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Direct Electron Microscopy Study on the Morphological Diversity of Bacteriophage Populations in Lake Plußsee

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Direct electron microscopy of bacteriophages adsorbed to a carbon film without prior enrichment by specific host strains or concentration by physical or chemical methods was used to study the morphological diversity of natural bacteriophage assemblages in a North German lake. All samples contained a mixture of morphologically different tailed viruses, which were regarded as bacteriophages. Most of them had isometric heads and long noncontractile tails, belonging to morphotype B1 (*Siphoviridae*). In addition, members of morphotypes A1 (*Myoviridae*), B2 (*Siphoviridae* with elongated heads), and C1 (*Podoviridae*) were present in lower numbers. Only one cubic virus was detected, while no filamentous or pleomorphic phages were found. Up to 11 different phages per sample, and a total of 39 phages when all samples were considered together, could be distinguished by morphological criteria. The total number of phages was estimated to be on the order of 10^8/ml.

Bacteriophages have been known since early in this century and have been isolated from all types of bacteria and from virtually any aquatic or terrestrial habitat where bacteria can exist. However, only in the last few years has it been recognized that viruses are extremely abundant in ocean and fresh waters (8, 28, 36, 37, 41) and may exceed the concentration of bacteria by up to 100-fold (8, 17, 28). This has resulted in a reevaluation of the role of viruses, and especially bacteriophages, in microbial ecology (10, 29, 36), particularly in marine environments, for which studies have focused on the distribution, abundance, and function of viruses.

Detailed studies on the morphology of phages from different natural habitats have examined phages which infect specific strains of host bacteria (6, 7, 15). Up to now, direct electron microscopy without enrichment was used only for general classifications of native marine viruses based on size (8, 11, 41) or morphology (16, 37). However, more detailed morphological descriptions of phage populations are available only for some very specific habitats, such as the rumen of cattle and sheep (20, 32), the ceca of birds (31), and sewage (13). Information on the morphology of native freshwater viruses is very scarce (21).

In order to circumvent the selectivity of enrichments with host strains, we used direct electron microscopy of unconcentrated water samples to study the morphology of native phages and the distribution of morphotypes within natural populations in a North German lake. The study revealed a high morphological diversity of phage populations at different depths and seasons.

MATERIALS AND METHODS

Sampling. All samples were taken from Lake Plußsee at the area of maximum depth (29 m). Water samples were collected between January and October 1990 in sterile glass bottles with a Sorokin sampler, either from 1 m or at the thermocline (7 m on 15 October 1990). Sediment cores ca. 3 cm in diameter were taken with a Züllig gravity sediment corer. Lake Plußsee is a eutrophic lake in the northern part of Germany (54°10.9'N, 10°20.7'E), with a diameter of 400 m, a surface area of 13.5 ha, and a mean depth of 9.4 m. Details on phyto-, zoo-, and bacterioplankton dynamics have been published previously (18, 19, 24, 26).

Electron microscopy. Specimens for electron microscopy were prepared by a modification of the method of Lembke et al. (22). The samples were not enriched, concentrated, or preserved. Only the January sample was fixed with 2% (final concentration) formaldehyde. Samples taken in July and August were filtered through 0.2-μm-pore-size cellulose-nitrate membranes (Schleicher & Schuell, Göttingen, Germany) to remove larger particles. As detritus, bacteria, and larger plankton did not adsorb to the carbon film, unfiltered samples were subsequently used to avoid adsorption of phages to the cellulose-nitrate membranes. For sediment samples, 2-cm layers were taken from the surface of a core. After vigorous shaking, the samples were centrifuged for 10 min at 7,000 × *g* and 10°C to remove larger sediment particles, and the supernatant was used for further preparation.

The phages were adsorbed by dipping a carbon-coated mica plate (ca. 2.5 by 2.5 mm) into 100 μl of the sample and floating the carbon film onto the drop. After 10 min, the film was washed twice in double-distilled water and finally transferred into uranyl acetate (2% [wt/vol], pH 4) for negative staining. After 30 to 60 s, the carbon film was picked up with a copper grid (3.05-mm diameter, 300 mesh; Plano, Marburg, Germany), and excess stain was removed with filter paper. The samples were examined with a Philips EM300 electron microscope at 80 kV and a magnification of ×114,000. The magnification of the negatives was ×37,620, as determined by photographing a cross-grating with a total width of 463 nm at the same magnification as the samples. Size measurements of phages were done on micrographs at a final magnification of ×189,000. The density of phage-like particles was between <1 and 15 per grid square. Morphological characterization was done from micrographs taken randomly to represent all different types of phages in a sample.

RESULTS

In every sample, phage concentrations were high enough (>10^8/ml) to prepare the electron microscopic grids without enrichment or concentration. The bacteriophage-like parti-

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cles were selectively adsorbed onto the carbon film. Detritus particles, colloids, bacteria, eucaryotic organisms, and other types of viruses were rarely detected.

Most phage heads were hexagonal in outline, with six-sided profiles which were regular, with three symmetrical axes (e.g., Fig. 1a to c); others were irregular, with only one symmetrical axis (e.g., Fig. 1l and 3a and b). However, these data are not adequate to differentiate between icosahedra, octahedra, and dodecahedra. Only one prolate-headed phage was detected (see Fig. 3i).

On average, the phages observed in water and sediment samples had head diameters of 41 to 117 nm and tail lengths of 11 to 710 nm (Table 1). Most had long tails of 74 to >600 nm. Some very large phages with isometric heads and long, noncontractile, flexible tails were 444 to 710 nm long (Fig. 2c to g and 3i).

At least 2 and up to 11 distinct morphotypes were observed in the different samples. In total, a minimum of 39 morphologically different phages could be distinguished (Table 2). Many (49.6%) of the phages revealed isometric heads and long noncontractile tails (morphotype B1 [5]; viruses from this group belong to the family *Siphoviridae* with isometric heads [14]). Only one tailed phage (Fig. 3i) belonged to morphotype B2 (*Siphoviridae* with prolate heads). Several phages (17.6%) possessed contractile tails of various shapes (morphotype A1, *Myoviridae* with isometric heads; e.g., Fig. 1f, i, k, and o and 3e). Others (12.8%) had tails that might be contractile, as deduced from the shape, but a neck was not clearly visible (e.g., Fig. 1j and p), and some (19.2%) had short tails (6 to 32 nm), resembling morphotype C1 (*Podoviridae* with isometric heads [Fig. 3j to n]). Only one cubic phage was found (Fig. 3o). Filamentous and pleomorphic phages were not observed in any of the samples.

Differences in the composition of the phage populations from different habitats or times were not obvious. Nevertheless, some phage types were found only in the sample taken in October 1990 at a depth of 1 m (Fig. 2f and 3e), at the thermocline (Fig. 3i), or in the sediment (Fig. 1p and 3g). The largest phages were observed in the unfiltered samples taken from 1 m in July, August, and October and in the filtered sample from April.

The resolution of the electron micrographs was good enough to reveal details in the fine structure of nearly all phages. Base plates, fibers, spikes, antennae, collars, capsomer structure of the head, and substructures of the tail could be seen. Several phages revealed distinct base plates (e.g., Fig. 1j and p). Long (Fig. 1g and o and 3l) or short (Fig. 1p) fibers were observed around the tips of some tails. Phages with a single fiber at the tip of the tail were also observed (Fig. 1c and 2d). The tailless phage in Fig. 3k possessed several spikes at the base of the head. Head structuring into regularly arranged capsomers was visible for the phages in Fig. 3c and o. The phage shown in Fig. 1b may have two short antennae extending from the edges of the head. Phages with a collar (e.g., Fig. 1e and m and 2b) were also detected. The collar of the phage in Fig. 3m is composed of at least two disks. The substructures of the tails were arranged in regular disks (e.g., Fig. 1b and g, 2b, and 3e), in a scale-like manner (e.g., Fig. 1f and 3f) or a criss-cross pattern (e.g., Fig. 2c and 3h). A long thin spiral filament like the one described for *Bacillus* phage G (3) surrounded the tail of one phage (Fig. 3e), and another (Fig. 1d) contained knob-like structures at the end of short filaments.

DISCUSSION

Great morphological variability was observed in the phage assemblages from Lake Plußsee. Similar results (data not shown) were obtained for Lake Kellersee (Holstein, Germany) and Lake Kinneret (Israel). With one exception, the phages possessed tails, suggesting that they were not viruses of eukaryotes. Tailed, virus-like particles have been observed in several eukaryotic algae (38), but their infectivity has not been confirmed (2). Cyanophages have to be considered possible elements of the virus communities in Lake Plußsee because cyanobacteria are important components of the phytoplankton in this lake (18, 24). They may regulate the density and distribution of their hosts in marine (35) and freshwater (27) environments. As the morphologies of cyanophages and other bacteriophages are similar (33), the techniques used here cannot be used to distinguish between them. Yet, as the two phage groups infect different trophic levels (photosynthetic carbon production versus heterotrophic carbon transformation), ultimately it will be important to distinguish between the two phage groups in studies on the role of viruses in aquatic carbon flow.

Every sample contained a mixture of morphologically different phages, belonging to the *Siphoviridae*, *Myoviridae*, and *Podoviridae* families (Table 2). In addition, one cubic phage was detected which could not be assigned to a specific morphotype, as no information on the nucleic acid type and structure of the envelope is available. The frequency of occurrence of these different morphological types is similar to that reported for the more than 4,000 cultivated phages from culture collections (1). Similar distributions were obtained for natural virus communities in water from Yaquina Bay, Oregon (37), and for 75 cultivated phages from a water sample from the North Atlantic (15). In these reports, tailed phages constituted more than 96% of the total number, noncontractile tails were more frequent than contractile tails, and isometric heads were more frequent than prolate ones. However, in direct microscopic studies of samples from the rumen of sheep and cattle, phages with contractile tails were the most frequent (20, 32).

In contrast to our observations for Lake Plußsee, in seawater from the Southern California Bight, small viruses (<60 nm) dominated (8–11, 41). Phages with tails or tail-like structures were less frequent in Norwegian fjords, Japanese coastal and offshore waters, and the Chesapeake Bay (8, 16, 41) and were classified predominantly into the large size classes (9). In Southern California Bay, tailed phage-like particles dominated in the 30- to 60-nm size class (11). So far, it is not clear whether this is a general difference between freshwater and marine phage populations. However, the high frequency of tailless viruses might also be due to a dominance of eukaryotic hosts or to preparation artifacts from ultrafiltration or ultracentrifugation onto specimen grids.

The average size of the phages observed in the samples from Lake Plußsee (head diameter, 37 to 180 nm; tail length, 3 to 539 nm) was typically in the range found for virus-like particles in the ultraoligotrophic Sproat Lake (21) and for 662 bacteriophages isolated from 112 bacterial species from diverse habitats (30). However, unusually large phages of 630 and 710 nm (Fig. 2f and g) were also detected. These are among the largest phages known, although there have been some reports of phages that are even larger (20, 32).

Since all RNA phages described to date are tailless and the viruses described in this study are tailed, most of them probably contained DNA. Similarly, as lipid-containing

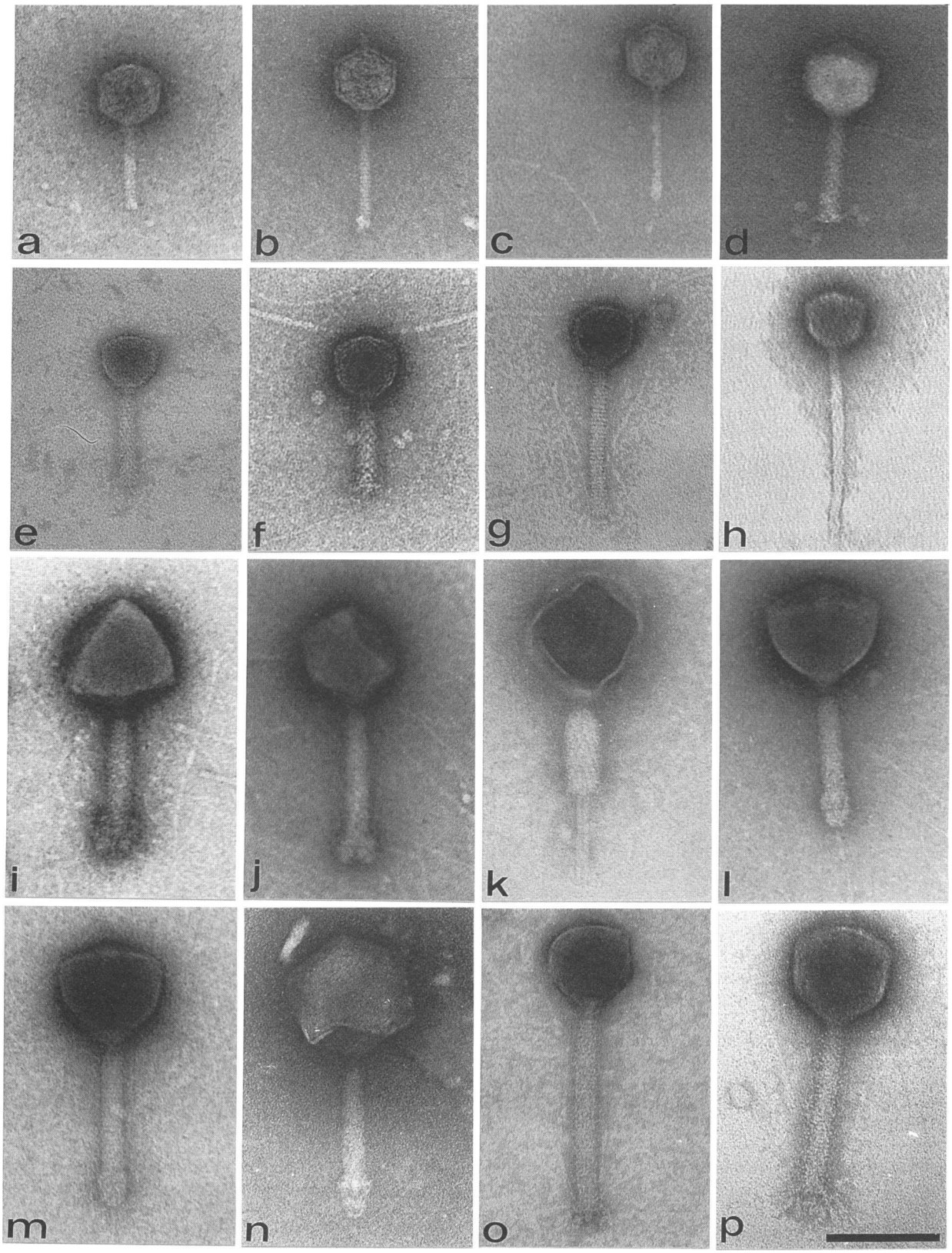

FIG. 1. Phage particles of morphotypes A1 and B1. Bar, 100 nm.

TABLE 1. Size range of tailed phages observed by electron microscopy

Sample date	Depth (m)	No. of observations	Head diam (nm)		Tail length[a] (nm)		Tail width, range (nm)	Total length (nm)	
			Range	Mean	Range	Mean		Range	Mean
January 22	1	2	53–58	56	90–106	98	8–24	148–159	154
April 9	1	31	42–106	59	80–450 (16–37)	153	6–21	130–510	208
July 7	1	27	41–117	73	74–143 (11–26)	124	8–26	120–292	203
August 13	1	15	53–106	74	74–572 (11–26)	208	8–26	127–630	287
October 10	1	15	53–106	76	90–636 (11)	229	8–26	180–710	314
	7	10	53–106	84	74–400	179	8–26	147–533	264
	27	25	48–117	89	106–400 (11–26)	192	11–32	196–485	268

[a] Values for phages belonging to morphotype C1 are shown in parentheses.

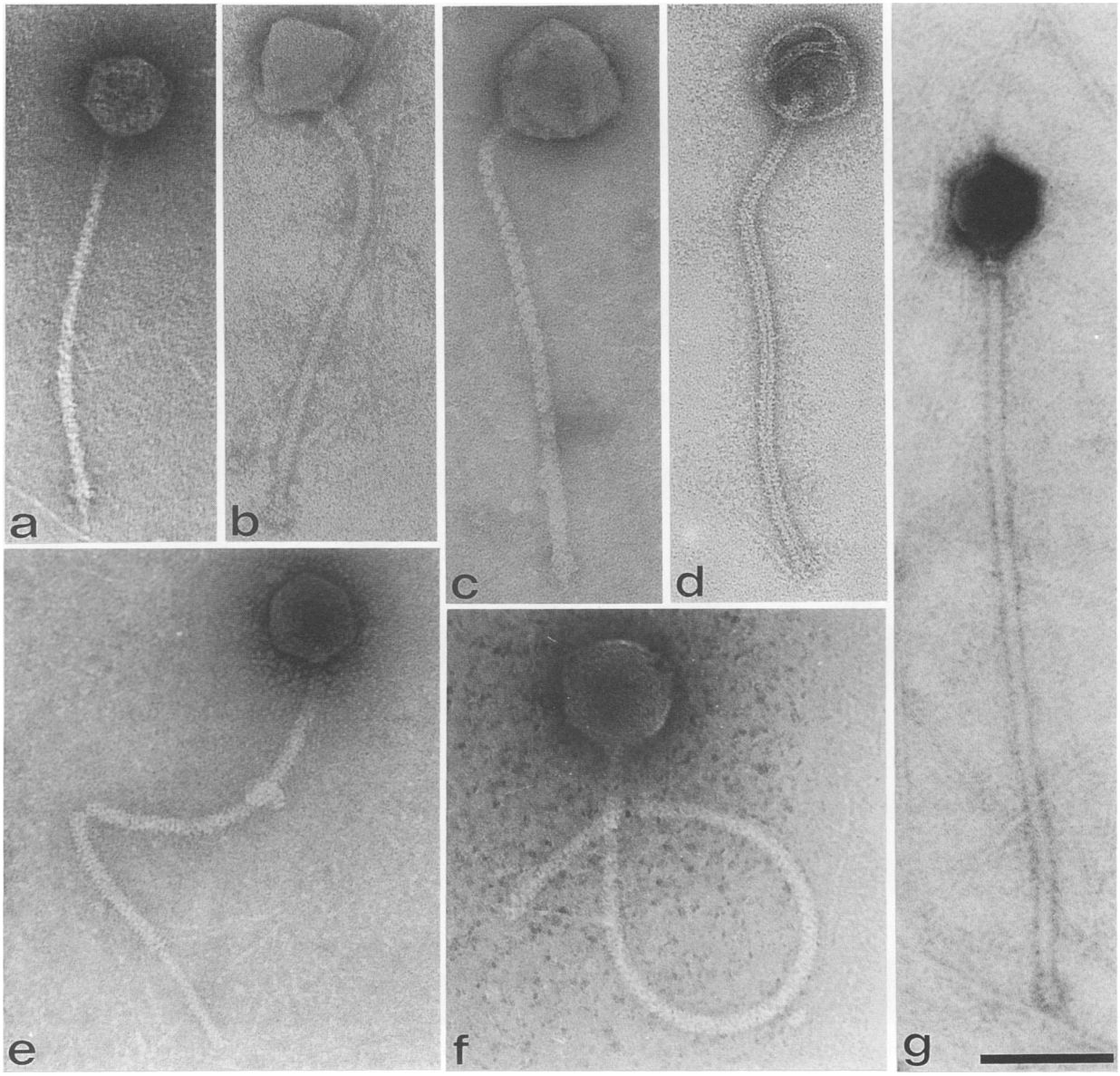

FIG. 2. Phage particles of morphotype B1, with isometric heads and long, flexible tails. Bar, 100 nm.

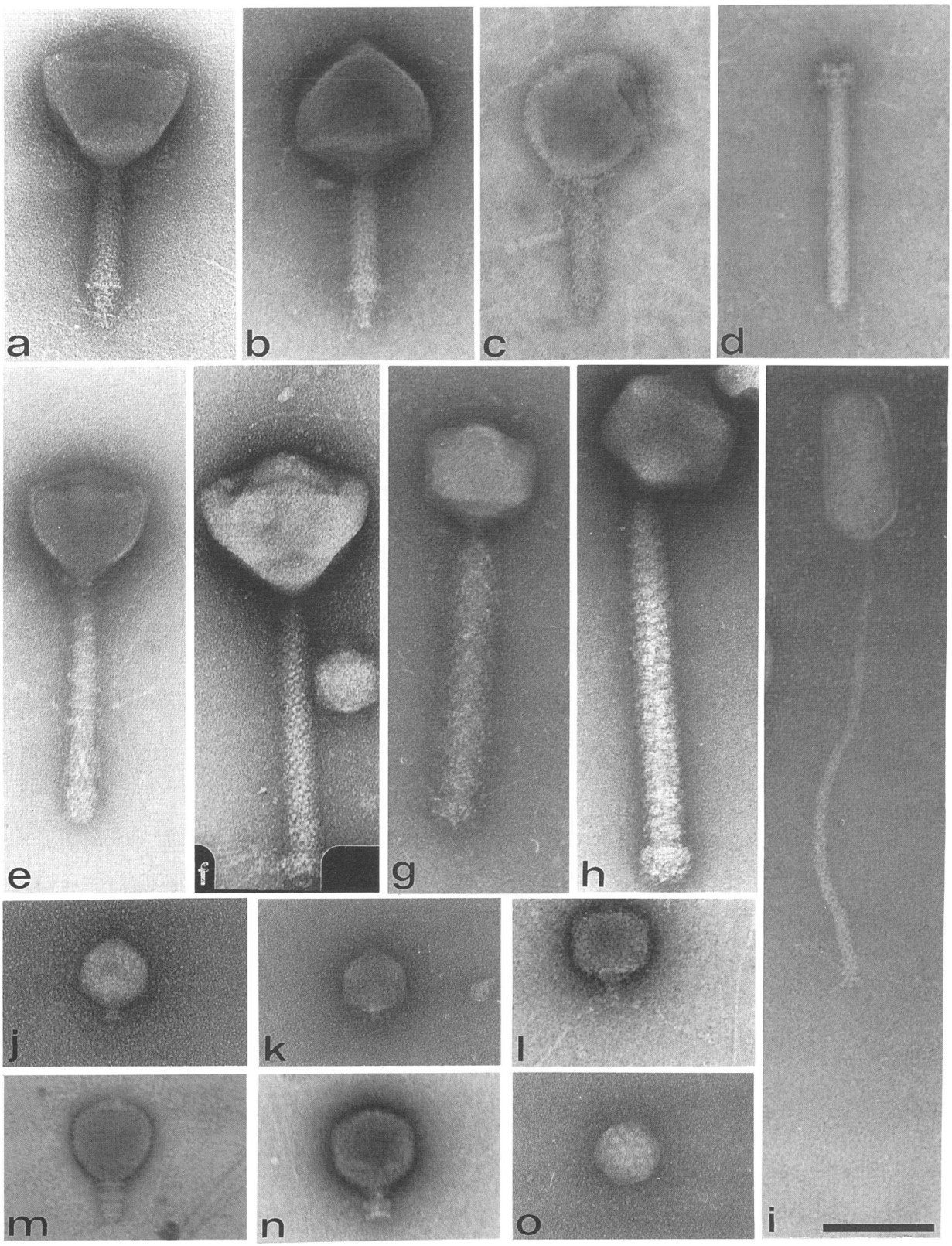

FIG. 3. Phage particles of morphotype A1/B1 (a to c and e to h), B2 (i), C1 (j to n), and D (o). Note tail-like particle in panel d. Bar, 100 nm.

TABLE 2. Frequency of morphotypes classified according to the scheme of Ackermann and Eisenstark (5)

Sample date	Depth (m)	No. of phages belonging to morphotype: A1	B1	A1/B1[a]	B2	C1	Total	No. of different phages
January 22	1	0	2	0	0	0	2	2
April 9	1	3	22	1	0	5	31	10
July 16	1	3	13	3	0	8	27	9
August 13	1	4	6	1	0	4	15	8
October 15	1	3	4	4	0	4	15	8
	7	0	6	3	1	0	10	7
	27	9	9	4	0	3	25	11
Total		22	62	16	1	24	125	39

[a] Classification uncertain.

phages lack tails (3), they were also probably not present or not detected. The absence of filamentous and pleomorphic phages in our samples is not surprising, since they represent rare groups infecting members of the family *Enterobacteriaceae*, archaebacteria, and *Mycoplasma* and *Pseudomonas*/*Xanthomonas* species (2). The possibility that some of the observed phages are of allochthonous origin (e.g., waterfowl, wild animals, or human activities in the catchment area) cannot be ruled out completely, because sewage (6) and the feces of birds (31) and ruminants (20) may contain a variety of different bacteriophages. However, it seems unlikely that they constitute a significant portion of the total assemblage of a natural lake like Plußsee without direct sewage influences, as the lake is situated in a small catchment area with little human impact.

The extent to which bacteriocins contribute to the different morphotypes in our samples is unknown. Bacteriocins may appear as complete phage-like particles or as phage tails (Fig. 3d) and may kill bacteria without replication (2). Therefore, they are sometimes indistinguishable by electron microscopy from bacteriophages.

The high morphological diversity of phage communities corresponds well with the great variability and dynamics of bacterial populations in Lake Plußsee (40). Since bacteriophages have been found in many host genera distributed over the entire bacterial world (2), it seems reasonable to assume that for any aquatic bacterial population, at least one appropriate phage population interacting and coexisting with it may exist in the same habitat; hence, the phage populations can be expected to be as dynamic and diverse as the bacterial populations. Only part of this variability can be described with morphological criteria, as immunological and molecular techniques have revealed serological, protein, and nucleic acid diversity among morphologically identical viruses (4, 12, 23).

Electron microscopy provides a direct insight into the morphological variability of phage populations without being dependent on the isolation of suitable host strains. This is important, as bacteria which are not cultivable under laboratory conditions usually dominate aquatic environments and may be important phage hosts. In any case, propagation with specific host strains is selective and will not detect all phages in samples with a high phage diversity (7). On the other hand, it has to be considered that different phages, especially filamentous, pleomorphic, and lipid-containing ones, might adsorb to the carbon film with different efficiencies. However, our experience with various phage lysates did not reveal marked differences in the adsorption of tailed and untailed phages of different morphologies.

Most of the phages from Lake Plußsee were intact, with distinct fine structures such as tail fibers, base plates, and other appendages which are important for the recognition of and interaction with the host cell. This may be an indication that a large proportion of the phages are suspended in the water as potentially infective particles.

As some bacteriophages can adsorb to clay minerals without losing their infectivity (34), when these particles sink, infective phages may become concentrated in the sediment. This may explain why we found the highest phage density in the sediment samples taken in October. It remains to be determined whether sediments are a reservoir of viruses that allow phage survival under unstable environmental conditions.

It was not the aim of this study to determine the number of phages in the water samples. Nevertheless, we compared the phage densities on the grids with those of phage lysates of known titer and roughly estimated the phage density in the water samples to be on the order of 10^8/ml. This is in the range observed for many other marine and freshwater habitats (8, 9, 16, 36).

A continuous supply of new phages from diverse bacterial populations is required to sustain the high numbers of phages and the numerous morphological types found in Lake Plußsee. This means that bacteriophages should interact dynamically with their hosts, significantly influencing the bacterial populations, as has been described for different aquatic habitats (10, 17, 25, 27, 35, 41). If the phages were in a more or less static state of coexistence with their hosts (39), without a permanent supply of new phages and consequently without substantial bacterial mortality, this high dynamism and diversity would be difficult to explain.

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