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ORIGINAL PAPER

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The diversity of bacteria, eukaryotic cells and viruses in an oligotrophic lake

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Abstract An in situ transmission electron microscopic study of biomass samples concentrated from oligotrophic lake water revealed a variety of virus-infected microbial cells and many free viruses and virus-like particles. The most abundant group of microorganisms in screened and filtered water-column samples were 2 μm or less in diameter, and included representatives of several oligotrophic genera, *Prosthecomicrobium*, *Ancyclobacter*, *Caulobacter* and *Hyphomicrobium*. Among the prokaryotic host cells, which included both heterotrophs and autotrophs, on the basis of electron microscope observations, approximately 17% were infected with bacteriophage or bore adherent phage particles on their surfaces. Several bacterial morphotypes were observed among the prokaryotic hosts. Water samples passed through a 20-μm Nitex screen allowed us to concentrate and examine the larger host cells as well, including several species of single-celled algae and two amoeba species. The infected algal cells included those *Chlorella*-like in appearance, photosynthetic flagellates and others that could not be positively identified. About one-third of the eukaryotic cells were infected by viruses that were larger (150–200 nm) and structurally more complex than bacteriophages (50–60 nm). None of the viruses have been isolated, but when 0.2 μm filtrate from a biomass sample was spotted onto lawns of four representative heterotrophs and a *Chlorella*, the clearing observed was taken as evidence of lysis. Cyanobacterial lawns showed no plaques. Thin sections of two amoeba showed food vacuoles containing what appeared to be virus particles of a type seen in certain prokaryotic and eukaryotic cells in the biomass.

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Introduction

A direct, in situ transmission electron microscopic (TEM) study of native microbial populations of aquatic samples has been used to determine the diversity of morphotypes and to estimate the relative numbers of each in waters of Lake Arthur, located in Black Rock Forest, Cornwall, N.Y. As in the earlier oceanic marine bacterial study by Johnson and Sieburth (1982), we have sought to explore the unique, ultrastructural features of picoplanktonic cells (Corpe and Jensen 1992). In addition, with the scanning/transmission mode (STEM) in conjunction with an energy-dispersive X-ray spectrometer one can readily determine the composition of cells (Jensen and Corpe 1993) and various associated particles (Jensen and Corpe 1994). Among the inorganic particles observed in a picoplanktonic biomass, recovered from oligotrophic lake water columns, were those with the morphological appearance of free bacteriophages and other virus particles. Viruses have been found in substantial numbers in lakes (Klut and Stockner 1990) and seawater (Sieburth, Johnson and Hargraves 1988; Murray and Eldridge 1994; Peduzzi and Weinbauer 1993).

Oligotrophic waters may be defined in terms of the average flux of nutrients (Poindexter 1981) where the flux does not exceed 0.1 mg C l^{-1} day^{-1}, in contrast to eutrophic lakes where the flux of organic C is in the range of 5 mg C l^{-1} day^{-1}. Oligotrophic bacteria are those adapted to uninterrupted organic nutrient limitation. The source of organic nutrients might be the result of active photosynthesis resulting in substantial growth of phototrophs or introduction of organic wastes from external sources. The latter can probably be ruled out in Lake Arthur because of successful water management, and perhaps only small amounts of readily metabolizable organic nutrients are supplied as run-off from surrounding forests. The failure of photosynthetic organisms to grow extensively in the lake has not so far been explained, but our encounter with viruses in bio-

mass samples caused us to examine their possible importance with the present study.

Materials and methods

The main thrust in this work was to exploit as much as possible the in situ study of thin sections of Epon-embedded biomass samples collected from lake water. The procedures employed have been described in detail (Corpe and Jensen 1992). In the study a total of six, 3-l samples were collected from Lake Arthur, one of several small lakes in the Black Rock Forest near Cornwall, NY. They were collected from the surface and at a depth of 4 m, 30 cm above the sediment, during 3 consecutive summer months. A part of each sample was air-dried onto Formvar-coated copper grids (Baxter and Jensen 1980). All samples were examined in a Hitachi H7000 TEM equipped with a STEM and PGT System-4-plus energy dispersive X-ray spectrometer, permitting elemental analysis of polyphosphate bodies. The 1992 study concentrated on the picoplankton, numerically the most abundant biological component. In the present study we report on nanoplankton (2–20 μm), which are important as hosts for viruses. Water samples fixed with glutaraldehyde (1% w/v) were filtered onto a black 0.2-μm Nucleopore polycarbonate membrane filter (Porter and Feig 1980) using gentle vacuum filtration (15 cm Hg). The filter was stained with 4′,6-diamidino-2-phenylindole, 0.001% final concentration Counting was done with an Olympus epifluorescence microscope.

Enrichment cultures for *Caulobacter* sp. were carried out according to procedures employed by Poindexter (1964). *Hyphomicrobium* enrichment cultures were prepared according to the method of Attwood and Harder (1972). An additional total count of nanoplankton, (protozoa and algae), providing numbers of each per unit volume, was estimated in a Sedgwick-Rafter chamber using an Olympus microscope with a final magnification of 400×. The 1% (w/v) glutaraldehyde-fixed sample, when necessary, was concentrated by centrifugation at 6000 *g* for 20 min and resuspended in a smaller volume.

Bacteriophage assays were carried out on unfixed lake-water samples using the method of Seeley and Primrose (1979). Several pure cultures of host organisms were used as the overlay including a laboratory culture of *Pseudomonas fluorescens*, and *Pseudomonas* sp., *Flavobacterium* sp., two cyanobacterial sp. and a *Chlorella* sp., all isolated from Lake Arthur. Since direct plaque assays of water samples were routinely unsuccessful, sterile Nucleopore filters of 0.2 μm pore size were used to prepare a "spotting mixture" from washed biomass samples. Filtrates were spotted on lawns of the host organisms in pure culture to determine their susceptibility to virus.

Results

Viruses in biomass from lake water

Some of the picoplanktonic cells (0.2–2.0 μm) contained in the biomass were infected with bacteriophage or had phage adsorbed to their surfaces (Fig. 1). Free hexagonal particles were also seen and recognized as bacteriophage. Other particles, not clearly hexagonal, were viewed as probable phage as well, because of the similarity in size and on account of the fact that the hexagonal appearance would only be manifest if the cut were through the center of the particle. The slightly curved gram-negative rods are probably a *Pseudomonas* sp. that was isolated in our earlier study (Corpe and Jensen 1992). No virus-infected picoplanktonic *Chlorella* were seen in thin sections studied nor did the cyanobacteria observed in sections show evidence of cell lysis. One methanotrophic morphotype and two heterotrophs, in addition to *Pseudomonas*, are shown as infected hosts (Fig. 1).

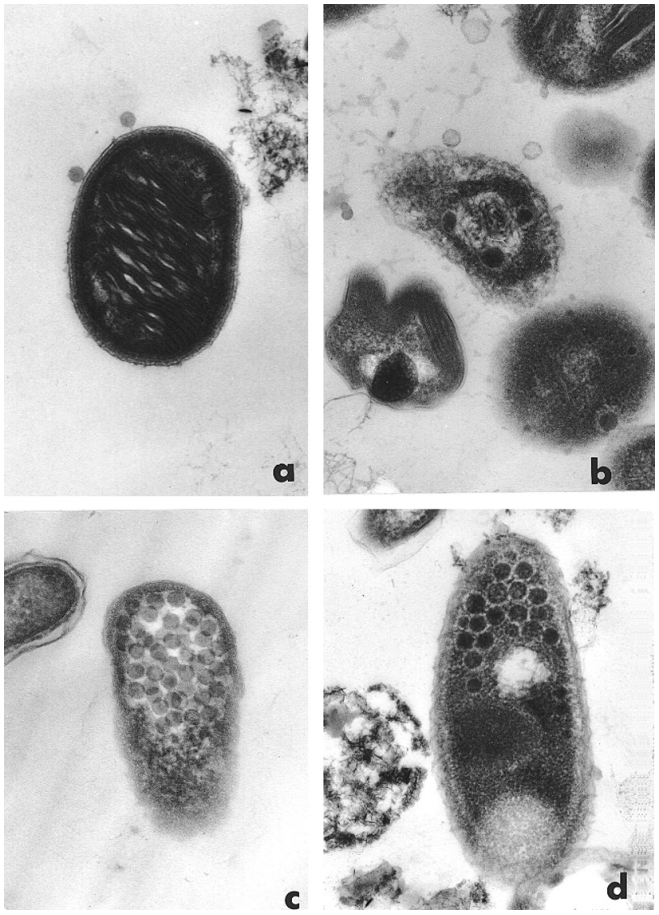

Fig. 1a–d Thin section showing examples of various picoplanktonic bacteria infected with bacteriophage. **a** Two phage particles attached to the surface of a gram-negative cell. 33 600×. **b** Two phage particles attached to a slightly curved, rod-shaped bacterium. 33 600×. **c** A rod-shaped gram-negative cell filled with phage particles. 33 600×. **d** Another rod-shaped cell with somewhat larger and more densely stained phage particles. 39 600×

An attempt to isolate viruses directly using soft overlay agar, inoculated with dilutions of water samples or pure cultures mentioned above, did not show plaque development on lawns of picoplankton isolates or nanoplanktonic *Chlorella*. We found that the *undiluted* picoplanktonic biomass filtrate caused some plaque development in *Chlorella* lawns.

The photomicrographs shown in Fig. 2 are examples of various free phage and virus-like particles. The bacteriophages observed ranged in size from 50 nm to 60 nm. However, occasionally larger hexagonal virus particles were seen that measured 160 nm. A search through our low-power (6000×) TEM photomicrographs revealed large hexagonal particles but they were

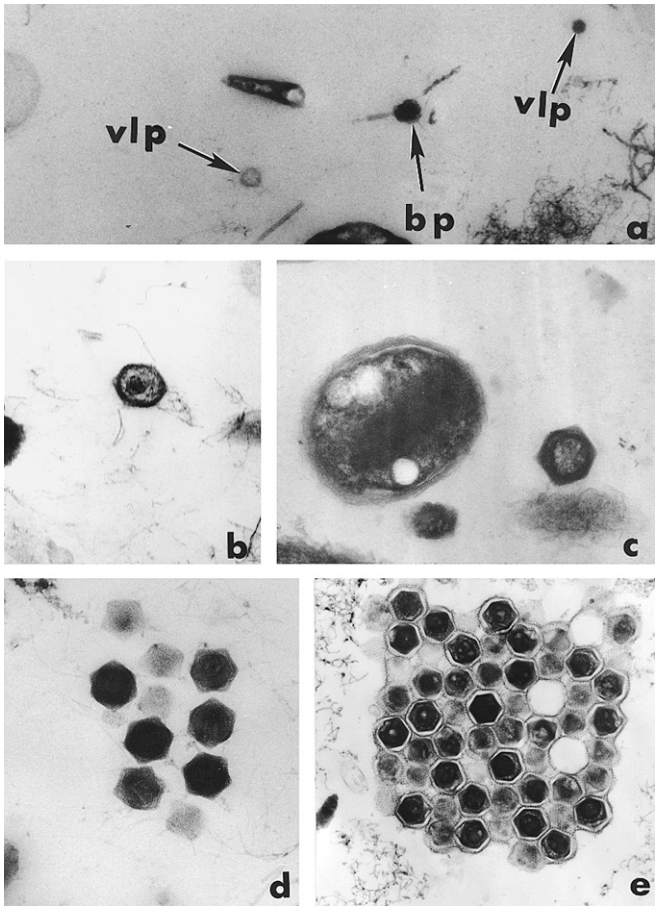

Fig. 2a–e Thin section showing a variety of virus-like particles. **a** Free bacteriophage (*bp*) and virus-like particles (*vlp*). 33 600×. **b** Free virus particle, hexagonal in shape with stained capsid and central granule. 33 600×. **c** A single virus, hexagonally shaped, showing stained layers and in association with a fibrous particle. 33 600×. **d** A cluster of virus particles, hexagonal with dark-staining circular layers. 33 600×. **e** A mass of virus particles in a structured arrangement. 15 300×

not as numerous as the bacteriophages. These suggested that other host cells had been separated from the picoplankton fractions with 5-μm screens and 2-μm filters.

Eukaryotic cells in lake water biomass

When the water samples filtered through 20-μm Nitex screens were fixed, concentrated and prepared as thin sections we were able to compare cells in greater detail. These contained not only the picoplankton but also nanoplankton, although the latter were present in very small numbers. Counting was done on low-power photomicrographs to estimate the relative abundance of each, which generally confirmed ratios of about 20:1, prokaryotic cells to eukaryotic cells (Fig. 3). Estimations of eukaryotic cells by epifluorescent techniques or counting in a Sedgwick-Rafter chamber showed that fewer than 1% of cells were eukaryotes.

In addition to the diversity of picoplanktonic morphotypes we found that, on the average, slightly fewer than 50% of the sectioned picoplankton cells contained one or more polyphosphate bodies. Examination of air-dried cells of the picoplankton biomass showed 70%–85% of the cells contained polyphosphate bodies PPBS. Air-dried preparations are of whole cells and all polyphosphate bodies present in a cell were observed.

Algae in lake water samples

The algae were characterized by size and a few descriptive terms, as shown in Fig. 4a–d. They are representative of many uninfected host cells of the various single-celled microalgae that were found in a biomass collected afterscreening with 20 μm Nitex. Virus-infected

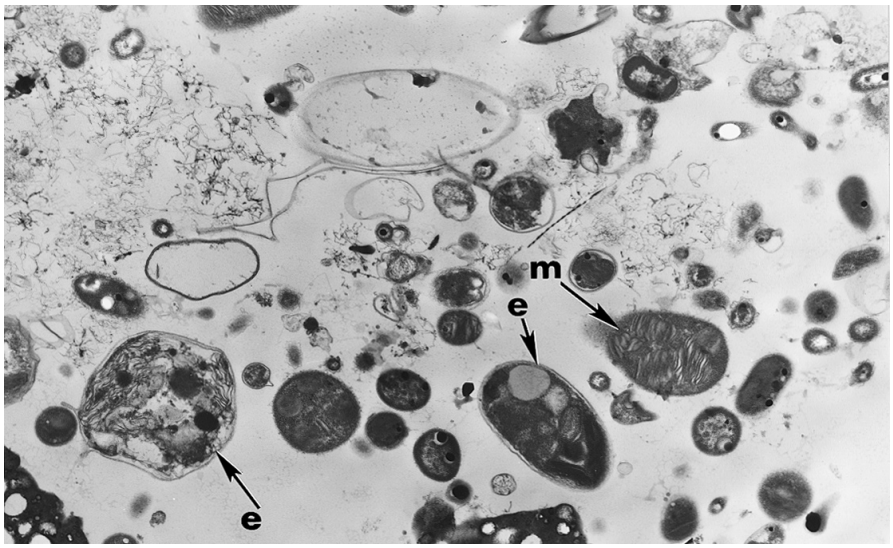

Fig. 3 Low-power photomicrograph derived from a 20-μm sieved filtrate of concentrated biomass, embedded and sectioned. 8 100×. The two eukaryotic cells (*e*) are not much larger than the methanotrophic bacterium (*m*). Both kinds of cells are surrounded by filamentous and particulate debris

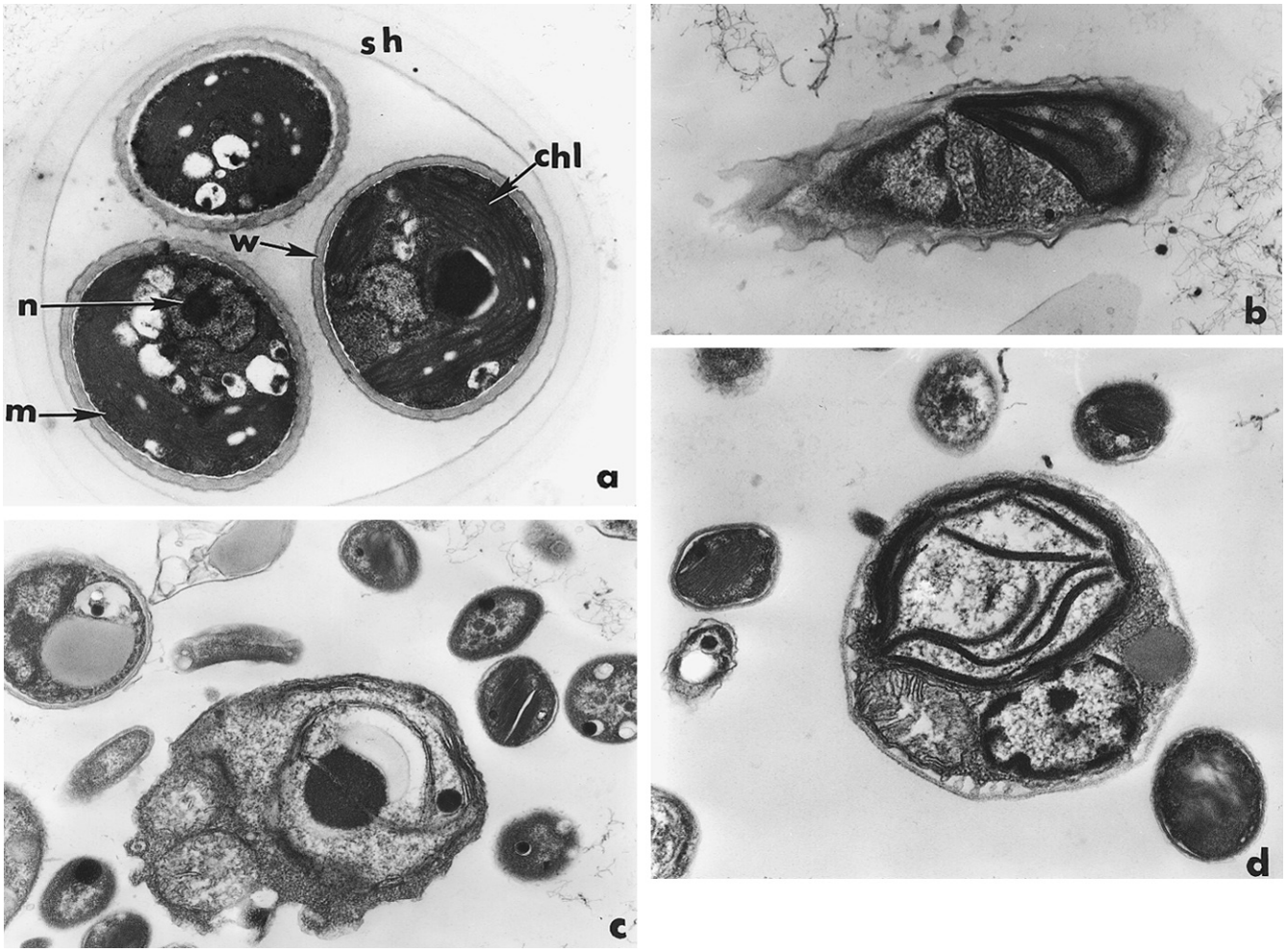

Fig. 4a–d Thin sections of several algal morphotypes found in Lake Arthur. **a** *Chlorella*-like cells with thick walls (*w*), three cells surrounded by thick sheath (*sh*), chloroplasts (*chl*), nucleus (*n*), mitochondria (*m*). 8 100×. **b** Elongated cell with a large chloroplast, an irregular shape, and a sculpted surface. 21 600×. **c** Phytoflagellate, showing chloroplast and flagella fragments. 13 200×. **d** Spherical-shaped cells with thin wall, a large chloroplast, and mitochondria. 20 400×

algal cells, shown in Fig. 5a–e, were not identifiable, at least with any certainty, since the virus particles were too numerous and often interfered with the host cell internal structure. *Chlorella*-like species were most numerous, as previously reported (Corpe and Jensen 1992). Extensive searching of biomass sections was required to locate the other representative uninfected and infected eukaryotic algae since their numbers were low, comprising much less than 1% on the basis of light-microscopic cell counting. The host cells that were present appeared healthy, although smaller in size than would have been anticipated.

Faster-growing, prokaryotic cells, heterotrophs, photo autotrophs and methylotrophs were also faced with destruction by phage, as suggested in Fig. 1. A major shared feature of these prokaryotes is the presence of polyphosphate bodies in over 70% of the cells.

Oligotrophic and prosthecate bacteria

In our study of several hundred photomicrographs in search of lysed cells and viruses, we began to recognize several bacterial morphotypes that have been reported to occur in oligotrophic waters but have not previously been noted by us (Corpe and Jensen 1992). The oligotrophs seen in greatest prominence were *Prosthecomicrobium* sp. (Fig. 6) and *Ancyclobacter* sp. Figure 6c shows a cell tentatively identified as *Caulobacter* sp. because of the presence of a partial stalk at one end of a slightly curved cell. The stalk portion in cross-section shows most cell envelope components to be continuous with those of the cell, as anticipated in *Caulobacter*. The outermost cell-surface component appears to be an S layer (Fig. 6c, d). Enrichment cultures were successfully prepared to confirm the presence of both stalked *Caulobacter* and budding *Hyphomicrobium*. Phase-contrast microscopic examination after at least 3 weeks of incubation of enrichment cultures established their presence (Poindexter 1964).

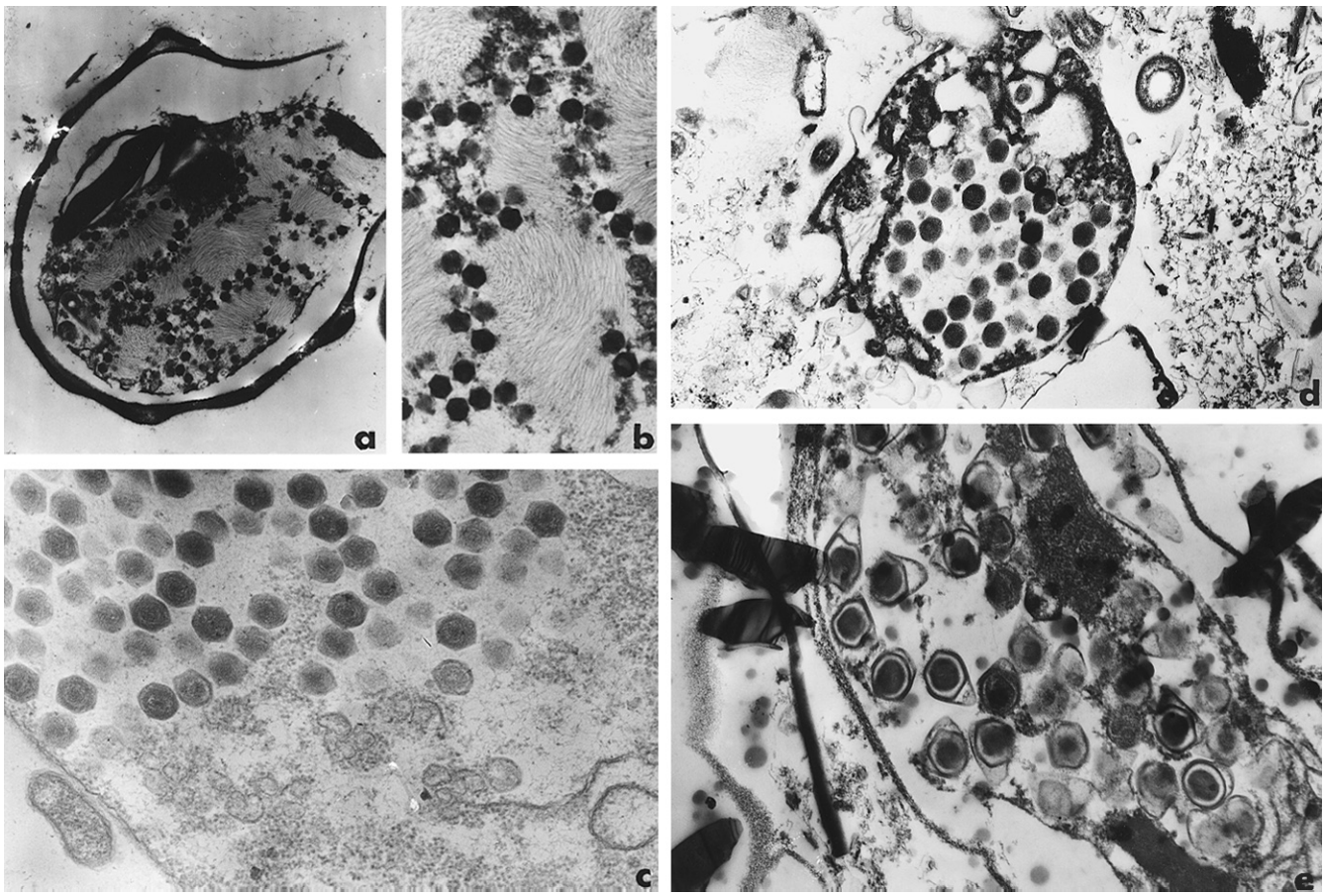

Fig. 5a–e Thin sections of virus-infected algal cells. **a** Cell within cup-shaped outer envelope containing a chloroplast. 9 600×. **b** Numerous virus particles distributed among an array of thin fibrillar material. 20 100×. **c** Elongated cell with thin wall showing structured viruses with heavily staining internal layers. 252 00×. **d** Virus-infected phytoflagellate. 19 200×. **e** Elongated cells largely decomposed with viruses. Virus is large with a layered envelope. 16 800×

Protozoa

Only two types of protozoans were observed in water samples. One of these was a flagellate, shown in Fig. 4c. The other were amoeba species. In thin sections of Lake Arthur biomass (Fig. 7) an amoeba is shown that is remarkable only because of the contents of its food vacuoles, and mainly the presence there of several types of viruses. The vacuoles are separated from each other by a membrane. As the sectioned cell is displayed, the vacuole on the right is filled with masses of bacteriophage particles similar in size and cross-section to those pictured in Fig. 1c in the section of a small gram-negative rod. Yet there appears to be no envelope residue associated with the vacuole. The vacuole on the left contains at least five hexagonally shaped virus particles.

Discussion

Oligotrophism

Lake Arthur and other small lakes or ponds of Black Rock Forest serve as a protected reservoir system for several towns in the lower Hudson Valley. In an earlier study Corpe and Jensen (1992) indicated the lakes were oligotrophic, as defined by Poindexter (1981). When the essential nutrients for microbial growth are available in such low supply the rate of growth is limited, and oligotrophic conditions develop. If sustained over a long period of time, ordinary aquatic bacteria become nutrient-stressed (Roszak and Colwell 1987). The organisms come to assume a smaller size and are rounded in form. In some cases at least, their ''normal'' appearance returns as nutrient is added back in a stepwise manner (Fry and Zia 1982). In contrast, other ''oligotrophic bacteria'' are those that are favored by sustained, low-nutrient conditions. These are a group of organisms, according to Semenov (1991) that have properties not found in eutrophic bacteria.

In the oligotrophic water of Lake Arthur we have recognized *Caulobacter* sp., *Ancyclobacter* sp. and *Prosthecomicrobium* sp. Still others, like *Hypomicrobium* sp. and *Blastobacter* sp., have rod shapes, so unless sections

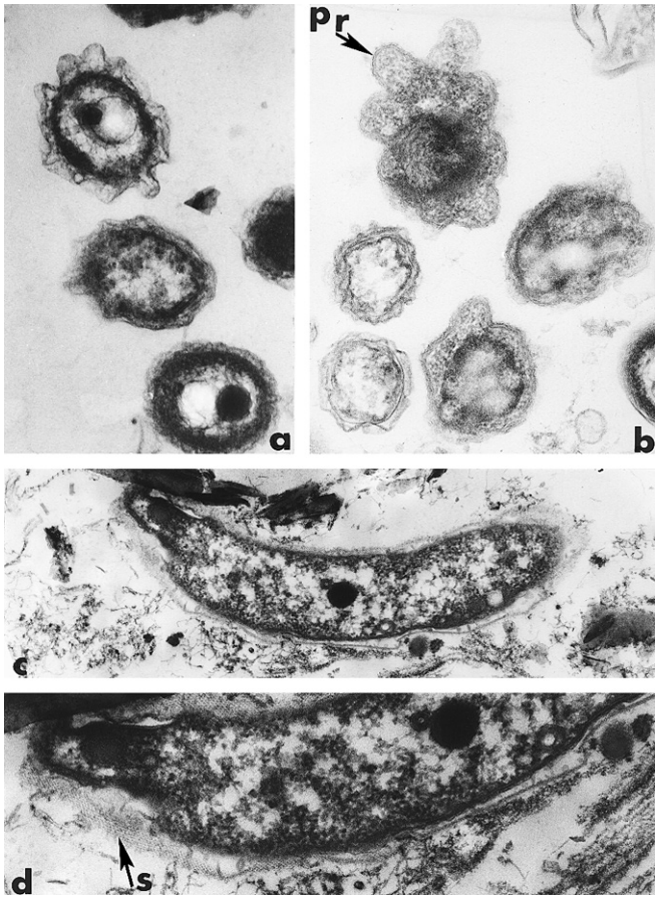

Fig. 6a, b Thin section of some oligotrophic bacteria in water column biomass. **a** A group of *Prosthecomicrobium* sp. cells showing (possibly) stages of development. 33 600×. **b** A second group of *Prosthecomicrobium* sp. with one cell showing fully developed prosthecae (*Pr*). 33 600×. **c** A single cell of *Caulobacter* sp. sectioned at such an angle as to show the beginning of the stalk and the outer covering S layer. 33 600×. **d** Enlargement of the stalk and the structured S layer more clearly. 50 400×

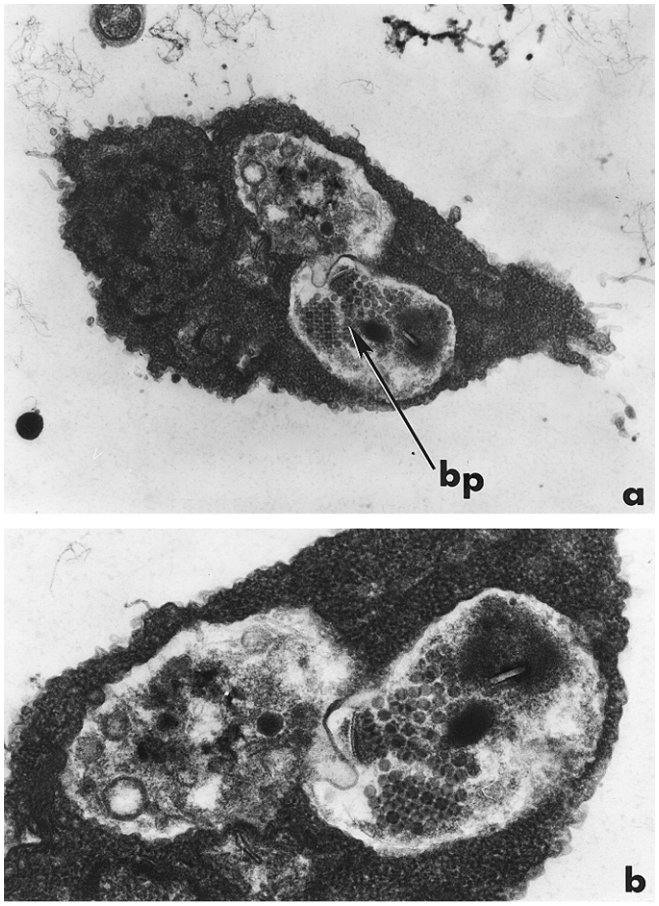

Fig. 7 a Thin section of an amoeba that is a part of the lake water biomass. The cell shows two large food vacuoles separated by a membrane. 20 400×. **b** An enlargement of the food vacuoles shows (in the one on the *left*) masses of bacteriophage (*bp*) each identical to the other in size and appearance. The vacuole on the *right* contains an array of structured particles including viruses and virus-like particles. 33 600×

were cut so as to expose hyphae, bud, unique cell arrangements or prosthecae one would not be able to distinguish them from other common bacteria. The only reason *Caulobacter* sp. could be identified was because the sectioned stalk or its tapered origin was observed. The cell surface was cut in such a way that the S layer (Sleytr et al. 1993) was exposed and one could observe its characteristic pattern (Fig. 6c, d). Since S layers are reported to be found on the surfaces of many prokaryotes it seems odd that we have seen them in situ only on the surface of *Caulobacter* sp.

It is significant that a large proportion of the minute cells in sections showed a highly convoluted and even lobed surface (Corpe and Jensen 1992). Whether these represent an adaptation by heterotrophs to stress represented by a low-nutrient environment or whether they are in fact a group of bacteria (Prosthecates) distinguished by small size has not been resolved, but certainly they are a significant part of the picoplankton population of Lake Arthur.

Bacteriophage

In a recent paper, Bergh et al. (1989) noted the considerable abundance of viruses in aquatic environments in relation to the number of prokaryotic cells present. They concluded that viruses might play an important role in bacterial mortality, thereby limiting their important biochemical activities but at the same time providing organic nutrients, phosphorus and nitrogen and other substrates for new bacterial growth. Similar results were found in the sea by Sieburth et al. (1988). Heldal and Bratbak (1991) and Bratbak et al. (1992) have suggested that viruses released at the time of lysis may themselves be degraded and possibly used by bacteria as a source of C, N and P. Our finding of free virus particles in waters of Lake Arthur, using the in situ electron microscopy method, was surprising, but efforts to make a direct estimation of bacteriophages by plaque counts against pure cultures of several prokaryotic organisms and *Chlorella* sp. did not meet with success. The

possibility that the free viruses observed in thin sections were not viable, as suggested above, was discounted, in part because plaques were developed on lawns of several heterotrophic bacteria and *Chlorella* but only when 0.2–0.3 ml 0.2-μm filtrates from a concentrated biomass was spotted onto a lawn surface.

Kokjohn et al. (1991) have indicated that interaction of phages with their hosts in natural environments is not well understood. In starved cells, for example, replication was significantly affected. In oligotrophic Lake Arthur, the cells seem to become smaller (Corpe and Jensen 1992) since 60%–75% of the cells ranged in diameter from 0.48 μm to 0.5 μm and the viability, heterotrophic and phototrophic, was only 25% of the total. We will not know the percentage of these that are susceptible to virus until we are able to recognize the specific host cells and carry out appropriate assays.

Klut and Stockner (1990) have reported the presence of virus-like particles with picoplankton in an oligotrophic lake on Vancouver Island and noted the possible effects on cyanobacterial populations, suggesting the ecological control of picoplanktonic microorganisms (Montegue and Philips 1991). Proctor and Fuhrman (1990) also suggested that bacteriophage infection may be a significant mechanism of mortality in bacteria and that understanding the mechanism is critical to understanding primary and secondary production of biomass.

Eukaryotic viruses

About 17 different algal morphotypes were found in water-column samples. The numbers of any one species are too few to have much significance. In fact, the total contribution of all species to the biomass is minor. In addition, the size of individual cells is small when compared to the species of similar morphology described in the literature (Zurek and Bucka 1994; Richardson and Stolzenbach 1995). The question concerning the *source* of host species and whether or not they developed a small size only after exposure to the water of the lake, with little opportunity for cell growth, has not yet been resolved.

Starvation conditions with regard to phosphorus and perhaps nitrogen exert an important population control on algal growth (Schindler 1977), and they rarely become important contributors to dissolved organic levels (Suttle et al. 1990). The amount of P_i found in Black Rock Forest lakes was reported to range from 0.008 mg/l to 0.75 mg/l (Adirondack Lake Survey Report 1987). Bacteria, by taking up phosphorus most effectively (Kortstee et al. 1994), only intensify the phosphorous limitation in algae (Moriarty and Bell 1993).

It would seem that algal host cells may become infected *after* they have adapted to the lake conditions and because they are unable to divide significantly for an extended period. For lack of P_i, they become reduced in size and the opportunity for infection and virus development increases. Since we know nothing about the diversity of the viruses, their effectiveness as pathogens or their possible host ranges at this time we cannot speculate further.

Chlorella species have been isolated from waters of Lake Arthur and other Black Rock Forest lakes, and are present in greater number than any other unicellular algae. *Chlorella*-like organisms were also observed in thin sections of picoplanktonic fractions from lake biomass. The isolates were normal-sized *Chlorella*-like organisms but those in the picoplanktonic biomass appeared to have the *Chlorella* form and eukaryotic ultrastructure (Corpe and Jensen 1992). The significance of algae and their viruses in aquatic environments is just beginning to be explored (Van Etten et al. 1991). Plaque assays for some viruses have made experiments possible (Van Etten et al. 1983). *Chlorella* strain NC64A viruses are ubiquitous in the USA, and in freshwaters in China (Van Etten et al. 1985; Zhang et al. 1988). Much of the information concerning details of algal virology has employed *Chlorella* sp. and their viruses. The *Chlorella* viruses isolated from freshwater bodies in Japan are reported to be very large: 125–200 nm and icosahedral, as reported by Yamada et al. (1991). Suttle (1994) has suggested that viruses in the marine environment are important in control of algal growth. Peduzzi and Weinbauer (1993) suggest that virus fractions (2–200 nm) influence patterns of planktonic community successions. Murray and Eldridge (1994) have shown that bacteriophages become incorporated into the membrane planktonic food web.

Starvation conditions, with regard to P and perhaps N, exert an important population control on algal growth in Lake Arthur to the extent that the algae do not become important contributors to the biomass nor to the dissolved organic matter in the lake. Cells enter from surrounding forest soils following a heavy rain or from a shallow area at the water/shoreline interface. We have not examined the sites of these sources but the work is currently underway.

Virus compared to protozoa as agents of population control

Wiggins and Alexander (1985) showed that a minimum density of susceptible host cells is required for bacteriophage replication. They suggested that phages have no significant impact on bacterial populations. Their work was done with pure cultures in the laboratory so may not relate to the complexity of conditions in the natural aquatic environments. On the other hand, the interactions of phages with their hosts in the natural environment, overall, is poorly understood (Kokjohn et al. 1991), but it has been shown that attachment and replication of phages are not impaired at host-cell densities equal to or lower than those frequently found in aquatic environments, when host cells are physiologically able to allow phage growth. Attachment of phage to active or to starved cells was not affected by river

water although replication in starved cells was significantly altered.

Suttle et al. (1990) found that natural marine waters contained 10^6–10^9 virus particles/ml, but the organisms they infect are largely unknown and thus their importance in the aquatic system is unknown as well. Our study of Lake Arthur viruses caused us to have a similar concern about their importance. Borsheim et al. (1990) showed that virus particles were an order of magnitude more abundant than bacteria in marine coastal waters. The smallest size class of marine bacteria predominated. The high proliferation rates they observed suggested that the viruses have the potential to act as "selection factors" for aquatic bacteria. The control of bacteriophage in nature may not be influenced by the presence of critical numbers of available host cells. Borsheim et al. (1990) suggest that viruses may adsorb to particles either by specific adsorption to hosts that are resistant or by nonspecific adsorption to other particles. Exoproteolytic enzyme activity in aquatic environments (Hoppe 1983) may catalyze breakdown in capsid proteins and produce a decline in virus numbers. Borsheim et al. (1990) have suggested that protozoa may be able to ingest viruses in natural communities, for which they show *no* evidence; since Sherr (1988) had shown that some protozoa are capable of ingesting high-molecular-mass substances, they reason that this may also include viruses. Our observations (Fig. 7) may be the first direct evidence of virus particle ingestion.

The relative abundance of amoebae in the water-column samples was very low, probably because amoebae require a solid surface so one would expect them to be more abundant on particulate matter, animal and plant residues and sediments (Rodriguez-Zaragoza 1994). Resuspension processes, such as gas bubbles (methane) rising from the bottom sediments to the air/water interface, might be sufficient to move and maintain amoebae and small particulates in the water column (Jensen and Corpe 1994). Our earlier data on feeding activities of isolated amoebae (unpublished) indicate that they could use several heterotrophic bacterial cells but showed no ability to feed on *Chlorella* or cyanobacteria, when washed cells were used on a 1% (w/v) agar surface. Figure 7 shows a thin section of an amoeba with a food vacuole containing two types of virus particles. It is unclear whether the viruses were in the bodies of lysing bacteria or whether the viruses were sought out as the desirable food. Two sizes of eukaryotic viruses were observed in the food vacuoles and, since *Chlorella* cells were not used as a food source, it remains possible that virus particles, perhaps adsorbed to prokaryotic cells or some organic complex, could be ingested.

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