DISTURBANCES OF BIOLOGICAL N-REMOVAL PROCESSES IN THE PRESENCE OF SULPHONAMIDES

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Abstract The environmental behaviour of selected sulphonamides was investigated. The research was focused on biodegradability and nitrification efficiency of sulphanilamide (SA), sulphacetamide (SCM), and p-toluenesulphonamide (p-TSA). The biodegradability was investigated using the Closed Bottle Test (OECD 301D). Nitrification inhibition assessment was carried out according to ISO guidelines (ISO 9509:1989). It was found that all tested sulphonamides are resistant to biodegradation. Biodegradation begins only after ten days of incubation and after twenty eight days it only reaches 8 -10 %. The highest biodegradation was observed for p-TSA (45% after forty nine days). Sulphonamides used in the test reveal an inhibition effect in relation to nitrification process. At their lowest concentration (1mg/l) the inhibition was in the range 4-27%, while at the highest concentration used (100 mg/l) it was about 20-45%. Thus, it can be concluded that these compounds are likely to cause problems during treatment of wastewater from the pharmaceutical industry.

Keywords: sulphonamides, biodegradation, nitrification inhibition, pharmaceutical wastewater

INTRODUCTION

Pharmaceutical agents used in veterinary and human therapy have aroused attention in recent years as potentially hazardous pollutants of the environment. Some of them are not readily biodegradable, some reveal genotoxic properties. Recently, some of pharmaceutical agents or their metabolites have been identified as endocrine disrupters (Larsen et al., 2004, Ternes and Joos, 2007).

Excretion is the major route by which human and veterinary medical compounds are introduced to the environment (Jjemba, 2002). Other sources are pharmaceutical wastewater containing large amounts of various organic compounds, which can inhibit microorganisms' growth. This can be a problem for the natural environment as well as for wastewater treatment plants. In the literature one can find papers which focus on the behaviour of therapeutic agents in the environment, usually in surface waters (Hirsch et al., 1999, Ingerslev et al., 2001) or in the soil (Jjemba, 2002).

The biodegradability and the effect on wastewater bacteria of selected antibiotics (Ciprofloxacin; Ofloxacin, Metronidazole) at concentrations typical of municipal as well as hospital wastewater were investigated by Kümmerer et al. (2000).

However, compared to municipal wastewater, the sewage from pharmaceutical industry contains much higher amounts of pharmaceuticals (e.g. antibiotics) and it may drastically change the results of their biodegradation and toxic effects if one considers biological treatment (Ternes et al., 1998). Examples of such compounds are sulphonamides, which have bacteriostatic and bactericidal properties. The bacteriostatic properties of sulphonamides were discovered in 1930. Since this time thousands of sulphonamides have been synthesized, but only few of them are applied in medicinal treatment. All of sulphonamides introduced as medicines are derivatives of p-aminebenzenesulphonamide. Sulphonamides are p-aminobenzoic acid (PABA) antagonists and have bacterioastatic effect. Sulphonamide consumption comprises ca. 2÷20 % of the total pharmaceutical use, depending on the country (in Poland it is about 15%) (Ferech et al., 2001). Some of the most important features of sulphonamides are the rapidly increasing resistance of bacteria to these substances, their toxic properties and their long half-time (up to 48 hours).

Their biodegradation is problematic, and the adaptation of microorganisms may be the only way to minimize their negative impact. For this reason knowledge of the environmental behaviour of sulphonamides is urgently needed.

The environmental effects of sulphonamides were investigated by Migliore and co-workers (Migliore et al., 1995, 1996, 1997). Based on their usage, the authors predicted concentrations of e.g. sulphadimetoxine and sulphametoxine in the soil in the range 4-266 μg per kg soil. From laboratory experiments, they found that sulphametoxine at a concentration of 300 mg/l significantly depresses the growth of some plants, due to its bioaccumulation in roots and foliage (Migliore et al., 1995, 1996). However, such high concentrations are unlikely to occur in the soil.

Previous investigations of the biotic transformation of sulphonamides have shown them to be persistent in model marine aquaculture sediment (Samuelson et al.1994). Al- Ahmad et al (1999) as well as Ingerslev and Halling –Sørensen (2000) have reported that sulphonamide substances are not readily biodegradable. No biodegradation was observed for sulphamethoxazole (Al –Ahmad, 1999). Sulphonamides adsorb only weakly to both to soil and activated sludge (Huang et al. 2001, Thiele, 2000, Fontaine et al. 1991, Ingerslev and Halling –Sørensen 2000). Moreover, sulphonamides were found to be susceptible to hydrolysis. Nevertheless, the hydrolysis of sulphonamides at neutral pH is very slow and may be considered as insignificant (Volmer and Hui, 1998).

Huang et al. (2001) stated that sulphamethoxazole is most likely present in wastewater effluent as well as sulphamethazine is present in agricultural runoff.

The wastewater predicted concentration of sulphamethoxazole in untreated wastewater treatment plant influent has been estimated at the level of 3.8 μg/l –excluding its metabolism and 3.2 μg/l including its metabolism. The daily waste of a swine is estimated to contain 496 mg/day-animal of tylosin and sulphamethazine.

Hartig et al (1999) developed the methodology for detection and identification of sulphonamide drugs in municipal wastewater. Liquid chromatography coupled with electrospray ionisaton tandem mass spectrometry is the method of choice. Fourteen different sulphonamide drugs (sulphonamide sulphacetamide sulphodiazine, sulphomethizole sulphamethoxazole etc.) were investigated. From grab samples of primary and secondary effluent from a municipal wastewater treatment plant (WWTP) in Berlin, the recoveries in extraction were, in general, high (50-90 %) with the exceptions of sulphanilamide and sulphacetamide. Their recoveries were from 18-40 % and from 32-40 % for sulphanilamide and sulphacetamide, respectively.

Sulphamethoxazole and sulphadiazine were detected in the range 30-2000 ng/l and 10-100 ng/l, respectively. Their occurrence in surface waters and secondary effluence emphasises their importance of sulphonamides as organic pollutants.

On the other hand, one should consider their effect on water selfpurification as well, as wastewater purification processes.

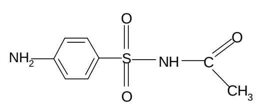
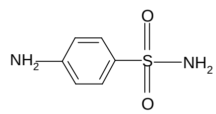
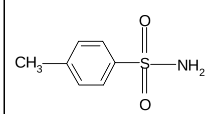
Nitrification is one of the most important process in the route of nitrogenous compounds transformation in the environment and their removal from wastewater. Its effectiveness in case of streams with high content of ammonium nitrogen is one of the crucial aspect when evaluate quality of the effluent. The transformation of $NH_4 – N$ into nitrates depends on the activity of nitrifying bacteria. They are very sensitive to various toxic substances, thus, there are some problems in case when wastewater pollutants are toxic. In the literature there are only a few reports on sulphonamides' toxicity to nitrifying bacteria (Ingerslev and Halling-Sørensen, 2000, Al- Ahmad et al. 1999) and only one report on the effect of these compounds to deammonification (Anammox) bacteria (Bąkowska et all., 2006). The inhibition of nitrification in the soil by various therapeutic agents has been reported by (Nimenya et al., 1999). However, other workers observed no impact (Warman, 1980).

Thus, further investigations of the inhibiting effect of sulphonamides on the nitrification process are needed.

In one pharmaceutical factory in Poland, the biological treatment of process wastewater was not effective, especially related to ammonia nitrogen removal. It was conjectured that the nitrification process could be retarded by sulphonamides present in wastewater. The discharge of insufficiently pre-treated pharmaceutical wastewater to a municipal treatment plant can later cause problems in the biological treatment process.
Thus, the current research aimed at the evaluation of the biodegradability of selected sulphonamides and their toxicity towards nitrifying bacteria.

Methodology
In the current work the environmental behaviour of some representatives of the sulphonamide group has been carried out. In this research, sulphanilamide (SA), sulphacetamide (SCM), and p-toluenesulphonamide (p-TSA) were used. SA and SCM are antimicrobials applied in medicine and veterinary treatment, but p-TSA is a metabolite of Chloramine T, used as a disinfectant against bacteria, viruses and fungi. Some properties of the tested sulphonamides are presented in Table 1. All sulphonamides were obtained from Sigma-Aldrich Co. Allyl thiourea (ATU) used in the nitrification inhibition test was supplied by Sigma Aldrich Co.
Inorganic salts, used for nutrient solution preparation were supplied by POCh S.A. The nutrient solution was prepared from aqueous solutions of individual salts. Salt solution A consisted of KH_2PO_4 (8.5 g dm^{-3}), K_2HPO_4 (21.75 g dm^{-3}), $Na_2HPO_4 \cdot 2H_2O$ (33.40 g dm^{-3}) and NH_4Cl (0.50 g dm^{-3}). Salt solutions B, C and D consisted of $CaCl_2$ (27.50 g dm^{-3}), $MgSO_4 \cdot 7H_2O$ (22.50 g dm^{-3}) and $FeCl_3 \cdot 7H_2O$ (0.25 g dm^{-3}) respectively. To prepare the nutrient solution one ml of each - A, B, C and D salt solution was diluted to one litre with distilled water.
Activated sludge (used in inhibition tests) and also supernatant liquor (used in biodegradation tests were sampled from the Municipal Waste Water Treatment Plant in Gdansk, Poland.

Table 1. Characteristics of sulphonamides used in the study

	Sulphacetamide (SCM)	**Sulphanilamide (SA)**	**p-toluene-sulphonamide (p-TSA)**
Nomenclature	N-[p- aminobenzene sulphonyl] – acetamide	4-aminobenzene sulphonamide	4-metylbenzene sulphonamide
Molecular weight [g/mol]	214.20	172.21	171.22
Molecular formula	$C_6H_{10}N_2O_3S$	$C_6H_8N_2O_2S$	$C_7H_9NO_2S$
Structural formula			
Solubility [g/l]	6.7	7.5	3.16
(theoretical oxygen demand) ThOD [mg O_2/mg]	1.9421	2.044	2.0558

Biodegradability measurements

The biodegradability was investigated by using Closed Bottle Test (OECD 301D). The test is recommended as a simple method that can be used to assess biodegradability of organic compounds (OECD, 1992).

Nutrient solution prepared as above, with added sulphonamides SA, SCM, p-TSA or TB, and inoculated with supernatant liquor (1 cm^3 dm^{-3}) was used in the test. The concentration of SA, SCM, p-TSA was 4 mg dm^{-3}. Closed Bottle Test OECD 301D was conducted according to the OECD guidelines in darkness and at constant incubation temperature 20±1 ºC . The standard time of incubation (28 days) was prolonged to 49 days (some more vessels were used) to examine a possible adaptation of the bacteria to the tested substances.

During biodegradation test the Dissolved Oxygen concentration (DO) was first measured each day and after one week – every seventh day. The biodegradability was calculated from equation (1) (OECD, 1992):

$$\%_{\deg radation} = \frac{BOD}{ThOD} \cdot 100 \qquad (1)$$

where BOD = biological oxygen demand. Its calculation was based on the change of dissolved oxygen concentration (DO). The values of the Theoretical Oxygen Demand (ThOD) were calculated from the chemical formulae and were corrected by including the nitrification process for the sulphonamides (as these compounds molecules contain nitrogen). The ThOD values after correction are presented in Table 1.

Inhibition of nitrification

In this research, the nitrification process was performed with using synthetic wastewater of high content of ammonium nitrogen (base solution containing 56 mg NH_4 – N/l , obtained by dissolution of the required amounts of ammonium sulphate and sodium bicarbonate).

The nitrification inhibition tests, without and with the presence of three sulphonamides (SA, SCM and p-TSA) were carried out according to the ISO guidelines (ISO 9509:1989).

Activated sludge with good nitrifying properties was taken from the WWTP in Gdansk. Before its use, the supernatant was discarded and the remaining sludge was rinsed with distilled water and further with the base solutions. The base solutions (containing 56 mg N/l) was used in all tests. The amount of activated sludge was adjusted so that MLSS (mixed-liquor suspended solids) was 3 g/l. Each sulphonamide was added to test flasks in a concentration series (five levels) ranging from 1 to 100 mg/l. The reference test was performed using allyl thiourea (ATU), a well-known nitrification process inhibitor. The control sample did not contain either tested sulphonamides or inhibitor (ATU). The tests were performed at constant temperature (usually in the range of 20 – 25^{0}C) and under dust-free and toxic vapours-free conditions. The flask content was aerated for 4 hours. The dissolved oxygen concentration was not lower than 2 mg/l. The tests were carried out in three replicates for each substance and each concentration.

According to procedure the inhibition effect of sulphonamides can be calculated from two different formulae (ISO 9509:1989). Equation (2) expresses the inhibition effect in relation to ammonium nitrogen removal:

$$\%_{inhibition} = \frac{C_i - C_e}{C_o - C_e} \text{ x } 100 \qquad (2)$$

where C_i = N_{NH4} concentration in test sample after incubation [mg/l]; C_e = N_{NH4} concentration in control sample after incubation [mg/l]; C_o = N_{NH4} initial concentration [mg/l].

Equation (3) expresses the inhibition effect in respect to formation of oxidized forms of nitrogen ($N_{OX} = N_{NO3} + N_{NO2}$):

$$\%_{inhibition} = \frac{C_c - C_t}{C_c - C_b} \text{ x } 100 \qquad (3)$$

where C_c = N_{OX} concentration in control sample after incubation [mg/l]; C_t = N_{OX} concentration in test sample after incubation [mg/l]; C_b = N_{OX} concentration in sample with ATU after incubation [mg/l].

RESULTS AND DISCUSSION

Biodegradability of sulphonamides

In Fig.1, the rate of biodegradation of the tested sulphonamides is presented. Each point is obtained from three independent experiments and represents the mean value. Ready biodegradability is judged based on the results after 28 days. The results have confirmed the observations of very low biodegradability of sulphonamides, mentioned by other researchers (Halling-Sørensen et al., 1998; Al.-Ahmed et al., 1999).

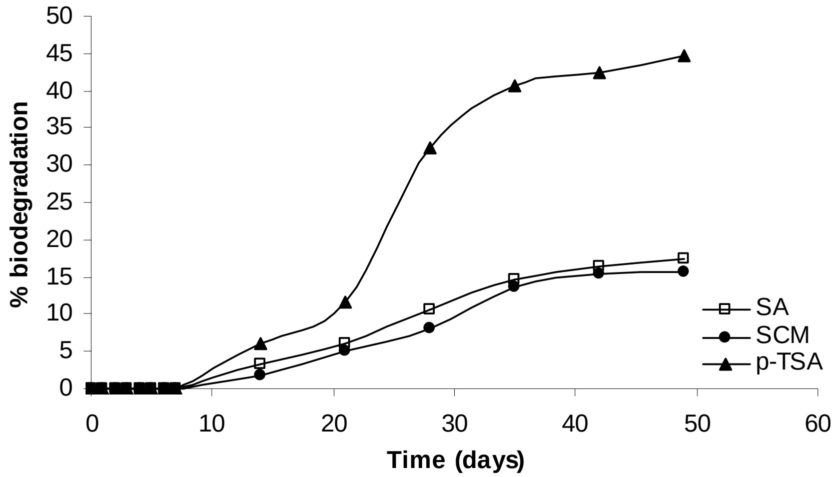


Figure 1. Biodegradation of sulphonamides

It can be seen that, even after 28 days, biodegradation of SCM and SA is very low (8% and 10.6%) and even for p-TSA, it is only 32%. According to OECD directives, a substance is susceptible to high biodegradability when its biodegradation level achieves 60% within 10 days, during the test time (28 days). However, if a biodegradation test is continued up to 49 days the results are slightly better (15.7%, 17.4% and 44.6%, respectively). This may suggest that adaptation of microorganisms may have happened, especially in the case of p-TSA. Biodegradation of p-TSA by *Pseudomonas sp.* was investigated by Van Haperen et al (2001). These bacteria have been isolated from activated sludge after its acclimation and they reveal excellent ability to use the p-TSA as a source of carbon, sulphur and nitrogen, what resulted in complete decay of this compound within few days. These results prove that adaptation of selected strains of bacteria to sulphonamides is possible. However, biodegradability of these compounds, measured by OECD test is not satisfied.

Inhibition of nitrification

In Figs 2-6 the results of inhibition effects of sulphonamides on nitrification process are presented. Each point represents the mean value from three independent experiments using the same activated sludge. In each series of experiments a new sludge was used (sludge indicated as A, B, C, D, E, F, G).

The results presented in Figs 2-4 relate to inhibition of ammonium nitrogen removal and were calculated according to eq. (2). They show the differences in the inhibition effect when use different sludge.

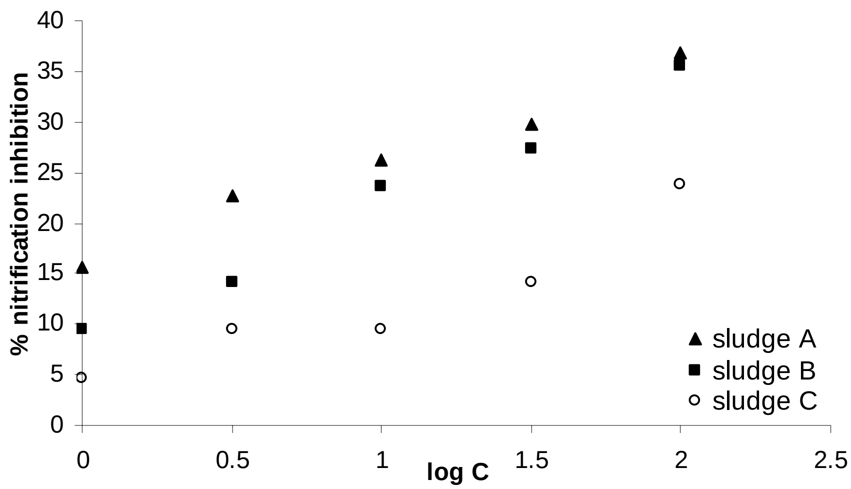


Figure 2. Inhibition of nitrification by sulphacetamide (SCM) calculated from eq. (2). Different sludge was used.

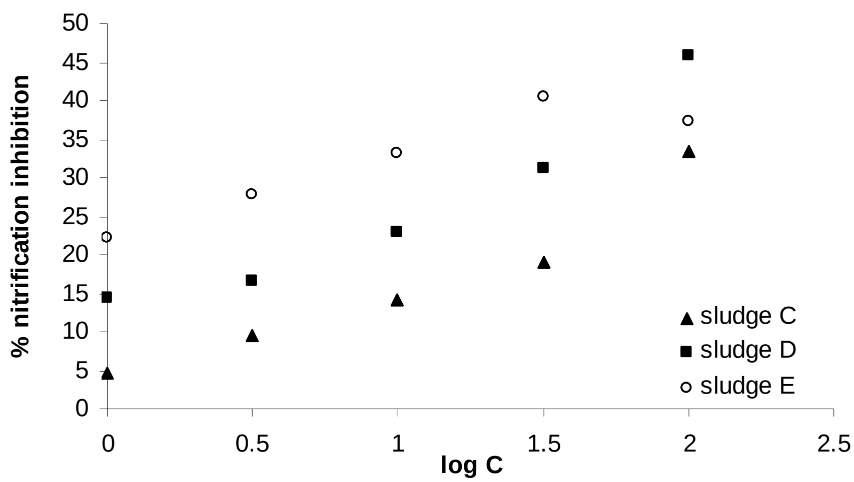


Figure 3. Inhibition of nitrification by sulphanilamide (SA) calculated from eq. (2). Different sludge was used.

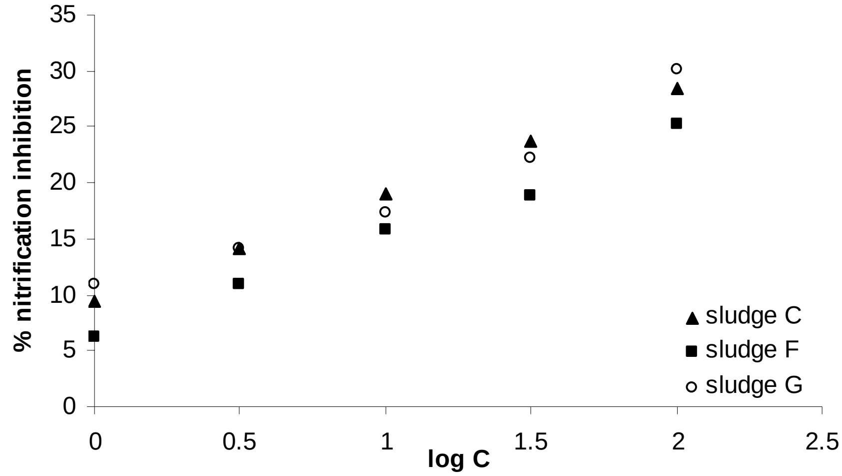


Figure 4. Inhibition of nitrification by p-toluenesulphonamide (p-TSA) calculated from eq. (2). Different sludge was used.

As can be seen from these results, the inhibition effect increases with the increase of sulphonamides concentration, and at 100 mg/l concentration it reaches 24-37% for SCM (Fig.2), 33-46 % for SA (Fig. 3) and 25-30% for p-TSA (Fig.4). Simultaneously, the reference inhibitor, ATU, reveals much higher toxicity, 62-89% of inhibition of NH_4 – N removal, depending on sludge (data not presented here and used only for calculations). In this paper there are not presented results for particular sulphonamides inhibition with respect to different sludge, calculated according to eq. (3). However, it has been stated that the inhibition of nitrogen oxidized forms formation is similar to inhibition of NH_4 – N removal. For example, at sulphonamides concentration 100 mg/l, the inhibition calculated from eq. (3) for SCM reaches 27-34%, for p-TSA 22 – 29% and only for SA it is lower (23-32%) than that calculated from eq.2 (33-46%).

One can expect that inhibition of nitrification expressed as % inhibition of NH_4 – N removal or as % inhibition of N_{OX} formation should be of a similar magnitude, thus the discrepancy observed in case of SA is difficult to explain. However, it should be noted that ammonia nitrogen removal comprises different processes - mainly nitrification and biomass synthesis. On the other hand, the reverse processes, e.g. microorganism decay and further biochemical ammonification can lead to an increase in the ammonia nitrogen concentration. Thus, the analysis of changes of all forms of nitrogen (including organic material) is necessary in order to understand the unbalanced inhibition results, as it was observed in case of SA.

The observed large variations in the results for particular sulphonamide (especially Figs. 2 and 3) result from the use of different samples of activated sludge, each of which can have a different nitrifying activity. In some cases the differences have reached even 25-30%. In such situation it was impossible to compare the inhibition effect, revealed by different sulphonamide. For this reason the series using the same activated sludge for all tested sulphonamides was performed. The results for one series (sludge C) are presented in Figs 5 and 6. In Fig 6 the results related to inhibition of formation of NO_3^- and NO_2^- during nitrification (calculated according to eq. (3)). are presented. The analysed concentration of NO_3^- was ten or more times higher than that of NO_2^-.

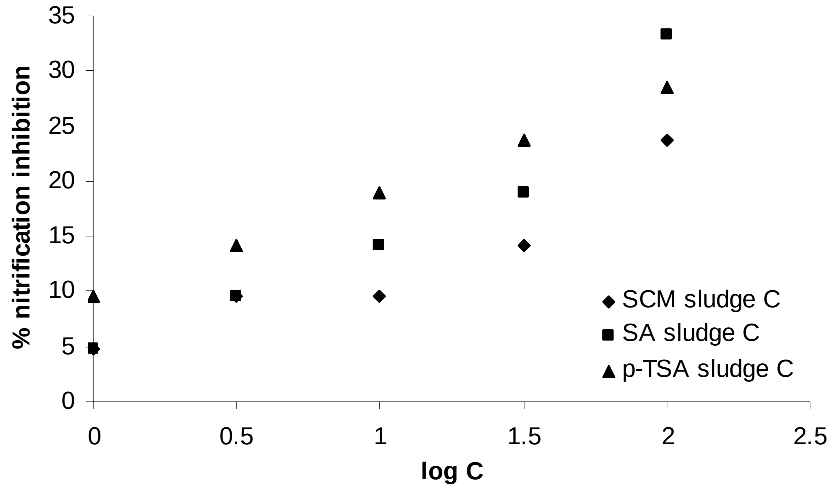


Figure 5. Inhibition of nitrification by different sulphonamides, calculated from eq. (2).

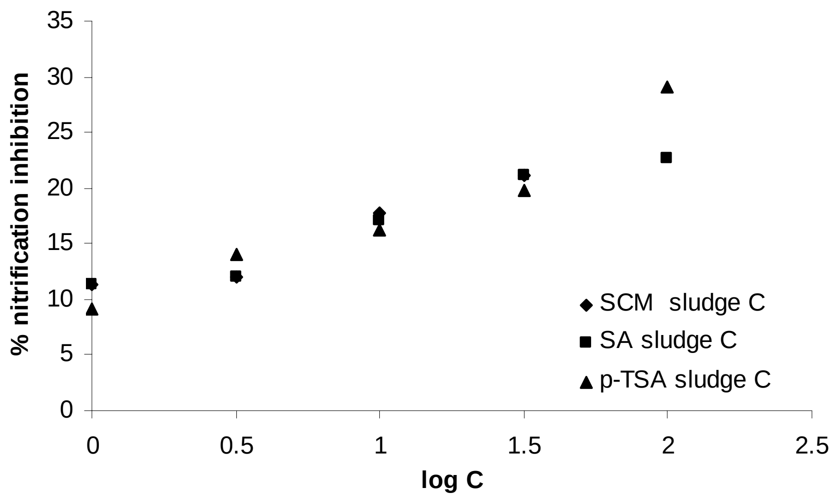


Figure 6. Inhibition of nitrification by different sulphonamides, calculated from eq. (3).

Analysing the results presented in Fig 5 one can clearly see that great variations are observed in case when inhibition was calculated from equation 2. Similar variations were observed in the series with other sludge (sludge H, results not presented here). On the other side, there are no substantial differences in formation of oxidized forms of nitrogen at the presence of all tested sulphonamides (inhibition calculated from eq.3, Fig.6). This discrepancy may result from the fact that N_{OX} formation is the effect of only ammonium nitrogen nitrification, while NH_4-N removal comprises the transformation of ammonium nitrogen by the oxidation process as well as its bioaccumulation in the biomass and the last one depends strongly on the activated sludge properties. It might be possible that biosynthesis of a new biomass is more sensitive to the presence and kind of sulphonamide. Thus, the ammonium nitrogen content may vary so much when different sulphonamides are added.

In order to assess the environmental risk as well as, the risk to wastewater bacteria, the EC_{50} value for each substance introduced to aquatic environment should be determined. The inhibition test procedure followed in this work employed a concentration range of tested substances from 1 mg/l to 100 mg/l (ISO 9509:1989). In the case of the sulphonamides tested the highest observed inhibition did not exceed 43%. However, it is visible in most cases that the trend in the inhibition effect is increasing when sulphonamides concentration approaches 100mg/l. Thus for determination of the EC_{50} value, the research have to be extended to region of higher concentration range.

CONCLUSIONS

On the basis of the presented results it can be stated that all tested sulphonamides are resistant to biodegradation. Only after ten days of incubation did any biodegradation become noticeable. The highest biodegradation was observed for p-TSA (45% after 49 days). However, none of the tested compounds achieved the level required by OECD (60% biodegradation within 10 days during a test of 28 days duration) (OECD, 1992). p-TSA, being a metabolite of Chloramine T (disinfectant against microorganisms) is probably less toxic than the parent compound. Thus, its biodegradability is better than that of the tested sulphonamides (SA, SCM). It would be interesting to find the EC_{50} value for all tested compounds, otherwise it will be difficult to compare their toxicities. However, it should be mentioned that the probable EC_{50} values will be far above the real concentrations of tested sulphonamides in aquatic environment and even in municipal wastewater. The only possible cases, where high sulphonamides content may be found are accidental leakages or high strength industrial wastewater, for example from pharmaceutical sector.

Sulphonamides used in the tests reveal high inhibition effects in relation to nitrification processes. Even at their lowest concentration (1 mg/l) the inhibition was in the range 4-27%, while at the highest concentration used (100 mg/l) it was about 20-40%, depending mainly on the activated sludge quality and also slightly on the compound under testing.

An interesting observation is that p-TSA is the substance whose biodegradability is highest among the tested sulphonamides and simultaneously its inhibition effect is slightly lower. Summarizing, one can expect that its environmental effect may be less negative than that of SA and SCM.

It may be suggested, that the effect of sulphonamides may vary, depending on type of bacteria, and especially the differences can be observed among the heterotrophic and nitrifying bacteria, as their sensitivity to toxic substances are different. So, it is possible that this difference may be reflected in the respiratory activity of these bacteria. Thus, further research should include the OUR (Oxygen Uptake Rate) tests in order to evaluate the negative effect of different sulphonamides to different types of bacteria.

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