

Cercetări Marine	I.R.C.M.	Nr.11	235 - 252	1978
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INTERACTION BETWEEN CHEMICAL POLLUTION COMPOUNDS AND MARINE UNICELLULAR ALGAE

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ABSTRACT:

The paper deals with the metabolic changes caused by several pollution compounds in marine unicellular algae. It presents data on cellular division, photosynthesis and utilization degree of pollution products by the algae.

Unicellular algae play a prominent part in the cycle of mineral and organic matter in water environments, by utilizing the compounds in their nutritional metabolism. Among the essential mineral elements, N has a very important place, its assimilation being possible in any of the reduced or oxidized states (3, 4, 5, 7) as well as from organic products, such as urea, uric acid, amino acids, amides, etc. (7).

A great number of organic compounds serve as carbon sources in both photosynthetic process and mixo- or heterotrophic processes. Of these, ethylic, n-propylic, n-butylic and amylic alcohols are certain carbon sources (1).

Consequently, occurrence of the above products in the environment, either as a result of organic matter decomposition, or from waste water, may signify increase of the available nutritive substances.

The present study is aimed to point out the effect of

N-NH₄, urea and methanol on algal metabolism and to determine the concentrations at which these compounds can be used by the algae.

MATERIAL AND METHODS

The algae Skeletonema costatum GREV. CL., Nitzschia tenuirostris MER. and Chlamydomonas sp. were selected for experiment, taking into account the following reasons. Skeletonema is one of the most widespread algae in the coastal phytoplankton and its development over the last years shows existence of the conditions required by this organism.

Species of the genus Nitzschia are typical for the media where organic decomposition and consequently, generation of ammoniacal nitrogen takes place. Laboratory experiments proved that the species Nitzschia closterium (EHR.) W. SM. can grow in media where the only nitrogen source was ammonia (3, 4). Nitzschia tenuirostris is a frequent organism in the phytoplankton of the coastal zone of the Black Sea, which shows a density increase tendency over the last years, simultaneously with the increase in total quantity of dissolved organic substance.

Chlamydomonas sp. belongs to the group of organisms the biomass of which began to increase in the coastal waters concurrently with the increase in waste water input. Its preference for ureic and ammoniacal forms of N, as well as for organic substances among which alcohols, are well known (2).

The experimented substratum was composed of waste waters which contained methanol, ammonia + urea, or both the former and the latter. Concentrations of chemical compounds varied within extremely wide limits: methanol 0.34 - 17 mg/l, ammonia 0.1-42 mg/l and urea 0.0019-1.52 mg/l.

All tests were performed on the same sea water - stored and pasteurized, according to the usual techniques.

The algae in the active division stage were separated from the growth medium, thoroughly washed with sterilized sea water (in which the experiments were to be done) and then resuspended in sea water to which different quantities of waste water had been added in order to obtain the proposed concentrations to be tested.

Algae growth conditions - bubbling, illumination (2,000 lx), temperature (19-21°C) were identical and constant with all

species.

The following determinations were made:

- cellular density/l, for pointing out stimulating and inhibiting concentrations of the tested compounds;
- concentration of existent compounds in the waste water during the experiments, for verifying the ability of the algae to utilize ammonia and urea, by SCHEINER's method (6), and methanol, by the chromotropic acid method that is used in wine industry and was adapted to sea water;
- P-PO₄, N-NO₃ and N-NO₂ concentration level (2) for establishing the metabolism activation degree under the influence of the upper called compounds;
- determinations of photosynthetic level and pH-measurements, for explaining the action characteristics of the investigated compounds.

RESULTS AND DISCUSSION

As it has mentioned, the products contained in the waste water were tested within very large concentration limits (Table 1).

Table 1

Concentrations of the compounds contained
in the waste water (mg/l)

Methanol	Study variants				
	Ammonia		Methanol	Mixture	
	Ammonia	Urea		Ammonia	Urea
0.34	0.10	0.0019	0.34	0.10	0.0019
0.60	0.20	0.0038	0.60	0.20	0.0038
0.85	0.30	0.0095	0.85	0.30	0.0095
1.70	0.70	0.0190	1.70	0.70	0.0190
6.80	3.50	0.0950	6.80	3.50	0.0950
17.00	7.00	0.1900	17.00	7.00	0.1900
34.00	10.00	0.3800	34.00	10.00	0.3800
68.00	21.00	0.7600	68.00	21.00	0.7600
173.00	42.00	1.5200	173.00	42.00	1.5200

CELLULAR DIVISION

a. Skeletonema costatum intensifies its division process when concentrations of the tested compounds have been reduced. Methanol, for example, in concentrations of 0.34-0.85 mg/l,

implies division stimulation exceeding the control by 8.78%, while higher concentrations restrict the division rate to its inhibition - which was the case of the variants with 68 and 173 mg/l of methanol (Fig.1).

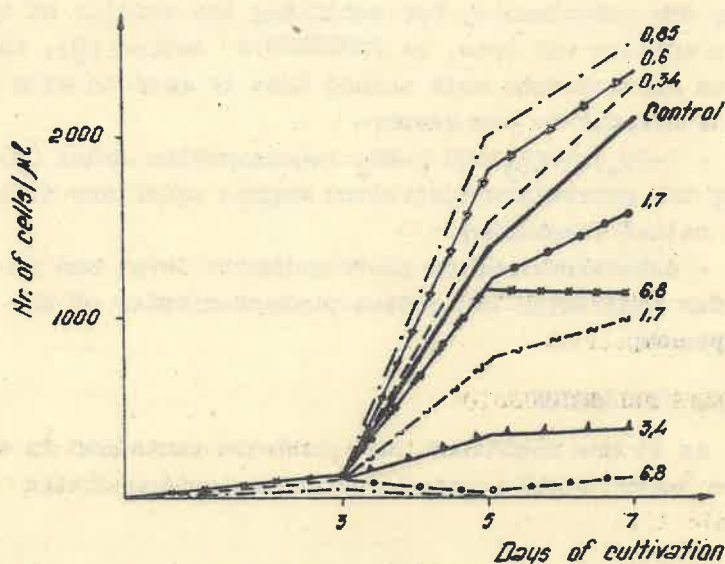


Fig. 1 - The development of *Skeletonema costatum* under the influence of waste waters charged with methanol (in mg/l; Control: sterilized sea water)

Ammonia and urea, between the limits of 0.1/0.002 and 0.7/0.02 mg/l (N-NH₄/urea) give an increase in cellular density by 30-73% of the control value (Fig.2); any concentration exceeding the cited upper limit inhibits division, causing the cells death during the time.

The mixture of the two sorts of waste water concentrations of ammonia, urea and methanol of 0.1/0.002/0.34 and 0.3/0.01/1.7 mg/l (ammonia/urea/methanol) gave an increase in cellular density by 23-73% greater than that obtained in the control (Fig.3). In this case the optimum increase is obtained for the smallest of the concentration values. Increase in concentration degree of pollution substances gives decrease in division rate (for instance, 0.7/0.02/6.8 mg/l), or much more it causes irre-

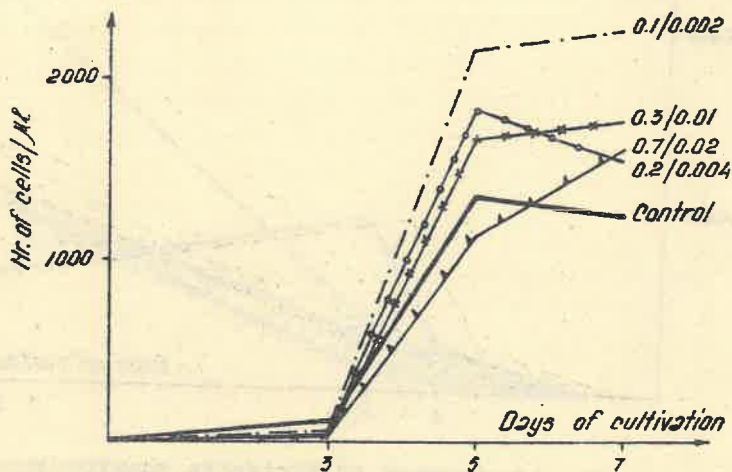


Fig. 2 - The development of *Skeletonema costatum* under the influence of waste waters charged with ammonia and urea (mg N-NH₃/mg urea; Control: sterilized sea water)

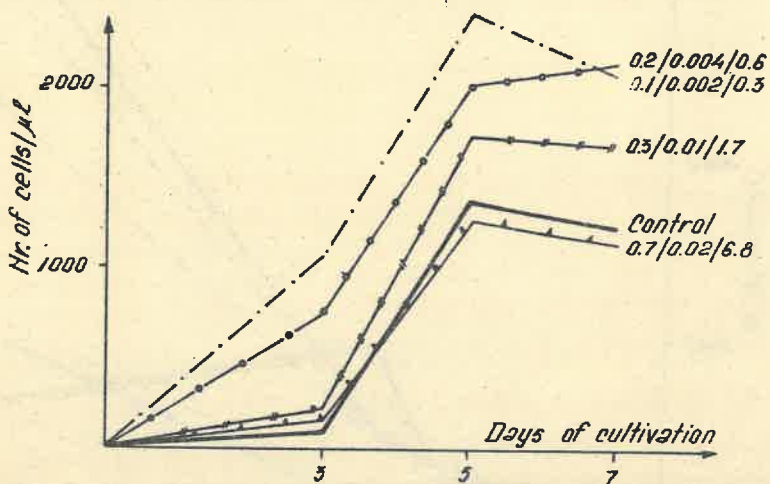


Fig. 3 - The development of *Skeletonema costatum* under the influence of waste waters charged with ammonia, urea and methanol (mg N-NH₄/mg urea/mg methanol %; Control: sterilized sea water)

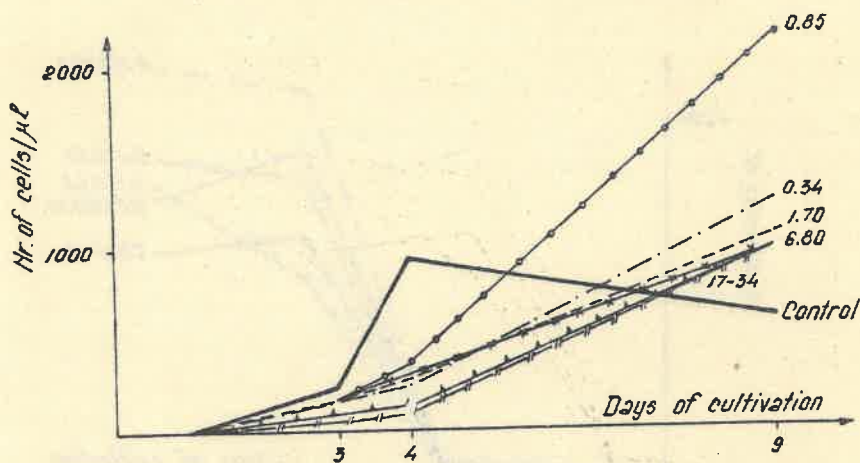


Fig. 4 - The development of *Nitzschia tenuirostris* under the influence of waste waters charged with methanol (in mg/l; Control: sterilized sea water)

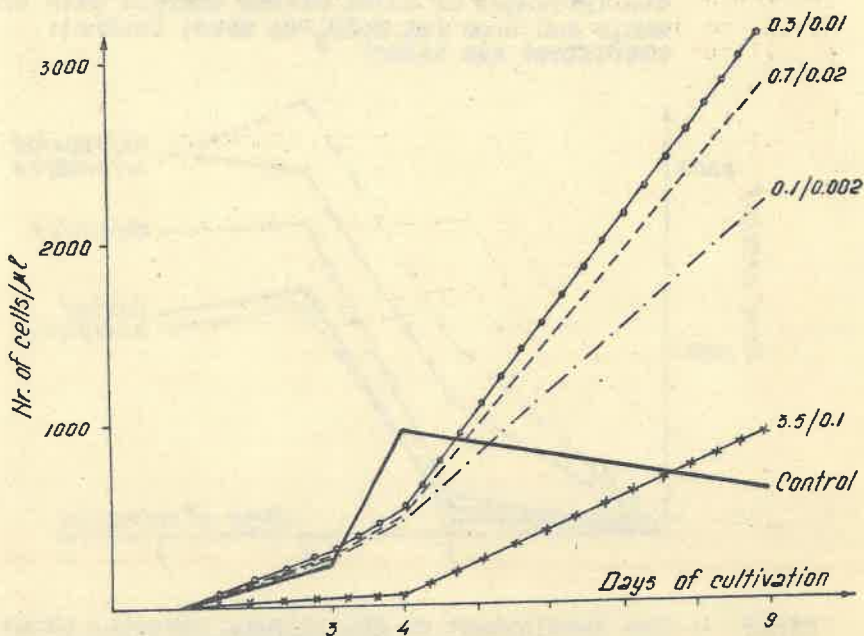


Fig. 5 - The development of *Nitzschia tenuirostris* under the influence of waste waters charged with ammonia and urea (mg N-NH₄/mg urea %; Control: sterilized sea water)

versible degradation of cells.

b. Nitzschia tenuirostris. When the tested products had, separately or in mixture, low concentration degrees, they caused stimulation of cellular division. Higher cellular density was obtained as compared with that of the control - by 45 - 100% for methanol, 100-200% for ammonia and urea, and 35-100% for the mixtures of these products (Figs. 4-6).

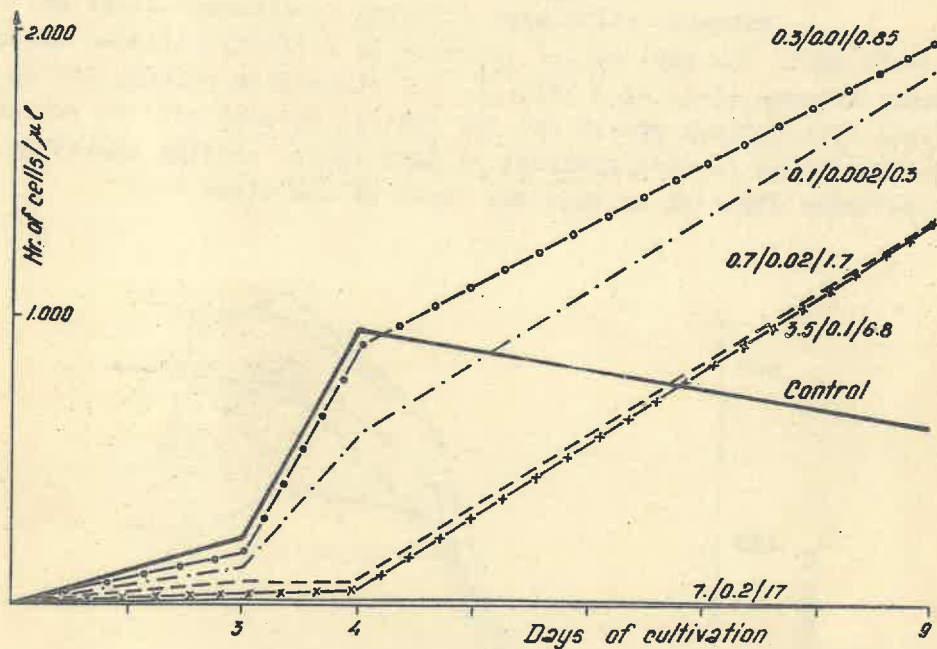


Fig. 6 - The development of Nitzschia tenuirostris under the influence of waste waters charged with ammonia, urea and methanol (mg N-NH₄/mg urea/mg methanol ‰; Control: sterilized sea water)

The stimulatory concentrations are 0.34-43 mg/l for methanol, with a maximum increase between 0.1 and 0.7 mg/l. For ammonia the limits are 0.1-0.7 mg/l, the maximum stimulation being produced at concentrations of 0.1-0.34 mg/l. To these ammonia concentrations the following urea levels correspond: 0.002 - 0.02 mg/l as stimulation limits, with maximum division between 0.002 - 0.01 mg/l.

When the two kinds of waste water are mixed, the concentrations of ammonia, urea and methanol (in this order) which favoured development were 0.1/0.002/0.34 to 0.7/0.02/1.7 mg/l and maximum growth was obtained when these products had concentrations from 0.1/0.002/0.34 to 0.3/0.01/0.85 mg/l.

c. Chlamydomonas sp.

Methanol stimulates division in concentrations of 0.34-34 mg/l. The most active increase in cellular division was obtained between 17-34 mg/l (Fig.7). The difference between the variants with optimum growth and the control reached 30%. At concentrations above 34 mg/l methanol is inhibitory, causing cessation of cellular division or even the death of the alga.

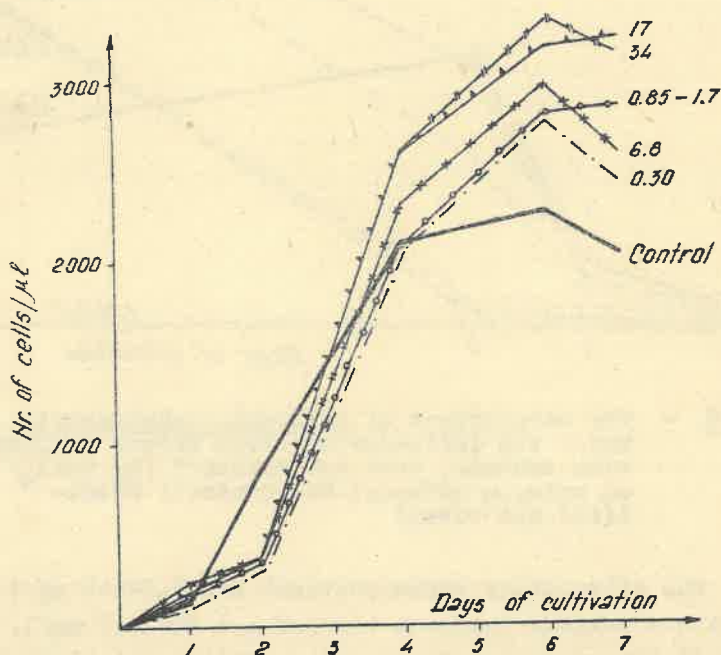


Fig. 7 - The development of Chlamydomonas sp. under the influence of waste waters charged with methanol (in mg/l; Control: sterilized sea water).

The waste water containing ammonia and urea has a stimulative effect only when the quantity of ammonia ranges within 0.1-7 mg/l and at the same time, urea takes concentrations between 0.02-0.2 mg/l. The optimum cellular division is reached in variants with 0.3-0.7 mg/l $N-NH_4$ and 0.01-0.02 mg/l urea. Within these limits, ammonia and urea make much better stimulative substratum than methanol, the optimum growth exceeding that of the control solution by about 50% (Fig.8). When ammonia exceeds 7 mg/l - correspondingly, urea 0.19 mg/l - ceasing of metabolic functions occurs, which is followed by 100% culture suppression.

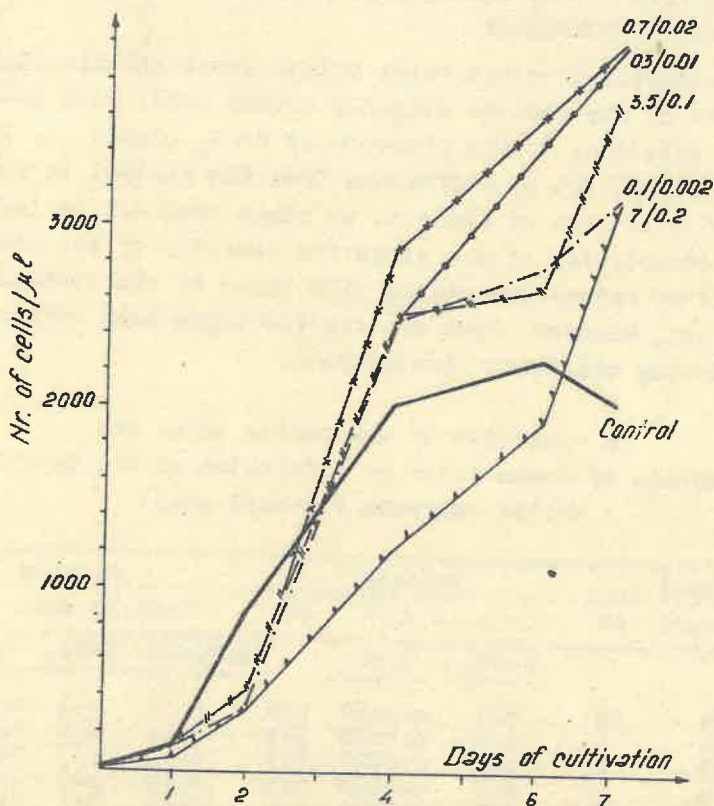


Fig. 8 - The development of Chlamydomonas sp. under the influence of waste waters charged with ammonia and urea (mg $N-NH_4$ /mg urea %; Control: sterilized sea water)

The presence of the three compounds gives a stimulating effect on division within the following concentration limits: methanol 0.34-17 mg/l, N-NH₄ 0.1-7 mg/l and urea 0.002-0.2 mg/l. However, the stimulation exceeds the control value only up to 30% (Fig.9). In consequence the effect on the algae is much similar to that which was obtained in the presence of ammonia and urea.

The maximum cellular division value was obtained at 0.3-7 mg/l N-NH₄, but at a lower gradient of methanol concentration: 6.8-17 mg/l, as compared to 17-34 mg/l, when methanol was tested alone.

THE INNER MOTIVE OF THE INTERRELATION BETWEEN ALGAE AND EXPERIMENTED COMPOUNDS

Addition of waste water brings about modifications in the reaction of the medium: slightly toward acid, with methanol, and toward alkaline, in the presence of N-NH₄ (Table 2). If, in case of methanol, the pH difference from the control is very small - 0.5 - in that of ammonia, pH rises from 6.5 to 10.5. But after the inoculation of the algae the reaction of the medium re-establishes at values coinciding with those of the control. It was found out, however, that the studied algae have different pH re-establishing abilities (Tables 2-5).

Table 2

pH variation of the medium under the influence of waste water as a function of the experimented variants (Control =6.5)

Methanol		Ammonia		Mixture				
Conc. in mg/l	pH	Conc. in mg/l	pH	Conc. in mg/l			pH	
		N-NH ₄	Urea	Methanol	N-NH ₄	Urea		
0.34	6	0.1	0.0019	6.5	0.34	0.1	0.0019	6.5
0.60	6	0.2	0.0038	7.0	0.60	0.2	0.0038	6.5
0.85	6	0.3	0.0095	7.5	0.85	0.3	0.0095	6.5
1.70	6	0.7	0.0190	8.0	1.70	0.7	0.0190	8.0
6.80	6	3.5	0.0950	8.5	6.80	3.5	0.0950	8.5
17.00	6	7.0	0.1900	9.0	17.00	7.0	0.1900	9.0
34.00	6	10.0	0.3800	9.0	34.00	10.0	0.3800	9.0
68.00	6	21.0	0.7600	9.5	68.00	21.0	0.7600	9.5
173.00	6	42.0	1.5200	10.5	173.00	42.0	1.5200	10.5

Table 3

pH modification in the experimented variants under the influence of the alga *Skeletonema costatum* at 24 hours (1) and 48 hours (2) after the insemination (Control =6.5-7)^{x/}

Methanol (mg/l)	pH		Ammonia (mg/l)	pH		Mixture (mg/l)		pH	
	1	2		1	2	Methan.	Ammonia	1	2
0.34	6	6.5	0.1	6.5-7	6.5	0.34	0.1	6.5-7	6.5
0.60	6	6.5	0.2	7	6.5	0.60	0.2	6.5-7	6.5
0.85	6	6.5	0.3	7	6.5	0.85	0.3	6.5-7	6.5
1.70	6	6.5	0.7	7-7.5	6.5	1.70	0.7	7	6.5
6.80	6	6.5	3.7	8-8.5	6.5	6.80	3.7	7.5	6.5
17.00	6	6.5	7.0	10	7	17.00	7.0	8	7
34.00	6	6.5	10.0	10	7.5	34.00	10.0	9	7

Table 4

pH modification in the experimented variants under the influence of the alga *Nitzschia tenuirostris* at 24 hours after the insemination (Control =6.5-7)

Methanol (mg/l)	pH	Ammonia (mg/l)	pH	Mixture (mg/l)		pH
				Methan.	Ammonia	
0.34	6.5-7	0.1	6.5-7	0.34	0.1	6.5-7
0.85	6.5-7	0.3	6.5-7	0.85	0.3	6.5-7
1.70	6.5-7	0.7	6.5-7	1.70	0.7	6.5-7
6.80	6.5-7	3.5	6.5-7	6.80	3.5	6.5-7
17.00	6.5-7	7.0	7.5	17.00	7.0	6.5-7
34.00	6.5-7	10.0	7.6-8	34.00	10.0	7

Table 5

pH modification in the experimented variants under the influence of the alga *Chlamydomonas* sp. at 24 hours after the insemination (Control =6.5-7)

Methanol (mg/l)	pH	Ammonia (mg/l)	pH	Mixture (mg/l)		pH
				Methan.	Ammonia	
0.34	6.5-7	0.1	6.5-7	0.34	0.1	6.5-7
0.85	6.5-7	0.3	6.5-7	0.85	0.3	6.5-7
1.70	6.5-7	0.7	6.5-7	1.70	0.7	6.5-7
6.80	6.5-7	3.5	6.5-7	6.80	3.5	6.5-7
17.00	6.5-7	7.0	7.5	17.00	7.0	6.5-7
34.00	6.5-7	10.0	7.5-8	34.00	10.0	7

^{x/} The quantities of urea corresponding to each variant are not mentioned in the tables.

Thus, the re-establishing of the reaction of the medium occurs after 48 hours cultivation for Skeletonema, while Nitzschia and Chlamydomonas needs only 24 hours.

Photosynthesis measurements on the algae Skeletonema and Nitzschia showed sharp reduction of the process after the initial 24 hours, from which correlative intensification of respiration is inferred. Using the formula:

$$D_x = 100 \times \frac{V_{1,2,x \dots} - M_0}{M - M_0}$$

where:

D_x = % O_2 /l as related to control,

$V_{1,2,x \dots}$ = O_2 /l in experimented variant,

M_0 = O_2 /l in sea water without algae,

M = O_2 /l in sea water with algae,

modifications of the photosynthetic process were obtained as presented in tables 6 and 7.

The shock induced by the tested products shows specificity as a function of the organism. Oxygen consume is Skeletonema is much intensive in the presence of $N-NH_4$ than of methanol; in Nitzschia, on the contrary, the most intense effect is produced by methanol. Moreover $N-NH_4$ concentrations of 0.1-0.3 mg/l stimulate photosynthesis; its reduction, along with intensifying respiration, takes place only when $N-NH_4$ concentrations exceed the above mentioned limits (Tables 6 and 7).

In both algae, the mixture of the tested compounds showed reduction of photosynthetic activity and respiration intensification in a much higher degree than in case of separately testing of each compound.

The comparing of pH modifications with those of photosynthesis shows that by changing the reaction of the medium the photosynthesis is reduced in the alga and their O_2 consumption by respiration is intensified, which explains the mechanism of pH re-establishment. Therefore, even if the algae do not survive the toxic concentrations of the experimented compounds, they exert a pH re-establishing effect on the medium.

For observing the metabolisation process of the experi-

Table 6

Modifications of the photosynthetic process in the alga *Skeletonema costatum* during the initial 24 hours of experiment (intensification or reduction of photosynthesis in % O_2/l as related to control = D_x)

Experimented variants					
Methanol		Ammonia		Mixture	
Conc. (mg/l)	D_x	Conc. (mg/l)	D_x	Conc. (mg/l)	D_x
0.34	- 32	0.1	- 89	0.34/0.1	- 103
0.85	- 129	0.3	- 289	0.85/0.3	- 450
1.70	- 153	0.7	- 326	1.70/0.7	- 465
6.80	- 426	3.5	- 371	6.80/3.5	- 478
17.00	- 471	7.0	- 392	17.00/7.0	- 498

Table 7

Modifications of the photosynthetic process in the alga *Nitzschia tenuirostris* during the initial 24 hours of experiment (intensification or reduction of photosynthesis in % mg O_2/l as related to control = D_x)

Experimented variants					
Methanol		Ammonia		Mixture	
Conc. (mg/l)	D_x	Conc. (mg/l)	D_x	Conc. (mg/l)	D_x
0.34	- 39	0.1	+ 141	0.34/0.1	- 130
0.85	- 134	0.3	+ 18	0.85/0.3	- 171
1.70	- 139	0.7	- 95	1.70/0.7	- 185
6.80	- 254	3.5	- 131	6.80/3.5	- 265
17.00	- 273	7.0	- 148	17.00/7.0	- 316
34.00	- 292	10.0	- 249	34.00/10.0	- 323

mented compounds, the alga *Chlamydomonas* sp. was chosen, as its growth showed the best stimulation by the waste waters.

The activation of the respiration process implies intense consumption of the energetic substratum. P-PO₄ dosage from the medium indicated its complete absorption after 24 hours of experiment. The recover of the energetic reserve is marked by the starting moment of the cellular division, during which the needs for nutrients are intensified. After 24 hours of observation the cellular division log phase starts and concurrently, the absorption of the trophic elements (Fig. 7-9 and Table 8).

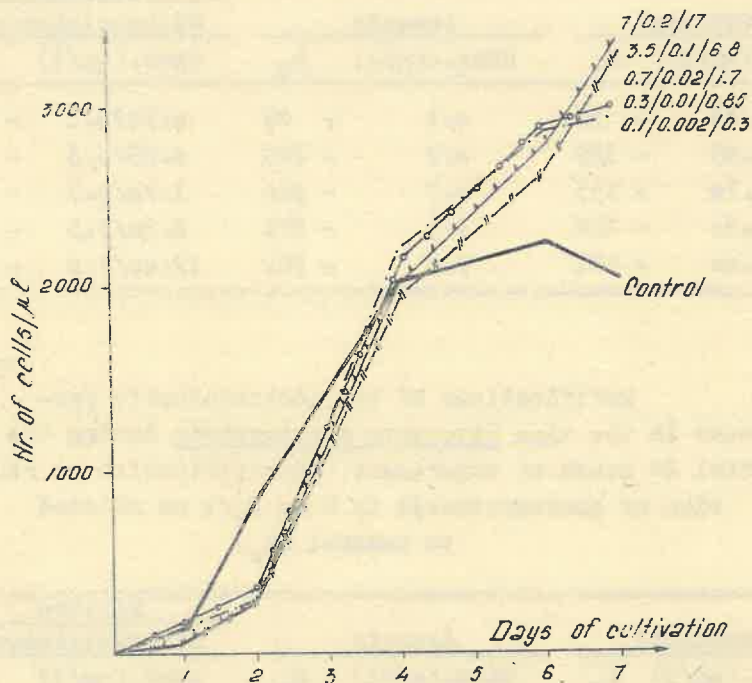


Fig. 9 - The development of *Chlamydomonas* sp. under the influence of waste waters charged with ammonia, urea and methanol (mg N-NH₄/mg urea/mg methanol %; Control: sterilized sea water)

The following of the N-NH₄ dynamics pointed out the possibility of its total elimination from the medium after four days of cultivation when tested concentrations reached 3.2 and 6.8 mg/l (Table 8). Take into account the N-NO₂ and N-NO₃ utilization both in control and in the experimented variants, it could be inf-

Table 8.

Utilization of ammoniacal nitrogen, nitrates and nitrites by the alga Chlamydomonas sp. ($\mu\text{g/l}$)

Variants	N - NH_4				N - NO_3				N - NO_2			
	Initial	1st day	2nd day	4th day	Initial	1st day	2nd day	4th day	Initial	1st day	2nd day	4th day
Ammonia	3200	2800	2500	0	758,5	671,6	667	513,7	7,49	8,20	16,20	8,76
Ammonia	6800	2600	2300	0	"	607,7	605	597,2	"	10,40	11,20	7,42
Mixture Ammonia Methanol (6,5 mg/l)	3400	1800	1400	0	"	645,1	616,7	543,6	"	10,03	17,10	16,4
Mixture Ammonia Methanol (16,6 mg/l)	6700	2300	2300	0	"	696,5	692	670,9	"	9,40	17,90	21
Methanol (6,5 mg/l)	0	0	0	0	"	423,6	20,3	12,7	"	4,50	0	0
Methanol (16,6 mg/l)	0	0	0	0	"	316,8	42,3	40,6	"	3,75	7,5	1,5
Control	0	0	0	0	"	511,8	128,7	23,2	"	10,40	12,7	4,76

ferred that algae have the possibility to oxidize or to reduce the dispoable forms of nitrogen. Hence, the $N-NH_4$ could be transformed in N oxidized forms. But it is possible, too, that $N-NH_4$ should be utilized directly; the quantities of $N-NO_3$ and $N-NO_2$ which remain nearly constant during the initial two days and diminish only together with the exhaustion of ammoniacal nitrogen may be a confirmation for the latter way of utilization.

It was noticed that the presence of methanol in concentration of 6.5 mg/l stimulates the process of $N-NH_4$ removing from the medium.

The methanol is absorbed by Chlamydomonas according to its concentration in the medium as well as the presence or absence of $N-NH_4$. Selecting two variants of optimum growth of the cells - 6.8 and 17 mg/l - it was noticed that methanol utilization in the

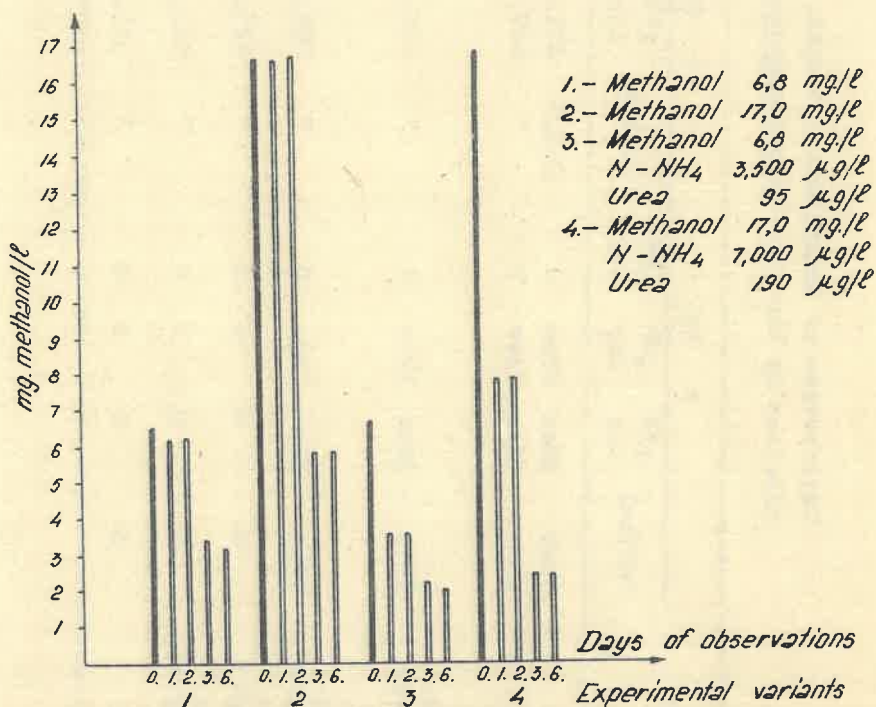


Fig. 10 - Methanol absorption by unicellular alga Chlamydomonas sp. under the influence of waste waters charged with methanol as well as those charged with methanol, ammonia and urea

absence of ammonia occurs after 3 days of cultivation, coming to 50% and respectively, 65% of the initial concentration (Fig. 10). The absorption value is proportional with methanol concentration. In the presence of $N-NH_4$, methanol absorption occurs from the first cultivating day, reaching 50%, and after 3 days of cultivation it exceeds 80% of the initial value.

CONCLUSIONS

1. Methanol and ammonia have a positive synergic action on the process of cellular division when the concentration of the tested compounds is low; the synergism is also valid as regards the toxic effect - a process occurring along with increasing the quantity studied compounds.

2. The values of methanol and ammonia (and urea, implicitly), concentrations which generated a pregnant growth of the algae range within 0.3-34 mg/l methanol, 0.1-7 mg/l $N-NH_4$ (corresponding to 0.002-0.2 mg/l urea) when these products are tested separately, and within 0.3-17 mg/l methanol, 0.1-7 mg/l ammonia (corresponding to 0.002-0.2 mg/l urea) when mixing the two categories of waste water.

3. Certain optimum concentrations of the three mentioned compounds correspond to each of the experimented algae.

4. The algae Chlamydomonas sp. and Skeletonema costatum show special affinity for methanol, tolerating high concentrations of this compound: 0.3-34 mg/l and 0.3-68 mg/l, respectively.

5. Ammonia is utilized within low concentration limits (0.1-7 mg/l), both separately and in mixture. The highest efficiency of $N-NH_4$ utilization occurred in Nitzschia, which developed cellular division by 100-200% more intensive than that of the control at concentrations between 0.1 and 0.7 mg/l; the best toleration of $N-NH_4$ was shown by Chlamydomonas sp., which was stimulated by concentrations of 0.1-7 mg/l.

6. The experimented algae have the ability to re-establish the pH of the medium by modifying the photosynthesis/respiration ratio.

7. The synergism of methanol and $N-NH_4$ also appears in the photosynthesis/respiration process, as well as in the ammonia utilization by the algae, but most of all in methanol utilization.

8. In the presence of ammonia, methanol absorption reaches 67.7-85% of the total quantity, as comparing with 50-65%, which is utilized in its absence.

9. Methanol utilization in the presence of $N-NH_4$ occurs after the first day of cultivation, whereas in the absence of $N-NH_4$, the process begins only after 3 days of cultivation.

10. In an environment where the total quantity of organic substance is steadily increased, it is possible to assume that one of its ways of utilization is through the primary decomposition products as $N-NH_4$, urea, and methanol.

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