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Imaging and quantifying virus fluorescence signals on aquatic aggregates: a new method and its implication for aquatic microbial ecology

Birgit Luef¹, Thomas R. Neu², and Peter Peduzzi¹

¹Department of Freshwater Ecology, Faculty of Life Sciences, University of Vienna, Vienna, Austria

²Department of River Ecology, Helmholtz Centre for Environmental Research – UFZ, Magdeburg, Germany

Abstract

The development of accurate methods to detect and enumerate viruses is an important issue in aquatic microbial ecology. In particular, viruses attached to floating aggregates are a largely ignored field both in marine and inland water ecology. Data on the total abundance and the colonization of aggregates by viruses are rare, mainly due to methodological difficulties. In the present study, we used confocal laser scanning microscopy (CLSM) to resolve fluorescence signals of single viruses and bacterial cells in a complex three-dimensional matrix of riverine aggregates. CLSM in combination with different fluorochromes is a very promising approach for obtaining information both on the aggregate architecture and on the spatial distribution of viruses attached to fully hydrated aggregates. Aggregates from the Danube River harbored up to 5.39×10^9 viruses cm^{-3} . We discuss the problems associated with different methods such as sonication or directly counting viruses on aggregates, both combined with epifluorescence microscopy and CLSM, to quantify viruses on suspended particles.

Keywords

virus; aggregate; extracellular polymeric substances; glycoconjugate; river; confocal laser scanning microscopy

Introduction

Viruses are highly abundant in aquatic systems, affect phyto- and bacterioplankton growth, may regulate the genetic diversity of their hosts and may potentially influence the cycling of organic carbon and nutrients (see reviews of Wommack & Colwell, 2000; Weinbauer, 2004; Suttle, 2007; Brussaard *et al.*, 2008). In order to understand the potential impact of viruses on microorganisms, it is important to detect and enumerate the viruses. Typically, viral abundance in aquatic systems ranges between $< 10^4$ and $> 10^8$ mL^{-1} (Weinbauer, 2004; Peduzzi & Luef, 2009). In general, fixed total water samples include particles and aggregates; however, it is likely that without specific techniques, viruses attached to aggregates are severely underestimated. Compared with free-living forms, viruses associated with aggregates and sediments have received little attention, perhaps largely due to

methodological difficulties. Nevertheless, the occurrence and importance of viruses in sediments (see the review of Danovaro *et al.*, 2008a, b) and on floating aggregates (Peduzzi & Weinbauer, 1993; Simon *et al.*, 2002; Luef *et al.*, 2007; Mari *et al.*, 2007; Peduzzi & Luef, 2008) is documented. So far, viruses associated with aggregates and sediments were analyzed using various techniques such as sonication (Danovaro *et al.*, 2001; Fischer *et al.*, 2005; Duhamel & Jacquet, 2006) or direct observation (Luef *et al.*, 2007), both combined with conventional epifluorescence microscopy (EFM), sonication in combination with flow cytometry (Duhamel & Jacquet, 2006; Riemann & Grossart, 2008) and transmission electron microscopy (TEM; Peduzzi & Weinbauer, 1993). Recently, Mari *et al.* (2007) described an indirect approach to enumerate viruses inside transparent exopolymeric particles using magnetic isolation and flow cytometry.

In recent years, confocal laser scanning microscopy (CLSM) has been introduced into the field of microbial ecology to investigate three-dimensional (3D) objects such as fully hydrated biofilms and aggregates in both freshwater and marine environments (e.g. Holloway & Cowen, 1997; Neu & Lawrence, 1999; Neu, 2000; Neu *et al.*, 2001). No attempt has yet been made to detect aggregate-associated viruses via CLSM. Significant advantages of CLSM include the elimination of out-of-focus information and the possibility for horizontal and vertical optical thin sectioning of thick, hydrated biological samples. Hence, our investigation applies CLSM in combination with SYBR staining to visualize the 3D distribution of viruses. In addition, we quantify the CLSM data sets of virus signals in fully hydrated riverine aggregates. We then discuss different methods for quantifying viruses associated with aquatic aggregates in combination with advanced imaging techniques.

Materials and methods

Sampling of aggregates

Water samples from the Danube River (Wildungsmauer, Austria, stream kilometer 1894) were taken in early May and late July 2005 at a sampling depth of *c.* 30 cm. In July, samples were taken after a flood event. Lotic aggregates were sampled in 1-L plexiglass bottles. The samples were always kept at +4 °C until analysis, which was completed within 24 h (Bura *et al.*, 1998).

Extraction of viruses by sonication

To confirm that viruses can also be properly detected by CLSM, we dislodged viruses from aggregates by sonication as follows: we adopted the method used for sediments by Weinbauer *et al.* (1998) and Danovaro *et al.* (2001) to enumerate viruses on floating aggregates. Water samples were filtered onto 3- μ m isopore membrane filters (TSTP, 25 mm diameter, Millipore) to obtain the particle-associated microbial fraction. Filters were stored in 4.5 mL of 0.02 μ m filtered water and fixed with formaldehyde (final concentration, 2%). Tetrasodium pyrophosphate (final concentration, 5 mM) was added to the samples, which were incubated for 1 h on a shaker. The samples were sonicated three times for 1 min on ice, pulsing at 40 W using a B. Braun Diessel Biotech Sonifier (needle diameter, 4 mm; Labsonic U, Melsungen, Germany). The sonication was interrupted for 30 s every minute. Afterwards, samples were filtered onto Whatman AnoDisc filters (pore size, 0.02 μ m; diameter, 25 mm, Whatman, Maidstone, UK) and stained with SYBR Green I (Invitrogen, Eugene, OR) according to Noble & Fuhrman (1998). Filters were mounted on glass slides with an antifade mounting solution (Citifluor AF1, Citifluor, London, UK). Viruses were detected under the CLSM.

Staining and CLSM

To label the matrix material of the riverine aggregates, the lectin from *Aleuria aurantia* (AAL, Vector Laboratories, Burlingame, CA) was used to stain the lectin-specific glycoconjugates of the extracellular polymeric substances (EPS) (Neu, 2000; Neu *et al.*, 2001; Staudt *et al.*, 2003). The lectins were self-labeled with the fluorochrome Cy5 according to the data sheet of the supplier under dim light (Amersham, Buckinghamshire, UK). Briefly, after the purification resin was packed into the column, the lectin-dye solution (50 μL of a 1 M sodium bicarbonate solution, 0.5 mL of a 2 mg mL⁻¹ lectin solution and the fluorescence dye were incubated for 1 h in the dark at room temperature) was carefully loaded onto the column. After the mixture entered the column resin, an elution buffer was added until the labeled lectin was eluted. Two colored bands, which represent the separation of the labeled lectin from unbound dye, appeared. The first band, which contained the labeled lectin, was collected.

Lectin staining of the EPS glycoconjugates (100 $\mu\text{g mL}^{-1}$) was performed as described previously (Neu, 2000). For staining, the lectin was diluted with deionized water to a final concentration of 0.1 mg mL⁻¹ protein. One hundred microliters of this solution were added to each sample and incubated for 20 min in the dark at room temperature. The aggregates (size range of 7–198 μm) were then carefully washed three times with tap water to remove unbound lectins. For this purpose, tap water was carefully added with a pipette and drawn off with filter paper to avoid resuspension and disruption of the aggregates. The aggregates were never allowed to dry in the air. To detect aggregate-associated bacteria and viruses on the fully hydrated aggregates, the samples were stained with the nucleic acid-specific stain SYBR Green I (Noble & Fuhrman, 1998). After staining the lectin-specific EPS compounds, SYBR Green I (1 $\mu\text{L mL}^{-1}$ deionized water) was directly applied to the aggregates and incubated for 5 min in the dark at room temperature. The stained samples were carefully transferred into cover well imaging chambers (0.5 mm spacer, Invitrogen), covered with tap water and immediately examined by CLSM.

CLSM was performed using a Leica TCS SP1, controlled by the LCS version 2.61 Build 1537 174192 (Leica, Heidelberg, Germany), equipped with an upright microscope. For detecting viruses, images were taken with a $\times 100$ 1.4 NA oil lens. The aggregate structure was analyzed by CLSM using visible lasers (488 and 633 nm). Emission signals were detected from 500 to 550 nm and from 650 to 750 nm. Optical sections of aggregates were taken every 0.2 μm .

Cryo-embedding, cryo-sections and CLSM

To quantify viruses and bacteria on riverine aggregates, cryo-sections were also performed. Aggregates from the Danube were embedded in liquid cryostat medium (Neg-50 by Richard-Allan Scientific), frozen at -26°C and physically sectioned (10 μm) with a cryomicrotome (Leica CM3050S) (Huang *et al.*, 1996). Thereafter, sections were stained with lectins and a nucleic acid stain, were embedded in water and covered with a coverslip before analysis. The sections of the aggregates were examined by CLSM using a TCS SP2 controlled by the LCS version 2.5 Build 1227 192162 (Leica). Images were collected using an inverted DM IRB microscope and a $\times 100$ 1.4 NA oil lens. Aggregate structure was analyzed by CLSM using visible lasers (488 and 633 nm). Emission signals were detected from 500 to 550 nm and from 650 to 750 nm.

Quantification and visualization

To quantify virus signals, the freely available software IMAGE J (<http://rsb.info.nih.gov/ij/>), developed in JAVA (Staudt *et al.*, 2004) or, alternatively, the IDL-based program CONFOCAL ANALYSIS

(CONAN) version 1.31, was applied. The software CONAN was developed by BioCom for the Helmholtz Centre for Environmental Research – UFZ in Magdeburg.

To quantify lectin signals, the software IMAGE J was used. For each aggregate, the threshold was set manually. Because of the very heterogeneous composition of the aggregates, automatic batch processing could not be applied.

To visualize 3D data sets, IMARIS 4.2 (Bitplane AG, Zurich, Switzerland) was used. Thresholds were set manually. Adobe Photoshop CS2 was used to insert calibration bars into the images.

Results

CLSM and visualization

In a first step, we had to confirm that virus signals can also be detected properly by CLSM. Therefore, samples were sonicated, filtered, stained and analyzed by CLSM (Fig. 1). Viruses and bacteria appeared bright and could be easily distinguished based on size and light intensity. Other than in conventional EFM, almost no background fluorescence was present when using CLSM. To obtain information about the spatial distribution of viruses attached to aggregates, the fully hydrated aggregates were analyzed directly by CLSM after staining. This is the first time that viral, bacterial and glycoconjugate distributions have been visualized together by means of CLSM in fully hydrated aggregates. The serial optical sections, as shown in Fig. 2, provide an insight into the arrangement of viruses, bacteria and the matrix of heterogeneous aggregates. In addition, Fig. 3 shows the presence of viruses and lectin-specific glycoconjugates, their location and also their colocalization in a 3D reconstruction.

CLSM and quantification

The software IMAGE J and CONAN enabled quantification of the lectin-specific glycoconjugates and of the bacteria, but not of the viruses. Although the diverse programs could not identify virus signals correctly, the human eye could easily distinguish between a virus and an occasional background pixel. Nonetheless, counting viruses manually in untreated aggregates was not manageable. The main obstacle was to identify whether the DNA signals were viruses or sections of bacteria: therefore, each section of an image stack had to be compared to determine whether the fluorescence signals were virus-derived or merely a section of a bacterium. Performing this comparison for each fluorescence signal is too laborious, especially for heavily colonized aggregates.

Cryo-sections, however, allowed detecting the distribution of viruses, bacteria and polymeric constituents inside the aggregate with more accurate resolution in a reasonable time (Fig. 4). Potential limitations of the CLSM technique due to scattering, laser penetration and diffusion of staining solutions can be overcome by analysis of cryo-sectioned poststained samples. Abundance data were therefore obtained from cryo-sections of aggregates that were counted manually. The abundances of bacteria and viruses associated with Danube aggregates are summarized in Table 1. In spring, for example, aggregates harbored on average 17.27×10^8 bacteria cm^{-3} and 18.77×10^8 viruses cm^{-3} lectin-specific glycoconjugate. In summer, after a flood event, the corresponding average values were 1.55×10^8 bacteria cm^{-3} and 2.48×10^8 viruses cm^{-3} lectin-specific glycoconjugate.

Discussion

CLSM, visualization and quantification

In recent years, CLSM has become an indispensable tool for studying 3D biofilm and aggregate architectures, their chemical compositions and associated microbial communities (e.g. Böckelmann *et al.*, 2002; Staudt *et al.*, 2003; Neu *et al.*, 2004). To our knowledge, no one has yet attempted to detect viruses attached to aquatic aggregates by CLSM. Generally, interactions between natural virus assemblages and suspended particulate matter are poorly documented. Particularly in riverine systems, where suspended matter is an important factor, very little information is available on the interaction of viruses with aggregates. We therefore used CLSM to resolve single virus signals and bacteria in a complex 3D matrix of riverine aggregates (Figs 2 and 3). In combination with different fluorochromes, this allowed us to obtain information on both the aggregate architecture and the spatial distribution of viruses in fully hydrated aggregates.

Because viruses attached to riverine aggregates were detectable, we attempted to quantify the virus signals. Ample software is available for quantifying CLSM data. This includes freely available as well as commercial software. None of the programs, however, is suitable for multipurpose applications.

One crucial step in digital image analysis is setting thresholds (Xavier *et al.*, 2001; Beyenal *et al.*, 2004). Thresholding is a segmentation method that essentially reduces 256 gray-scale level images to binary images in order to separate the image into biomass (signal) and interstitial space (background). To extract statistically meaningful parameters from image series, the thresholding has to be reproducible. There are no general rules for setting thresholds. The operator uses his or her best judgment, setting the gray-scale level such that the binary image appears to capture the essence of the EPS structure, viral and bacterial signals. Setting the thresholds manually is quite time consuming and variability between operators in choosing the threshold adversely affects the measurements obtained from the binary image. Most computer programs are equipped with automatic image-thresholding procedures. Such procedures must be tested carefully before use. Changes in thresholding can alter the numerical values of diverse parameters (Beyenal *et al.*, 2004). We tried different automated methods of thresholding. As the aggregate structures were so heterogeneous, the computer programs did not have a sufficiently precise automatic procedure to yield reproducible results compared with the human operator.

Setting the thresholds manually, the computer programs *CONAN* and *IMAGEJ* allowed us to estimate bacterial and EPS volumes, but it was not possible to quantify viruses accurately. Two important points have to be considered for digital image analysis and especially for virus quantification: firstly, virus signal intensity vs. background signal, and secondly, virus signal size vs. pixel size. The above computer programs failed to distinguish between virus and background pixels, probably because the algorithms were insufficiently sensitive. Viruses and bacteria attached to aggregates were therefore counted manually. Nevertheless, CLSM is recommended as the method of choice to resolve single viruses in a complex 3D matrix of fully hydrated riverine aggregates. Although more sensitive and precise algorithms for thresholding may become available, thresholding remains difficult because riverine aggregates have a very complex composition.

Comparison of different methods to quantify viruses and bacteria attached to riverine aggregates

Direct counts provide a wealth of basic information on viruses in aquatic ecosystems. This calls for accurate methods to detect and enumerate viruses on particles as well. At least three approaches have been used for such enumerations: (1) TEM, (2) DNA/RNA staining

techniques using EFM or flow cytometry and (3) magnetic isolation combined with flow cytometry.

Using TEM, for example, riverine aggregates can be embedded in Nanoplast in combination with uranylacetate counter-staining (Leppard *et al.*, 1996). TEM offers a high resolution and therefore clearly identifies accumulations of viruses. However, TEM requires fixation and/or dehydration in order to examine the 3D aggregate structure. Both processes can produce severe artifacts, especially in highly hydrated samples. Moreover, TEM is very time consuming and expensive.

Sonication is widely used to dislodge viruses from sediments, aggregates, etc., combined with EFM and flow cytometry (e.g. see the review of Danovaro *et al.*, 2008a; Riemann & Grossart, 2008). Using CLSM, we also accurately detected dislodged viruses (Fig. 1). However, sonication appears to be a useful method for reasonably quantifying viruses on floating aggregates, but does not supply any information on the spatial distribution of viruses on the aggregates.

Luef *et al.* (2007) directly observed particle-attached viruses and bacteria in combination with conventional EFM. This technique has limitations in distinguishing between stained viruses and background fluorescence from deeper layers of the aggregate. Occasional high densities of attached bacteria also hampered the precise detection of single virus signals. Examining a sample by EFM required frequently adjusting the plane of focus. Furthermore, bacteria and viruses underneath the aggregates or deeper inside were undetectable.

The major disadvantage of fluorescence microscope techniques is image degradation by out-of-focus information originating from focal planes located above or below the objects of interest. CLSM systems provide the opportunity to extend light microscopy studies beyond the limitations of traditional EFM (Lawrence *et al.*, 2002, 2007).

For CLSM-based quantification, the entire aggregates were not analyzed. Riverine aggregates incorporate a high amount of noncellular matter such as sand, clay and detritus, which limit diffusion of the staining solution, laser penetration and detection of emission signals in thick samples. Thus, large aggregates had to be embedded and physically sectioned into slices using cryo-sectioning. Cryo-sections allow much better detection of the distribution of viruses, bacteria and polymeric constituents inside the aggregates. From each slice, bacterial and viral abundances were counted manually due to the above-mentioned difficulties when using digital image analysis.

CLSM allowed clear recognition of virus accumulations (Fig. 5), although sometimes the quantification of single virus signals and clear identification within such accumulations was still hampered. It was also occasionally difficult to distinguish single virus particles in aggregates containing high densities of stained cells and/or a matrix that was also stainable with nucleic acid dyes. Nevertheless, CLSM is a very promising approach for obtaining the abundances of viruses attached to or within aggregates.

The resolution of any linear imaging system is given by its point spread function, which quantifies the blur of an object point in the image. The sharper the point spread function, the better the resolution. In 1873, Ernst Abbe discovered that lens-based optical microscopes cannot resolve objects that are closer together than half of the wavelength of light. Recently, however, Schmidt *et al.* (2008) introduced stimulated emission depletion microscopy with 4π geometry that creates nearly spherical focal spots 40–45 nm ($\lambda/16$) in diameter. The combination of these techniques will probably push the z resolution to < 10 nm (Hell, 2007). Very likely, such an approach, which sharpens the point spread function, will yield even better resolution/images of viruses attached to aggregates.

Interactions of viruses and particulate material

The literature on the interaction between suspended matter and the natural assemblage of viruses infecting microplankton organisms, such as bacteria and phytoplankton, is surprisingly scarce. Particularly in freshwater systems, where suspended matter is often a prominent factor, very little information is available on the interaction of viruses with aggregate-associated host bacteria. However, even for marine systems, we know little about virus–particle interactions.

Few studies on the abundance of viruses attached to or within organic aggregates are available (Peduzzi & Weinbauer, 1993; Simon *et al.*, 2002; Luef *et al.*, 2007; Mari *et al.*, 2007; Peduzzi & Luef, 2008). Marine snow particles from the Northern Adriatic Sea harbored 5.6×10^{10} viral particles cm^{-3} aggregate based on ultrathin sections and subsequent TEM investigations (Peduzzi & Weinbauer, 1993). In the present study, up to 5.39×10^9 viruses cm^{-3} lectin-specific glycoconjugate were found in the Danube when analyzed by CLSM (Table 1). A comprehensive overview of studies on viruses attached to different substrata in inland waters is given by Peduzzi & Luef (2009). For example, Luef *et al.* (2007) and Peduzzi & Luef (2008) showed that $0.01\text{--}0.89 \times 10^7$ viruses mL^{-1} water were attached to particles in the Danube and its floodplain system. In the Talladega Wetland, Alabama, viruses attached to surfaces ranged from 1.3×10^6 virus particles cm^{-2} on macrophytes to 1.1×10^7 virus particles cm^{-2} on wood (Farnell-Jackson & Ward, 2003). In the Mahoning River, Ohio, viral abundances ranged from 1.65 to 6.68×10^8 g^{-1} ash-free dry mass (Lemke *et al.*, 1997), leaves harbored $4.81\text{--}21.8 \times 10^8$ viruses g^{-1} and sediment samples $4.71\text{--}8.91 \times 10^6$ viruses g^{-1} (Baker & Leff, 2004). At Lake Hallwil, Switzerland, $0.2\text{--}0.9 \times 10^8$ viruses cm^{-2} biofilm, $3.5\text{--}10.6 \times 10^7$ viruses mg^{-1} C_{org} plant litter and $1.9\text{--}5.3 \times 10^9$ viruses cm^{-3} sediment were found (Filippini *et al.*, 2006). Furthermore, quite recently, Danovaro *et al.* (2008a) gave a broad overview of viruses in both freshwater and marine sediments. In inland waters, viral abundances range from > 0.01 to 203.3×10^8 viruses g^{-1} dry sediment. A literature comparison of viral abundances associated with aggregates, biofilms, sediments, etc. is very difficult due to the different methods used and the variable units presented. Nonetheless, such associated viruses apparently reveal some dependence on the type of the particulate matter, such as particle size and quality (Lemke *et al.*, 1997; Farnell-Jackson & Ward, 2003; Luef *et al.*, 2007).

The presence of solid surfaces in the water column implies several ecological consequences. Flood & Ashbolt (2000) observed that wetland biofilms could entrap viral-sized particles and concentrate them over 100-fold compared with abundances in the surrounding water column.

In a study on viral decay in the Gulf of Mexico, free-living viruses may bind irreversibly to loosely associated aggregates, thereby losing their infectivity (Suttle & Chen, 1992). On the other hand, viral association with colloidal and particulate materials can prolong their survival (Kapusinski & Michell, 1980), and phage production and transduction frequencies can increase in the presence of particulate matter (Kokjohn *et al.*, 1991; Ripp & Miller, 1995).

Using a model system, Riemann & Grossart (2008) showed that a marine phage may effectively lyse surface-attached bacteria. Enhanced lytic production of viruses may also occur in natural systems with high particle loads, due to elevated viral abundances found on transparent exopolymeric particles (Mari *et al.*, 2007) and on some riverine particles (Luef *et al.*, 2007). Therefore, aggregates may represent hot spots for viral infection of bacteria. Bacterial lysis mediated by viruses on aggregates may be an important mechanism to release dissolved organic matter into the water column. Furthermore, increased viral production on aggregates may also lead to increased release into the surrounding water, which may

significantly alter the distribution and community composition of viruses in the whole system. However, solid surfaces can also adsorb virus particles, thus reducing viral infection while increasing prokaryotic production (L. Kernegger, I. Zweimüller & P. Peduzzi, unpublished data).

Viral infection of bacterial cells attached to aggregates may be impeded by various structures, including the bacterial extracellular polymers. Specific phages can degrade susceptible biofilms and continue to infect biofilm bacteria during the degradation of EPS (Hughes *et al.*, 1998a, b). Many phages, but not all, may use polysaccharases or polysaccharide lyases (Hughes *et al.*, 1998a, b; Sutherland *et al.*, 2004). Viruses can indirectly produce EPS by lysing bacteria and phytoplankton biomass and may play an important role in flocculation processes (Peduzzi & Weinbauer, 1993). The formation of aggregates or the attachment of viruses can also have biogeochemical consequences when considering vertical or horizontal transport of such material in the various aquatic systems. Thus, viruses certainly can be nanoscale drivers in ecological and biogeochemical processes.

Based on the above-outlined information, it becomes evident that visualizing and quantifying virus distribution on particles is important in microbial ecology of floating aggregates in aquatic systems. This new technique should contribute, among others, to elucidate the significance of viruses on suspended matter.

Conclusions

CLSM, in combination with different fluorochromes, represents an *in situ* approach that yields information both on the architecture of aggregates and on the spatial distribution of bacteria, viruses and polymeric constituents. For the first time, viral, bacterial and glycoconjugate distributions could be visualized by means of CLSM in fully hydrated riverine aggregates. Although some difficulties occurred when quantifying viruses attached to aggregates, CLSM is proposed as the method of choice for investigating multiple constituents in fully hydrated suspended matter.

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Fig. 1. CLSM maximum intensity projection showing particle-derived viruses and bacteria on a filter after sonication. White arrows point to viruses, and yellow ones to bacteria. Calibration bar: 5 μm .

Fig. 2. CLSM image series of a riverine aggregate in the axial direction. Dual channel presentation of specific glycoconjugates, bacteria and viruses. Arrow points to viruses. Color allocation: green, nucleic acid; red, glycoconjugates; yellow, bacteria and viruses in or in contact with lectin-specific EPS. Calibration bar: 30 μm.

Fig. 3. 3D volume reconstructions from a riverine aggregate. (a) and (b) represent different views of the aggregate. Arrows point to viruses. Color allocation: green, nucleic acid; red, glycoconjugates; yellow, bacteria and viruses in or in contact with lectin-specific EPS. Calibration grid: 5 μm .

Fig. 4.
(a) Single scan of a cryo-section showing the distribution of viruses, bacteria and glycoconjugates inside an aggregate. (b) View of one channel to present bacteria and viruses. White arrows point to viruses. Color allocation: green, nucleic acid; red, glycoconjugates; yellow, bacteria and viruses in or in contact with lectin-specific EPS. Calibration bar: 10 μm .

Fig. 5. 3D volume reconstruction from a riverine aggregate. For deconvolution, the method of classic maximum likelihood estimation was applied. The program HUYGENS 3.0.0, SVI (Scientific Volume Imaging B.V., the Netherlands) was used. Arrows point to viruses. Color allocation: green, nucleic acid; red, glycoconjugates; yellow, bacteria and viruses in or in contact with lectin-specific EPS. Calibration grid: 5 μm .

Table 1

Quantification of viruses and bacteria on riverine aggregates on cryo-sections

	<i>n</i>	Minimum	Maximum	Mean	SE
Spring					
Bacteria (× 10 ⁸ cm ⁻³ specific glycoconjugate)	34	0.35	96.02	17.27	3.39
Viruses (× 10 ⁸ cm ⁻³ specific glycoconjugate)	34	1.75	53.86	18.77	2.42
VBR	34	0.39	5.00	1.65	0.18
Summer					
Bacteria (× 10 ⁸ cm ⁻³ specific glycoconjugate)	33	0.20	7.45	1.55	0.25
Viruses (× 10 ⁸ cm ⁻³ specific glycoconjugate)	33	0.31	11.47	2.48	0.40
VBR	33	0.24	8.00	2.25	0.32

VBR, virus to bacterium ratio; *n*, number of analyzed cryo-sections.