THE EFFECT OF SULPHONAMIDES ON ACTIVATED SLUDGE DEHYDROGENASE ACTIVITY

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Abstract The effect of sulphonamides on activity of activated sludge dehydrogenase was studied. Three sulphonamides, such as sulphacetamide (SCM), sulphanilamide (SA), p-toluene-sulphonamide (p-TSA), were selected for investigations and their concentrations ranged from 10 to 100 mg/l. Dehydrogenase activity was evaluated with the TTC test. Twelve samples of activated sludge collected from the Gdansk wastewater treatment plant were used. Dehydrogenase activity, its inhibition and degree of toxicity at the presence of sulphonamides have been studied and evaluated. It has been found that the dehydrogenase activity decreases with the increase of a sulphonamides concentration, and the effect was the highest for p-TSA. On the other side, the maximal toxicity value was the highest in case of SA and the lowest for p-TSA.
Keywords Activated sludge; dehydrogenase activity; sulphonamides

INTRODUCTION

Wastewater may contain many different chemical substances, which can inhibit microorganisms' growth and cause various troubles during biological treatment. These harmful substances, depending on their physical and chemical properties, can be not readily biodegradable and remain toxic during the entire wastewater treatment process (Schalk *et al.*, 1998; Halling-Sørensen *et al.,* 2000). Examples of such compounds are different pharmaceuticals such as sulphonamides, which are used in human therapy and animal husbandry. Sulphonamides are entering into the environment as effluents from pharmaceutical industry, municipal wastewater treatment plant as well as fish- and stock-farming. Different sulphonamides such as sulphamethazine, sulphamethoxazole and sulphadiazine concentrations were found in wastewater treatment plant effluents and surface water; they ranged from 10 to 2000 ng/dm^3 (Hartig *et al.,* 1999; Hirsch *et al.,* 1999). These compounds are not readily biodegraded and they reveal weak adsorption both, to soil and activated sludge (Ingerslev and Halling–Sørensen, 2000; Huang *et al*., 2001).

Due to sulphonamides properties, their migration in the environment may result in contamination of drinking water and food products. The inhibition effect of the sulphonamides on microorganisms growth in activated sludge or biofilm has been reported by Jjemba (2002). Besides, in the literature there are a few reports on sulphonamides' toxicity to nitrifying bacteria (Ingerslev and Halling-Sørensen, 2000; Al- Ahmad *et al*., 1999). Therefore, these compounds can induce serious trouble in self-purification of water as well as in biological wastewater treatment. Their removal during wastewater treatment, even in modern treatment plants is not sufficient and purified streams still contain these compounds.

Investigation of microbial activity with the use of artificial acceptors

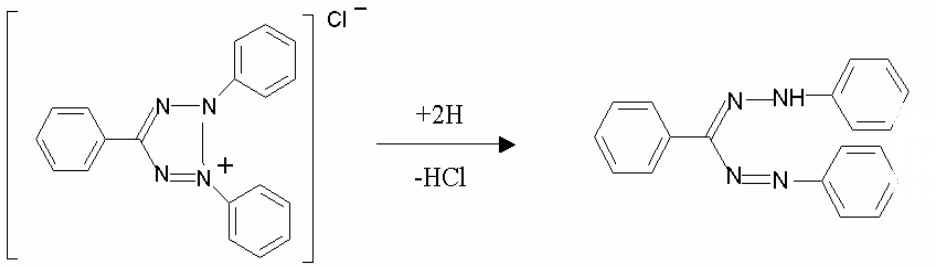
Degradation reactions of pollutants, which could be find in activated sludge, take place in the presence of the microorganisms enzymes. Therefore the substrate oxidation efficiency depends on enzymes activity of the respiration chain. One can state it indirectly by the use of the OUR test

(oxygen uptake rate). The direct methods of measuring of enzymatic activity rely on evaluation of catalase activity, which is an intracellular enzyme, participating in decomposition of toxic hydrogen dioxide, appearing in the final stage of cellular respiration of many types of microorganisms (Pawlaczyk-Szpilowa, 1980, Glikeboom, 1999).

The activity of activated sludge microorganisms can be evaluated also by investigation of dehydrogenase activity. Dehydgrogenases are enzymes catalysing oxidation of organic material. They catalyse reaction of dehydration of substrate and transfer of protons and electrons to further stages of respiratory chain. Dehydrogenase activity analysis can be useful for control of wastewater treatment and for evaluation of activated sludge adaptation to industrial effluents.

There are numerous methods of dehydrogenase activity investigations. One of them is INT method, developed by Benefield et al. (Benfield et al., 1977) and Trevors et al. (Trevors et al., 1982). They have used INT (2-p-iodophenyl-3-p-nitrophenyl-5-phenylotetrazolium chloride) as hydrogen acceptor when determine dehydrogenase activity. Mosher et al. (Mosher et al., 2003) improved this method. They have used acetonitrile for extraction of INTF (reduced form of INT) from the sludge, what minimize negative effect caused by of PAH (poly-aromatic hydrocarbons) or heavy metals, present in the sludge.

However, the widest application have found method with the use of 2,3,5-trifenyltetrazolium chloride (TTC). The reaction of organic material oxidation catalysed by dehydrogenases may be detected in reaction of TTC reduction to the reduced form - TF ($TTCH_2$ - trifenylformazane), called TTC test (equation 1). Comparison of INT and TTC tests shows that INT test is more sensitive (Gong, 1997), but still TTC is used preferably. TTC Test was introduced by Lenhard (Lenhard, 1956), and the procedure was developed by Casida et al. (Casida et al., 1964) and Thalmann (Thalmann, 1968). Light and oxygen disturb determination.



(1)

TTC **TF**

The reaction of substrate dehydration catalysed by dehydrogenase takes place during incubation of deoxygenated samples The intensity of sample colour (due to TF formation) is determined spectrophotometrically and the result is expressed as the ratio of TF to SS content (suspended solids). From investigations of Upadhyaya and Eckenfelder (Upadhyaya and Eckenfelder, 1975) it results that TF concentration rises with the increase of the load of activated sludge and reaches 20–70 μmol TF/g dms and depends on type of organic matter. Zhao and Li (Zhao i Li, 1999) have applied TTC test for determination of inhibition of activated sludge microorganisms by leakages from landfills. The leakage contained high ammonium nitrogen concentration (up to 5 gN/l). It has been found that dehydrogenase activity decreases substantially (from 11 do 4,2 μg TF/ MLSS, mixed liquor suspended solids) with the increase of ammonium nitrogen content (from 50 to 800

mgN/l). This lead to conclusion that the leakage should be purified from nitrogen prior to classical biological treatment.
Le Bihan and Lessard (Le Bihan and Lessard, 1998) applied TTC test for determination of dehydrogenase activity during watewater treatment on biofilters. They have stated that dehydrogenase activity depends on biomas content as well as, on substrate concentration and filtration rate.

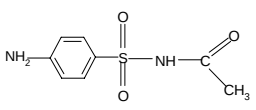
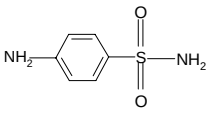
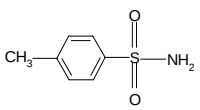
The aim of this research was evaluation the effect of selected sulphonamides on activated sludge dehydrogenase activity. The scope of the investigations covers experiments without and with sulphonamides. The degree of sulphonamides toxicity was also calculated.

MATERIALS AND METHODS

In this study the TTC test was applied. During organic material oxidation, catalysed by dehydrogenase, TTC is reduced to TF (Eq.1). The TF concentration (determined spectrophotometrically at 490 nm) is a measure of dehydrogenase activity. To perform TTC tests the appropriate chemicals and acivated sludge from biological treatment plant were used. The chemicals were prepared according to Polish norm PN-82/C-04616.08.

The characteristic of sulphonamides is presented in Table 1. The experiments were carried out at sulphonamides concentrations: 0, 10, 20, 30, 40, 60, 80, 100 mg/l.

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
Structural formula			

Selection of test parameters

At first, the tests without sulphonamides were performed in order to define the amount of TTC and the amount of sludge necessary to use in the tests. The reagents and activated sludge of volumes given in Table 2 were poured into the tubes. Duplicate analysis were performed for each concentration.

Table 2. Volumes of reagents used in TTC tests [ml]

Reagent	**Tube no.**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Solution of Na_2SO_3	1	1	1	1	1	1	1	1
Solution of TTC [ml] **[µmol]**	0 0	0.1 0.072	0.2 0.144	0.3 0.216	0.4 0.288	0.6 0.36	0.8 0.504	1 0.72
H_2O	1	0.9	0.8	0.7	0.6	0.4	0.2	-
Activated sludge	8	8	8	8	8	8	8	8

After filling, the tubes were shaken and then incubated for 30 min in dark at 37ºC to keep the optimal conditions for microorganisms development [PN-82/C-04616.08]. After incubation, samples were centrifuged at 5000 rpm and 23ºC and supernatant was discarded. To each tube 8 ml of methanol was introduced and then after shaking tubes were centrifuged for 6 min. Supernatant was taken for absorbance analysis at 490 nm and from a calibration curve the TF concentration was found. The results were expressed in relation to VSS mass.

Activated sludge characteristics

In the studies, the activated sludge from the Gdansk „Wschod" Wastewater Treatment Plant was used. First, the sludge was washed three times with a buffered water, in order to keep a proper pH. Then it was diluted with a distilled water and homogenized to release dehydroganese from bacteria cells. Twelve different samples of activated sludge were used in TTC tests and their characteristics is presented in Table 3.

Table 3. Characteristics of activated sludge used in TTC tests

Activated sludge sample	COD [mg/l]	Suspended solids (SS) [g/l]
1	91	2.56
2	93	2.31
3	111	2.09
4	120	2.40
5	97	2.29
6	116	2.44
7	103	2.22
8	130	2.60
9	104	2.35
10	124	2.15
11	132	2.43
12	116	2.36
Mean value	**111.4**	**2.4**
Standard deviation	13.9	0.2

Selection of activated sludge concentration

The dehydrogenase activity may depend on the amount of microbial cells disintegrated for enzyme release. Thus it was necessary to establish an optimal concentration of activated sludge for tests performance. In the experiments the sludge was used both without dilution and diluted with water in ratios of 1:1 and 1:3. The results obtained for diluted sludge are presented. in Figure 1.

The results suggest that determined activity of dehydrogenase (TF concentration) was higher at lower sludge dilution, as bacteria cells deliver more enzyme. This was advantageous because of the higher sensitivity and better precision of the spectrophotometric method (regression value is higher). Thus, for further research of sulphonamides effect on dehydrogenase activity the sludge was diluted with water in 1:1 ratio.

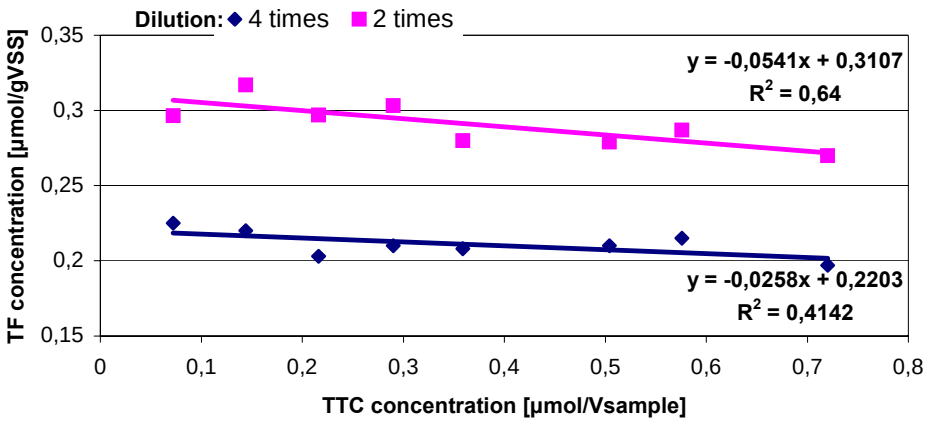


Fig.1. TF concentration versus TTC amount in the sample. Sludge sample - 1

Selection of TTC concentration

It was very important to select a proper amount of reagents, especially TTC. The concentration of TF formed during a test reaction shouldn't depend on the amount of TTC added. So, first the tests were performed without sulphonamides and with different amount of TTC (from 0.072 to 0.72 µmol of TTC). It was stated that from 0.144 µmol of TTC up to 0.72 µmol of TTC the amount of TF was similar (about 1.2-1.3 µmol/gVSS) and only the concentration 0.072 was too low. Finally, it was decided that in the tests with sulphonamides the highest concentration of TTC (0.72 µmol) will be used.

TTC tests with sulphonamides

Tests with sulphonamides were performed at seven different concentrations of these compounds (Table 4). Sulphonamides concentration in initial solution was 1g/l. The procedure was the same as in tests without sulphonamides. Duplicates were performed for each sulphonamide concentration.

Table 4. Volumes of reagents used in TTC tests at the presence of sulphonamides [ml]

Reagent	**Tube no.**							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Solution of Na_2SO_3	1	1	1	1	1	1	1	1
Solution of TTC [ml]	1	1	1	1	1	1	1	1
Solution of sulfonamide	-	0.1	0.2	0.3	0.4	0.6	0.8	1
H_2O	1	0.9	0.8	0.7	0.6	0.4	0.2	0
Activated sludge	7	7	7	7	7	7	7	7

Calculations

After performed experiments and estimation of the TF value, calculations were done according to the equations presented in Table 5. Dehydrogenase activity (AD) was calculated for the following concentrations of the sulphonamides: 10, 20, 30, 40, 60, 80, 100 (AD_{10}, AD_{20} …).

Table 5. Calculations of dehydrogenase activity, its inhibition and degree of toxicity

No	Name	Equation	Parameters
1	Dehydrogenase activity (AD)	$AD = \frac{TF_2 - TF_1}{T_2 - T_1}$	TF_2 – TF concentration after 30 min. of incubation [mgTF/l], TF_1 – TF concentration after 5 min. of incubation [mgTF/l], T_1- time equal to 5 min [h], T_2 - time equal to 30 min [h].
2	Inhibition of dehydrogenase activity by sulphonamides (in %)	$\%_{inhibition} =$ $[(TF_0 - TF_S) / TF_0] \times 100\%$	TF_0 - TF concentration for sludge without sulphonamides, TF_S - TF concentration for sludge with sulphonamides
3	Degree of toxicity (ST)	$ST = (AD_0 - AD_T) / AD$	AD_0 - dehydrogenase activity without sulphonamides; AD_T - dehydrogenase activity at different concentrations of sulphonamides; for example AD_{10}, AD_{20}

For estimation of the maximum value for degree of toxicity, $ST_{max.}$, the diagram for the relationship beetween 1/ST and 1/C has been done for each supphonamide. As can be observed from Figure 2 linear reggresion was found and a value for the $1/ST_{max}$ could be read from the diagram.

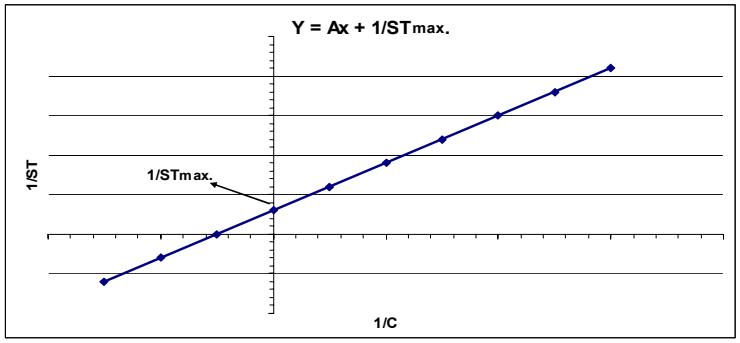


Fig. 2. Relationship between 1/ST and 1/C scheme

RESULTS

Dehydrogenase activity at the presence of sulphonamides

The effect of sulphonamides on activated sludge dehydrogenase activity has been studied for SA, SCM, p-TSA and results are presented in Figure 3. Inhibition caused by sulphonamides, calculated according to the equation 2 from Table 4, is presented in Figure 4. It shows the average values obtained for 3 or 4 different samples of activated sludge used in the tests with each sulphonamide.

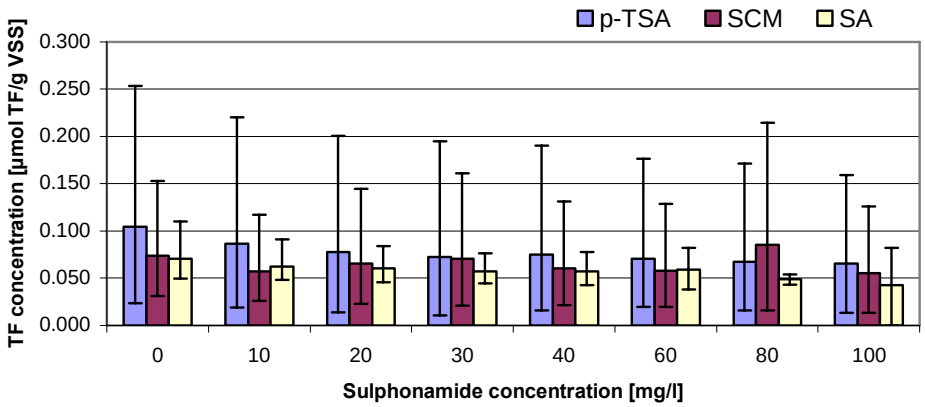


Fig. 3. The effect of sulphonamides on activated sludge dehydrogenase activity

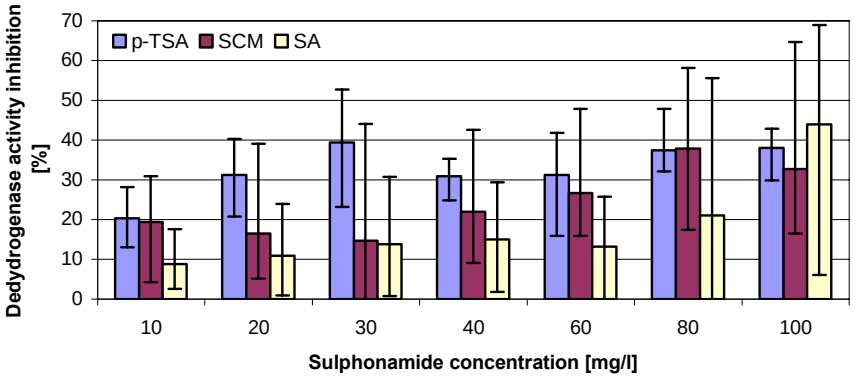


Fig. 4. Inhibition of activated sludge dehydrogenase activity by sulphonamides

The drop in the activated sludge dehydrogenase activity with the increase of SA concentration was observed. For a concentration of 100 mg SA/l activated sludge average dehydrogenase activity was reduced with the value of $2{,}77 \cdot 10^{-2}$ µmol TF/g VSS (activity decrease by 39%), if compared to the control sample. A substantial decrease of dehydrogenase activity was also observed with the increase of p-TSA concentration. The average decrease in dehydrogenase activity of $3{,}87 \cdot 10^{-2}$ µmol TF/g VSS (37%) for the sample of 100 mg p-TSA/l was obtained. This tendency was not found for SCM. In the presence of this sulphonamide big fluctuations were observed.

Inhibition of dehydrogenase activity by the sulphonamides varied between 5 and 40 % and increased with the concentration (Figure 4). The highest values was observed for p-TSA within almost the whole spectrum of tested concentrations.

Degree of toxicity of sulphonamides

Measurements of dehydrogenase activity were used for the calculation of degree of toxicity (ST) according to the equation 3 (Table 4). The realtionships between 1/ST and 1/C for SA, SCM and p-TSA are presented in figures 5-7.

The calculated maximal values of toxicity are presented in Table 6.

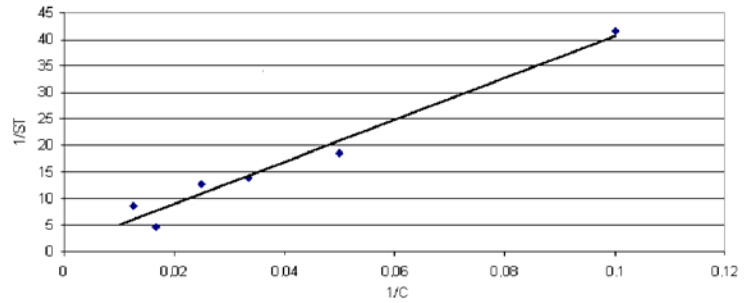


Fig. 5. Relationship between 1/ST and 1/C for activated sludge dehydrogenase activity (SA, activated sludge No 4)
$y = 395\ x + 1.0451;\ R^2 = 0.96;\ 1/ST_{max.} = 1.0451;\ ST_{max.} = 0.9568$

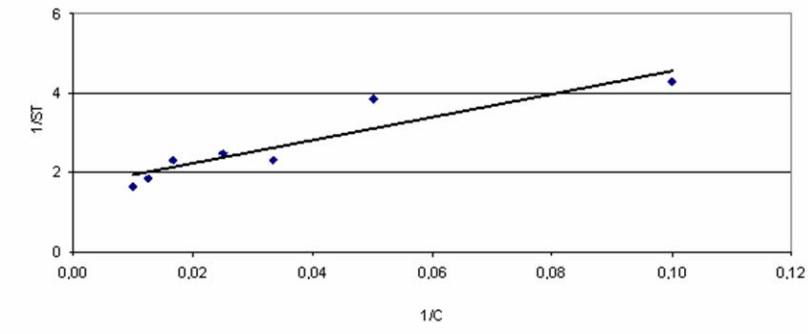


Fig. 6. Relationship between 1/ST and 1/C for activated sludge dehydrogenase activity (SCM, activated sludge No 6)
$y = 29.104\ x + 1.6509;\ R^2 = 0.85;\ \ 1/ST_{max.} = 1.6509;\ ST_{max.} = 0.6057$

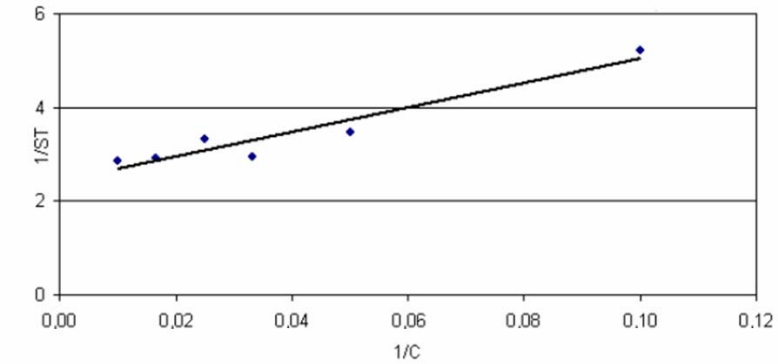


Fig. 7. Relationship between 1/ST and 1/C for activated sludge dehydrogenase activity (pTSA, activated sludge No 12)
$y = 26.219\ x + 2.4339;\ R^2 = 0.94;\ \ 1/ST_{max.} = 2.4339;\ \ ST_{max.} = 0.4109$

Table 6. Sulphonamides toxicity vs. activated sludge dehydrogenase activity

Acitivated sludge	Sulphonamide	$1/ST_{max.}$	$ST_{max.}$	Average $ST_{max.}$
3	***SA***	1.150	0.870	0.805
4		1.045	0.957	
5		1.705	0.588	
6	***SCM***	1.651	0.606	0.672
7		1.036	0.965	
8		2.239	0.446	
9	***pTSA***	2.434	0.411	0.398
10		2.215	0.451	
11		4.578	0.218	
12		1.954	0.512	

CONCLUSIONS

Comparison of the TF concentration in the control sample and in the sample with sulphonamides showed that dehydrogenase activity is reduced with the increase of p-TSA and SA concentrations.
The maximum values calculated for degree of toxicity $ST_{max.}$ proved that sulphanilamide is the most toxic compound. SCM showed lower values of $ST_{max.}$, but toxicity of p-TSA is almost 50% lower than of SA (Table 4).
Taking into account the incresase of toxicity the following can be concluded:

$SA \leq SCM < p\text{-}TSA$ (inhibition of dehydrogenase activity by sulphonamides, TF)

$p\text{-}TSA << SCM < SA$ (degree of toxicity against activated sludge, ST_{max})

It turned out that ST_{max} seemed to be more useful than dehydrogenase activity or values of % inhibition because they allowed for the evaluation of the results obtained for the sludge with different characteristics.

REFERENCES

Al-Almad A., Daschner F.D., Kümmerer K (1999). Biodegradability of Cefotiam, Ciprofloxatin, Meropenem, Penicilin G and Sulfamethoxazole and inhibition of wastewater bacteria. *Archives of Environmental Contamination and Toxicology*, **37**, 158-163.
Casida L. E., Klein D.A., Santaro T. (1964). Soil dehydrogenase activity, *Soil Science,* **98**, 371-376.
Gilkeboom D. H., Van Buijsen H. J. J. (1999). Podręcznik mikroskopowego badania osadu czynnego, Wydawnictwo Seidel – Przywecki, Szczecin.
Gong P. (1997). Dehydrogenase activity in soil: a comparison between the TTC and INT assay under their optimum conditions, *Soil Biol. Biochem*., **29**, 211-214.
Halling- Sørensen, B. (2000). Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere*, **40**, 731-739.
Hartig C, Storm T., M Jekel (1999). Detection and identification of sulphonamide drugs in municipal wastewater by liquid chromatogaphy coupled with electrospray ionization tandem mass spectrometry, *Journal of Chromatography A,* **854**, 163-173.
Hirsch R., Ternes T., Haberer K., Kratz K.-L. (1999). Occurrence of antibiotics in the aquatic environment, *The Science of The Environment,* 225, 109-118.
Huang C-H., Renew J.E., Pinkston K., Sedlak D.L. (2001). Occurrence and fate of antibiotics compounds in municipal wastewater and animal waste, *Water Environment Federation (WEFTEC), Conferential Proceedings.*
Ingerslev F. , Halling –Sørensen (2000). Biodegradability properties of sulfonamides in activated sludge. *Environmental Toxicology and Chemistry*, 19, 2467-2473.

Jjemba, P.K., (2002). The potential impact of veterinary and human therapeutic agents in manure and biosolids on plant grown on arable land: a review. *Agriculture Ecosystems & Environment*, **93**, 267-278.
Le Bihan Y., Lessard P. (1998). Influence of operational variables on enzymatic tests applied to monitor the microbial biomass activity of a biofilter, Elsevier Science, 37, 199 - 201.
Lenhard G., (1956). Die Dehydrogenaseaktivität des Bodens als Maß für die Microorganismentätigkeit im Boden, *Z. Pflanzanernnähr. Düng. Bodenk.*, **73**, 1-11.
Mosher J. J., Levison B. S., Johnston C. G., (2003). A simplified dehydrogenaz enzyme assay in contaminated sediment using 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride, *Journal of Microbiological Methods*, **53**, 411-415.
Pawlaczyk - Szpilowa M. (1980). Ćwiczenia z mikrobiologii wody i ścieków. PWN Warszawa.
Schalk J., de Vries S., Kuenen J. G., Jetten M.S. M. (2000). Involvement of a Novel Hydroxylamine Oxidoreductase in Anaerobic Ammonium Oxidation Autores: Biochemistry, **39**, 5405-5412.
Trevors J. T., Mayfield C. I., Inniss W. E. (1982). Measurement of electron transport system (ETS) activity in soil, *Microbial Ecology*, 8, 163-168.
Thalmann A.,(1968). Zur Methodik der Bestimmung der Dehydrogenase-aktivität im Boden mittels TTC, Landwirtsch Forsch., 21, 249-257.
Upadhyaya A. K., Eckenfelder W. W. (1975). Biodegradable fraction as an activity parameter of activated sludge, *Water Research,* 9,691.
Zhao Q. L., Li X. Z. (1999). Inhibition of microbial activity of activated sludge by ammonia in leachate, *Elsevier science,* **25**, 961-968.