can be directly caused by the toxicity of single substances or mixtures of substances. AOB and NOB are known sensitive organisms with respect to environmental conditions such as dissolved oxygen (DO), pH, temperature, alkalinity, and the presence of toxic compounds. As a result of changes in these parameters, the coupling in rates between AOB and NOB can be easily disturbed. Du et al. [12] investigated the influence of erythromycin on AOB and NOB using batch and continuous experiments. During batch experiments, the impact of erythromycin was strictly dose-dependent for AOB and NOB. The continuous addition of 1 mg L^{-1} of erythromycin in the long-term exposure experiment made NOB sensitive, whereas AOB was minimally sensitive. After a prolonged exposure to concentrations, even up to 50 mg L^{-1}, both NOB and AOB became insensitive to increases in erythromycin. In several studies, the role of AOB in the removal of MPs was investigated by selectively inhibiting the enzyme AMO with allylthiourea (ATU) in AOB-enriched activated sludge. The results obtained indicate better removal with active (usually >70% removal) than inhibited (<40% removal) AMOs, including several pharmaceuticals and synthetic estrogens [13–15], artificial sweeteners [16], and micropollutants such as bisphenol A (BPA), nonylphenol, and triclosan [17,18], suggesting that this enzyme is involved in the degradation of these compounds. Blum and Speece studied the toxicity of organic chemicals to different groups of bacteria used in treatment processes and observed that the nitrifiers were growing more slowly and were more sensitive to toxic compounds compared to heterotrophic bacteria, increasing their vulnerability to disturbances at WWTPs [19].

EE2, DCF, and 4-NP belong to the most common micropollutants occurring in municipal WWTPs. According to Liwarska-Bizukojć et al. [20], the highest concentrations in raw municipal wastewater were reported for nonylphenol and diclofenac; they were found at high concentrations in raw municipal wastewater and in surface water. For 4-NP, the observed levels exceeded 100 μg L^{-1}. The reported concentrations of diclofenac varied from below 0.001 to 94.2 μg L^{-1}. Steroid hormones also belong to the group of micropollutants, and their concentrations in wastewater usually do not exceed 1 μg L^{-1}. A synthetic estrogen, EE2, is widely used in contraceptive pills, and it was estimated that about 40% of the total EE2 used by one person, i.e., about 10.5 μg day^{-1}, reaches the sewage influent. The removal of these MPs from wastewater has previously been studied, but knowledge about their effect on the biochemical activity of nitrifiers is still not clear.

SQAS represent a new class of antimicrobial surfactants and it is important to check, before being introduced on the market, whether they should be classified as environmentally relevant emerging contaminants. To the best of our knowledge, there are no studies concerning the influence of sophorolipid quaternary ammonium salts on the biochemical activity of nitrifiers. Delbeke et al. observed that four of them, SQAS1–SQAS4, are more active than the antibiotic gentamicin sulfate against the Gram-negative strains *Escherichia coli* LMG 8063 and *Klebsiella pneumoniae* LMG 2095, and the Gram-positive strains *Staphylococcus aureus* LMG 8064 and *Bacillus subtilis* LMG 13579 [21].

This work focused on the influence of SQASs and three micropollutants: the two pharmaceuticals DCP and EE2, and one of the most common alkylphenols in wastewater, i.e., 4-NP on nitrification and the biochemical activity of nitrifiers (AOB and NOB). The aim of the study was to determine the effect of the micropollutants and potential micropollutants on the activity of three functional groups of activated sludge microorganisms, that is, heterotrophic bacteria (HET), AOB, and NOB. The description comprises the determina-

tion of the oxygen uptake rate (OUR), the specific oxygen uptake rates (SOUR), and the calculation of the degrees of inhibition for each group of microorganisms studied.

2. Materials and Methods

All experiments were conducted according to a previously published operating procedure [7,22]. The methodology of this test is based upon the determination of $N-NH_4^+$ and $N-NO_2^-$ oxidation rates by measuring the OUR of activated sludge samples before and after the addition of the selective nitrification inhibitors. The experiments were carried out in a 200 mL closed batch reactor vessel equipped with a ProODOTM optical DO -meter (YSI Environmental, Yellow Springs, OH, USA), connected to a computer to register changes in the DO concentration and the temperature over time. The tests were performed at a constant temperature of 22 ± 1 °C; samples were mixed continuously by means of a magnetic stirrer at 150 rpm. Activated sludge was obtained from the aerated part of the biological reactor in the Combined Wastewater Treatment Plant in Lodz (Poland) operating in Modified University of Cape Town mode and were acclimatized, for 7 days in activated sludge chambers, to the composition of the synthetic municipal wastewater. The synthetic wastewater used in the acclimatization studies contained: 300 mg peptone, 100 mg CH_3COONa, 50 mg K_2HPO_4, 50 mg $NaHCO_3$, 50 mg $(NH_4)_2HPO_4$; 5 mg $MgSO_4 \bullet 7H_2O$, and 5 mg NaCl per liter. The average synthetic wastewater flow was 7 L day^{-1}.

Activated sludge after acclimatization had typical properties, with total suspended solids (TSS) and volatile suspended solids (VSS) of 3.6 ± 0.2 g L^{-1} and 2.1 ± 0.1 g L^{-1}, respectively. The sludge volume index (SVI) was at the level of 112 ± 8 mL g TSS^{-1}. Soluble chemical oxygen demand (COD) and ammonium nitrogen were at the levels of 46 ± 5 mg O_2 L^{-1} and 1.27 ± 0.22 mg N L^{-1}, respectively. The physicochemical analyses were performed in agreement with standard methodologies [23].

SQAS and MPs were mixed with activated sludge biomass at the appropriate concentration. The suspension was then transferred to the reaction vessel. It was saturated with oxygen by intensive agitation (which enabled to obtain the initial dissolved oxygen concentration of 7–8 mg O_2 L^{-1}); the agitation speed was then kept constant at 150 rpm. The consumption of DO was observed. The test was performed using allylthiourea (ATU) and $NaClO_3$, well-known nitrification process inhibitors. OUR measurements started the moment the oxygen electrode was immersed in the reactor. First, the total OUR was determined. The first inhibitor $NaClO_3$ was added to the reaction mixture after several minutes (depending on the test progress) when the DO concentration decreased about 2 mg L^{-1}. After some time, when the concentration of DO decreased again about 2 mg L^{-1}, the second inhibitor ATU was added and the remaining OUR was measured. Parallel control tests without additive SQAS and MPs were performed. The inhibitor concentrations in the reaction mixture were 20 mM for $NaClO_3$ and 5 mg L^{-1} for ATU. Oxygen consumption determination was performed in three replicates. The dissolved oxygen uptake rate was determined by linear regression from the slope of the oxygen utilization curve. The difference between the total OUR and the OUR after $NaClO_3$ addition was considered as the oxygen uptake due to nitrite oxidation (associated with NOB); the difference in OUR measured in the presence of $NaClO_3$ and ATU represents the oxygen uptake due to ammonium oxidation (associated with AOB). The remaining OUR measured after $NaClO_3$ and ATU addition reflected the oxygen consumption of the heterotrophs [7,22].

The degree of SQAS and MPs inhibition is defined as:

$$\%Inhibition = \frac{OUR_0 - OUR_{comp}}{OUR_0} * 100\% \quad (1)$$

where OUR_0 is the oxygen uptake rate without SQAS and MPs, and OUR_{comp} is the oxygen uptake rate under certain concentrations of SQAS and MPs (mg O_2 L^{-1} h^{-1}).

To determine the activity of three functional groups of activated sludge microorganisms, SOUR was calculated according to Park and Lee [24]:

$$\text{SOUR} = \frac{\text{OUR}}{\text{VSS}},\ \text{mg O}_2\ \text{g VSS}^{-1}\ \text{h}^{-1} \quad (2)$$

where OUR is the oxygen uptake rate (mg O_2 L^{-1} h^{-1}), and VSS is the volatile suspended solids concentration of activated sludge (g VSS^{-1} h^{-1}) for each operating condition. SOUR may also be referred to as the respiration rate (RR). The SOUR normalizes the response to the mass of organisms and allows the comparison of oxygen response for different mixed liquors for each gram of organisms [25].

2.1. Statistical Elaboration of the Results

The basic statistical elaboration of the results of the tests performed, including the calculation of the mean values and the standard deviation(s), was conducted with the use of MS Excel (Microsoft Corporation, Washington, DC, USA). A one-way analysis of the variance (ANOVA) was applied to estimate if the values of the biochemical activity of the appropriate group of microorganisms exposed to SQAS or MPs, and the biochemical activity of the appropriate group of microorganisms not exposed to SQAS or MPs (control tests), were statistically equal. A null hypothesis stating that they were equal was assumed. The ANOVA implemented in MS Excel (Analysis ToolPak) software was used. A confidence level of 95% was assumed.

2.2. Chemicals

In the current work, the influence of four sophorolipid quaternary ammonium salts, synthesized at the Department of Green Chemistry and Technology (Ghent University, Belgium): SQAS1—N,N-dimethyl,N-octadecyl-(8-L-[(2,3,3,4,4,6,6-heptaacetoxy-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide, SQAS2—N-benzyl,N-methyl, N-octadecyl-(8-L-[(2,3,3,4,4,6,6-heptaacetoxy-2-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide, SQAS3—N,N-dimethyl,N-octadecyl-(8-L-[(2β-O-β-D-glucopyranosyl-β-D- glucopyranosyl)-oxy])nonan-1-ammonium iodide, and SQAS4—N-benzyl,N-methyl,N-octadecyl-(8-L-[(2β-O-β-D-glucopyranosyl-β-D-glucopyranosyl)-oxy])nonan-1-ammonium iodide, and three micropollutants purchased from Sigma-Aldrich (17α-ethynylestradiol (EE2), $C_{20}H_{24}O_2$, CAS Number 57-63-6, purity ≥ 98%; diclofenac (DCF), $C_{14}H_{10}C_{l2}NNaO_2$, CAS Number 15307-79-6, purity ≥ 98%, and 4-nonylphenol (4-NP), $C_{15}H_{24}O$, CAS Number 104-40-5, analytical standard) on the biochemical activity of nitrifiers was carried out. More details about SQAS physicochemical properties were described by Delbeke et al. [21]. The stock solutions of inhibitors were freshly prepared in concentrations 125 mg L^{-1} for ATU and 0.5 M for $NaClO_3$.

3. Results and Discussion

Our previous tests [26] showed that that SQAS does not inhibit the respiration activity of activated sludge bacteria to a high extent, i.e., at a higher level than 50%, when the SQAS concentration did not exceed 100 mg L^{-1}. In this study, we decided to check the influence of the SQAS and the MPs at a concentration of 100 mg L^{-1}.

Tables 1 and 2 demonstrate general trends in the distribution of the bacterial activities during the process in the presence of SQAS and micropollutants. The total respiratory activities of both the microbial communities and nitrifying bacteria decreased after sludge exposure to both SQAS and MPs. For all SQAS, the respiratory activity of both the AOB and NOB decreased dramatically; for SQAS2, NOB activity decreased more than AOB. Comparing the results obtained by adding MPs, we noted that the influence of inhibitors on the specific oxygen uptake rates was much higher on the NOB than on the AOB. The effect of all the test substances on the respiratory activity of the heterotrophs was also evident; however, in this case, the decrease in SOUR was the smallest.

Table 1. Influence of SQAS on the specific oxygen uptake rates determined at a concentration of 100 mg L^{-1}.

Tested Compound	$SOUR_{TOT}$ (mg O_2 g VSS^{-1} h^{-1})	$SOUR_{AOB}$ (mg O_2 g VSS^{-1} h^{-1})	$SOUR_{NOB}$ (mg O_2 g VSS^{-1} h^{-1})	$SOUR_{HET}$ (mg O_2 g VSS^{-1} h^{-1})
SQAS1	4.86	0.54	0.50	3.82
SQAS2	5.76	1.26	0.50	4.00
SQAS3	4.86	0.36	0.40	4.10
SQAS4	4.68	0.18	0.15	4.35
None (Control)	8.64	1.78	1.96	4.90

Table 2. Influence of MPs on the specific oxygen uptake rates determined at a concentration of 100 mg L^{-1}.

Tested Compound	$SOUR_{TOT}$ (mg O_2 g VSS^{-1} h^{-1})	$SOUR_{AOB}$ (mg O_2 g VSS^{-1} h^{-1})	$SOUR_{NOB}$ (mg O_2 g VSS^{-1} h^{-1})	$SOUR_{HET}$ (mg O_2 g VSS^{-1} h^{-1})
4-NP	5.50	0.79	0.30	4.41
DCF	5.90	0.86	0.18	4.86
EE2	5.04	0.89	1.24	2.91
None (Control)	8.64	1.78	1.96	4.90

In this study, the inhibitory effect of SQAS and MPs at the concentration of 100 mg L^{-1} was tested (Figure 1). The degree of inhibition determined for the biochemical activity of nitrifying microorganisms higher than 50% indicated the significant effect of these compounds on nitrifiers [27]. Among the SQAS, only SQAS2 did not inhibit the first stage of nitrification and, as a consequence, the activity of NOB. The most inhibitory effect on nitrification was observed for SQAS4 at about 90%, and at about 85% for SQAS3. However, among the micropollutants tested, EE2 significantly inhibited only the second stage of nitrification, while no significant inhibitory effect on nitrification at any stage was found for 4-NP ($p > 0.05$). The most inhibitory effect on nitrification was observed for DCF, at about 75%. Comparing the inhibition percentages for the two pharmaceuticals tested, DCF had a higher negative effect on the nitrification process than EE2. After considering the results obtained for EE2, only its significant inhibitory effect on the second stage of nitrification of about 70% was indicated ($p < 0.05$). It is well-known that AOB and NOB bacteria are less tolerant than heterotrophic microorganisms of a large range of organic toxicants in activated sludge. Experiments with a dozen compounds [28] also showed that chlorate, cyanate, azide, and hydrazine are more inhibitory of NOB's oxidation than of AOB's oxidation. He and Bishop [29] stated that Acid Orange 7, an azo dye commonly used in textile, pharmaceutical, food, and cosmetic industries, inhibits all stages of the nitrification process, although the NOB were more sensitive than the AOB.

From a practical point of view, if the activity of the NOB is inhibited to a greater extent than the activity of the AOB [30], an imbalance may occur. Wastewater treatment plants frequently fail to establish stable nitrification, which is often attributed to the slow growth of nitrifying bacteria; an accumulation of nitrite often results from a modification of the growth kinetics of AOB and NOB. Notably, nitrifiers are obligate aerobes, so an important factor for nitrification is DO concentration. Maximum nitrification occurs at a DO level 3.0 mg L^{-1}. Significant nitrification occurs at a DO level of 2.0 to 2.9 mg L^{-1}. Nitrification stopped at DO levels of <0.5 mg L^{-1}. Theoretically, 4.57 g of oxygen is needed to completely oxidize 1 g of ammonia-nitrogen (ammonia-N) into nitrate, with 3.43 gO_2 $g^{-1}N$ for the first-step nitrification (ammonia oxidation) and 1.14 gO_2 $g^{-1}N$ for the second-step nitrification (nitrite oxidation) [31]. The correct determination of oxygen requirement in the activated sludge process is also essential for the effective and cost-efficient operation of a wastewater treatment plant. Inhibiting the oxidation of nitrites by NOB allows for a 25% reduction in oxygen demand in relation to full oxidation. In practice, it is desirable to achieve a

high nitrification rate at the lowest possible cost, and it must be controlled in each case. The dissolved oxygen concentration is usually kept higher than 2 mg L^{-1} in conventional WWTPs to prevent oxygen depletion [32]. As a result, a large amount of energy is used for aeration. In the aeration tank, the required aeration depends on the actual oxygen demand and the oxygen transfer efficiency. Therefore, two directions can reduce aeration need: improve the oxygen transfer efficiency, or reduce the actual oxygen demand. Theoretically, if the aeration tank is running with a DO of 0.5 mg L^{-1} instead of 2 mg L^{-1}, the oxygen transfer efficiency would be enhanced by about 16% [31]. Jayamohan et al. showed that continuous nitrification under low DO leads to a high nitrite accumulation, and by limiting the amount of oxygen in the nitrification process, accumulation occurs only partially [33].

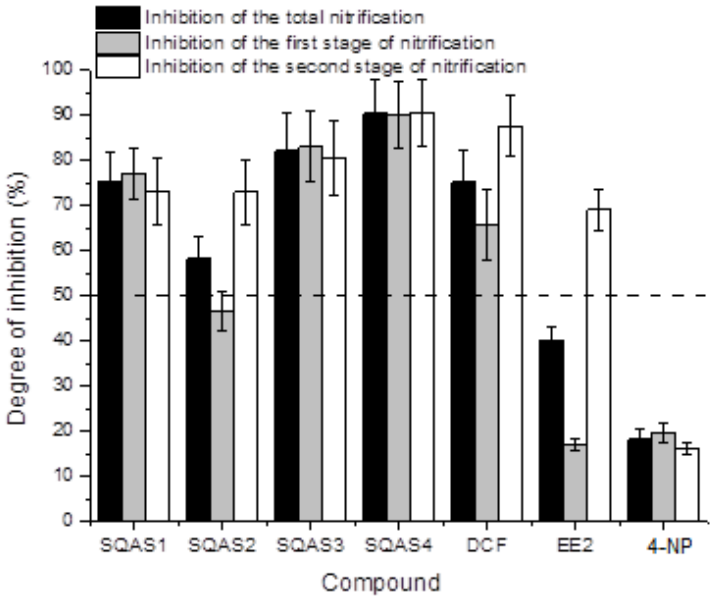
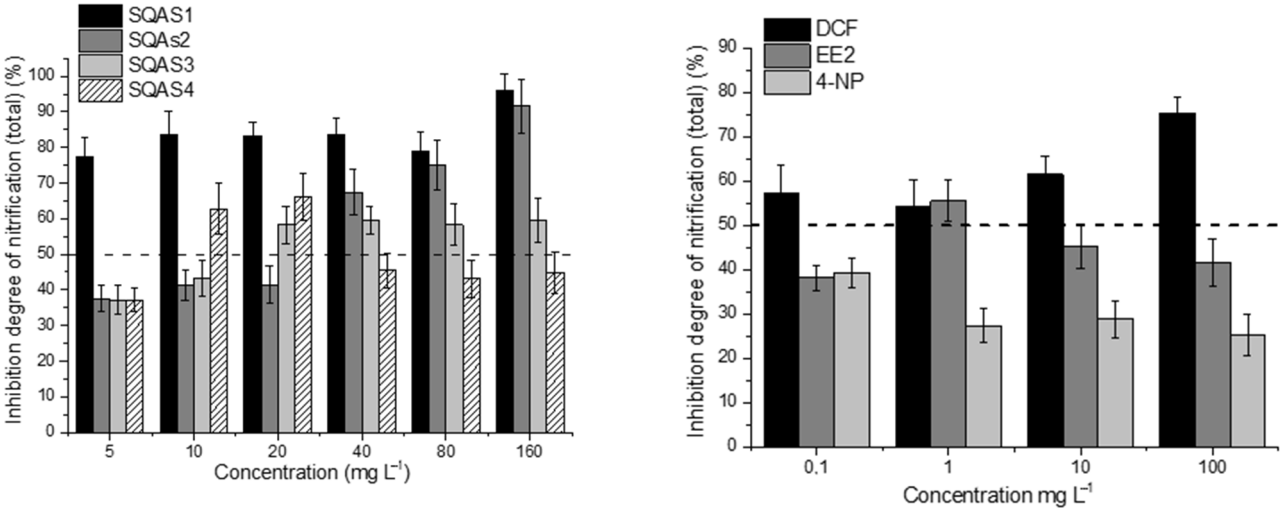


Figure 1. Comparison of the inhibition of nitrification for SQAS and the MPs determined at the same concentration of 100 mg L^{-1}.

Analyzing the results presented in Figure 2a,b, it can be clearly seen that the SQAS and MPs used in the study showed inhibitory effects in relation to the nitrification processes. SQAS caused inhibition of nitrification, even at their lowest concentration (5 mg L^{-1}). The inhibition degree was in the range of 37–78%, while at the highest concentration used (160 mg L^{-1}), it was about 45–96%, depending mainly on the tested compound. In most cases, the degree of inhibition increased when the SQAS concentration approached 80–160 mg L^{-1}. The highest inhibition effect of the nitrification process was observed for SQAS1. In the case of SQAS2, a significant effect on the respiratory activity of microorganisms and a direct relationship between the degree of inhibition and the concentration were observed at concentrations of 40–160 mg L^{-1} ($p < 0.05$). Significant inhibition was determined for SQAS3 for the concentrations from 20 to 160 mg L^{-1}, and for SQAS4 for the concentration range of 10–20 mg L^{-1} ($p < 0.05$). When analyzing the effect of SQAS and MPs on the biochemical activity of two groups of nitrifying microorganisms, it was also observed during the experiments (Figure 3) that SQAS1 exhibited a strong inhibitory effect on both AOB and NOB in the complete concentration range. SQAS2 had a significant inhibitory effect on AOB only for the concentration 40–160 mg L^{-1} and on NOB over the entire concentration range ($p < 0.05$). SQAS3 had no significant effect on the AOB, but its influence on NOB was significant across the concentration range tested ($p > 0.05$). SQAS4, at concentrations above 20 mg L^{-1}, influenced bacteria from the first phase and in low concentrations. SQAS4 (10–20 mg L^{-1}) also had a significant inhibitory effect on bacteria in the second phase of nitrification ($p < 0.05$). In the case of SQAS, this inhibition of AOB varied from 11–96%; for NOB, it was in the range of 21–95%.



(**a**) (**b**)

Figure 2. (**a**) Effect of SQAS on the nitrification and (**b**) effect of selected MPs on nitrification.

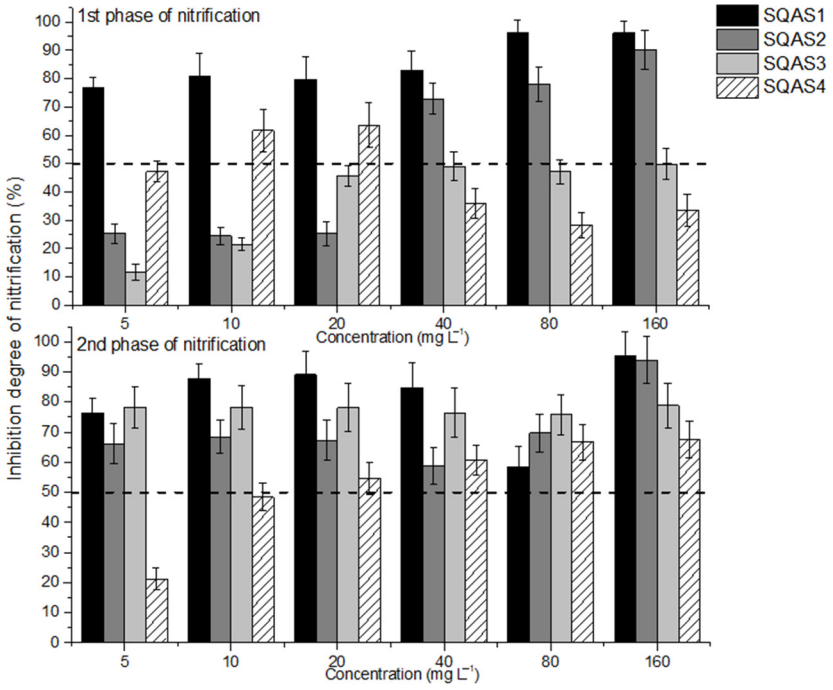


Figure 3. Effect of SQAS on the first and second stages of nitrification.

Analyzing the effect of the MPs on the degree of inhibition (Figure 2b), it is found that in the lowest tested MPs concentration (0.1 mg L^{-1}), the inhibition was in the range of 37–57%, while at the highest concentration used (100 mg L^{-1}), it was about 25–75%. MPs influenced the activity of nitrifiers to a lower extent than was the case with SQAS. For DCF, more than 50% inhibition of nitrification (total) was observed over the entire concentration range tested, and for EE2, only at the concentration of 1 mg L^{-1}. 4-NP did not significantly affect nitrification, but the inhibition varied from 25–40% ($p > 0.05$).

NOB (Figure 4) was more sensitive to the presence of MPs than AOB; this was observed for DCF and EE2. A significant degree of inhibition for AOB was noticeable only for DCF (50–65%) over the entire concentration range tested, and for EE2, at a concentration of

100 mg L^{-1} ($p < 0.05$). DCF and EE2 had significant effects on NOB across the concentration range tested ($p < 0.05$). 4-NP had no significant effect on either AOB or NOB activity ($p > 0.05$). In the case of MPs, the inhibition of AOB varied from 20–37%; for NOB, it was in the range of 25–87%. This agrees with the results presented by Liwarska-Bizukojc et al., who reported that the presence of micropollutants did not adversely affect biological wastewater treatment processes [34]. Additionally, according to Margot et al., autotrophic nitrifying organisms (especially AOB) do not play a significant role in micropollutant removal, except for a few compounds: BPA, NPX, iohexol, irgarol, and terbutryn [35]. Falas et al. observed that the removal rate of pharmaceuticals depends on the properties of the compounds and the bacteria composition of the sludge. They also noticed that the inhibition of ammonia monooxygenase by ATU most often has a limited effect on the removal of pharmaceutical compounds. Their results indicated that the removal of ibuprofen, naproxen, and ketoprofen in municipal activated sludge processes is highly dependent on the heterotrophic bacteria community and that AOB may only play a minor role [36].

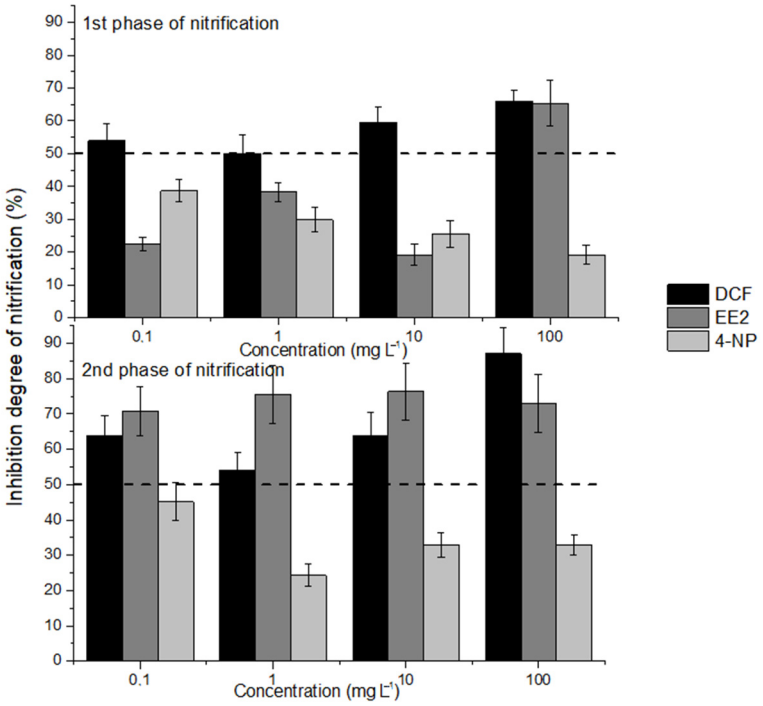


Figure 4. Effect of MPs on the first and second stages of nitrification.

4. Conclusions

Nitrifying bacteria, both AOB and NOB, are sensitive to inhibitory compounds, which can be present in various industrial wastewaters. It is therefore important not to allow wastewater that may contain inhibitors to enter WWTPs.

1. We found that both SQAS and MPs have an inhibitory effect on the biochemical activity of nitrifiers and on the degree of inhibition. The influence of all the tested substances is much larger on NOB than on AOB, and smallest on the respiratory activity of heterotrophs.
2. Among the examined SQAS, SQAS1, demonstrated the highest impact on the biochemical activity of the nitrifiers in the complete concentration range.
3. Three tested MPs also presented an inhibitory effect on nitrification but influenced the activity of nitrifiers to a lower extent than SQAS. DCF inhibits both AOB and NOB; EE2 inhibits the process of nitrification more. In the case of 4-NP, nitrification is inhibited to a minimum extent.

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