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BOGUMIŁA ORŁOWSKA*, TYMOTEUSZ JAROSZYŃSKI**, EDWARD GOMÓŁKA**

CHARACTERISTICS OF ACTIVATED SLUDGE ADAPTED TO DIOXANE AND N-METHYLPYRROLIDINE

The results of investigations show that dioxane has no adverse effect on the action of activated sludge, when occurring at a concentration of 300 g/m³. This has been substantiated by microbiological analysis which confirms the presence of protozoa and rotifera, as well as of miscellaneous bacterial flora of which *Bacillus pumilus*, Yersinia sp., Flavobacterium rigense, and F. aquatile were predominant. The presence of B. brevis, B. firmus, Kurthia zopfii, Nocardia sp., Actinobacillus equuli, Alcaligenes faecalis, Moraxella sp., and Plesionnas sp. was also confirmed.

N-methylpyrrolidine exerted a toxic effect at a concentration higher than 900 g/m³. Increase in the concentration of this substance in the activated sludge eliminated gradually protozoa and rotifera until at a concentration of 2000 g/m³ the sludge consisted of bacterial populations alone. *Flavobacterium* sp. (various species) accounted for more than 50% of the bacterial flora. Less numerous were the rods of *Aeromonas hydrophila* subsp. *aerogenes*. The remaining strains were identified as *Corynebacterium* sp., *Aeromonas salmonicida* subsp. *achromogenes*, and *Alcaligenes faecalis*.

1. INTRODUCTION

Activated sludge process belongs to the treatment methods most widely employed in engineering practice. The efficiency of the activated sludge process is characterized by a number of technological parameters, such as: BOD_5 and dichromate COD in the influent and effluent, degree of oxidation, degree of removal of certain pollutans, and Mohlman Index. In the evaluation of the process efficiency, biological observations of the microflora and microfauna, which occur in the sludge, and the growth of their biomass are of great importance.

^{*} Institute of Inorganic Chemistry and Metallurgy of Rare Elements, Technical University of Wrocław, ul. Smoluchowskiego 23, 50-372 Wrocław, Poland.

^{**} Institute of Environment Protection Engineering, Technical University of Wrocław, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland.

Intensification of the activated sludge process is also a problem of great interest. Taking advantage of the fluctuation in enzymatic activity of the microorganisms, we can grow such ones that will be able to oxidize not only compounds resistant to biodegradation, but toxic substances as well. This may be of particular utility in the treatment of industrial wastewaters, as their composition is highly differentiated and varies from one industry to another. It seems therefore reasonable to study more extensively the effect of various substances on the action of activated sludge.

This paper shows the effect of 1,4-dioxane (dioxane) and N-methylpyrrolidine (NMP) on activated sludge. General characteristics of the two compounds, as well as their resistance to biodegradation, are reported in an earlier paper (JAROSZYŃSKI, GOMÓŁKA [5]).

Laboratory-scale technological investigations were carried out to adapt the activated sludge to dioxane and NMP. Biological analysis included microscopic examinations of the biomass and quantitative determinations of the various kinds of bacteria, namely those participating in the ammonification, nitrification and denitrification processes, those capable of growing on enriched agar-agar as well as actinomycetes and yeasts. The bacteria isolated from the agar-agar medium were identified.

2. MATERIAL AND METHODS

The experiments were conducted in ejector-type through-flow reactors (the volumeof about 2 dm³) (JAROSZYŃSKI [4]). The activated sludge under test was generated in the Świebodzice-Ciernie Sewage Treatment Plant of the Wałbrzych Coal Region. Adaptation to dioxane and NMP was achieved by increasing the concentration of either compound in the influent. For this purpose artificial wastewater was used. After 37 days of adaptation, the experiments were conducted for over 200 days using artificial wastewater containing both pollutans, then municipal sewage and mineral wastewater. Daily analyses of physicochemical composition were conducted both for the influent and the effluent. More than once a week the sludge quantity and Mohlman Index were measured, and microscopic examinations were carried out, the emphasis being given to the shape of the sludge flocs, as well as to the presence of free bacteria, protozoa, fungi, and worms. Microfauna was identified according to KLIMOWICZ [6, 7, 8], KLIMOWICZ, 1977, and TUROBOYSKI [14].

Samples for bacteriological analyses were taken after 250 days of dioxane adaptation (when its concentration had reached 300 g/m³) and after 266 days of NMP adaptation. (when its concentration had approached 2000 g/m³). Quantitative determinations of ammonification, nitrification, and denitrification bacteria were conducted by the serial dilution method. The most probable number of organisms was determined according to Mc Credy's tables in a 3-test tube system (PALUCH [9]). For determining ammonification bacteria peptone water was used, nitrification bacteria of the first and second phases being determined in Winogradski's media No. 1 and No. 2. Denitrification bacteria were identified in the Giltay medium. Determinations of ammonia nitrogen and nitrites involved Nessler's

reagent and Griess's reagent, respectively, brucine and sulphuric acid being used for the determination of nitrates (RODINA [12]).

Heterotrophic bacteria, actinomycetes and yeasts were examined in agarized media, and their number was assessed in the plate method. For bacteria, enriched Bio-Med agar was used, actinomycetes were grown in an organic Gauze medium and a peptonemaize-starch agar (RODINA [17]). For yeast growth, Bio-Med Sabouraud medium treated with penicillin (1000 units/cm³) and streptomycin (1 mg/cm³) was used.

Isolated bacterial strains were identified and classified according to COWAN and STEEL [2] and BREED et al. [1]. The experimental media and reagents, as well as the biochemical tests were prepared and conducted by means of Cowan-Steel's methods.

The resistance of selected strains to dioxane and NMP was studied by the serial dilution method (in geometrical progression) in enriched broth. Bacteriocidal activity was checked by inoculation of the culture (which had been exposed for 24 hours to the action of the two solvents in question) on a fresh agar-agar medium.

3. RESULTS

The parameters of the activated sludge process employed for the treatment of artificial wastewaters, municipal sewage and mineral wastewaters (each of them containing dioxane and NMP) are given in tab. 1. Table 2 shows the compositions of the influent and effluent. Variations in the biological composition of the sludge are listed in tab. 3. As can be seen from this table, there was only a partial elimination of protozoa and rotifers from wastewaters containing dioxane, so the effluent was quite clarified. Unlike dioxane,

Table 1

Process parameters for the treatment of dioxane and NMP-containing wastewaters in the activated sludge process

Parametry procesu oczyszczania ścieków zawierających dwuoksan i N-metylopirolidynę przy zastosowaniu osadu czynnego

Solvent	Type of wastewater	Duration	Concen- tration of solvent	Time of aeration	Hydraulic loading of tank	BOD ₅ loading of sludge	Sludge concentration in tank
-		days	g/m³	h	$m^3/m^3 imes d$	$\begin{array}{c} g \ O_2/g \\ dry \ sub. \times d \end{array}$	kg/m ³
	artificial	266	5-600	10.9	2.2	0.11-0.53	1.4-6.0
Dioxane	municipal	42	50-300	12.3	1.9	0.03-0.17	4.0-4.7
	mineral	24	300	12.3	1.9	0.0006-0.001	2.3-3.6
	artificial	266	5-2000	12.4	1.9	0.07-0.64	3.6-17.8
NMP	municipal	42	100-1500	10.0	2.4	0.11-1.12	4.1-12.6
	mineral	43	1500-2500	10.0	2.4	0.33-0.86	6.9-19.1

NMP was found to exert a toxic effect on the activated sludge. Increase in NMP content to 900 g/m^3 in the entering wastewater resulted in elimination of protozoa and rotifers. Once this concentration value is reached (and exceeded), the sludge consists of bacteria alone and the turbidity of the effluent continues to increase.

Table 2

Solvent	Type of	Measured in	Concentration of solvent	Dichromate COD	Total alkalinity	Ammonia nitrogen
R. C. D	wastewater	-	g×m ⁻³	$g O_2 \times m^{-3}$	g $CaCO_3 \times m^{-3}$	g $N\!\times\!m^{-3}$
-	artificial	influent	5-600	503-1645	115-530	6–16
	artinciai	effluent	_	150-1063	15-330	0-70
Diovane	municipal	influent	50-300	153-1169	190-360	10-50
DIOXane	municipai	effluent	35-262	107-542	45-215	0.1-12
	mineral	influent	300	595-614	165-190	14-18
	millerar	effluent	268-292	463-580	40-70	0.2-2.8
	artificial	influent	5-2000	507-3860	180-425	2.5-16
	artinolar	effluent		49-540	30-1080	0-300
NMD	municipal	influent	110-1500	260-3360	200-380	10-45
	mumerpar	effluent		75-96	68-780	5-90
	mineral	influent	1500-2500	2797-4455	170-646	10-16
1	minoral	effluent	51-800	104-2060	550-995	0.7-100

Physicochemical characteristics of the influent and effluent Fizykochemiczne właściwości wcieku i odcieku

The presence of dioxane and NMP also affects the composition of bacterial flora. Table 4 gives quantitative results for selected physiological groups of microorganisms present in the activated sludge. Thus, in the presence of dioxane the number of ammonification bacteria is greater than in the presence of NMP. Of the nitrification bacteria only those responsible for the first phase of the process (i. e. those converting ammonium compounds to nitrites) are found in both dioxane and NMP sludges. Bacteria participating in the second phase of the nitrification process (i. e. those accounting for the oxidation of nitrites to nitrates) are found in small numbers in dioxane sludge alone. On the contrary, denitrification bacteria are numerous in the biocenosis of both dioxane and NMP sludges and the number of cells approaches $10^8/\text{cm}^3$.

Quantitative determination of bacteria capable of growing on enriched agar were carried out by the plate method. The objective was not only to determine their number, but also to isolate and subject to taxonomic analyses all the strains that had been found to occur most frequently. Thus, their numbers amounted to 9×10^8 /cm³ and 8.7×10^7 /cm³ for dioxane sludge and NMP sludge, respectively. Actinomycetes occurred in dioxane sludge alone and were represented by several strains of the genus *Nocardia*. Yeasts and yeast-like organisms in NMP sludge were not very frequent. In dioxane sludge their number was almost the same as in any other polluted aquatic medium.

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Biological composition of activated sludge during treatment of wastewaters containing dioxane and NMP (ejector system)

Skład biologiczny osadu czynnego podczas oczyszczania ścieków zawierających dwuoksan i N-metylopirolidynę (system ejektorowy)

				Schizon	ny-		011		Nen	nathelm	intes	Oligo-
Day of	Concen-	Type of	Sludge	cetes			Cinata		Rotate	oria Ner	natoda	chaeta
experi- ment	of solvent $g \times m^{-3}$	waste- water	index cm ³ ×g ⁻¹	Fila- men- tous	Vorti- cella sp.	Epis- tilis sp.	Sty- lony- chia sp.	Lino- tus sp.	Roti- fer sp.	Cel- urella sp.	Bip- loga- ster sp.	Styla- ria sp.
				Г	Dioxane							
0	0	artificial	94	+	+	+	+	+	++	++		++
54	400	atrificial	(332)	+	_	+	· -		+			
138	600	artificial	364	++	+		+	+	+	_		
80	300	mineral	107	+	·				+	_	_	
					NMP							
0	0	artificial	76	+	+	-+-	· _ · ·		++	2 <u>71</u> - 191 -	<u></u>	
20	50	artificial	55	+	+	+	84 <u>0 -</u> 1	1 <u>-</u> 10 -	-			
140	700	artificial	129		+				· _	_	-	- /
181	900	artificial	90	-	+		_		_		_	_
-222	1200	artificial	83	_		_	121	<u> </u>				
1	acout											

+ present. ++ numerous.

- absent.

Table 4

Quantitative composition of microflora in activated sludge adapted to dioxane and NMP Ilościowy skład mikroflory w osadzie czynnym zaadaptowanym do dwuoksanu i N-metylopirolidyny

Selected groups of microorganisms	Dioxane* NMP** cells/cm ³						
Ammonification bacteria		9.5×10 ⁸	9.5×10^{7}				
Nitrification bacteria (first phase)		3.6×10 ⁶	4.5×10^{3}				
Nitrification bacteria (second phase)		0.6×10 ¹	absent				
Denitrification bacteria		9.5×10 ⁷	9.5×10 ⁸				
Heterotrophic bacteria		1.9×10 ⁸	8.7×10 ⁷				
Actinomycetes		single colonies	absent				
Yeasts and yeast-like organisms		$2.1 imes 10^3$	0.6×10 ¹				

* Concentration of 300 g/m³.

** Concentration of 2000 g/m3.

The bacterial flora grown in the two types of sludge under study is listed in tab. 5. As follows from this table, dioxane sludge exhibits miscellaneous bacterial cultures. Grampositive bacteria were mostly represented by *Bacillus pumilus* which accounted for 28% of the total population active in this medium. Less frequent were *B. brevis*, *B. firmus*, *Kurthia zopfii* and *Nocardia* sp., all of them accounting for 2% of the total population. From among gram-negative bacteria, the rods classified as *Yersinia* sp. were the most numerous, accounting for 20% of the total population. The genus *Flavobacterium* (with

Table 5

Bacterial flora in activated sludge adapted to dioxane and NMP Flora bakteryjna w osadzie czynnym zaadaptowanym do dwuoksanu i N-metylopirolidyny

Dioxane*	2	%
Bacillus pumilus, Meyer and Gottheil, 1910		
Bacillus brevis, Migula 1900		27.0
Bacillus firmus, Bredeman and Werner,1933		
Corvnebacterium sp., Lehman and Neumann, 1895		
Kurthia zonfii. (Kurth) Trevisan, 1885		2.0
Nocardia sp., Castellani and Chalmers, 1919		
Versinia sp. van Loghen, 1944		20.8
Elavobactorium rigense Bergev et al. 1923		10.1
Elavobacterium aquatile Frankland and Frankland, 1889		8.6
Moraxella osloensis, Bovre and Henrikensen, 1967		2.9
Actinobacillus equuli. (van Strauten) Haupt, 1934		
Alcaligenes faecalis Castellani and Chalmers, 1919		
Moravella sp. I woff 1939		28.6
Plasiomongs sp. Habs and Schubert 1962		
Unidentified		
NMP**		%
Elevendesterium sp. Frankland and Frankland, 1889		52.0
Accompany hydrofile subsp. anderogenes. Schubert, 1964		22.0
Corynebacterium sp., Lehman and Neumann, 1896		10.0
Agromonas salmonicida subsp. achromogenes, (Smith) Schubert 1	967	
Alcaligenes faecalis, Castellani and Chalmers 1919		16.0

* Concentration of 300 g/m³.
** Concentration of 2000 g/m³.

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F. rigense -10% and F. aquatile -8.6%) ranked second. Organisms of the genus Moraxella osloensis formed about 3% of the microflora. The remaining microorganisms belonged to the Actinobacillus equuli, Alcaligenes faecalis, Moraxella sp., and Plesiomonas sp. Some of the gram-negative bacterial strains remained unidentified.

In NMP sludge a relatively small number of bacterial groups was isolated. Of grampositive bacteria only *Corynebacterium* sp. was identified, it accounted for 10% of the total population. In this type of sludge the genus *Flavobacterium* was predominant, accounting for as much as 52%, *Aeromonas hydrophila* subsp. *achromogenes* (22%) ranked second. The remaining 16% included microorganisms determined as *Aeromonas salmonicida* subsp. *achromogenes*, a small number of *A. faecalis* and single unidentified strains. The resistance of the most frequent strains to dioxane and NMP is given in tabs. 6 and 7.

Table 6

Strains isolated from	Ba	Con cterio	centratio cidic	n of solvent Bacteriostatic		
		%			%	
Bacillus pumilus		32			4	1.
Flavobacterium aquatile		16			4	
Flavobacterium rigense		16			4	
Moraxella osloensis		8			4	
Alcaligenes faecalis		4			2	
Plesiomonas sp.		16			4	
Yersinia sp.		16			4	
Strains isolated from other sludge						
Strains house nom other strage						
Aeromonas salomonicida subsp. anaeroganes		16			8	
Aeromonas hydrophila subsp. achromogenes		32			4	
Alcaligenes faecalis		4			2	
Plesiomonas sp.		16			4	
Gram(-)rod		16			8	
Gram(-)rod		32			8	
Gram(-)rod		16			8	
Bucillus sp.		16			4	

Resistance of selected strains to dioxane Oporność wybranych szczepów na dwuoksan

Most of the cells in the biomass adapted to dioxane (tab. 6) were killed in 16% (wt./vol.) solution of this compound. Only *B. pumilus* was capable of tolerating high concentrations of dioxane probably due to the production of spores. *M. osloensis* and *A. faecalis* were less resistant to dioxane. Bacteriostatic action became pronounced at low concentrations of this solvent (2-4\%). A comparative study of resistance performed for strains isolated from other sources than sludge adapted to dioxane gave similar results.

Resi	istance	of	selected	strai	ns	to	NMP
Oporność	wybran	ych	szczepów	v na	N-	met	ylopirolidynę

		Concentration of so							
Strains isolated from sludge adapted to NMP	В	acterio	cidic	Bacteriostatic					
Strains isolated from shuge daped to this		%		%	-				
Commentarium sp. No. 1	n T	4		4	-				
Corynebacterium sp. No. 2		16		8					
Coryneodclerium sp. 100. 2		4		4					
Flavobacterium sp.		16		8					
Aeromonas hydrophila subsp. anderogenes No. 1		8		4					
Aeromonas salmonicida subsp. achromogenes No. 2		8		4					
Aeromonas salmonicida subsp. achromogenes 140. 2		16		8					
Strains isolated from other sludges									
Pacillus numillus		16	<u>.</u>	8					
Elementarium aquatile		8		4					
Flavobacterium ugaatte		16		8					
Flavodacierium rigense		16		8					
Gram(-)rod		32		8					
Gram(-)rod		16		8					
Gram(-) rod		16		4					
Bacillus sp.		10		-					

The resistance of the strains under study to NMP are given in tab. 7. Like dioxane, NMP was also found to exert a bacteriocidal action at relatively high concentrations (8-16%). Its bacteriostatic effect became evident at the concentrations ranging from 4 to 8%. This holds not only for sludges adapted to NMP, but also to other sludges, except for *Corynebacterium* sp. and *Flavobacterium* sp. which were found to be less resistant to NMP.

4. DISCUSSION

Wastewater treatment in the activated sludge process involves mineralization of the organic substances contained in the biomass prior to the discharge procedure. The mineralization process is catalyzed by the presence of microorganisms and depends on the constituents of waste to be treated and on the specific properties of the microorganisms growing in the sludge.

The bacterial flora of industrial and biological sludges was investigated by many workers both quantitatively and qualitatively. Most of the determinations were carried out for physiological groups (e. g. nitrification, denitrification and cellulolytic bacteria) by the method of serial dilutions. For this purpose poor media were used (e. g. sewage agar), assumed that they are very similar to the natural habitat and, therefore, optimal ones (DIAS, BHAT [3] and PRAKASAM [11]). Other investigators used enriched media to grow their bacterial populations (e. g. peptone-glucose agar) and obtained better results (SEILER, BUSSE [13] and PIKE, CURDS [10]).

The objective of this report was to determine the number of selected physiological groups and a taxonomy of strains isolated from the sludges tested. Considerations were focussed on aerobic bacteria alone, because the treatment process employed ejectors providing a good degree of aeration so that only aerobes or facultative anaerobes could be of importance in this treatment procedure.

The results obtained indicate that the composition of the bacterial flora is substantially affected by the quality of the sludge. Sludge adapted to dioxane displayed a richer and more differentiated flora than NMP adapted sludge. In NMP sludge two genera — *Flavobacterium* and *Aeromonas* were predominant. The fact that no *Pseudomonas* growth was found in the sludge samples under test is worth mentioning, the more so that the presence of *Pseudomonas* is known to be typical of wastewaters and activated sludges. Their absence might be due to the specific biocoenosis of activated sludges adapted to dioxane and NMP, as the adaptation process is very long. It may also be attributed to the inadequate nomenclature for microorganisms belonging to the genus *Pseudomonas*.

The bacterial strains isolated from the experimental sludges were resistant to dioxane and NMP at concentrations considerably higher than those employed in the experiments. It has been found, however, that in NMP concentrations higher than 0.09% the microfauna was removed from the activated sludge, which deteriorated the treatment efficiency. Thus, the well known observation has again been confirmed, namely that the efficiency of the activated sludge depends on its biocoenosis as a whole and not on its particular components.

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CHARAKTERYSTYKA OSADU CZYNNEGO ZAADAPTOWANEGO DO DWUOKSANU i N-METYLOPIROLIDYNY

Wyniki badań wykazały, że dwuoksan w stężeniu 300 g/m³ nie wywiera szkodliwego wpływu na osad czynny. Analiza bakteriologiczna wykazała obecność pierwotniaków i wrotków oraz różnorodnej flory bakteryjnej, wśród której dominowały: Bacillus pumilus, Yersinia sp., Flavobacterium rigense i F. aquatile. Stwierdzono też obecność B. brevis, B. firmus, Kurthia zopfii, Nocardia sp., Actinobacillus equuli, Alcaligenes faecalis, Moraxella sp. i Plesiomonas sp.

N-metylopirolidyna jest toksyczna w stężeniu większym niż 900 g/m³. Wzrost stężenia tej substancji do 2000 g/m³ w osadzie czynnym eliminuje stopniowo pierwotniaki i wrotki, tak że w osadzie pozostają jedynie populacje bakteryjne. Flavobacterium sp. (różne gatunki) stanowiło ponad 50% flory bakteryjnej. Mniej liczne były pałeczki Aeromonas hydrophila subsp. aerogenes. Pozostałe szczepy oznaczono jako: Corynebacterium sp., Aeromonas salmonicida subsp. achromogenes i Alcaligenes faecalis.

ХАРАКТЕРИСТИКА АКТИВНОГО ИЛА, АДАПТИВИРОВАННОГО ДЛЯ ДИОКСАНА И N-МЕТИЛПИРРОЛИДИНА

Результаты исследований показали, что диоксан в концентрации 300 г/м³ не оказывает вредного влияния на активный ил. Микробиологический анализ обнаружил наличие простейших и коловратков, а также разнообразной бактериальной флоры, среди которой доминировали: Bacillus pumilus, Yersinia sp., Flavobacterium rigense, F. aquatile. Выявлено также наличие B. brevis, B. firmus, Kurthia zopfii, Nocardia sp., Actinobacillus equuli, Alcaligenes faecalis, Moraxella sp., Plesiomonas sp.

N-метилпирролидин токсичен в концентрации, большей чем 900 г/м³. Повышение концентрации этого вещества до 2000 г/м³ в активном иле постепнно элиминирует первейшие и коловратки, так что в иле остаются только бактериальные популяции. Flavobacterium sp. (различные виды) составляли свыше 50% бактериальной флоры, Менее многочисленны были палочковидные бактерии Aeromonas hydrophila subsp. aerogenes. Остальные штаммы обозначены как Corynebacterium sp., Aeromonas salmonicida subsp. achromogenes и Alcaligenes faecalis.

ZUM ABBAU VON DIOXAN UND N-METHYLPYROLIDIN ADAPTIERTER BELEBTSCHALMM

In Konzentrationen bis zu 3000 g/m³ übt Dioxan keinen hemmenden Einfluß auf den Belebtschlamm. Eine mikrobiologische Analyse erwies die Anwesenheit von Protozoen und Rädertierchen sowie ein reiches Bakterienspektrum in dem *Bacillus pumilus*, Yersinia sp., Flavobacterium rigense und F. aquatile dominierten. Amwesend waren auch B. brevis, B. firmus, Kurthia zopfii, Nocardia sp., Actinobacillus equuli, Alcaligenes faecalis, Moraxella sp. und Plesiomonas sp.

N-Methylpirolidin wirkt toxisch in Konzentrationen die höher als 900 g/m³ sind. Eine Konzentrationserhöhung bis zu 2000 g/m³ eliminiert allmählich aus dem Belebtschlamm die Protozoen und Rädertierchen so daß nur noch Bakterien verbleiben. *Flavobacterium* sp., (verschiedene Arten) machten mehr als 50% der gesamten Bakterienflora aus. Weniger zahlreich waren Stäbchenbakterien von Aeromonas hydrophila subsp. aerogenes. Den Rest bildeten Stämme von Corynebacterium sp., Aeromonas salmonicida subsp. achromogenes und Alcaligenes faecalis.