Morphology and distribution of phage-like particles in a eutrophic boreal lagoon*

SIGITAS SULCIUS1,*
JUOZAS STANIULIS2
RičARDAS PASKAUŠKAS1,2

1 Coastal Research and Planning Institute, Klaipėda University, H. Manto 84, Klaipėda 92294, Lithuania;
e-mail: sigas@corpi.ku.lt
*corresponding author

2 Institute of Botany, Nature Research Centre, Žaliųjų ežerų 49, Vilnius 2021, Lithuania

Received 9 February 2011, revised 25 March 2011, accepted 28 April 2011.

Abstract

In this paper we present the results of direct observations of the morphology and size of phage-like particles by means of transmission electron microscopy (TEM) as a function of their spatial distribution in the shallow highly productive Curonian Lagoon of the Baltic Sea. In total, 26 morphologically different forms of phage-like particles were found. Different trends of distribution in terms of abundance, size and shape of virus-like particles were demonstrated. The total abundance of viruses varied from 1.91 \times 10^7 \text{ ml}^{-1} to 5.06 \times 10^7 \text{ ml}^{-1}. The virus to bacteria ratio (VBR) changed from 15.6 to 49 and was negatively associated with total bacterial numbers (r = −0.60; p < 0.05). The phages of family Myoviridae were the most diverse and were dominant at all stations.

* This study was funded by a grant (No. T-66/05) from the Lithuanian State Sciences and Studies Foundation.

The complete text of the paper is available at http://www.iopan.gda.pl/oceanologia/
1. Introduction

Electron microscopy remains a prime instrument in phage ecology studies of most unexplored aquatic ecosystems (Pearce & Wilson 2003, Drucker & Dutova 2006). Morphological investigations of virioplankton range from descriptions of new phages to illustrations of the distribution of biodiversity (Ackermann 2001, Castberg et al. 2002). Despite the advantages of relatively new approaches such as epifluorescence microscopy (Noble & Fuhrman 1998) and flow cytometry (Brussaard et al. 2000), the application of transmission electron microscopy (TEM) in virioplankton studies allows more accurate information about virus morphology and size distribution to be obtained (Børsheim et al. 1990).

The taxonomic structuring of phage-like particles has been proposed by several authors (Bradley 1967, Ackermann & Eisenstark 1974, Wichels et al. 1998) and approved by the International Committee on Taxonomy of Viruses (ICTV). Studies with the aim of grouping viruses into size classes have shown that morphological types of viruses are distributed widely in different pelagic ecosystems (Weinbauer 2004). The vast majority of phages belong to the order Caudovirales and have a broad range of isometric heads varying from 20 to 200 nm, with the 30–60 nm size class phages dominant in marine (Wommack et al. 1992) and 60–90 nm phages prevalent in fluvial and lacustrine ecosystems (Mathias et al. 1995, Drucker & Dutova 2006).

Recent studies, particularly in unexplored aquatic areas, lack morphological analyses of viruses. Molecular analyses and virus genome sequencing are often used in virus research and identification, but genome size can provide only a rough estimate of the rates of ecological interactions between predator and prey, and synergistic or antagonistic relations among predators (grazers and viruses). The same genome size viruses could possibly exhibit different morphological forms. Holmfeldt et al. (2007) showed two different morphological forms with a very similar genome size. Furthermore, it is still impossible to define the phenotype of a virus solely from its genomic sequence (Büchen-Osmond 2003); meanwhile, TEM analysis may help to distinguish between different phage isolates with a high degree of sequence identity (Jenkins & Hayes 2006). Thus, different phage phenotypes could lead to shifts in virus infection rate, virus burst size or even virus grazing rate. In general, the capsid size of viruses could be a more important criterion in studying microbial predator-prey interactions within a multiple community than the criteria of genome size of viruses, which could be more important on a particular predator-prey occasion. However, genome size analyses (i.e. pulsed field gel electrophoresis) and morphological descriptions of viruses, if used independently, severely underestimate the total diversity.
of the viral community, even though they yield complementary results (Auguet et al. 2006).

The importance of virioplankton studies in eutrophic ecosystems is sustained not only by the assumptions that viruses are the main contributors to bacteria and phytoplankton mortality (Suttle & Chan 1994), but also that they are produced more intensively than in less productive environments (Wilcox & Fuhrman 1994). Despite the recent enhanced interest in the ecology of freshwater viruses (Middelboe et al. 2008), delineation of the distribution of morphological types of phages is still rare. Even less is being done in the coastal freshwater lagoons of the Baltic Sea. No aspects of virus ecology in the Curonian Lagoon have yet been studied. The quantification and a detailed survey of the occurrence of viruses could serve as a proper introductory step for elucidating interactions between viruses and their hosts in these environments. The aim of this paper is to provide patterns of the spatial distribution of abundance, size and morphological diversity of virioplankton in the eutrophic Curonian Lagoon of the Baltic Sea.

2. Material and methods

**Description of the study area and sampling strategy.** The Curonian Lagoon lies along the Baltic coast of Lithuania and the Kaliningrad Region of the Russian Federation. It is a shallow (av. depth 3.7 m), eutrophic, freshwater body typical of the south-eastern coast of the Baltic Sea. The discharge of the River Nemunas in the central part of the lagoon comprises 96% of the average annual runoff. The lagoon is connected to the Baltic Sea in the north by a narrow strait, where seawater intrusion may raise the salinity to 8 PSU (Pustelnikovas 1998). As a result of this salinity intrusion, therefore, the Curonian Lagoon can be divided into two (not strictly delimited) parts, where the community structure follows the fluctuation in seawater inflows (Gasiūnaitė 2000). Salinity, wind direction and variations in hydraulic forcing are considered to be very important factors for the succession of plankton communities in the Curonian Lagoon (Pilkaitytė & Razinkovas 2006, Ferrarin et al. 2008).

A number of studies have been performed in the Curonian Lagoon over the past two decades (Gasiūnaitė et al. 2008). They have revealed various patterns of distribution, functions and interactions among different groups of plankton under various environmental conditions, where cyanobacteria blooms usually occur from June to the end of October (Pilkaitytė 2007) and phytoplankton production depends on the extent of the sea water intrusion during midsummer. Our study was performed at 13 sites (Figure 1) in the Lithuanian part of the Curonian Lagoon during a two-day cruise at the end of July 2005. Samples were collected from the surface water (0.5 m depth)
Figure 1. Scheme of sampling stations along the Lithuanian part of the Curonian Lagoon. Densely populated areas are coloured grey

with a Ruttner collector and treated according to standard requirements. Physicochemical parameters, chlorophyll $a$ concentration (representing phytoplankton biomass) and bacteria abundance were determined at each station. Salinity was measured in situ with a WTW MultiLine F/Set 3 portable universal meter; chlorophyll $a$ was extracted with 90% acetone and analysed spectrophotometrically (Jeffrey & Humphrey 1975).

Transmission Electron Microscopy. The material for virioplankton morphological studies (1000 ml) was collected in PE bottles rinsed with water from the study sites and kept cold ($+4^\circ$C) until further processing. In the laboratory the samples were passed through a 0.45 $\mu$m pore size membrane filter to remove larger particles. Viruses were concentrated 200 times by filtration onto Pragopor 11 nitrocellulose filters under vacuum and stored at $+4^\circ$C until analysis. The particles from the filter surface were resuspended by ablution with a new dose (5 ml) of 1% glutaraldehyde aqueous solution. Three microlitres of the concentrated phage stock preparation were placed onto a Formvar-carbon-coated 400-mesh palladium grid and allowed to adsorb on the grid until complete evaporation. The grid was then immediately stained with 1 drop of a 2% (wt/vol) aqueous
uranyl acetate solution for 30 s and blotted with filter paper. At least 10 fields and 200 phage-like particles were examined under a JEOL JEM-100S transmission electron microscope at an accelerating voltage of 60 kV and 10–25,000x instrumental magnification. Different types of particles were recognized on the basis of size, head morphology and tail characteristics (if present) from all the randomly taken micrographs. Estimates of particle abundance were based on a count of the virus-like particles on the calculable area of the screen. This calculation was performed assuming that 0.425 µl of the concentrated solution was applied onto 1 mm² of the grid area. The virus-like particles were counted on the area of the whole EM screen (45.36 cm²). The original volume of the corresponding liquid was calculated by multiplying the picture area and the magnification.

**Epifluorescence Microscopy.** Samples (50 ml) for bacteria abundance were collected in PE bottles and immediately fixed with 0.2-µ-pore-size pre-filtered 37% formaldehyde (to a final concentration of 1%) and stored at −20°C until processing. Direct counts of bacteria were obtained using epifluorescence microscopy (OLYMPUS IX70 with a long-pass (LP) green-emission filter at 488 nm wavelengths to take close-ups at 1000× magnifications) by the examination of at least 10 randomly selected fields per slide, as described in Noble & Fuhrman (1998).

**Statistical Analysis.** Multi-dimensional scaling (MDS) was used for morphological distribution, and cluster analysis for size distribution. Analysis of similarity (ANOSIM) and similarity percentage (SIMPER) tests were used to verify whether all the most similar samples were within the same groups and to identify the contribution of each size group to the observed dissimilarity between samples. The statistical analyses were performed using the PRIMER v5 software package.

3. Results

3.1. Morphological types of phages

All samples contained a mixture of morphologically different phage-like particles, and at least three different morphotypes per sample were found. Filamentous or other morphological types of phages were absent. At least 26 forms of phages could be distinguished by morphological criteria, including the relative proportions of phage head and tail (if present). Many of the phages (Figure 2) had isometric heads and contractile tails and could be assigned to the family *Myoviridae* to be further subdivided into morphotype A1 (icosahedral capsid) and A2 (elongated capsid) according to head shape (see Figures 2c and 2a respectively). Morphotype A3 (a relatively more elongated capsid than A2) was absent
Figure 2. Morphological diversity of phage-like particles observed in the pelagic part of the eutrophic Curonian Lagoon: (a–n; p, ag, ai) different types of Myoviridae; (o, q–w, ae–af, ah, ad) different types of Siphoviridae; (x–ac) different types of Podoviridae; (aa) non-tailed phage-like particle of diameter 200 nm. Scale bar 100 nm

in all samples. Most phages were icosahedral with three symmetrical axes, whereas phages with one symmetrical axis (Figure 2m) were present only in some samples. Bacteriophages with isometric heads and short tails were attributed to the family Podoviridae (e.g. Figure 2y) and constituted the second largest (19%) group of bacteriophages found in the Curonian Lagoon. The spatial distribution of these viruses tended to decrease toward the central (freshwater) part of the lagoon. Only one type (C1, icosahedral capsid; e.g. Figure 2y) of these subgroups was found at all the stations; phage-like particles belonging to subtypes C2 (elongated capsid) or C3 (a relatively more elongated capsid than C2) were not observed. Phages belonging to the Siphoviridae and to subgroups B1 (icosahedral capsid; e.g. Figure 2r) and B3 (elongated capsid; e.g. Figures 2a,d) were also observed and tended to increase toward the central part of the lagoon.

Multi-dimensional scaling (MDS) analysis revealed that the relative distribution of different families was dependent on their location (Figure 3).
Morphology and distribution of phage-like particles...

Figure 3. Multi-dimensional scaling (MDS) analysis revealed different patterns of family distribution, based on the relative differences between all stations, which could be separated into 4 groups. Circle 2 includes ‘offshore’ stations of the lagoon, while circles 1, 3 and 4 include stations located near densely populated areas.

Moreover, stations located at different points on the lagoon showed a different relation to the proportional distribution of families (Table 1).

The dominance of Myoviridae (no less than 65%) was evident at the study sites located near densely populated areas with potentially elevated municipal loads (Figure 1 and Table 1). Analysis of family contributions (SIMPER) to the differences between stations (Figure 3) located closer to populated areas (stations 1 and 2; 4 and 5; 12 and 13) and stations at offshore sites (stations 3; 6–11) showed that the differences between the stations in groups 1 and 2 could be attributed to Siphoviridae (46.9%), those between the stations of groups 2 and 3 to Myoviridae (46.5%), and those between the stations of groups 2 and 4 to Podoviridae (48.2%). The differences between the stations located close to populated areas were related mainly to the distribution of two families in a sample. Podoviridae (47.7%) and Myoviridae (37.9%) contributed mostly to the differences between groups 1 and 3, Siphoviridae (46.4%) and Podoviridae (43.3%) to the differences between groups 1 and 4, and Siphoviridae (46.2%) to the differences between groups 3 and 4. Significant differences were observed between all the groups located close to populated areas and the groups in offshore stations in the lagoon (p < 0.05).

In general, tailed phages made up more than 97% of the total number of phages detected, and long-tail phages were dominant, with tail lengths from 20 nm to 630 nm (Table 1). Phages with isometric heads were more frequent than prolate phages, and phages with contractile tails were more frequent than phages with non-contractile tails.
Table 1. Numerical and relative distribution of virus-like particles, bacteria and chlorophyll a along the Lithuanian part of the Curonian Lagoon. VLP – Virus-like Particles, VA – Virus Abundance [10^6 ml^-1], CS – Capsid Size (± standard deviation), TL – Tail Length (± standard deviation), VBR – Virus-to-bacteria ratio

<table>
<thead>
<tr>
<th>St.</th>
<th>Total VLP VA</th>
<th>CS</th>
<th>TL</th>
<th>Bacteria</th>
<th>VBR</th>
<th>Chl a</th>
<th>Myoviridae</th>
<th>Siphoviridae</th>
<th>Podoviridae</th>
<th>Non-tailed</th>
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<tr>
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<td>107.44±90.74</td>
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<td>114.93±76.35</td>
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<td>22</td>
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<td>110.71</td>
<td>65.09</td>
<td>25.83</td>
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3.2. Size distribution

In earlier reports all phages were considered to form size groups (Bratbak et al. 1990, Cochlan et al. 1993, Mathias et al. 1995, etc.). We placed all the observed phages into 5 size classes (30–60 nm; 60–80 nm; 80–100 nm; 100–120 nm; 120–160 nm), and the relative distribution of these classes was examined at all the study sites. Cluster analysis (75% Bray-Curtis similarity) revealed that all the study sites in the Curonian Lagoon could be divided into three different groups corresponding to size classes (Figure 4) or three zones corresponding to geographical distribution. Group I, which was dominated by the 30–60 nm and 60–80 nm size fractions, covered 4 stations with elevated water salinity recorded at the time of the study, which shows that mixing with different water bodies took place. Groups II and III represented the distribution of capsid sizes in the freshwater part of the lagoon. Group III covered two stations located in the open part of the lagoon and was dominated by the 30–60 nm size fraction (up to 48%). In group II, the 30–60 nm size fraction did not exceed 10%; the group was dominated by 80–100 nm and 100–120 nm capsid size phages. Both the latter size fractions constituted from 48% to 70% per station respectively. Phage-like particles of 200 nm capsid size (Figure 2aa) were found at stations 1, 8 and 11 with respective frequencies of 1, 1 and 2. These phages were not included in the cluster analysis as outliers.

Figure 4. Based on the relative dominance of capsid size classes (30–60 nm; 60–80 nm; 80–100 nm; 100–120 nm; 120–160 nm), all stations form three zones (groups) in the Curonian lagoon

Analysis of size class contributions (SIMPER) to the differences between groups (in Figure 4) revealed that group I (sea water) differed from group II (freshwater) mainly in the 30–60 nm capsid size fraction (57.2%). Differences between the conditionally marine group I and the freshwater
group III were due to 80–100 nm (34.9%) capsid phages. The difference between the two freshwater groups was due to the much higher relative abundance of 30–60 nm size fraction phages in group III (52%). Analysis of similarity (ANOSIM, based on Bray-Curtis similarity) revealed significant differences between groups I and III and between groups II and III (p < 0.05), whereas the differences between groups I and II were not so obvious (p = 0.067).

3.3. Abundance of viruses, bacteria and chlorophyll a content

The total abundance of viruses determined by means of electron microscopy ranged from $1.91 \times 10^7$ ml$^{-1}$ to $5.06 \times 10^7$ ml$^{-1}$ without significant differences (p = 0.15; df 11) between the freshwater and saline zones of the lagoon. In terms of abundance, *Myoviridae* were dominant at all the study sites (Table 1), and the numerical distribution of this family as well as of *Podoviridae* and non-tailed phages between the freshwater and saline parts of the lagoon was insignificant (p > 0.05; df 11). However, the distribution of *Siphoviridae* did differ significantly (p = 0.002; df 11) between the northern and central parts of the lagoon.

The minimum (45.70 µg l$^{-1}$) chlorophyll a concentration was recorded in the saline part of the lagoon, the maximum (186.60 µg l$^{-1}$) in its freshwater part. The differences (p > 0.05) between these zones were random and did not correlate with the total number of viruses, but were positively correlated (r = 0.89; p < 0.001) with the abundance of *Myoviridae*. The total bacterial abundance varied between $0.64 \times 10^6$ ml$^{-1}$ and $1.66 \times 10^6$ ml$^{-1}$ and did not differ between fresh and saline waters either. The virus to bacteria ratio (VBR) varied from 15.6 to 49 at different stations, without a significant increase in the freshwater part of the Curonian Lagoon. However, VBR was negatively correlated with the total number of bacteria (r = −0.60; p < 0.05). It should be noted that only *Podoviridae* were positively correlated (r = 0.57; p = 0.052) with VBR, whereas the total number of phages were not correlated with VBR or the total abundance of bacteria.

4. Discussion

Twenty-six different phages from the Curonian Lagoon are described on the basis of morphological properties. The importance of this phenotypic diversity is of interest not only within a particular aquatic environment or at a particular time but could be useful in considerations of annual shifts of interactions between phages and their hosts and for comparisons between similar environments. There are still no data on the diversity of phage-like particles from other Baltic Sea lagoons. However, the morphology of the members of the *Podoviridae* found in the Curonian Lagoon was similar to
that observed by Wichels et al. (1998) and less diverse than the morphology of the members of the *Myoviridae* and *Siphoviridae*. Most of the phages possessed tails, which suggests that they are not viruses of eukaryotes. However, tailless phage-like particles ca 200 nm in size were found very occasionally at three different sites (1, 8 and 11; Figure 2aa). Sommaruga et al. (1995) described similar phage-like particles with sizes between 195 and 210 nm from a eutrophic water body, suggesting an association between the occurrence of these particles and anthropogenic impact. In our case it was hard to define the occurrence of these large phage-like particles owing to their low frequency of occurrence and distribution throughout the study area. The dominance of the *Myoviridae* at the sites located near densely populated areas (not less than 65%) might be associated with greater municipal loads. This is in agreement with Muniesa et al. (1999), who demonstrated the dominance of myovirid coliphages in anthropogenically polluted areas. Some densely populated sites close to the Curonian Lagoon (Figure 1) had no water treatment facilities, so municipal discharges could be a potential source of the elevated numbers of myoviruses in such areas.

On the other hand, the size range of phages was shown to be related to the morphology (Weinbauer & Peduzzi 1994) and community structure of the hosts (Mathias et al. 1995). Cyanobacteria make a significant contribution to phytoplankton in the shallow, low-salinity lagoons of the Baltic Sea (Carsten et al. 2004). According to Safferman et al. (1983), cyanophages range in size between 50 and 100 nm and most of them (up to 80%) belong to the family *Myoviridae*. Their high morphological diversity was shown to depend on salinity (Lu et al. 2001). The Curonian Lagoon was dominated by cyanobacteria (particularly the filamentous *Aphanizomenon flos-aquae*) during the survey (Olenina 2006). Electron micrograph analysis showed the *A. flos-aquae* virus to be of 50–60 nm capsid size with a 20–30 nm contractile tail in eutrophic lakes (Granhall 1972). According to these descriptions *A. flos-aquae* viruses tend to belong to the family *Podoviridae*. Moreover, the *A. flos-aquae* virus was found to appear only in the active growing season of these cyanobacteria and seems to regulate bloom termination (Granhall 1972). The considerable role of viruses in terminating blooms was shown in other studies (Jacquet et al. 2002), and the ‘kill the winner’ hypothesis was proposed (Thingstad & Lignell 1997). However, the quantitative evaluation of viral impact, and particularly of cyanophages, on host community structure and activity as well as in mass cyanobacteria development needs to be determined in further investigations of the Curonian Lagoon.

The 80–100 nm and 100–120 nm size fractions of viruses were dominant in the freshwater part of the Curonian Lagoon, while an increase in the 30
–60 nm size fraction was observed in the northern part (possibly due to the sea water intrusion and mixing of water masses). Such a distribution could imply active virus interaction within microbial communities in different zones of the lagoon. The larger viruses show a smaller burst size (Weinbauer & Peduzzi 1994), and consequently lower production and infection rates (Murray & Jackson 1992). Moreover, larger viruses tend to be grazed more efficiently than smaller ones (Gonzalez & Suttle 1993). Hence, the relative importance of larger size-fraction viruses is limited by the physiological state of the host (e.g. cell size) and increased top-down pressures. Since the size fraction of less than 60 nm is prevalent in aquatic environments (Wommack & Colwell 2000), to be smaller but more abundant for the possibility of assembling more capsids within the cell and thus attaining a larger burst size could be a living strategy for viruses. A similar strategy was shown for bacteria to prevent both grazing and virus encounter rate (Weinbauer & Höfe 1998), while Cochlan et al. (1993) argued that the numerical dominance of the virioplankton community by small viruses occurs because larger viruses are produced at relatively slower rates and/or are degraded at higher rates. Moreover, in highly eutrophic freshwaters phagotrophic protists, including flagellates and ciliates, are strictly controlled by larger zooplankton (Stoecker & Capuzzo 1990). Thus, viruses as well as bacteria are partially released from protist pressure. Consequently, it is possible that a larger size fraction of viruses can become dominant in such an environment (Weinbauer 2004). The dominance of relatively larger size class phages in the Curonian Lagoon supports this scenario.

The widely accepted assumption that the majority of viruses are phages is based on their morphology and size, as well as on correlations with abundance of heterotrophic bacteria and cyanobacteria (Proctor & Fuhrman 1990, Wommack et al. 1992). Moreover, the abundance and diversity of viruses depend on the density and activity of host cells (Murray & Jackson 1992) and on the seasonal dynamics of environmental variables (Lymer et al. 2008). If these changes favour the domination of specific host species, an increase in viral abundance and their role in the regulation of host populations (Jacquet et al. 2002) and a decrease in viral morphological (but not necessarily genetic) diversity can be expected. The total number of viruses (1.91×10^7 ml⁻¹ to 5.06×10^7 ml⁻¹), taken as a single parameter, did not reveal any likely associations with hosts (either with total bacterial abundance or with chlorophyll a) and was homogeneous in the lagoon. However, the overall predominance of myoviruses and a positive, strong correlation between Myoviridae and chlorophyll a was observed (r = 0.89; p < 0.001). In the manner of a correlation between variables (Boehme et al.
1993), these results imply that myoviruses are an active component of the plankton community at least at a particular time of the annual succession.

The virus to bacteria ratio (VBR) is considered an important variable, indicating the potential importance of viruses in the control of bacterial abundance and has been shown to be higher in freshwater and more nutrient-rich environments. The average VBR for the Curonian Lagoon was 28.2 and did not differ greatly from the average ratio reported for freshwaters (Maranger & Bird 1995). In most cases VBR values remain consistent over changes in bacteria and virus abundance (Hara et al. 1991). Therefore, it is a useful variable for obtaining an overall impression of possible interactions between viruses and the host community. Bratbak & Heldal (1995) suggested that the negative correlation between VBR and total bacterial numbers indicates the level of bacterial community diversity. A positive correlation is thought to depend directly on virus production (Hara et al. 1996). In the case of the Curonian Lagoon, it is difficult to infer virus impact on the bacterial community, since the morphologies of cyanophages and other bacteriophages attributed to Myoviridae are similar (Safferman et al. 1983) and cannot be distinguished solely on the basis of electron micrographs. On the other hand, VBR depends on infection rates and virus burst sizes. The latter variable is known to depend on virus capsid size (Weinbauer & Peduzzi 1994). Thus, the dominance of a larger size fraction of viruses could result in a decrease of VBR.

Although we cannot predict many important virus-host interactions, such as the role of phages in the genomic diversity of hosts or the rate of gene transfer, based only on morphology or size distribution, the different patterns of all three parameters reflecting virioplankton, i.e. size, shape and abundance, provided a more accurate picture of the spatial distribution of phage-like particles in the Curonian Lagoon than could have been revealed from a single variable. Finally, the morphology and size analysis of phage-like particles may be useful to explain the variation of such parameters as virus burst size (e.g. larger viruses tend to have a smaller burst size) or at least serve as a good basis for the further planning of research and experiments.

Acknowledgements

The authors would also like to express their appreciation to the staff of the Department of Immunology and Cell Biology (Institute of Biotechnology, Vilnius) for their valuable support with microscopic techniques. Early phases of the research were considerably enhanced by the participation of Kristina Slavuckyte, whose contribution is greatly acknowledged. We thank Viaceslav Jurkin (Klaipeda University, Coastal Research and Planning
Institute) for his help with the preparation of Figure 1. Special thanks go to the anonymous reviewers, whose comments and suggestions resulted in a significantly improved manuscript.

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