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CADMIUM AND COPPER TOXICITY ASSESSMENT IN ACTIVATED SLUDGE USING TTC BIOASSAY

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Abstract: The aim of this work was to determine the effect of various cadmium and copper concentrations on the activated sludge dehydrogenase activity. The investigations were carried out in six aerated chambers with activated sludge, volume of 1L each, by the continuous culture method (one control chamber, not contaminated with heavy metals and five with 0.5; 1; 2; 4; 8 mg L^{-1} Cu^{+2} and 0.1; 0.3; 0.9; 2.7; 8.1 mg L^{-1} Cd^{2+}). Cadmium sulfate and copper sulfate as a source of heavy metals were used. The concentrations of these metal ions, causing 50% dehydrogenase activity inhibition were determined. The particular attention was paid to the toxic effect of metal ions, as well as the variations of the microbial respiration activity proceeded during toxins exposition. The investigation showed that even the lowest concentration of the investigated metal ions caused significant changes of the activated sludge dehydrogenases activity. Copper ions showed to be more toxic than cadmium ions.

INTRODUCTION

The studies on the heavy metals properties, in particular their toxicity and the role they play in biochemical processes, raise the researchers' interest towards them, as it is shown in the number of publications [1, 2, 3].

Both copper and cadmium are heavy metals. Due to their wide application, high concentrations of these elements are present in wastewaters, particularly in industrial regions [4, 5]. The literature data suggest that the toxic load of both, cadmium and copper, may reach many wastewater treatment plants [6, 4, 7, 8]. It may exert adverse effect on the processes of biological oxidation in the microbial cells (for instance, the effect on COD removal, Sludge Biotic Index change) [9, 10, 33].

Organic compounds degradation is catalyzed by enzymes, among others by oxidoreductases transferring electrons from the oxidized organic substrates onto electron acceptors [11, 12]. Numerous methods of the toxic effect estimation of metal ions on organisms [13, 14] are described. Standard toxicological tests, using such organisms as: *Vibrio fischeri* [15] or *Daphnia magna* are commonly used [3]. The toxic effect of a compound may be also evaluated by measuring the inhibition of selected enzymes, such as ATP-ases, dehydrogenases and catalases [12]. Dehydrogenases transfer hydrogen and electrons through a chain of intermediate electron carriers to oxygen as a final electron ac-

ceptor. The activity of dehydrogenases reflects the general physiological state of microorganisms. For this reason its determination enables fast and relatively simple evaluation of the total activity of microorganisms. The activity of dehydrogenases may be determined using various methods, including the TTC and INT tests [12]. The triphenyltetrazolium chloride (TTC) is water soluble heterocyclic organic salt that can be easily reduced to red, water insoluble product triphenyl formazan TF. The TTC has been used in this work.

There are many studies on the effect of organic pollutants and heavy metals on the enzymatic activity, but the majority of these investigations concern soil microorganisms [1]. Relatively scarce papers are devoted to the cadmium and copper effect on the enzymes of the activated sludge microorganisms [16]. It is well known that the processes performed in the reactor with the activated sludge and in the natural environment are similar and almost all species that are present in the bottom sediments appear also in the treatment plant. For this reason, the activated sludge investigations give the information useful for technologists to predict the environmental results of the presence of the hazardous toxic compounds and in selecting the appropriate preventive actions.

The aim of this work was the estimation of the copper and cadmium ions effect on the microorganisms of the activated sludge. The toxic effect was determined measuring the activity of dehydrogenases of the microorganisms.

MATERIALS AND METHODS

Cultivation of the activated sludge

The activated sludge used in the experiments was obtained from the "Śródmieście" mechanical-biological wastewater treatment plant in Zabrze (Poland) operating in the "Bardenpho" system. Twenty liters of activated sludge samples from the radial secondary settling tanks (pumped to a mechanical concentrating unit), were taken. Before placing in the experimental chamber (volume: V = 20 L) the sediments were filtered through a sieve to remove large particles. The cultivation of the activated sludge was continuous and basic parameters were determined directly before the measurements of the dehydrogenases activity. The hydraulic retention time of culture medium in the reactor was 6.5 h; load of culture medium: 571.2 mg COD $L^{-1}day^{-1}$. Other parameters were as follows: culture medium chemical oxygen demand: COD = 156 mg L^{-1} O_2; culture medium flow: Q = 36.7 $Lday^{-1}$, activated sludge density in all samples: X = 3.5 gL^{-1}.

Materials

Cadmium sulfate ($CdSO_4 \times 8\ H_2O$) and copper sulfate ($CuSO_4 \times 5\ H_2O$) were obtained from Sigma-Aldrich, Poland (≥ 99%). Both salts were dissolved separately in one liter of ultrapure water (Milli-Q) before introduction into the activated sludge chambers. Dissolution of the salts used was performed at 70ºC, in the case of copper sulfate 392.97 gL^{-1} and in the case of cadmium sulfate 228.25 gL^{-1} was used. Sodium sulfite (bioultra, anhydrous > 98%), methyl alcohol (biotech. brade > 99.9%) were obtained from Sigma-Aldrich, Poland and 2,3,5-triphenyltetrazole chloride (TTC) from Biomedicals Inc.

Enzymatic assays

The activity of dehydrogenases was measured using the reaction with 2,3,5-triphenyltetrazole chloride (TTC) as the final artificial hydrogen acceptor in the respiratory

chain [16, 17]. Dehydrogenases activity corresponds with the concentration of red - triphenyl formazan (TF), a reduced form of TTC, produced in the reaction. One mL of TTC in its optimal concentration (8 gL^{-1}) and 1 mL 0.2% Na_2SO_3 were added to 8 mL activated sludge, gently mixed and incubated at 25ºC for 30 min. The incubated samples were centrifuged (5000 rpm, 5 min) in order to separate the microorganisms from the liquid media. The supernatant was discarded. Afterwards, the formed TF was extracted by addition of 10 mL methyl alcohol per sample. The samples were intensively shaken for 20 min and centrifuged again. The absorbance of the supernatant was measured at 485 nm with the UV Spectrometer, Hewlett-Packard type 8452A/diode array. The experiments were run with three replications.

Optimal TTC concentration

The optimal TTC concentration was measured directly before each experiment. It was the concentration at which the activity of dehydrogenases (measured as TF concentration) was the highest after 30 min of incubation at 25ºC. Eight test tubes were prepared with the following concentrations of tetrazolium chloride: 0.2; 0.4; 0.6; 0.8; 1.0; 1.4; 1.6 and 2.0% TTC. After that 1 mL of Na_2SO_3 (0.2%) and 8 mL of activated sludge (X = 3.5 gL^{-1}) were added to each tube, gently mixed and incubated at 25ºC for 30 min. Than the test tubes were centrifuged (at 5000 rpm, 5 min) and the supernatant was discarded. Afterwards, the formed TF was extracted by the addition of 10 mL methyl alcohol to the test-tube. The samples were intensively shaken for 20 min and centrifuged again. The absorbance of the supernatant was measured at 485 nm. The TTC concentration with the highest absorbance after 30 min of incubation was chosen for future experiments. The experiments were run with three replications.

Toxicological studies - determination of cadmium concentration causing the 50% inhibition of the activity of dehydrogenases of the activated sludge

The symbol EC_{50} denotes the cadmium/copper concentration causing the 50% inhibition of activated sludge dehydrogenases activity. The EC_{50} determination for a given compound was performed at two stages (Fig. 1).

The first was the range-finding stage. Four 1-L, aerated chambers were used. One was a control chamber with non contaminated activated sludge, and the other three contained activated sludge (X = 3.5 gL^{-1}) intoxicated by copper sulfate or cadmium sulfate in a volume which allowed to obtained metal concentration equal to 1, 10 and 100 mg Me^{2+} per one liter of activated sludge. After 5 minute the TF concentration was measured in each sample and compared with the TF concentration in the control. In this way, the range of concentrations in which 50% inhibition of the dehydrogenases activity took place after 5 minutes was determined.

Afterwards, from the determined range of concentrations 5 values were chosen to estimate EC_{50} value. For copper sulfate: 0.5; 1; 2; 4; 8 mg L^{-1} Cu^{2+} (7.87; 15.74; 31.47; 62.95; 125.89 µmolL^{-1} Cu^{2+}) and for cadmium sulfate: 0.1; 0.3; 0.9; 2.7; 8.1 mg L^{-1} Cd^{2+} (0.89; 2.67; 8.01; 24.02; 72.06 µmolL^{-1} Cd^{2+}) were chosen. The activated sludge was placed into 1-L aerated chambers and intoxicated by these salts (Fig. 1). The control was uncontaminated activated sludge. The dehydrogenase activity of the activated sludge was determined after 5, 90, 150, 200 and 290 minutes of heavy metal exposition and the value of EC_{50} for each time was estimated. To compare the toxicity of copper and cadmium molar concentration of metal ions was calculated (Tab. 1 and 2).

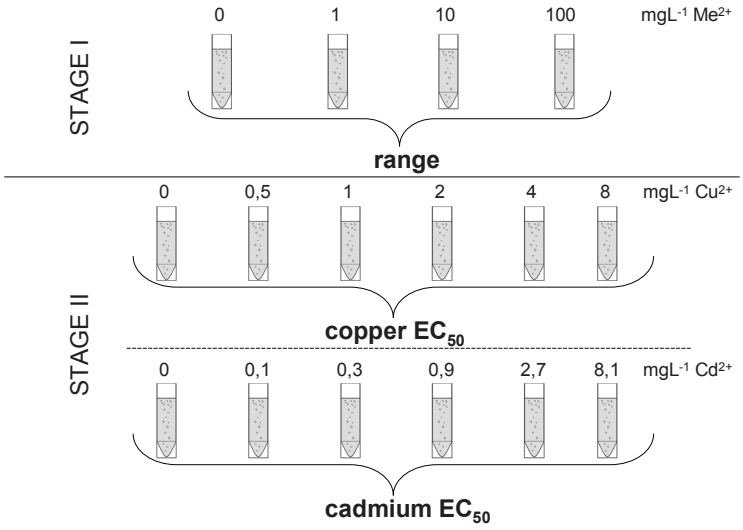

Fig. 1. Determination of cadmium/copper concentration causing the 50% inhibition of the activity of dehydrogenases of the activated sludge microorganisms

Statistic analysis methods

The statistic analysis of the significance differences between the control and contaminated samples was performed with Student's test with 95% confidence interval ($\alpha = 0.05$). EC_{50} was estimated by log-probit method [18]. The probits were given as a function of the log10 of the molar concentration. The best fit linear regression was performed on the probit and log10 (concentration): $y = ax + b$, where: y – is the probit; x – is the log10 of concentration [19]. The parameters: "a" (slope) and "b" (intercept) were calculated using the method of least squares [20].

RESULTS AND DISCUSSION

The value of the optimum TTC concentration varies and depends on the actual composition, morphology, and physiological state of the studied biocenosis. The determination of this concentration compromises the contradicting requirements: such TTC concentration that enables its penetration into the intracellular structures showing the dehydrogenase activity and the TTC concentration below its toxic activity [17]. Therefore, it is recommended to find the proper concentration for a particular biocenosis.

The results of studies are presented in Tables 1 and 2 and in Figures 2 and 3.

The average TF concentrations and their variances, the absolute value of the td coefficient in the t-Student test, the inhibition expressed in percents and probits, as well as the parameters of the lines used to determine EC_{50} values were calculated for the particular metal molar concentration and time of the toxin contact with the activated sludge. Too low inhibition of dehydrogenases activity after the 5 minute contact of cadmium sulfate with the activated sludge (Table 1) made the evaluation of EC_{50} impossible.

The literature data show that cadmium and copper ions present toxic influence on organisms. They adversely affect many processes, including nitrification [21, 22] and

Table 1. Cadmium sulfate

Time [min]	Cadmium Concentration		TF concentration after 30 incubation time	Variance	|td|	% Inhibition	probits	log C	Linear model parameters			log EC50	EC50
	[mgL^{-1} Cd^{2+}]	mol/l *10^6	[mgL^{-1} TF]						a	b	R^2		[$\mu molL^{-1}$ Cd^{2+}]
Control:			10.373	0.250									
5	0.1	0.89	10.036	0.056	1.05	-	-	-7.051	-	-	-	-	-
	0.3	2.67	10.118	0.153	0.69	-	-	-6.574					
	0.9	8.01	9.873	0.074	1.52	-	-	-6.097					
	2.7	24.02	8.736	0.036	5.30	15.78%	4.006	-5.619					
	8.1	72.06	6.473	0.008	13.29	37.60%	4.695	-5.142					
90	0.1	0.89	9.618	0.040	2.43	7.27%	3.524	-7.051	0.809	8.367	0.955	-4.162	68.9
	0.3	2.67	8.918	0.070	4.46	14.02%	3.920	-6.574					
	0.9	8.01	8.409	0.002	6.77	18.93%	4.122	-6.097					
	2.7	24.02	7.273	0.000	10.73	29.89%	4.476	-5.619					
	8.1	72.06	4.427	0.079	17.94	57.32%	5.176	-5.142					
150	0.1	0.89	7.927	0.040	7.87	23.58%	4.294	-7.051	0.432	6.861	0.921	-4.311	48.9
	0.3	2.67	7.173	0.000	11.08	30.85%	4.504	-6.574					
	0.9	8.01	7.018	0.005	11.50	32.34%	4.532	-6.097					
	2.7	24.02	5.973	0.010	14.95	42.42%	4.798	-5.619					
	8.1	72.06	3.773	0.044	21.09	63.63%	5.176	-5.142					
200	0.1	0.89	8.091	0.003	7.86	22.00%	4.228	-7.051	0.691	8.165	0.838	-4.577	26.5
	0.3	2.67	8.155	0.167	5.95	21.38%	4.194	-6.574					
	0.9	8.01	7.636	0.027	9.01	26.38%	4.357	-6.097					
	2.7	24.02	5.600	0.008	16.27	46.01%	4.900	-5.619					
	8.1	72.06	3.164	0.001	24.91	69.50%	5.524	-5.142					
290	0.1	0.89	8.318	0.070	6.30	19.81%	4.158	-7.051	0.799	8.674	0.783	-4.596	25.4
	0.3	2.67	8.545	0.027	7.12	17.62%	4.085	-6.574					
	0.9	8.01	8.355	0.030	7.68	19.46%	4.122	-6.097					
	2.7	24.02	4.936	0.019	19.38	52.41%	5.050	-5.619					
	8.1	72.06	2.945	0.146	20.77	71.60%	5.583	-5.142					

|td| - the value compared with the critical value for the 0.95 confidence interval in the t-Student test
*lack of significant differences (|td| < t) between the control activity and the activity of a sample is denoted in gray, t- critical value for $\alpha = 0.5$ and n = 4; t = 2.132, n – number of degrees of freedom, n = n1+n2 -2, n1, n2 – number of samples, log C – decimal logarithm of toxin concentration, a, b – parameters of lines: probits = a * log C + b, R^2 – fitting coefficient for the lines, EC_{50} / log EC_{50} – concentration causing a 50% inhibition of the activated sludge dehydrogenases / decimal logarithm of this value

Table 2. Copper sulfate

Time [min]	Copper Concentration		TF concentration after 30 incubation time	Variance	|td|	% Inhibition	probits	log C	Linear model parameters			log EC50	EC50 [µmolL^{-1} Cd^{2+}]
	[mgL^{-1} Cd^{2+}]	mol/l *10^6	[mgL^{-1} TF]						a	b	R^2		
Control:			10.373					0.250					
5	0.5	7.87	8.291	0.005	23.14	27.04%	4.387	-6.104	1.435	11.770	0.986	-4.720	19.1
	1	15.74	6.209	0.007	38.31	45.36%	4.874	-5.803					
	2	31.47	3.864	0.004	57.16	66.00%	5.413	-5.502					
	4	62.95	2.436	0.001	69.91	78.56%	5.806	-5.201					
	8	125.89	1.564	0.001	76.74	86.24%	6.080	-4.900					
90	0.5	7.87	10.627	0.001	5.80	6.48%	3.445	-6.104	2.041	14.174	0.919	-4.495	32.0
	1	15.74	7.682	0.139	14.77	32.40%	4.532	-5.803					
	2	31.47	4.345	0.003	54.05	61.76%	5.306	-5.502					
	4	62.95	2.664	0.007	64.67	76.56%	5.733	-5.201					
	8	125.89	2.091	0.003	71.41	81.60%	5.916	-4.900					
150	0.5	7.87	13.527	0.056	11.65	-19.04%	-	-6.104	1.956	13.666	0.933	-4.431	37.1
	1	15.74	9.355	0.000	15.94	17.68%	4.085	-5.803					
	2	31.47	5.373	0.001	47.19	52.72%	5.075	-5.502					
	4	62.95	3.164	0.016	56.23	72.16%	5.583	-5.201					
	8	125.89	2.164	0.005	69.29	80.96%	5.878	-4.900					
200	0.5	7.87	11.673	0.175	1.14	-	-	-6.104	1.832	13.181	0.924	-4.466	34.2
	1	15.74	8.955	0.001	18.98	21.20%	4.194	-5.803					
	2	31.47	5.027	0.010	45.73	55.76%	5.151	-5.502					
	4	62.95	3.036	0.005	62.71	73.28%	5.613	-5.201					
	8	125.89	2.191	0.014	64.03	80.72%	5.878	-4.900					
290	0.5	7.87	11.255	0.001	0.85	-	-	-6.104	1.582	12.162	0.960	-4.528	29.6
	1	15.74	8.118	0.014	22.65	28.56%	4.447	-5.803					
	2	31.47	4.727	0.003	51.11	58.40%	5.202	-5.502					
	4	62.95	3.318	0.000	63.81	70.80%	5.553	-5.201					
	8	125.89	2.073	0.000	73.50	81.76%	5.916	-4.900					

|td| - the value compared with the critical value for the 0.95 confidence interval in the t-Student test

*lack of significant differences (|td| < t) between the control activity and the activity of a sample is denoted in gray, t- critical value for α = 0.5 and n = 4; t = 2.132, n – number of degrees of freedom. n = n1+n2 -2, n1, n2 – number of samples, log C – decimal logarithm of toxin concentration, a, b – parameters of lines: probits = a * log C + b, R^2 – fitting coefficient for the lines, EC_{50} / log EC_{50} – concentration causing a 50% inhibition of the activated sludge dehydrogenases / decimal logarithm of this value

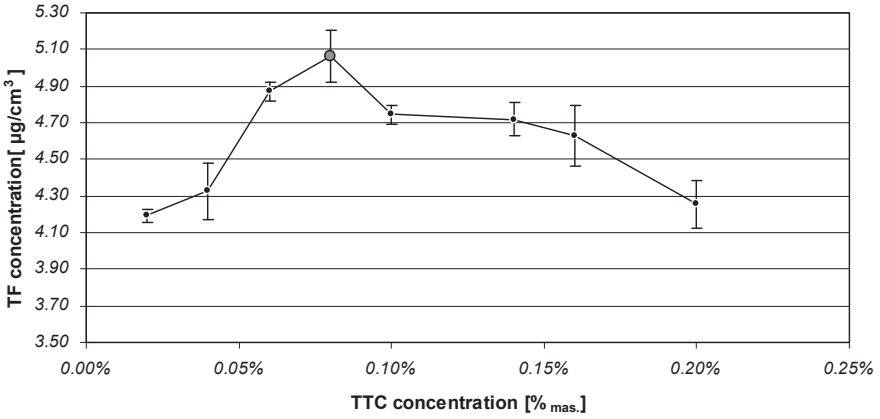

Fig. 2. Dependence of TF concentration on the quantity of added TTC, after 30 min. incubation

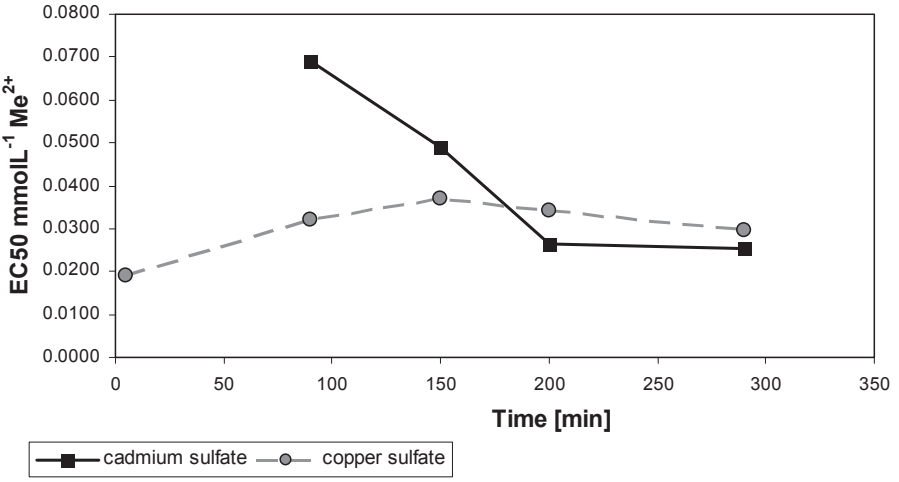

Fig. 3. Variations of EC_{50} values for cadmium sulfate and copper sulfate in time

COD removal from wastewater [10]. At particular concentrations they are lethal to the organisms, significantly changing the composition of the species of various biocenosis, also the activated sludge [5]. Our studies show the significant effect of cadmium and copper ions on the activity of dehydrogenases of the activated sludge organisms, as well as on the total state of these organisms (Tables 1 and 2, Fig. 3). This influence was not observed after 5 minute contact of the activated sludge with cadmium at 0.1; 0.3; 0.9 mg L^{-1} Cd^{2+} (0.89; 2.67; 8.01 µmolL^{-1} Cd^{2+}) and with copper at 0.5 mg L^{-1} Cd^{2+} (7.87 µmolL^{-1} Cu^{2+}) after 200 and 290 minutes incubation with the activated sludge (Tabs 1 and 2). The reactions observed may result from the processes proceeding both outside and inside the cells. The direct cause of the inhibition of dehydrogenases activity may be the blocking of active centers belonging to the respiratory chain by the studied metal cations [8, 10]. Cysteine and histidine, amino acids present in the enzymes, show high affinity to metal ions [11]. The toxic effect of cadmium and copper may also be a result of the cell mem-

branes permeability change. Copper is rapidly bound by amino acids present in the cell membrane and wall, enhancing the excretion of cations, for instance: K^+ and PO_4^{3-} [2, 23].

The fact that the activated sludge is a good biosorbent of Cd^{2+} and Cu^{2+} ions [24, 25] may also affect the results. The sorption of metals is different for each species of microorganisms and the removal of metal ions from the solution may require less than 20 seconds [2, 7, 26, 27, 28]. There are reports that metal ions penetrate the cells up to the active center of dehydrogenases [1]. Our studies show that copper and cadmium ions affect activity of dehydrogenases rapidly. The toxic effect was observed very fast, already after 5 minute contact between the active sludge and cadmium at the concentrations of 2.7 and 8.1 mg L^{-1} Cd^{2+} (24.02; 72.06 μmolL^{-1} Cd^{2+}) and copper at 0.5; 1; 2; 4; 8 mg L^{-1} Cu^{2+} (7.87; 15.74; 31.47; 62.95; 125.89 μmolL^{-1} Cu^{2+}) (Tabs 1 and 2).

The considerable preventive role against the toxic action of heavy metals is played by low molecular, sulfur-containing proteins. Metallothioneine, which enjoys a growing interest among many researchers, is an example of such protein [29, 30]. The metallothioneine contains numerous sulfhydryl groups [11] and resembles proteins present in many species of vertebrates, invertebrates, plants, fungi, and even in *Procaryota* [11]. The high affinity of cadmium and copper to sulfur causes that these metals are bound by metallothioneine. This process may slow down their toxic effect or inhibit it completely.

The obtained results show the considerable influence of time on EC_{50} variations (Fig. 3). Similar observations were done also by Hatano and Shoji [31]. The response of microorganisms to a particular substance is a combined result of the effects exerted on all species present in the activated sludge that contain dehydrogenases. The obtained relationship 'concentration – response' has the features of the normal distribution. The percent of dehydrogenases activity inhibition was increasing with the concentration of the toxic substance after the definite time of the toxic substance contact with the activated sludge. This increase was linear for the relationship between the dehydrogenases activity inhibition expressed in probits and the decimal logarithm of copper/cadmium ions concentration. The R^2 coefficients exceeded 0.8 in the majority of cases (Table 1 and 2).

Significant variations of EC_{50} values for the studied ions after 90 and 150 minutes were observed. These results indicate that copper ions are more toxic than cadmium ones after this time. Similar results were obtained by Madoni and Romero [32], who studied the effect of cadmium and copper on the freshwater ciliated protists, as well as Chaperon and Sauve [1] in the studies of the activity of dehydrogenases of soil organisms. After 200 minutes EC_{50} values of examined salts were similar while earlier differences between EC_{50} values for sulfate copper and sulfate cadmium were significant.

CONCLUSIONS

Our studies showed a considerable variation of dehydrogenases activity after a short-time contact of microorganisms with copper and cadmium ions. It suggests the rapid penetration of these ions into cell structures. The inhibition of microbial dehydrogenases of the active sludge depended on the heavy metal exposition time. The linear type of the variations in the system. i.e., inhibition expressed in probits vs. decimal logarithm of the studied ions concentration was observed. It was shown that copper is more toxic than cadmium in 5–150 min time interval.

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WYKORZYSTANIE TESTU TTC DO OCENY TOKSYCZNOŚCI KADMU I MIEDZI W OSADZIE CZYNNYM

Celem niniejszej pracy było określenie wpływu różnych stężeń kadmu i miedzi na aktywność dehydrogenaz mikroorganizmów osadu czynnego. Badania przeprowadzono z wykorzystaniem sześciu komór z napowietrzanym osadem czynnym, każda o objętości 1L (niezanieczyszczona próba stanowiła kontrolę, a pozostałe zawierały: 0,5; 1; 2; 4; 8 mg L^{-1} Cu^{+2} oraz 0,1; 0,3; 0,9; 2,7; 8,1 mg L^{-1} Cd^{2+}). Metale wprowadzano do zawiesiny w postaci siarczanu kadmu i siarczanu miedzi. Wyznaczono stężenia jonów powodujące 50% zahamowanie aktywności dehydrogenaz. Szczególną uwagę zwrócono na porównanie toksyczności jonów obu metali oraz na zachodzące w czasie pod ich wpływem zmiany aktywności oddechowej mikroorganizmów. Badania wykazały, że nawet najniższe z zastosowanych stężeń badanych związków powodowały istotne zmiany w aktywności dehydrogenaz osadu. Jony miedzi okazały się być bardziej toksyczne niż jony kadmu.