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Changes of Bacterial Community Structure in Experimental Ponds after Contamination of Phosphorus and Cadmium

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Abstract: Features of the structure of bacterioplankton and bacteriobenthos were investigated in seasonal dynamics in experimental ponds with loads of phosphates and cadmium. Adding of cadmium at the background of phosphates decreased the number of cells of bacterioplankton containing polyphosphates and percent polyphosphate-accumulating bacteria in the bacteriobenthos. Based on the known mechanisms for failure of polyphosphates in the presence of heavy metals, it has been suggested that the polyphosphates accumulated by cells of bacterioplankton and bacteriobenthos were used for detoxification of cadmium after its inclusion in the experimental ponds. In open experimental pond, the load for cadmium at the background of phosphates caused a major change in the composition of bacterioplankton. Probably in an overgrown experimental pond, the main role in the accumulation of cadmium belonged to higher aquatic plants. It was found that higher aquatic plants had a strong impact on the complex structure of bacterioplankton and bacteriobenthos. Due to the presence of cattail in experimental ponds in conditions of declining of trophic ability, we detected a quite serious changes directed towards increasing the proportion of polyphosphate-accumulating bacteria in the structure of bacterial communities.

Key words: Bacterial community · Experimental ponds · Phosphorus and cadmium

INTRODUCTION

Bacteria resistant to heavy metal ions are widely distributed in nature. Their strains were isolated from industrial wastewater, soil and waters contaminated with heavy metals [1-4].

Despite the fact that heavy metals such as cadmium normally toxic even at low concentrations [5, 6], microbes possess a spectrum of mechanisms of resistance to them, these mechanisms prevent ingress of metal or promote active cell their removal from the cell. One such mechanism is intracellular chelation of heavy metals by polyphosphates [7-13].

Polyphosphates are linear polymers containing from tens to hundreds of orthophosphate residues associated with high-energetic phospho-anhydride bonds. The intracellular polyphosphates mediate the ability of prokaryotic organisms to survive during starvation, these compounds are able to replace ATP in kinase reaction and act as metal chelators. They are also associated with a number of important microbiological processes such as motility, biofilm formation, competence and virulence [13-18].

It has been found that bacteria accumulate considerably more heavy metals during growth on a medium rich in phosphate than during growth on low phosphate medium [7, 19]. A number of scientific studies have established that polyphosphate granules synthesized in the bacterial cells are destroyed during the growth of bacteria in the presence of metals [8, 10, 12, 20]. It was found that not only the high intracellular content of polyphosphate granules but mainly, the ability of prokaryotes to synthesize and destroy polyphosphates is important for the tolerance to heavy metals [8]. A model was proposed for detoxification of heavy metals, according to which metal ions stimulate polyphosphate hydrolysis. In turn, phosphates bind metals to insoluble metal-phosphates complexes, which are transported from the cells and precipitated on their surface [9, 21, 22].

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It is known that not only the bacteria are involved in the accumulation of heavy metals. Macrophytes also have the ability to accumulate heavy metals [23]. It has been established that the interactions between macrophytes and rhizosphere bacteria are very important in the process of absorption of heavy metals, particularly cadmium by plants [24]. Rhizospheric bacteria may enhance the absorption of heavy metals by plants by stimulating the metal-transport proteins and increase of root biomass of plants. Addition of heavy metals can also change the species composition of the rhizosphere microflora [24].

By this reason, investigations of the mechanisms of interactions between rhizosphere microorganisms and macrophytes, as well as an understanding of how environmental conditions affect the natural metabolism of polyphosphates, are becoming increasingly important.

MATERIALS AND METHODS

The studies were conducted in experimental ponds, including 5-cm layer of soil, natural water capacity of 30 liters with accompanying hydrobionts clumps of narrow-leaved cattail (*Typha angustipholia*) from Sredniy Kaban Lake. It is located on the territory of the Republic of Tatarstan, Kazan, Russia. We simulated two types of experimental ponds - overgrown (with narrow-leaved cattail) and open (no cattail).

Mineral phosphorus KH_2PO_4 at a concentration of 10 mg / l and inorganic phosphorus in the same concentration and cadmium as $Cd(NO_3)_2$ at a concentration of 0.25 mg / l were added once to experimental ponds. Cadmium, separately of phosphate, was added to the same experimental ponds, but data for these studies are not shown, due to the fact that the introduction of cadmium led to a drastic decrease in cell size of bacterioplankton therefore it was not possible to determine the amount of polyphosphate-accumulating bacteria.

Research was conducted in seasonal dynamics from June to October. Water and soil samples were taken to determine the total number of bacteria, the number of polyphosphate-accumulating bacteria (PAB), nitrogen fixers, oligocarbophilic bacteria, autotrophic and heterotrophic nitrifying bacteria.

The total number of bacterioplankton, bacteriobenthos and polyphosphate-accumulating bacteria were determined by direct counting on membrane filters using a microscope MBI-4 [25].

These water samples were placed to sterile flasks and formalin was added to a final concentration of 2%. Soil samples of 1 g were selected from a depth of 0.5-1 cm, placed into sterile vials with distilled water and formalin was added to a final concentration of 2%. For the separation of particles adsorbed on the soil bacteria, we were guided by the methods described by several authors [26, 27]. We simulated the processing conditions for treatment of soil samples using ultrasonic device UZV-1,3TTS 35 kHz ("Sapphire", Russia) for maximal separation of bacteria from soil particles. The suspension was homogenized in an ultrasonic device for 6 minutes, with an interval of 30 seconds. Then it was washed once with distilled water, the necessary volume was selected and the necessary dilutions with distilled water were carried out. All the water and soil samples were filtered through filters Vladipor (Russia), with a pore size of 0.2 microns.

After that, the filters were painted over with toluidine blue for obtaining of metachromatic color of volutin (polyphosphate) granules [28], a total number of bacteria and volutin-containing bacteria were calculated. Each filter with over 500 cells in 10 visual fields was analyzed.

We evaluated the state of hydroecosystems in experimental ponds using the total number of bacterioplankton according to nomenclature of Roshydromet (Table 1) [29].

All other groups of microorganisms were determined by plating on selective growth media. Nitrogen-fixing bacteria determined in Ashby's medium (per 1 liter of distilled water): K_2HPO_4 0.2 g, $MgSO_4$ 0,2 g, NaCl 0,2 g, KH_2PO_4 0.1 g, $CaCO_3$ 5 g, mannitol 20 g, agar 20 g [30]. To determine oligocarbophilic bacteria, we used Gorbenko medium of the following composition (per 1 liter of distilled water): 0.5 g of nutrient agar, agar-agar, 13.5 g. To identify autotrophic nitrifying bacteria (ammonium-oxidizing bacteria) we used Vinogradski medium (per 1 liter of distilled water): $(NH_4)_2SO_4$ 2 g, 1 g K_2HPO_4, $MgSO_4$ 0,5 g, NaCl 2 g, $FeSO_4$ 0,4 g, $CaCO_3$ 5 g [31], the presence of nitrite in the medium was determined by Griess reagent. For the quantitative determination of nitrifying bacteria we used a table of McCready. To determine heterotrophic nitrifying bacteria, we used a medium of the following composition (for 1 liter of distilled water): 3.5 g of nutrient agar, $(NH_4)_2SO_4$ 0,5 g, agar 13.5 g [32], Griess reagent was used as indicator of change of bacterial colonies color to determine the presence of nitrite in the medium.

RESULTS

As a result of our studies, it was found that seasonal changes in the general population as well as in the structure (i.e., the number of separate groups of microorganisms) of bacterioplankton and bacteriobenthos occurred in all the experimental ponds.

So, in the end of June, an increase in the total number of bacterioplankton and bacteriobenthos in all control and experimental ponds was detected. The state of water in open habitats was characterized as "dirty", in overgrown ones - as "polluted" (Fig. 1, Table 1). The total number of bacteriobenthos during this period increased in open habitats on the order of magnitude, but in overgrown – in 2-3 times only (Fig. 2).

In autumn, by the end of the experiment, the total number of bacterioplankton decreased. Water quality in all open experimental ponds was described as "moderately polluted" or "polluted" while in the overgrown ones water quality has improved to the category of "clean" or "pure" - "moderately polluted" (Fig. 1, Table 1). The total number of bacteriobenthos also decreased at the end of the experiment in all open and overgrown experimental ponds (Fig. 2).

That is, the burden on phosphates did not affect the total number of bacterioplankton and bacteriobenthos in the open and overgrown biotope. The load on cadmium at the background of phosphate in the first two days of experiment resulted in a decrease of about half the total number of bacterioplankton in the open experimental pond and bacteriobenthos in the open and overgrown experimental ponds (Fig. 1, 2, indicated by arrows). Then, for five days the number was recovered (Fig. 1, 2).

Polyphosphate-accumulating bacteria (PAB) have evolved in the experimental ponds in conditions of phosphate load as well as phosphate and cadmium (Fig. 3, 4). In late June, there was an increase in their numbers in the bacterioplankton in a few times, but as part of bacteriobenthos an order of magnitude. In autumn, there were changes in a number of polyphosphate-accumulating bacteria, especially in the bacteriobenthos (Fig.3-4).

It should be noted that in the open biotope two days after the introduction of phosphate and cadmium, there was 2-fold decrease in the number of PAB in bacterioplankton (Fig. 3, indicated by arrow). For next five days the number of PAB increased to the original level (Fig.3).

Addition of phosphates and cadmium resulted in an increase in the percent of PAB in bacterioplankton and bacteriobenthos (Fig.5-6).

The increase in the percent of polyphosphate-accumulating bacteriobenthos to 8-11% in all habitats with loads occurred in the first two days of the experiment (Fig. 6). Then, for five days in all habitats with the load for phosphates and cadmium PAB percentage decreased to 5% (Fig. 6, arrows) and in habitats with a load just for phosphates remained at the same level. In late June, the percentage of the PAB in the bacteriobenthos in open habitats decreased to 4.2% while in the overgrown remains at the level of 10-14%, which is probably due to the higher trophic status (Fig. 1, 2,6) of open habitats compared to overgrown [33] in this period.

In Autumn, in overgrown and open experimental ponds with loads there was the highest percentage of the PAB in the bacterioplankton and bacteriobenthos - 23% and 10-16% on average, respectively (Fig. 5, 6).

We were unable to identify any patterns in the seasonal changes in the number of nitrogen-fixing bacteria, oligocarbophilic bacteria, autotrophic and heterotrophic nitrifying bacteria revealed under the influence of loads (Table 2, 3). This is not consistent with the results of several authors, according to which these groups of bacteria are the most sensitive to contamination by heavy metals [3] (Oliveira and Pampulha, 2006; Nweke *et al*., 2007; Park and Ely, 2008).

Table 1: Classifier of water quality by Roshydromet on bacterioplankton (Roshydromet, 1992).

Class of water quality	Degree of water purity	Total number of bacteria, 10^6 cell/ml	Number of saprophytic bacteria,10^3 cell/ml	The ratio of the total number of bacteria to saprophytic bacteria
I	Very pure	<0,5	<0,5	$>10^3$
II	Pure	0,5-1,0	0,5-5,0	$>10^3$
III	Slightly polluted	1,1-3,0	5,1-10,0	10^3-10^2
IV	Polluted	3,1-5,0	10,1-50,0	$<10^2$
V	Dirty	5,1-10,0	50,1-100,0	$<10^2$
VI	Very dirty	>10	>100	$<10^2$

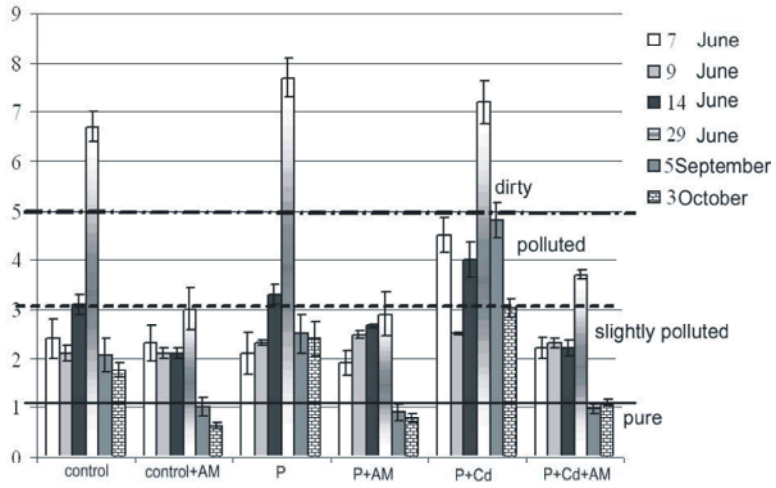

Fig. 1: Total number of bacterioplankton (cell/ml x 10^6, axis Y). Class of water quality is presented according to Roshydromet. P – mineral phosphorus, AM – aquatic macrophytes.

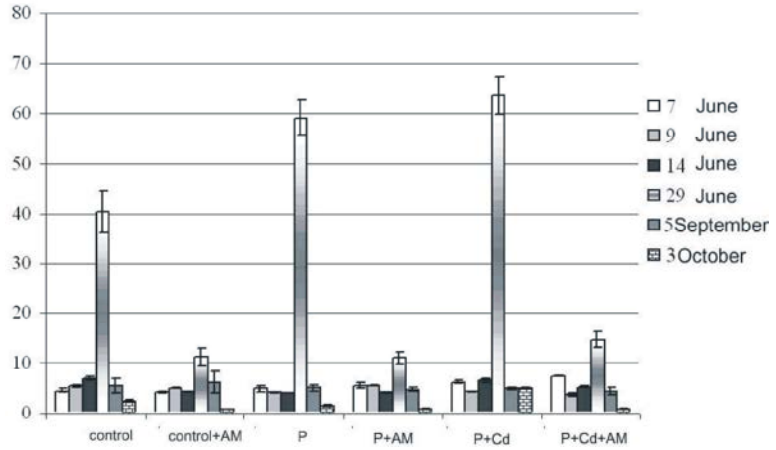

Fig. 2: Total number of bacteriobenthos (cell/g x 10^8, axis Y). P – mineral phosphorus, AM – aquatic macrophytes.

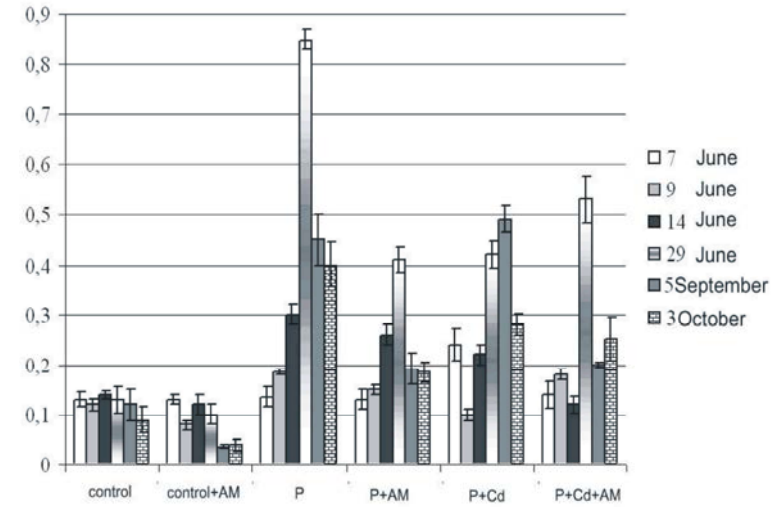

Fig. 3: Total number of polyphosphate-accumulating bacteria with volutin (cell/ml x 10^6, axis Y). P – mineral phosphorus, AM – aquatic macrophytes.

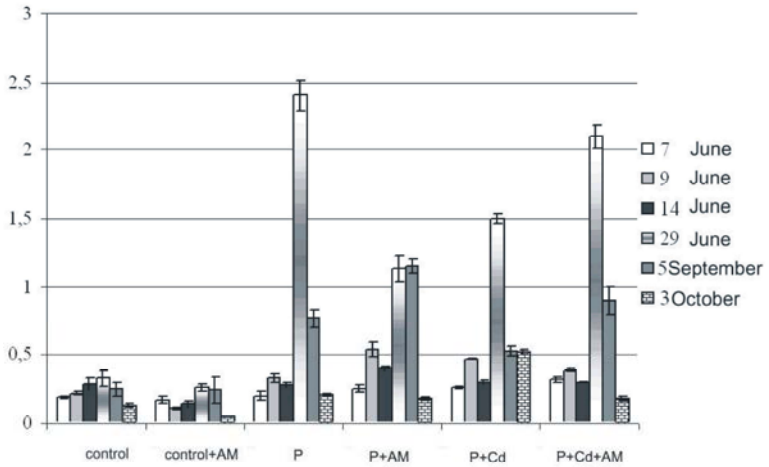

Fig. 4: Total number of polyphosphate-accumulating bacteria with volutin in soil (cell/g x 10^8, axis Y). P – mineral phosphorus, AM – aquatic macrophytes.

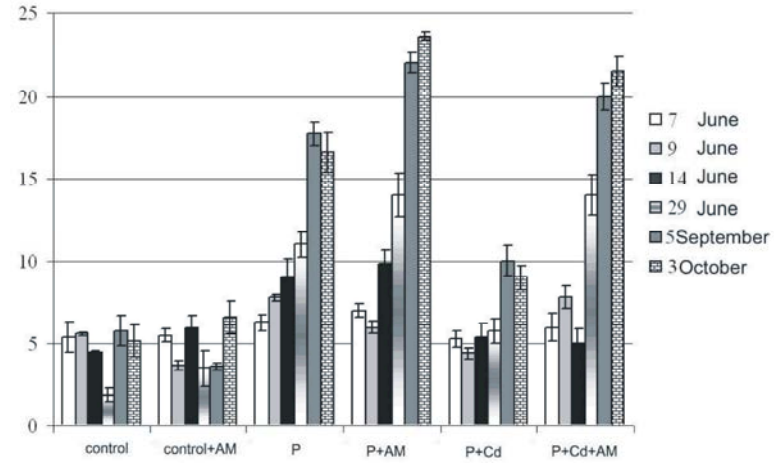

Fig. 5: Percentage of polyphosphate-accumulating bacteria with volutin (axis Y) of total number of bacterioplankton. P – mineral phosphorus, AM – aquatic macrophytes.

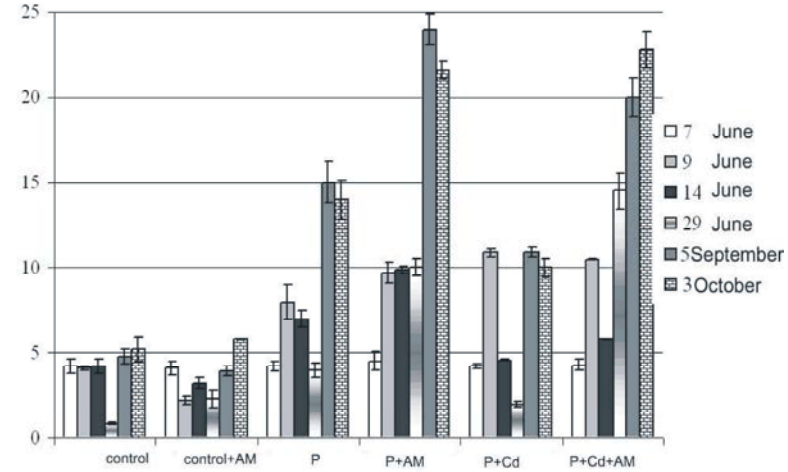

Fig. 6: Percentage of polyphosphate-accumulating bacteria with volutin (axis Y) of total number of bacteriobenthos. P – mineral phosphorus, AM – aquatic macrophytes.

Table 2: The number of microorganisms of individual trophic groups of bacterioplankton x1000 cells / ml

	Control	Control + AM	P	P+AM	Cd+ P	Cd+ P+ AM
Nitrogen-fixing bacteria						
7 June	0,1±0,00	20±2,32	0,1±0,00	10±1,77	1±0,00	30±1,32
14 June	0,5±0,00	32±2,36	2,1±0,01	20±0,00	0,7±0,00	20±0,02
29 June	10,3±0,87	7,4±0,02	7±0,01	10±0,15	12,4±0,83	10,4±0,95
5 September	7±0,17	15±1,70	0,5±0,00	10±0,31	7±0,11	15±2,11
3 October	23±0,12	17±0,05	15,6±0,81	45±4,12	43±2,66	60±3,64
Nitrifying bacteria*						
7 June	0,6	6	0,6	0,6	0,6	0,6
14 June	6	60	6	0,6	60	6
29 June	6	6	60	60	6	6
5 September	60	60	60	60	60	6
3 October	6	60	60	6	60	60
Heterotrophic nitrifying bacteria						
7 June	8,3±0,44	7±0,21	3±0,22	5,8±0,41	6,7±0,58	3,1±0,37
14 June	5,1±0,13	2,3±0,51	1,4±0,11	4,7±0,37	2,1±0,13	2,8±0,46
29 June	1±0,04	1±0,07	0,9±0,09	4±0,25	0,2±0,00	21±1,11
5 September	0,1±0,00	14±1,21	1,6±0,54	3,6±0,23	0,01±0,00	0,1±0,00
3 October	0,1±0,00	0,3±0,00	1±0,00	0,3±0,00	8,4±1,22	0,23±0,00
Oligocarbophilic bacteria						
7 June	8,6±0,02	2,5±0,03	1,2±0,01	4,3±0,02	3,9±0,00	1,8±0,00
14 June	0,4±0,00	3±0,01	0,5±0,00	2,4±0,03	1,4±0,00	4,9±0,55
29 June	2,3±0,05	0,01±0,00	0,01±0,00	0,6±0,00	0,01±0,00	2,8±0,01
5 September	95±4,80	10±0,20	80±6,53	10±0,12	8±0,23	63±6,20
3 October	93±5,40	29±0,90	75±7,32	14±0,93	15±1,01	68±5,42

* – the most probable number of microbial cells

Table 3: The number of microorganisms of individual trophic groups of bacteriobenthos, x1000 cells / g of wet soil

	Control	Control + AM	P	P+AM	Cd+ P	Cd+ P + AM
Nitrogen-fixing bacteria						
7 June	80±3,24	580±23,56	230±22,18	110±9,91	210±11,32	160±10,16
14 June	44±11,24	137±19,23	32±11,23	171±18,93	64±8,45	150±14,56
29 June	53±3,45	490±22,36	10,2±0,56	280±18,52	21±2,36	310±23,46
5 September	63±4,34	460±33,12	90±10,45	330±23,56	70±16,78	480±33,94
3 October	600±36,25	530±22,45	230±33,12	500±42,15	480±22,56	940±12,45
Nitrifying bacteria*						
7 June	0,6	0,6	0,6	6	60	60
14 June	6	6	60	60	60	6
29 June	600	600	60	600	60	600
5 September	600	60	600	600	60	60
3 October	60	600	600	600	600	600
Heterotrophic nitrifying bacteria						
7 June	50±5,61	420±12,22	220±10,45	40±8,75	310±14,52	270±22,14
14 June	24±2,45	207±12,45	104±16,24	26±8,75	174±23,56	128±23,46
29 June	1±0,00	1±0,00	1±0,02	12±1,45	17±0,97	6±0,00
5 September	18±1,40	30±2,56	37±5,16	28±7,33	58±8,39	90±9,23
3 October	1±0,00	4,2±0,11	20±1,56	4±0,00	10±0,32	8±0,04
Oligocarbophilic bacteria						
7 June	11±0,23	128±12,36	34±9,36	161±23,42	51±9,86	62±9,23
14 June	8±0,07	150±9,34	35±2,35	26±5,63	132±10,45	73±3,46
29 June	1,9±0,00	8±0,45	1±0,00	4,6±0,12	1,9±0,02	21±0,78
5 September	100±17,13	200±22,12	125±9,21	300±16,23	320±22,11	100±15,63
3 October	140±23,56	370±31,23	191±25,23	112±14,56	102±12,45	317±26,33

* – the most probable number of microbial cells.

Table 4: The concentration of cadmium, phosphates and total phosphorus in the experimental ponds

	Cd concentrations, mg/dm^3						Phosphate concentrations, mg/dm^3						Total phosphorus concentratuions, mg/dm^3					
	control	Control +AM	P	P+ AM	Cd +P	Cd +P + AM	control	Control +AM	P	P+ AM	Cd +P	Cd +P + AM	control	Control +AM	P	P+ AM	Cd +P	Cd+ P+ AM
7 June	0,001	0,001	0,001	0,001	0,001	0,001	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,04	0,04
9 June	0,001	0,001	-	-	0,058	0,053	0,05	0,05	23,7	24,8	23,9	23,4	0,04	0,04	7,25	7,97	7,59	7,42
14 èþíÿ	0,001	0,001	-	-	0,008	0,012	0,05	0,05	8,21	7,95	8,95	9,99	0,04	0,04	2,58	2,77	3,16	3,62
29 June	0,001	0,001	-	-	0,003	0,0017	0,05	0,05	2,4	1,27	3,79	2,84	0,04	0,04	0,875	0,43	1,2	0,9
5 September	0,001	0,001	-	-	0,001	0,001	0,05	0,05	0,05	0,05	0,309	0,05	0,047	0,049	0,052	0,04	0,186	0,049
3 October	0,001	0,001	-	-	0,001	0,001	0,05	0,05	0,05	0,05	0,05	0,05	0,04	0,04	0,04	0,04	0,048	0,04

All of the control and experimental habitats displayed the same seasonal changes in the number of these groups of bacteria. At the end of June (during a period of eutrophication), there was a decrease in number of oligocarbophilic bacteria in all experimental ponds. In autumn their numbers have increased by 1-3 orders of magnitude in the bacterioplankton and bacteriobenthos. Also in the autumn in all experimental and control ponds a number of heterotrophic nitrifying bacteria decreased, while the number of autotrophic nitrifying bacteria increased by 1-3 orders of magnitude in the bacterioplankton and bacteriobenthos, the number of nitrogen-fixing bacteria also increased (Table 2, 3). That is, by the end of the observation period, the number of nitrogen-fixing, oligocarbophilic and nitrifying bacteria, as well as their percentage in the bacterioplankton and bacteriobenthos was increased; also, we detected increasing the percentage of polyphosphate-accumulating bacteria. A positive correlation was found between the two groups of bacteria in all habitats with loads.

In the open experimental pond with loads for phosphates and cadmium we observed a negative correlation between the concentration of cadmium and a number of indicators, namely the total number of bacterioplankton, number and percentage of polyphosphate-accumulating bacteria.

In all habitats with loads, a number of phosphates, total phosphorus and cadmium decreased rapidly. It was established that in the end of June, the concentration of phosphates and cadmium in overgrown habitats were slightly lower than in the open ones. In Autumn, their concentrations in all habitats with the load reached a level of controls (Table 4).

DISCUSSION

First of all, it is worth noting that in conditions of the load on mineral phosphorus, the number of polyphosphate-accumulating bacteria in the bacterioplankton and bacteriobenthos was increased, which is consistent with previous studies [37, 38].

Load for cadmium significantly more influenced the structure of bacterioplankton in open experimental ponds than in overgrown ones, as only for the open habitat we identified a negative correlation between the concentration of cadmium, the total number of bacterioplankton and the number of polyphosphate-accumulating bacteria.

We believe that the observed reduction in the number of bacteria containing granules of volutin (Fig. 3) in experimental open pond in the first two days after addition of cadmium is the result of the preemptive use of their energy for activation of hydrolysis of polyphosphates in bacterioplankton cells to eliminate toxic effects of cadmium. As it was mentioned above, metal ions entering the cell, are able to stimulate the hydrolysis of the polyphosphate, resulting in formation of insoluble metal-phosphate complexes, which are transported out of the cell and are deposited on the surface of the cell wall of bacteria [9, 12, 13, 22]. Well soluble toxic form of cadmium nitrate owing to bacterial polyphosphates, becomes to insoluble complex of cadmium phosphate, which is deposited on the surface of the bacterial cell wall and then gradually settles in the benthos with bacterioplankton cells.

The observed decrease in the percentage polyphosphate-accumulating bacteria in the bacteriobenthos of open and overgrown experimental ponds during the first week after the introduction of cadmium, is also associated with the use of intracellular polyphosphate for detoxifying of accumulated cadmium ions (Fig. 6). As for the open habitat we detected a very low percentage of polyphosphate-accumulating bacteria in the bacterioplankton and bacteriobenthos that even during Autumn did not exceed 10-10,9% (Fig. 5, 6).

It should be noted that the concentration of cadmium decreased more rapidly in the experimental pond in the presence of higher aquatic macrophytes. Probably in the overgrown biotope, the major role in the accumulation of cadmium belonged to cattail, which is able to accumulate heavy metals in their biomass.

In addition, in September-October, there were significant changes in the structure of bacterial communities. First, there was a decrease of trophic status of hydroecosystems in experimental ponds, as evidenced by the decrease in the total number of bacterioplankton and bacteriobenthos (Fig. 1, 2). Reducing the number of heterotrophic nitrifying bacteria and an increase in autotrophic nitrifying bacteria and oligocarbophiles by the end of the observation period was considered as an indirect measure of reducing the concentration of easily decomposed organic compounds in hydroecosystems (Table 2-3). Second, there was an increase in the percentage of PAB within bacterial communities (Fig. 3, 4), which is consistent with our recent studies [37, 38].

That is, in conditions of declining of trophic status of experimental pond's hydroecosystems and low concentrations of biogenic elements, oligocarbophilic bacteria have evolved and started to play an important role in the accumulation of polyphosphate, which was also indicated by a high correlation between the percentage of PAB and oligocarbophiles. We found a positive correlation between the number of autotrophic nitrifying bacteria and PAB that suggests on the favorable conditions, including a sufficient amount of dissolved oxygen in the habitats, to ensure that these processes occur simultaneously.

Higher aquatic macrophytes, as in the previous model experiments [37, 38] has provided a strong complex effect on the size and structure of bacterial communities. In experimental ponds with aquatic macrophytes, in conditions of declining of trophic status of hydroecosystems, there were significant changes in the structure of bacterial communities, geared toward increasing the proportion of polyphosphate-accumulating bacteria. The ability of bacterioplankton and bacteriobenthos to accumulate and retain phosphorus in the ecosystem for planktonic and benthic communities was most pronounced in the experimental ponds with cattails.

In conclusion it should be noted that the study of features of polyphosphate metabolism in bacterial cells under the influence of environmental conditions, will greatly improve the existing biological processes for the purification and methods of indication of contaminated hydroecosystems.

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