Accumulation of bacteriophage T4 by bivalves *Unio pictorum* and *Anodonta cygnea* in model experiments

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Abstract. Bivalve mollusks are filter feeders that accumulate various particles suspended in water, including viruses. Shellfish also accumulate bacteriophages, but, unfortunately, there is little information about this, despite the fact that these organisms make a significant contribution to the ecology of aquatic communities of living organisms and can be used as a good object for biomonitoring. This knowledge gap prompted us to study in more detail the bioaccumulation of phages by mollusks. As a result of the research, it was revealed that bacteriophages, despite differences in species of mollusk, show a similar bioaccumulation model, where their titer depends on the activity of the mollusk and changes in cycles. The bacteriophage multiplies due to the natural microflora of the animal and can be retained by it due to its filtration type of nutrition.

1 Introduction

Bioaccumulation is the process of accumulation of substances and particles from the environment in living organisms, in which the organism absorbs this substance faster than it is released from it [1]. *Bivalvia* (type *Mollusca*) or bivalve mollusks (Linnaeus, 1758) are filter feeders. This feature allows them to accumulate various particles suspended in water, including virus virions [2, 3]. Due to this, such mollusks are already used in environmental monitoring to detect chemical and biological pollution [4]. For example, they are used to monitor the presence of *E. coli* and for the detection of water pollution in general. In addition, bivalve mollusks are consumed by humans, which only increases the attention of scientists to these organisms [5]. Quite often, shellfish are eaten raw, which increases the risk of infection with various intestinal pathogens, and the overall globalization of the food industry leads to the rapid spread of these infections [6]. Filter type of nutrition clams can induce outbreaks of certain diseases. They retain various pathogenic viruses, such as norovirus, adenovirus and hepatitis A virus [7].

Shellfish also accumulate bacterial viruses (bacteriophages), but, unfortunately, there is little information about this. However, they can be a good indicator for monitoring infections. They are already used to assess the efficiency of wastewater treatment plants, because their number can correlate with the number of eukaryotic viruses [8]. Bacteriophages used in the assessment of sanitary quality of recreational waters [9]. The use of bacteriophages is included in the Russian rules for assessing water safety. For example, SanPiN 2.1.4.1074-01 includes a requirement for mandatory water analysis for the content of coliphages [10]. The most suitable bacteriophages for tracking pathogens that cause gastrointestinal diseases are those that infect *E. coli* and other coliform bacteria. According to research, phages of *Straboviridae* and *Siphoviridae* families (order *Caudovirales*) are most often found in wastewater, including *Escherichia virus* T4 (family *Straboviridae*) [11, 12]. There is evidence that bacteriophages can be used for food control because they persist longer in food samples than bacteria [13].

The role of bacteriophages in the ecosystem cannot be ignored. Bacteriophages influence the abundance of bacteria and their evolution, making a huge contribution to horizontal gene transfer between bacteria [14]. Phages are an important part of the food chain and contribute to the preservation of biodiversity among microorganisms by reducing the population size of the most numerous and fastest-growing bacteria [15]. They also take part in the cycle of various substances, such as nitrogen and carbon. [16]. Among other things, phages affect the microflora of mollusks by parasitizing the host bacteria living in them. Studying the behavior of bacteriophages in aquatic environments and organisms allows us to learn more about the structure and life cycles of these and other viruses, as well as to develop a more accurate picture of their role in the ecological community.

All these factors prompted us to study the bioaccumulation of phages by mollusks in more detail.

2 Materials, organisms, bacteriophages and methods

Unio pictorium (Linnaeus, 1758) and *Anodonta cygnea* (Linnaeus, 1758) were used as laboratory animals (Fig.1) [2]. They belong to family *Unionidae*, order *Unionida*, class *Bivalvia*. *Unio pictorium* measured from 6.8 to 7.5 cm in length, while representatives of *Anodonta cygnea* reached 8.3 – 9 cm. The mollusks were collected in shallow waters in the Oka River near the city of Pushchino from May to August 2023 by A.A. Zimin and N.A Nikulin. The mollusks were kept in conditions close to natural in glass aquariums volume from 15 to 40 liters for 1 to 8 months with dechlorinated tap water, where good aeration was maintained. They were fed with detritus once every two weeks, and before the final experiment, they were abundantly fed with a diluted culture of *Chlorella vulgaris,* included in order - *Chlorellales* family - *Chlorellaceae* genus - *Chlorella* (Beijerinck, 1890) [17]. A chlorella culture for feeding bivalve mollusks was grown in a saline environment using a modified method presented in the article by B. P. Matos [18]. The cultivation was carried out in a 250-liter volume under natural light for 10 hours with the addition of 4 hours of artificial overhead lighting at 4000 lumens per 250 liters of medium. Experiments were conducted using bacteriophage *Escherichia virus* T4. The phage was grown on *E. coli B* strain.

Fig. 1. Temporary maintenance of *Unio pictorium* and *Anodonta cygnea* in an aquarium with a water level of 30 cm. 1 - *Unio pictorium*, 2 - *Anodonta cygnea*, the aquarium is equipped with an air-lift filter (3) and additional aeration (4).

Each of the mollusks was placed in a glass with 200 ml of water from the aquarium where the mollusk previously lived (Fig.2). The water was sterilized to eliminate phages and bacteria by autoclaving under a pressure of 0.05-0.2 MPa. The sterility of the water was checked by inoculation it on LB medium. Aeration of the water was achieved using an air compressor, which recreated the habitual habitat of the studied animal and allowed to distribute bacteriophages evenly in the water. After mollusk's shell was open, a phage suspension was added at an initial concentration in water of $1*10^{3}$ or $1*10^{4}$ PFU/ml in an amount of 2 ml. Water samples (1 ml) were taken immediately after adding the phage suspension, as well as after 1, 3, 6 and 24 hours using a sterile pipette and a sterile tube. Samples were collected 3-fold repetition.

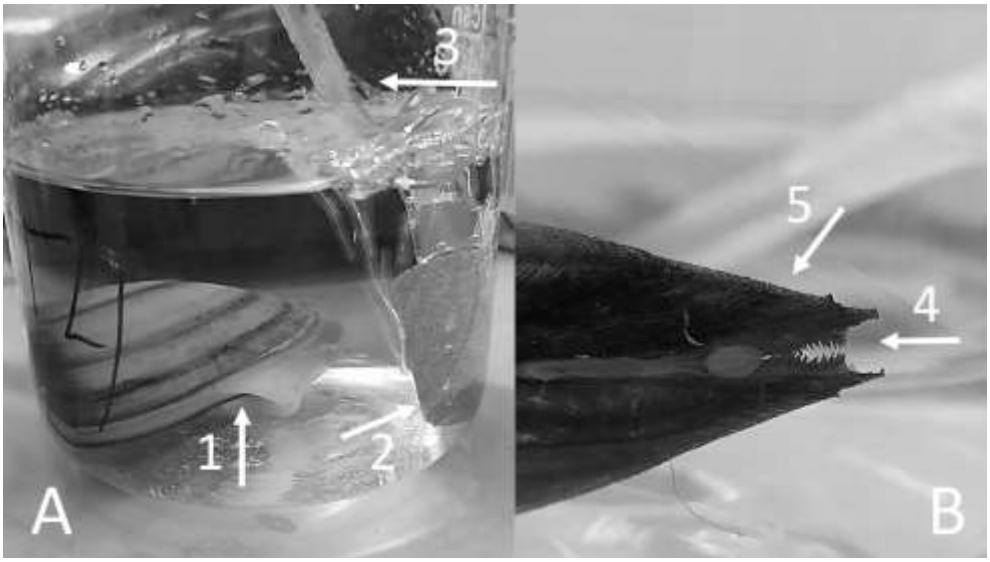

Fig. 2. *Unio pictorium* under experimental conditions 1 - mollusk leg. 2 - air compressor, 3 - beaker, 4 - siphon, 5 - shell. The opening of the siphon by a mollusk indicates favorable conditions for it.

Sterile aquarium water was used as a control, into which a suspension of bacteriophage was added in the same concentration and volume. Samples were taken according to the scheme presented above.

All samples were titrated using the Grazia method, added to LB medium in Petri dishes and cultured overnight at 37C°. Then the resulting plaques were counted, after which the average value for each sample was calculated; these data were used as the basis for the graphs.

3 Results

Based on our results, we noticed several features of bacteriophage bioaccumulation in the mollusk. We observed the a cyclic pattern, where the titer increased after an hour, then after 3 hours it decreased with the same dynamics maintained after 6 and 24 hours from the start of the experiment (Table 1, Fig. 3). We also carried out cultures for the presence of *E. coli*, where we recorded not only its presence, but also some correlation with the experiments.

We associate the change in the bacteriophage titer curve with the fact that mollusk microflora contains with *E. coli*, affecting the titer of bacteriophages, due to which the phage multiplied in the environment. 1 hour after the start of the experiment, the mollusk may release a portion of water and active reproduction of the phage begins. The bacteriophage circulates in waves - this pattern consistent across all experiments. It is likely that the bacteriophage circulation is associated with the activity of the mollusk. After 24 hours, the phage probably stops circulating in the water and is retained by the mollusk. Despite the high level of similarity of experimental results, the phage circulation in animals and the environment may vary between organisms due to the unique natural microflora present in each mollusk. Individual animal activity may also play a role in this variation.

In the control, where phage was added to sterile water from the aquarium, the titer did not change significantly, but decreased towards the end of the experiment. The phage appears to begin to settle after 24 hours, which correlates with the results of experiments with shellfish (Fig. 3).

Table 1. Change in bacteriophage titer over time in one of the experiments using *Unio pictorium* .

Phage	Bacteriophage titer before an experiment	Phage titer in the samples taken in various time intervals after the experiment start PFU/mL				
		0 h	1 h	3 h	6 h	24 h
T4	0	$3.7*10^1$	$1.5*10^2$	$6.3*10^1$	$1.23*10^2$	$6*10^1$

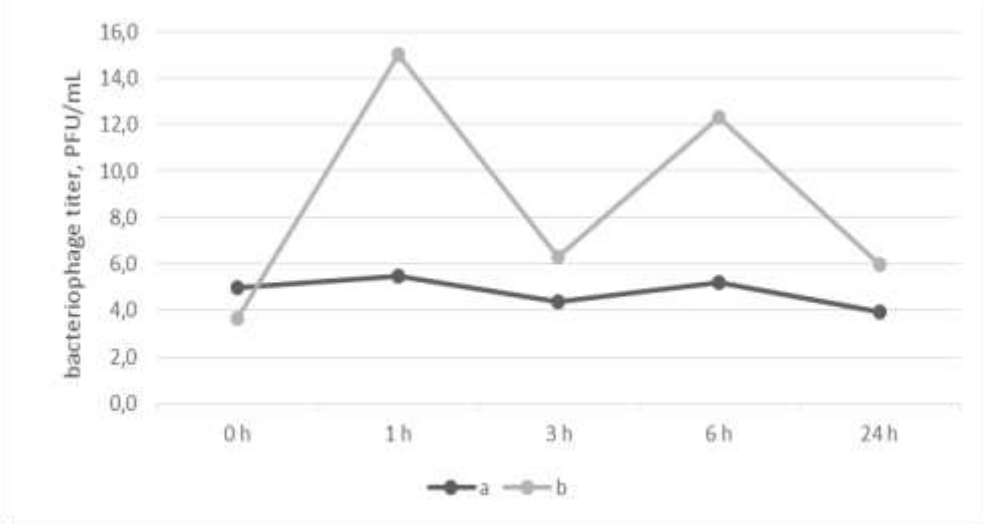

Fig. 3. Change in the titer of bacteriophage T4 depending on time in experiments. a) – with *Unio pictorium*, b) – with sterilized aquarium water.

A result similar to the experiments with *Unio pictorum* was also obtained in experiments with *Anodonta cygnea*. Comparing the results of the experiments, a similar cyclical change in the titer of the bacteriophage depending on time was observed, where a decrease and increase in the titer of the virus alternated. It can be inferred that the bioaccumulation of bacteriophage T4 by different representatives of bivalve class mollusks involves a similar mechanism through which the phage circulates in water and in the mollusks.

The activity of the mollusk can also be influenced by type of a nutrition. As described in section 2, feeding was done with either dendrite or a chlorella culture, which is a more nutritious food for these mollusks. In the first graph, prominent cycles are evident: the titer increased after an hour, then after 3 hours it decreased, after 6 and 24 hours from the start of the experiment the same dynamics were maintained (Fig. 4, a). In the second graph, this feature is not so clearly expressed; after an hour, the phage titer increased, then after three hours it decreased, but then only increased slightly by the end of the experiment (Fig. 4, b).

When feeding was done with a diluted culture of *Chlorella vulgaris,* the mollusks showed greater activity.

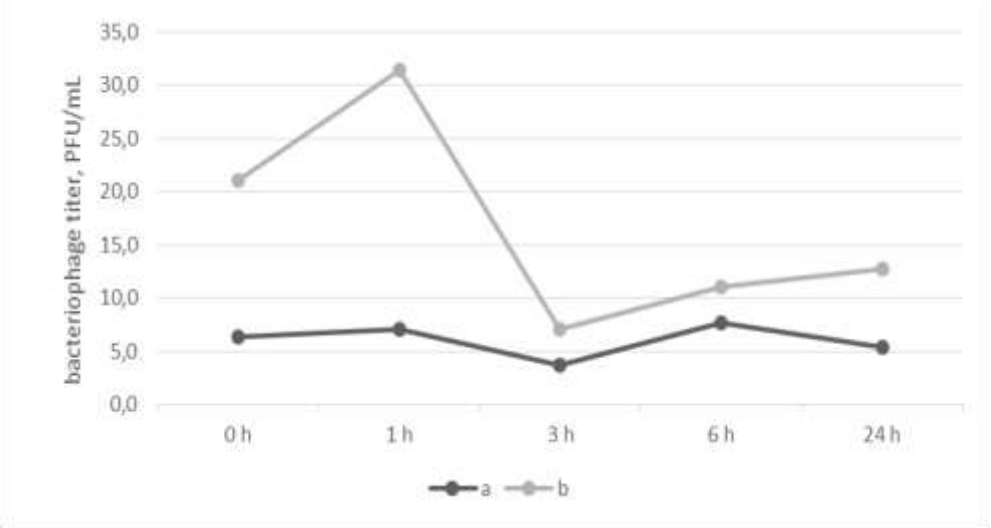

Fig. 4. Dynamics of changes in the titer of bacteriophage T4 depending on the feeding characteristics, affecting the activity of *Unio pictorium*. a) – feeding with a diluted culture of *Chlorella vulgaris*, b) - feeding with detritus.

In 2020, we published the first such study in this area , and upon comparison, with it, some similarities can be observed [19]. The phage population multiplied because of *E. coli,* which is contained in the mollusk microflora. Cycles can also be discerned: the titer alternately rises and falls over time. However, some differences between studies can be identified. The phage titer increased after a longer period of time from the start of the experiment (from 3 to 24 hours). There is no increase in titer an hour after the start of the experiment. Cycles are observed, but not as distinctly apparent.

4 Summary

When generalizing all the data we collected, it can be noted that, despite the differences in conditions and animal species, bacteriophages exhibit a similar pattern of bioaccumulation, where their bacteriophage titer depends on the mollusk activity and changes in cycles, representing an alternating increase and decrease in phage titer over time. The bacteriophage multiplies due to natural microflora of the animal and can be retained by it due to its filtration type of nutrition. Differences in results are due to the characteristics of natural microflora and activity of each mollusk. The activity of the animal is also influenced by the feeding specifics - with more nutritious food, mollusks are more active. Bioaccumulation of bacteriophages in different representatives of class of bivalve mollusks has some similar mechanism by which the phage circulates in water and mollusks.

However, to complete the picture, it is necessary to conduct more experiments using other species of bivalve mollusks, as well as experiments that take into account other factors affecting the animal, such as temperature and pH. It is also worth increasing the experimental time and studying bioaccumulation of the phage after 24 hours. It is also possible to introduce other strains of bacteriophages.

The authors express their gratitude for preparing microbiological media and laboratory glassware to U.N. Druchkova, for preparing and transferring the original T4 strain to A.N. Nikulina, and for collecting mollusks to N.A Nikulin.

The research was supported by the Russian Science Foundation grant No. 24-26-00205, https://rscf.ru/project/24-26-00205/

References

1. D.E. Alexander , Geological Magazine, 697–711 http://dx.doi.org/10.1017/S0016756899393328 (1999).
2. C. Linnaeus *Systema naturae per regna tria naturae, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis. Tomus I. Editio decima, reformata*, 645, (Impensis Direct. Laurentii Salvii, Stockholm, 1758).
3. D. Lees International Journal of Food Microbiology, **59**, 81-116 https://doi.org/10.1016/S0168-1605(00)00248-8 (2000).
4. S. Capizzi-Banas et all, Water, **13**, 904 https://doi.org/10.3390/w13070904 (2021).
5. M.D. Sobsey LA Jaykus *Human enteric viruses and depuration of bivalve molluscs*, http://dx.doi.org/10.1201/9781351074810-9 (1991).
6. J.L. Romalde et all, A. Bosch International journal of food microbiology, **74**, 119–130. https://doi.org/10.1016/S0168-1605(01)00744-9 (2002).
7. M. Bellou, P. Kokkinos, A. Vantarakis, Food Environ Virol. **5**, 13-23 https://doi.org/10.1007/s12560-012-9097-6 (2013).
8. J. Mendez et all, J. Water Health **2**, 201–214 https://doi.org/10.1016/j.jviromet.2003.11.013 (2004).
9. J.M. Colford et al, SB Weisberg, Epidemiology **18**, 27–35 https://doi.org/10.1097/01.ede.0000249425.32990.b9 (2007).
10. SanPiN 2.1.4.1074-01. *Drinking water. Hygienic requirements for water quality of centralized drinking water supply systems. Quality control* , 100 (Russian Ministry of Health , Moscow, 2002)
11. M. Muniesa, F. Lucena, J. Jofre, J. Appl. Microbiol. **87**, 402–409 https://doi.org/10.1046/j.1365-2672.1999.00833.x (1999).
12. J. Jofre J. Appl. Microbiol. **106**, 1059–1069 https://doi.org/10.1111/j.1365-2672.2008.03957.x (2009).
13. D. Toribio-Avedillo, A. R. Blanch, M. Muniesa, L. Rodríguez-Rubio, Viruses, **13**, 1089 https://doi.org/10.3390/v13061089 (2021).
14. KEWommack, RR Colwell, Microbiol Mol Biol Rev, **64**, 69-114 https://doi.org/10.1128/mmbr.64.1.69-114.2000 (2000).
15. S. Maslov, K. Sneppen Sci. Rep. **7**, 39642 https://doi.org/10.1103/physreve.96.022412 (2017).
16. N.Y. Ankrah et all , The ISME Journal, **8**, 1089–1100 https://doi.org/10.1038/ismej.2013.216 (2014).
17. M. W. Beijerinck, Bot. Zeitung. **48**, 781–785 (1890).
18. A. P. Matos et all, Brazilian Journal of Chemical Engineering, **35**, 1183-1192 https://dx.doi.org/10.1590/0104-6632.20180354s20170338 (2018).
19. A. Karmanova, A. Zimin, Journal of Physics: Conference Series, **1701,** 12012 https://doi.org/10.1088/1742-6596/1701/1/012012 , eLIBRARY ID: 60732883 EDN: EHNZSR (2020).