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## The ecology of *Paraphysomonas imperforata* based on studies employing oligonucleotide probe identification in coastal water samples and enrichment cultures

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### Abstract

The geographical distribution and seasonal abundance of the cosmopolitan heterotrophic flagellate *Paraphysomonas imperforata* in several coastal waters was examined using species-specific oligonucleotide hybridization probes which target small subunit ribosomal RNA. *P. imperforata* was found to occur in several coastal environments, but at very low abundances (typically  $<50$  cells  $\text{ml}^{-1}$ ). The seasonal abundance of *P. imperforata* examined at one sampling site remained consistently low and constituted no more than 1% of the total nanoplankton at any time during a 17-month sampling period. In contrast to the low abundances observed in natural water samples, *P. imperforata* frequently dominated heterotrophic enrichment cultures prepared from these same samples, comprising up to 98% of the total nanoplankton. Based on these findings, we conclude that *P. imperforata* is an opportunistic species capable of growing rapidly to high abundances when prey concentrations are high. Water and enrichment temperature as well as the temperature tolerance range of *P. imperforata* appear to have played a role in the seasonal differences observed in *P. imperforata* dominance. Experiments with enrichment cultures indicated that the absolute abundances of *P. imperforata* in the water samples and the activity of consumers of nanoplankton also influenced the degree to which *P. imperforata* dominated the heterotrophic nanoplankton assemblages of enrichment cultures. Seasonal changes in water temperature might also affect these latter factors, and, as a consequence, indirectly influence the ability of *P. imperforata* populations to dominate enrichments. Our results support the notion that enrichment cultivation of heterotrophic flagellates, and perhaps incubations in general, can select for species such as *P. imperforata* that may not be representative of nanoplanktonic protists that numerically dominate natural assemblages.

Heterotrophic nanoflagellates (2–20  $\mu\text{m}$  in diameter) play a major role in the flow of energy in planktonic food webs, as the primary grazers of bacteria and other picoplankton and as prey for larger protists and metazoa (Fenchel 1982*b,c*; Sieburth and Davis 1982; Azam et al. 1983; Sieburth 1984; Sherr and Sherr 1994). These heterotrophs are also potential sources of nutrients and dissolved organic material, which are regenerated as a result of their grazing activities (Goldman and Caron 1985; Caron and Goldman 1990). Although our understanding of the role of these microorganisms in aquatic ecosystems has improved considerably, very little is known about the spatial and temporal abundance and dynamics of individual species in the water column. The abundance of total nanoplanktonic protists in nature can be routinely enumerated by standard staining and counting methods using fluorochrome dyes and epifluorescence microscopy (Sherr et al. 1993), but these methods do not reveal ultrastructural features for identifying individual flagellate taxa.

Several investigators have recently begun to document the diversity and distribution of heterotrophic nanoflagellates in different marine waters, but such studies have not focused on obtaining quantitative information for individual species (Vørs 1992, 1993*a,b*). Studies documenting the diversity and distribution of individual species of nanoflagellates have not

been quantitative because identification of these taxa usually involves enrichment cultivation and/or electron microscopy. Samples must be concentrated or enriched for this work because nanoplanktonic flagellates usually do not occur in sufficient densities in natural waters for direct observation. As a result, abundances of these microorganisms are difficult to obtain from electron microscopical investigations. The absolute and relative abundances of nanoflagellates in these preparations are either biased (e.g., in enrichment cultures) or no longer representative of their original densities because of cell loss during preparation for electron microscopy.

A recent microscopical approach that enables simultaneous identification and enumeration of specific microorganismal taxa in water samples without isolation or culture is in situ hybridization of whole cells with rRNA-targeted oligonucleotide probes (Stahl and Amann 1991; Lim et al. 1993; Amann et al. 1995; Lim 1996; Lim et al. 1996). Oligonucleotide probes are designed to bind to short, complementary sequence regions of the rRNA that are unique to the taxa of interest. These probes are labeled with fluorescent dyes (either directly or indirectly via a secondary reporter molecule) so that cells hybridized with these probes may be directly observed by epifluorescence microscopy, confocal laser scanning microscopy, or flow cytometry (DeLong et al. 1989; Amann et al. 1990*b*; Lim et al. 1993; Manz et al. 1995; Simon et al. 1995). When used in combination with a recently developed quantitative in situ hybridization method, oligonucleotide probes have been shown to be applicable for detecting and quantifying small protists in field samples by epifluorescence microscopy (Lim et al. 1996).

*P. imperforata* is a heterotrophic, free-living flagellate

### Acknowledgments

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within the class Chrysophyceae. The cells are typically spherical in shape and range in size from 4 to 8  $\mu\text{m}$  in diameter. This species and other members of the genus are identified by the siliceous scales that cover the organism. *P. imperforata* was chosen as the target species for this study because it has been observed commonly in enrichment cultures established from a wide variety of environmental water samples in all seasons. Both freshwater and marine forms appear to be cosmopolitan (Preisig and Hibberd 1982a,b; Preisig et al. 1991; Vørs 1993a,b). Moreover, numerous laboratory studies have employed *Paraphysomonas* as an experimental organism that is representative of the heterotrophic nanoplankton, and thus extensive physiological information exists for this organism (Fenchel 1982a,b; Caron et al. 1985; Goldman and Caron 1985; Goldman et al. 1985; Andersen et al. 1986; Caron et al. 1986; Landry et al. 1991; Choi and Peters 1992).

In this paper, we describe the occurrence and seasonal abundance of *P. imperforata* in coastal waters based on counts obtained by probing water samples with *P. imperforata*-specific oligonucleotide probes (Caron et al. in prep.). The seasonal abundance of *P. imperforata* relative to the total nanoplankton in natural samples was compared to its abundance in enrichment cultures to assess the effect of nutrient enrichment on *P. imperforata* dominance. Experiments with enrichment cultures were further performed to examine the importance of absolute *P. imperforata* abundance, nanoplankton grazers, and prey availability in controlling *P. imperforata* abundance and distribution in mixed plankton assemblages.

## Methods

*Sampling sites and sampling schedule*—Water samples were collected from several coastal environments to examine the geographical distribution and abundance of *P. imperforata* among the nanoplankton. The sampling locations were Vineyard Sound (Falmouth) and Great Harbor (Woods Hole) in Massachusetts, seven estuarine sampling sites on Long Island (Orient Harbor, West Neck Bay, Coecles Harbor, Northwest Creek, Northwest Harbor, Quantuck Creek, and Quantuck Canal) in New York, and Ferry Reach, Bermuda. The Massachusetts and Bermuda water samples were mesotrophic, while the Long Island water samples were eutrophic and contained high algal biomass. At each site, samples were collected at a depth of approximately 0.1–0.2 m and preserved with formaldehyde to a final concentration of 3.7%. Samples were stored at 4°C and processed within 1 week of collection as described below.

Seasonal changes in the abundance of *P. imperforata* were examined by sampling at the Vineyard Sound site for a period of 17 months beginning in June 1995. Water samples were collected on the 1st and the 15th of every month (except for the 2 June and 16 August 1995 sampling days) as described above. Samples from June 1995 were preserved as whole seawater samples, but subsequent samples were filtered through 20- $\mu\text{m}$  Nitex screening to remove larger plankton and detrital material before preservation in formaldehyde.

*Experiments with enrichment cultures*—Vineyard Sound seawater from the same samples collected for analysis of *P. imperforata* seasonal abundance were used for heterotrophic nanoflagellate enrichments twice a month over the 17-month sampling period. These enrichment cultures were established to assess the effect of enrichment on *P. imperforata* abundance compared to the TNAN. Yeast extract was added directly to 250 ml of Vineyard Sound seawater at a final concentration of 0.005% (to promote growth of the natural bacterial assemblage) and incubated in the dark at 20°C. These enrichments were sampled 4 d later for enumeration of TNAN and *P. imperforata*. In our experience, this incubation period allowed peak nanoplankton abundances to develop (data not shown). Eight of the enrichment cultures established over the 17-month period were also sampled on days 2 and 3 to follow changes in the abundances of TNAN and *P. imperforata* during the 4-d period. All the enrichments were performed in duplicate, except for those established from June 1995 to January 1996.

Three experiments with enrichment cultures were conducted in parallel to the routine enrichment cultures to assess whether factors such as initial *P. imperforata* abundance, grazing by nanoplankton consumers, or food limitation affected *P. imperforata* dominance. Subsamples used in these experiments were taken from the same water samples collected for monitoring the seasonal abundance of *P. imperforata* in Vineyard Sound and for establishing the routine enrichment cultures.

We hypothesized that the absolute abundance of *P. imperforata*, although it was low in most samples, might affect the degree to which *P. imperforata* dominated enrichment cultures. To test this hypothesis, *P. imperforata* (Clone VS1, isolated from Vineyard Sound in 1987) was added to enrichment cultures on 15 August 1996 to increase the initial abundance of *P. imperforata* relative to its natural density. The target concentration of *P. imperforata* after addition was approximately 1% of the TNAN. The addition was performed in duplicate. Based on the results of this experiment, we repeated this treatment on 1 September 1996 and included a second treatment (*P. imperforata* also was added at a concentration of <1% of the TNAN). All the enrichment cultures were sampled immediately following enrichment with *P. imperforata* and on days 3 and 4 for the enumeration of TNAN and *P. imperforata*.

In the second experiment, we examined whether grazing by nanoplankton consumers might play a role in determining *P. imperforata* dominance. Grazing experiments with enrichment cultures were conducted using Vineyard Sound seawater collected on three occasions (15 April, 15 May, and 15 June 1996). Water samples were divided into three treatments, each consisting of 0.5 liter of seawater contained in 1-liter polycarbonate bottles, performed in duplicate: unfiltered seawater, 20- $\mu\text{m}$  filtrate of seawater, and 5- $\mu\text{m}$  filtrate. The unfiltered seawater treatment contained the entire grazer assemblage, the 20- $\mu\text{m}$  filtrate included only nanoplanktonic (2–20  $\mu\text{m}$ ) grazers, and the 5- $\mu\text{m}$  filtrate presumably contained only small protists and picoplanktonic cells but excluded consumers >5  $\mu\text{m}$  that might feed on *P. imperforata*. Changes in the abundance of TNAN and *P. imperforata* in the <20- $\mu\text{m}$  and unfiltered seawater treatments relative to

the <5- $\mu\text{m}$  treatment therefore should reflect grazing by nanoplankton and/or micro- and macrozooplankton. Yeast extract was added to each bottle at a concentration of 0.005%. All the treatments were incubated in the dark at 20°C and sampled on the day of enrichment and on days 4, 6, and 7 for counts of TNAN and *P. imperforata*.

The effect of food limitation on *P. imperforata* dominance in enrichment cultures was assessed on two occasions (15 September and 1 October 1996). In each experiment, one set of enrichment cultures was supplemented with bacteria as prey (after the heterotrophic nanoplankton in these cultures reached late exponential growth phase and had significantly removed the original enriched bacterial population) and compared to another set of enrichment cultures that was not supplemented with bacteria. The bacterial concentrate was prepared by enriching the natural bacterial assemblage in a 1-liter filtrate (Whatman GF/F filters) of Vineyard Sound seawater on 0.5% yeast extract for 2 d. The bacteria were pelleted by centrifugation (10,000 rpm for 15 min) and then resuspended in approximately 50 ml of sterile Vineyard Sound seawater. A 7-ml aliquot of this bacterial concentrate was added to duplicate enrichment cultures, beginning on day 3 (when the heterotrophic nanoplankton reached late exponential growth) and every day thereafter until day 6. The final concentration of bacteria in the cultures was estimated to be approximately  $10^8$  cells  $\text{ml}^{-1}$  after the additions. The enrichment cultures were sampled on days 1, 2, and/or 3, 4, 5, and 7 for enumeration of TNAN and *P. imperforata*.

*Oligonucleotide probe preparation, detection, and enumeration of P. imperforata and total nanoplankton*—Oligonucleotides were synthesized with an amino group or a biotin at the 5' terminus (Operon Technologies, Eppendorf, Oligos). The amino groups of amino-modified oligonucleotides were coupled with biotin (Molecular Probes), purified through Sephadex G-25 columns, and finally purified by polyacrylamide gel electrophoresis as previously described (Lim et al. 1993).

Detection and enumeration of TNAN were performed using oligonucleotide probes complementary to regions on the small subunit (SSU) ribosomal RNA that are conserved for all eukaryotes. The probes were as follows (the numbers correspond to *Escherichia coli* SSU rRNA base positions): EUK 1209 (5'-GGG CAT CAC AGA CCT G-3') (Giovannoni et al. 1988), EUK 502 (5'-ACC AGA CTT GCC CTC C-3') (Amann et al. 1990a), and EUK 309 (5'-TCA GGC TCC CTC TCC GG-3') (Sogin and Gunderson 1987).

Probes specific for *P. imperforata* were designed based on SSU rRNA sequences obtained from clonal cultures of *P. imperforata* originally isolated from Vineyard Sound seawater (Caron et al. in prep.). A combination of three oligonucleotide probes with the following sequences were used for enumeration of *P. imperforata* (the numbers also correspond to *E. coli* SSU rRNA base positions): PIMP 635 (5'-TGA GGG GCG GAC CGG TCG CC-3'), PIMP 663 (5'-GGA CGC AGA GAC CAG GTG CAC A-3'), and PIMP 1683 (5'-CCA AGC CGC AGT CCG AGA-3'). The specificity of each probe was tested against several clones of *P. imperforata* and a variety of closely and distantly related protists in slot blot hybridization experiments (Caron et al.

in prep.). The *P. imperforata* clones used for probe testing originated from Vineyard Sound and Waquoit Bay, Massachusetts; Sakonnet River, Rhode Island; Santa Barbara Channel, California; and England. Wash temperatures for appropriate specificity were determined empirically by slot blot and whole cell hybridizations. The *P. imperforata* probes were also tested against three other species of *Paraphysomonas*, *P. bandaiensis*, *P. vestita*, and *P. butcheri*; 13 other species of chrysophytes spanning six families; three species of green algae; two species of synurophytes, prymnesiophytes, and diatoms; and one species of prasinophyte, dinoflagellate, red alga, bodonid flagellate, and ciliate.

Total nanoplankton and *P. imperforata* were enumerated by the quantitative in situ hybridization method using biotinylated probes and fluorescein (FITC)-labeled avidin (Lim et al. 1996). Briefly, samples were vacuum filtered onto 0.4- $\mu\text{m}$  polycarbonate filters of Transwell tissue culture inserts (Costar), dehydrated in a series of ethanol washes, and pre-hybridized in hybridization buffer (10 $\times$  Denhardt's solution, 0.1 mg  $\text{ml}^{-1}$  of polyadenylic acid, 5 $\times$  SET buffer [750 mM NaCl, 100 mM Tris-HCl {pH 7.8}, 5 mM EDTA], 0.1% sodium dodecyl sulfate) for at least 45 min at 40°C. Oligonucleotide probes were then added to the samples and hybridized overnight at 40°C. Each probe was used at a final concentration of 2.5 ng  $\mu\text{l}^{-1}$  in all the hybridizations. Following hybridization, samples were washed at 45°C in 0.2 $\times$  SET buffer (30 mM NaCl, 4 mM Tris-HCl [pH 7.8], 0.2 mM EDTA) for 10 min, incubated with FITC-labeled avidin (20  $\mu\text{g}$   $\text{ml}^{-1}$  in 100 mM  $\text{NaHCO}_3$ -buffered saline [pH 8.2]), and washed with cold  $\text{NaHCO}_3$ -buffered saline to remove unincorporated FITC-labeled avidin. Control hybridizations incubated with FITC-labeled avidin only were also performed for all the samples examined to check for background binding. Filters were finally cut out of the Transwells and mounted on glass slides for observation by epifluorescence microscopy.

The volume of unenriched seawater samples filtered for counts of total nanoplankton and *P. imperforata* ranged from 15 to 25 ml for Vineyard Sound seawater and from 3 to 10 ml for Long Island seawater. A volume of 50 ml was filtered for the sample collected from Bermuda. The volumes of enriched seawater samples filtered ranged from 0.5 to 5 ml, depending on the nanoplankton densities. One hybridization was carried out for each sample. Fluorescence observation and enumeration of FITC-labeled cells were performed at  $\times 630$  magnification with a Zeiss Axioskop using a BP450-490 exciter filter and an LP520 barrier filter. Approximately 15–30 fields were observed for enumeration of total nanoplankton in unenriched seawater samples, but *P. imperforata* cell counts were always obtained from a total of 50 fields. The number of fields observed for enumeration of cells in enrichment samples ranged from 10 to 30 fields. Epifluorescence micrographs of the samples were taken with a Zeiss MC100 camera with Ektachrome 200 ASA color film.

## Results

*Abundances and distribution of P. imperforata at various collecting sites*—*P. imperforata* was either undetectable or

Table 1. Abundances of total nanoplankton (TNAN) and *P. imperforata* in coastal waters of Massachusetts; Long Island, New York; and Bermuda.

Location	Total nanoplankton (cells ml <sup>-1</sup> )	<i>P. imperforata</i>	
		(cells ml <sup>-1</sup> )	% of TNAN
Vineyard Sound, MA (34 sampling d)	$1.96 \times 10^3$ – $1.28 \times 10^4$	$<5.00 \times 10^0$ – $4.93 \times 10^1$	1%
Great Harbor, MA	$4.51 \times 10^3$	$2.10 \times 10^1$	<1%
Long Island, NY (seven sampling sites)	$1.32 \times 10^4$ – $1.17 \times 10^5$	$<1.30 \times 10^1$ – $4.03 \times 10^2$	1%
Bermuda	$2.02 \times 10^3$	$<3.00 \times 10^0$	<1%

at very low abundances in all the coastal water samples examined in this study (Table 1). Samples in which *P. imperforata* was not detected were denoted as  $<3$ – $5$  cells ml<sup>-1</sup> for Vineyard Sound and Bermuda samples and as  $<13$  cells ml<sup>-1</sup> for Long Island water samples. These values represent the limit of detection for *P. imperforata* based on the volume of sample filtered, magnification, and area of the filter examined by epifluorescence microscopy. The highest abundance of *P. imperforata* detected, 400 cells ml<sup>-1</sup>, originated from the Quantuck Canal sampling site on Long Island, New York. However, typical abundances of *P. imperforata* in all the samples examined were 50 cells ml<sup>-1</sup>. In all the samples, *P. imperforata* constituted  $\leq 1\%$  of the total nanoplankton.

*Seasonal abundances of P. imperforata and TNAN in Vineyard Sound seawater*—Total nanoplankton abundances in Vineyard Sound seawater during the 17-month sampling pe-

riod oscillated between  $2 \times 10^3$  and  $1 \times 10^4$  cells ml<sup>-1</sup> (Table 1; Fig. 1). *P. imperforata* was often not detected in the water samples, but given that the detection limit of the counting procedure employed was 3–5 cells ml<sup>-1</sup> (denoted with the  $\downarrow$  symbol in Fig. 1), it was not possible to determine whether *P. imperforata* was actually absent or at very low abundances. When detected, typical abundances of *P. imperforata* were  $\leq 10$  cells ml<sup>-1</sup>, and the highest abundance observed was 49 cells ml<sup>-1</sup>. These abundances represented  $\leq 1\%$  of the TNAN in Vineyard Sound seawater. The abundances of heterotrophic nanoplankton (HNAN) could not be distinguished from phototrophic nanoplankton (PNAN) in this study because ethanol washes during sample preparation bleached photosynthetic pigments and reduced their autofluorescence. Autofluorescence is instrumental for discriminating PNAN (Sherr et al. 1993). However, it is unlikely that *P. imperforata* constituted a large percentage of the HNAN

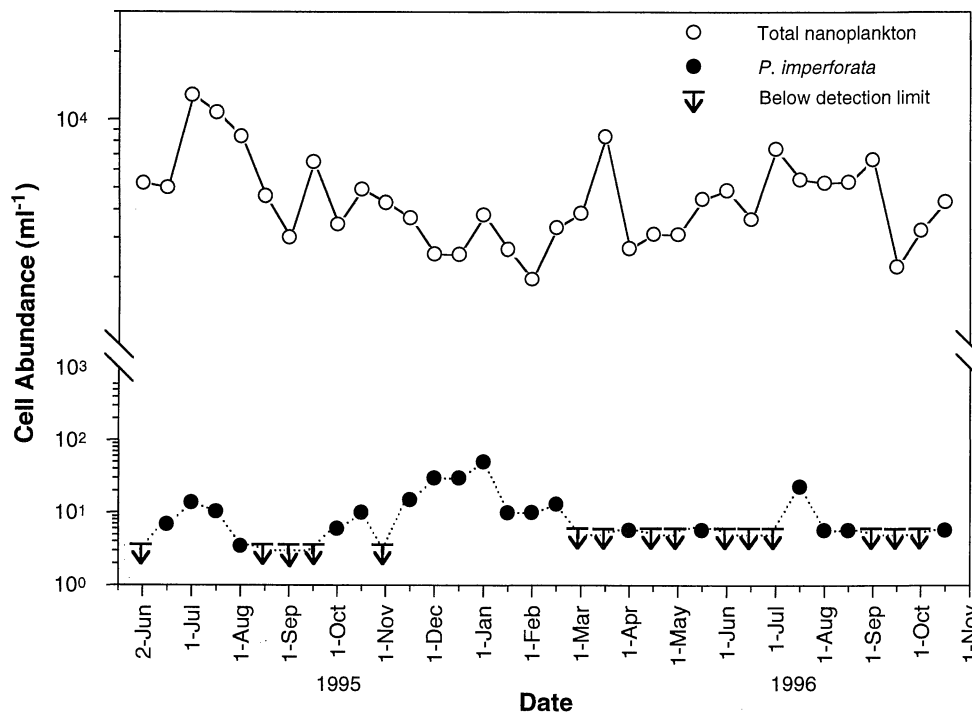


Fig. 1. Seasonal abundances of *P. imperforata* and total nanoplankton in Vineyard Sound seawater. The symbol  $\downarrow$  denotes the detection limit of *P. imperforata* based on the volume of sample filtered, the magnification, and the area of the filter examined.

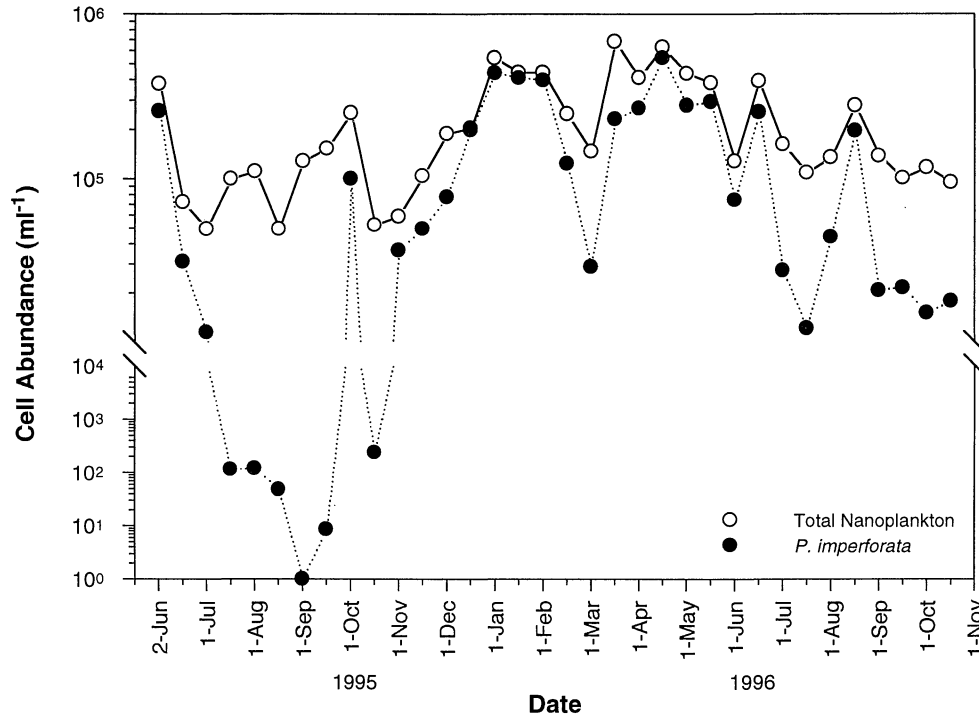


Fig. 2. Seasonal abundances of *P. imperforata* and total nanoplankton in enrichment cultures started with Vineyard Sound seawater.

either. Previous studies of HNAN in Vineyard Sound indicated that they were typically 50% of the TNAN abundances (Caron 1983). Thus, *P. imperforata* would have constituted 2% of the HNAN at most.

*Seasonal abundances of P. imperforata and TNAN in enrichment cultures*—The addition of yeast extract to natural seawater samples collected from Vineyard Sound stimulated growth of the bacterial assemblage within 24 h. The enriched bacterial densities, approximately  $10^8$  cells  $\text{ml}^{-1}$ , were two orders of magnitude higher than natural densities. Heterotrophic nanoplankton, which consisted largely of flagellated protozoa, grew in response to the elevated bacterial densities, and by the fourth day of incubation, nanoplankton abundances ranged between  $5 \times 10^4$  and  $7 \times 10^5$  cells  $\text{ml}^{-1}$  during the entire sampling period (Fig. 2).

*P. imperforata* abundances in enrichment cultures started during the winter and spring months (from November 1995 to June 1996) constituted a significant fraction of the TNAN abundances and reached densities of  $>10^5$  cells  $\text{ml}^{-1}$  (Figs. 2, 3). The lowest abundances of *P. imperforata* were observed in enrichment cultures during the summer and fall of 1995 and 1996 (from July to October of each year). *P. imperforata* abundances were  $2 \times 10^2$  cells  $\text{ml}^{-1}$  and approximately  $2 \times 10^4$  cells  $\text{ml}^{-1}$  during these periods in 1995 and 1996, respectively (with the exception of one sampling day in each year).

In general, the highest *P. imperforata* abundances were obtained in enrichments begun with water samples that were  $10^\circ\text{C}$  or colder (Fig. 4). Five out of the six enrichment cultures in which *P. imperforata* comprised  $>75\%$  of the TNAN (up to 98% of the TNAN) were established from

water samples with in situ temperatures of  $10^\circ\text{C}$ . *P. imperforata* averaged  $<1$  and 20% of TNAN in enrichment cultures during the late summer and fall of 1995 and 1996, respectively (Fig. 4). These periods of low *P. imperforata* abundance in enrichment cultures coincided with the warmest ambient water temperatures of each year, when water temperature approached  $20^\circ\text{C}$  (July through mid-October). Overall, the water temperature during the summer and fall of 1995 (maximum temperature =  $24.5^\circ\text{C}$ ) was higher than during 1996 (maximum temperature =  $21^\circ\text{C}$ ). Maximal abundances of *P. imperforata* and their percent contributions to TNAN were lower during the summer and fall of 1995 than during the same period of 1996 (Fig. 4).

Changes in population abundances of TNAN and *P. imperforata* (also expressed as a percent of TNAN) were monitored every day (except for day 1) for 4 d on 8 of the 34 sampling dates. The population changes measured on 15 December 1995 and on 15 January, 1 September, and 15 October 1996 are shown in Fig. 5 and are representative of the types of changes observed in other enrichment cultures. Abundances of nanoplankton and *P. imperforata* reached maximum densities on the third or fourth day of incubation. The maximum density of *P. imperforata* in these enrichment cultures also coincided with the highest percent contribution of *P. imperforata* to TNAN. High abundances were usually maintained through day 4 (Fig. 5: 15 December 1995 and 15 January and 1 September 1996), but in a few cases, they decreased significantly by the fourth day (Fig. 5: 15 October 1996).

Net growth rates of *P. imperforata* in the enrichment cultures were calculated to compare them to values reported for *P. imperforata* in the literature. The growth rates of *P. im-*

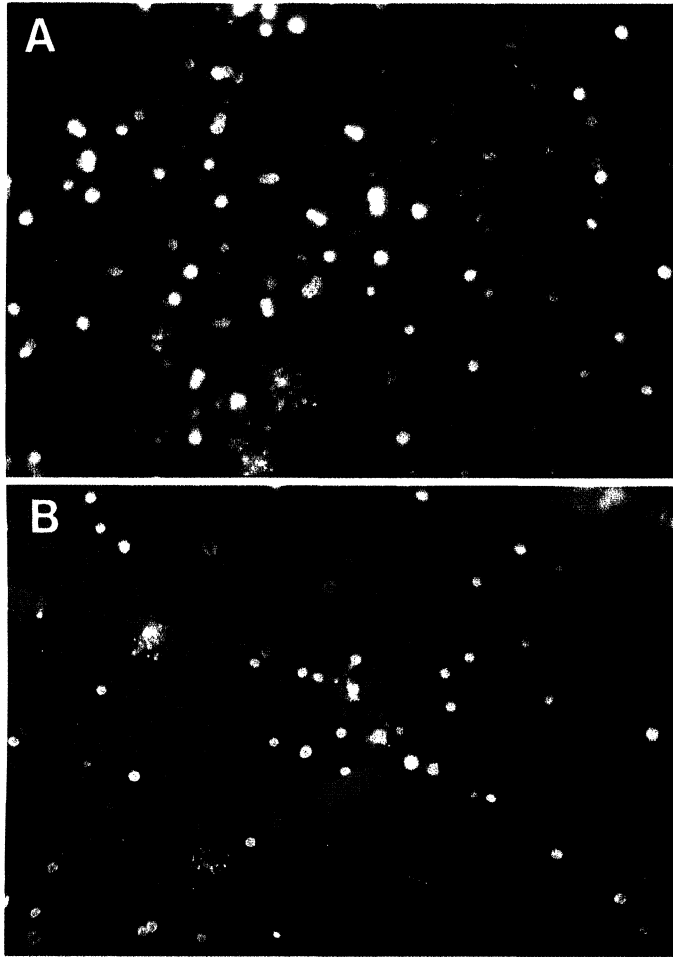


Fig. 3. Epifluorescence micrographs of *P. imperforata* in enrichment cultures. (A) and (B): *P. imperforata* cells labeled by *P. imperforata*-specific probes are the bright, yellow-green round cells (as a result of FITC fluorescence). Unidentified flagellates and ciliates (oblong cells in B) that were not labeled by *P. imperforata* probes possess a dull, brownish-yellow autofluorescence, which clearly distinguishes them from the probed cells.

*perforata* were determined from changes in their abundances by assuming exponential growth during the first 3 d of incubation (the period of rapid changes in abundance for most of the cultures; Fig. 5). These calculations were performed on samples that had measurable abundances of *P. imperforata* at the time of enrichment (19 samples). Growth rates of *P. imperforata* in enrichment cultures determined in this manner throughout the seasonal study ranged from 1.1 to 3.9  $\text{d}^{-1}$  and averaged approximately 3.0  $\text{d}^{-1}$ .

**Effect of initial *P. imperforata* abundance on *P. imperforata* dominance**—We hypothesized that one explanation for whether or not *P. imperforata* dominated the enrichment cultures in our seasonal study might be due to the abundances of this species at the times of collection and enrichment. A number of our samples had initial densities of *P. imperforata* that were below the limit of detection for this method and thus might be too low to allow this species to increase its abundance fast enough to dominate the enrichments. The

importance of the initial abundance of *P. imperforata* for determining its contribution to TNAN in enrichment cultures was examined on two dates by comparing enrichment cultures with various starting densities of *P. imperforata*. *P. imperforata* from a laboratory culture (Clone VS1) was added to enrichment cultures to increase the initial abundance of *P. imperforata* relative to its natural density. Natural densities of *P. imperforata* before enrichment were 5 cells  $\text{ml}^{-1}$  on these two dates (<1% of the TNAN). Following additions of *P. imperforata* Clone VS1, the initial abundances of *P. imperforata* were 180 cells  $\text{ml}^{-1}$  (3% of the TNAN: 15 August 1996) and 15 cells  $\text{ml}^{-1}$  and 90 cells  $\text{ml}^{-1}$  (<1 and 1% of the TNAN, respectively; 1 September 1996).

Enrichment cultures with higher starting densities of *P. imperforata* as a consequence of the addition of Clone VS1 were dominated by *P. imperforata* to a greater extent than enrichment cultures with lower initial *P. imperforata* densities (Fig. 6). Thus, the initial densities of *P. imperforata* in the samples appeared to play a role in whether or not enrichments would become dominated by this protozoan. The maximum densities of *P. imperforata* in treatments with *P. imperforata* Clone VS1 were approximately two- to five-fold higher than in enrichment cultures without *P. imperforata* added. *P. imperforata* during the 15 August experiment constituted up to 95% of the TNAN in the treatments supplemented with *P. imperforata* compared to 75% of the TNAN in enrichment cultures without *P. imperforata* added (Fig. 6). Starting concentrations of *P. imperforata* in the 1 September experiment were undetectable (<5 cells  $\text{ml}^{-1}$  natural abundance), 15 cells  $\text{ml}^{-1}$  and 90 cells  $\text{ml}^{-1}$ . *P. imperforata* in these enrichment cultures constituted 22, 79, and 90% of TNAN, respectively, after 3 d of incubation (Fig. 6).

**Effect of nanoplankton grazers on *P. imperforata* dominance**—Enrichment cultures were performed with whole (unfiltered) seawater, <20- $\mu\text{m}$  filtered seawater, and <5- $\mu\text{m}$  filtered seawater to test whether nanoplankton grazers controlled the degree to which *P. imperforata* dominated the enrichment cultures. We hypothesized that selective feeding by nanoplankton consumers could alter the competitive ability of *P. imperforata* relative to other bacterivorous HNAN in the water samples. The whole seawater and the <20- $\mu\text{m}$  enrichment cultures were expected to contain zooplankton larger than 5  $\mu\text{m}$  that could potentially consume *P. imperforata*, but the <5- $\mu\text{m}$  enrichment cultures should contain only small protists such as *P. imperforata* and other bacterivorous species.

Increases in the TNAN among whole seawater and the <20- and <5- $\mu\text{m}$  fractions were similar after 4 d of enrichment in all three experiments conducted on 15 April, 15 May, and 15 June 1996 (Fig. 7). *P. imperforata* comprised similar percentages of the TNAN in the <5- $\mu\text{m}$  fractions, the <20- $\mu\text{m}$  fractions, and the whole seawater enrichment cultures on day 4 in each of the three experiments, although that percentage was somewhat different for the three dates (90, 85, and 60%). Removal of larger nanoplankton, microzooplankton, and macrozooplankton therefore did not affect growth of the TNAN or the ability of *P. imperforata* to dominate the TNAN during the first 4 d of enrichment. *P. imperforata* constituted a higher percentage of the TNAN in

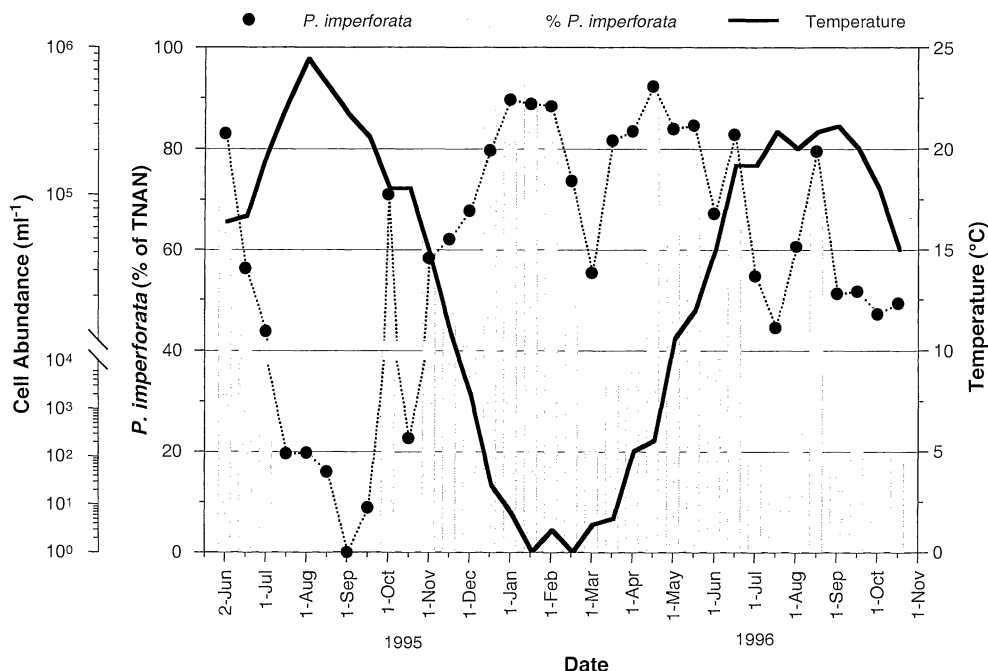


Fig. 4. Seasonal abundance of *P. imperforata* in absolute cell densities and as a percentage of total nanoplankton (TNAN) in enrichment cultures, and seasonal water temperature in Vineyard Sound, Massachusetts.

the  $<5\text{-}\mu\text{m}$  filtrates than in the whole seawater samples, however, by day 6 during the 15 April and 15 June experiments and on day 7 in the 15 May experiment.

*Effect of food availability on P. imperforata dominance*—Bacteria were added to enrichment cultures after 72 h (3 d) of incubation and every 24 h thereafter until day 6 on two dates to examine whether food limitation affected the contribution of *P. imperforata* to TNAN in the enrichments. TNAN abundances increased to approximately  $2 \times 10^5$  cells  $\text{ml}^{-1}$  on day 3 following the initial enrichments, and *P. imperforata* constituted ca. 60% of TNAN in both experiments (Fig. 8). TNAN in the enrichment cultures remained constant (15 September experiment) or increased slightly (1 October experiment) from day 3 to day 7 when bacteria were added daily, but TNAN in the enrichment cultures without addition of bacteria decreased by ca. 10-fold during this period.

The abundances of *P. imperforata* (percentage of TNAN) in the enrichment cultures with and without the addition of bacteria, however, decreased dramatically between day 3 and day 7 in both experiments (bars in Fig. 8). *P. imperforata* decreased to approximately 20–30% of the TNAN after the fourth day of incubation in both experiments and was no longer detected by day 6 despite the continued availability of bacterial prey. Food supply apparently was not able to maintain numerical dominance of *P. imperforata* in the enrichment cultures after the initial, rapid increase in TNAN abundance.

## Discussion

Oligonucleotide hybridization probes that target the rRNA provide a means of directly assessing the abundances of spe-

cies or groups of microorganisms in natural water samples as well as in cultures. When applied to natural water samples using a new quantitative approach (Lim et al. 1996), hybridization with eukaryote-specific probes has been found to yield nanoplankton abundances that were comparable to counts obtained by conventional 4', 6-diamidino-2-phenylindole (DAPI) and acridine orange staining of protistan assemblages (Sherr et al. 1993). The use of species-specific oligonucleotides further increases the usefulness of the quantitative in situ hybridization method by identifying individual species in mixed assemblages of small protists. In this study, oligonucleotide probes specific for the heterotrophic nanoflagellate, *P. imperforata*, were used for identifying and determining the abundances of this species in coastal environments. Our results demonstrate the feasibility of employing species-specific probes for documenting the quantitative occurrence of small protistan species and thus obtaining information on their spatial and temporal distribution in aquatic environments.

Natural abundances of *P. imperforata* in coastal water samples during this study were extremely low irrespective of the location or the time of year. *P. imperforata* abundances in the relatively eutrophic estuaries of Long Island, New York, did not differ greatly from abundances found in more mesotrophic, coastal waters such as Vineyard Sound and Great Harbor, Massachusetts, with the exception of Quantuck Canal, Long Island (Table 1), where the abundance of *P. imperforata* was 400 cells  $\text{ml}^{-1}$ . At this site and all others in this study, however, *P. imperforata* still constituted not more than 1% of the TNAN. Low abundances of *P. imperforata* therefore appear to be common phenomena based on the coastal water samples examined in the present



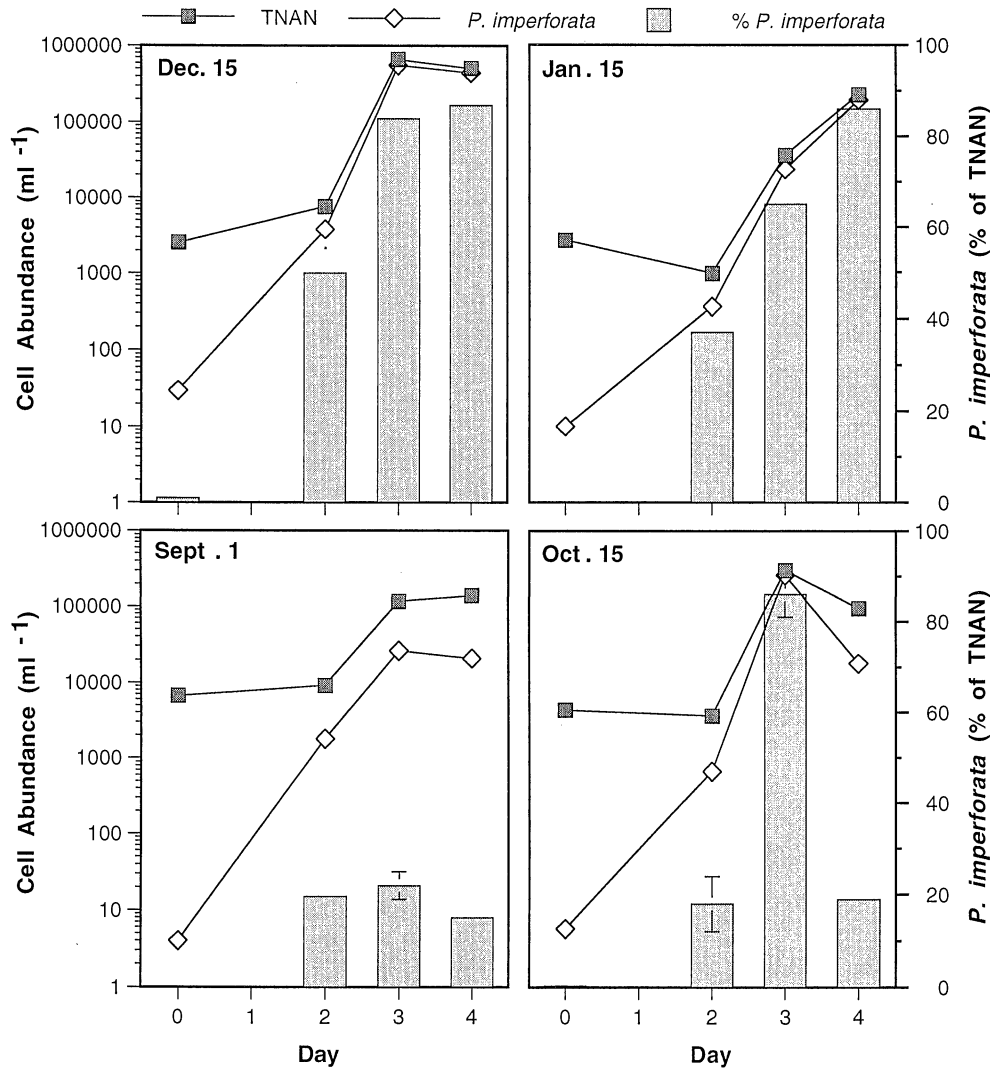


Fig. 5. Examples of changes in the abundance of *P. imperforata* and TNAN represented by enrichment cultures started on 15 December 1995 and on 15 January, 1 September, and 15 October 1996. Error bars represent the standard error of the mean.

study. Similarly, seasonal abundances of *P. imperforata* examined at the Vineyard Sound sampling site remained consistently low and constituted no more than 1% of TNAN over a 17-month sampling period (Table 1; Fig. 1).

Our finding that *P. imperforata* occurred at low absolute and relative abundances in natural samples was unexpected because this species commonly occurred at high abundances in enrichment cultures of natural samples. We therefore questioned whether the fluorescent in situ hybridization method with *P. imperforata* probes underestimated the abundances of *P. imperforata* in field samples. The abundances of this species enumerated by probe hybridization could have been lower than actual abundances if the rRNA content of *P. imperforata* cells in field populations was extremely low. This situation might occur, for example, if cells in situ were in poor physiological condition due to starvation. Low rRNA content would result in lower probe binding and thus, reduced fluorescent signal, so that some individuals of the target species would not be apparent as probed cells during

microscopical examination. It is also possible that the probes were only targeting certain strains of *P. imperforata* and that a substantial number of *P. imperforata* strains undetectable by the probes exist in nature.

However, several lines of evidence indicate that the oligonucleotide probes and the in situ hybridization method employed in this study for enumerating *P. imperforata* provided accurate estimates of *P. imperforata* in our field samples. The probes accurately detected different strains of *P. imperforata* isolated from several different marine habitats in slot blot hybridizations and whole cell hybridizations (see section in "Methods" for *P. imperforata* clones tested in specificity studies; see also Caron et al. in prep.). This result does not eliminate the possibility that the probes missed certain strains of *P. imperforata* in the field samples. Our previous studies, however, provided good evidence that the probes were capable of detecting different strains of *P. imperforata* that might have been present in the natural samples. In addition, when applied to seawater samples inocu-

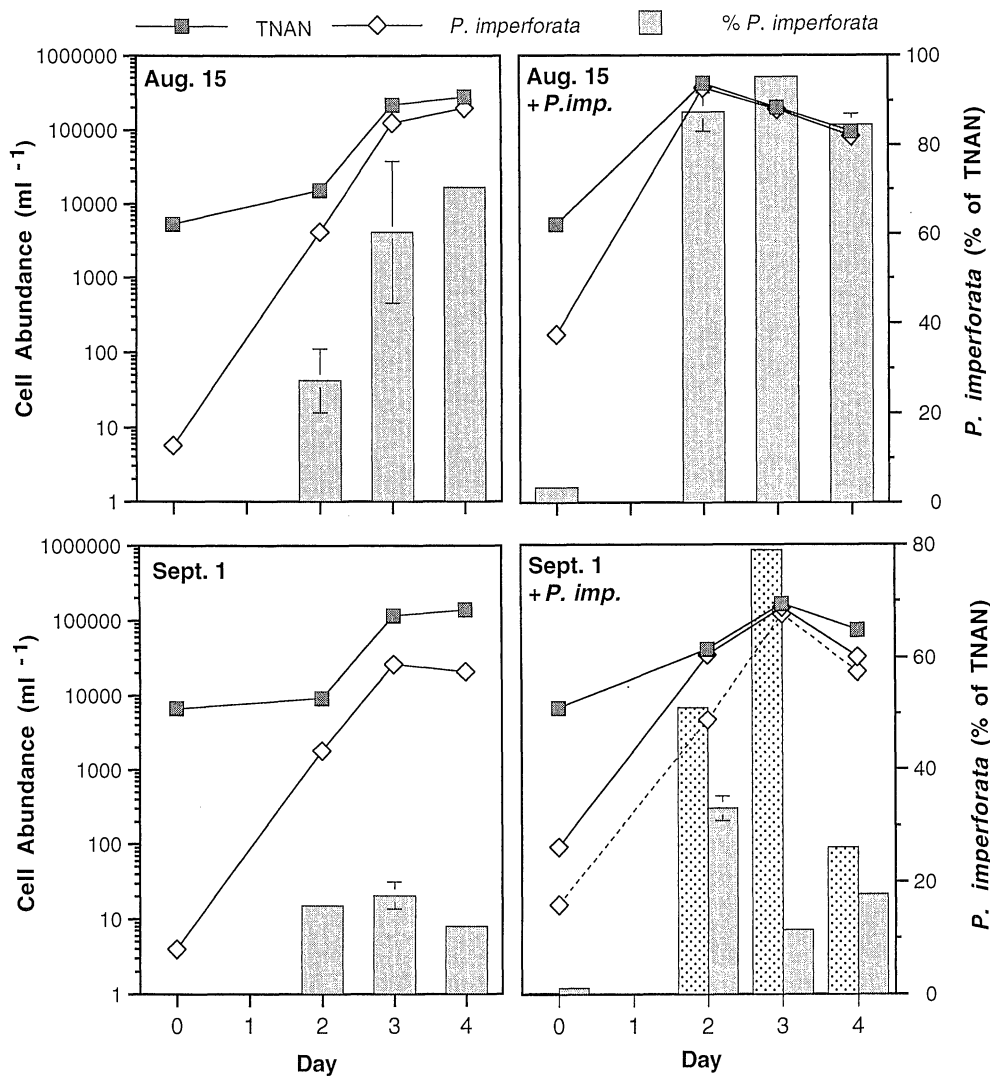


Fig. 6. Changes in the abundance of *P. imperforata* and TNAN in enrichment cultures with and without additions of *P. imperforata* Clone VS1. Experiments were performed with enrichment cultures started on 15 August and 1 September 1996. Panels on the left show protistan abundances in enrichments without added *P. imperforata*. Panels on the right indicate abundances in enrichments with added *P. imperforata*; the solid line/open diamond and shaded bars pertain to cultures that were supplemented with *P. imperforata* to a final density of 90 cells ml<sup>-1</sup> (1% of TNAN). The dotted bars and dotted line in the lower right panel represent changes in *P. imperforata* abundance in enrichment cultures that were supplemented with *P. imperforata* to a final density of 15 cells ml<sup>-1</sup> (<1% of TNAN). Error bars represent the standard error of the mean. Error bars that are not visible are within the size of data points.

lated with known concentrations of *P. imperforata*, oligonucleotide probe counts yielded accurate abundances of *P. imperforata* (Fig. 6). These additions of *P. imperforata* served as "internal standards" and indicated that sample preservation, storage, and processing did not affect our estimates of *P. imperforata* abundance in the natural samples. Finally, we demonstrated previously that the simultaneous use of three probes and the biotin-avidin detection system in hybridizations (as used in the present study) allowed detection of protists maintained under prolonged starvation conditions (Lim et al. 1993). Therefore, the physiological condition of *P. imperforata* in the natural samples of the present

study should not have caused underestimation of their abundances.

We also examined the validity of our counts of *P. imperforata* in natural seawater samples by predicting the abundances of this species in Vineyard Sound samples at the time of establishment of the enrichment cultures. For this prediction, we assumed that the protists present in the cultures at the time of peak abundances were in good physiological condition and could be probed effectively. We therefore used the abundance estimates of *P. imperforata* in the cultures after 3 d of enrichment and the observed growth rates of *P. imperforata* Clone VS1 added to enrichment cultures on 15

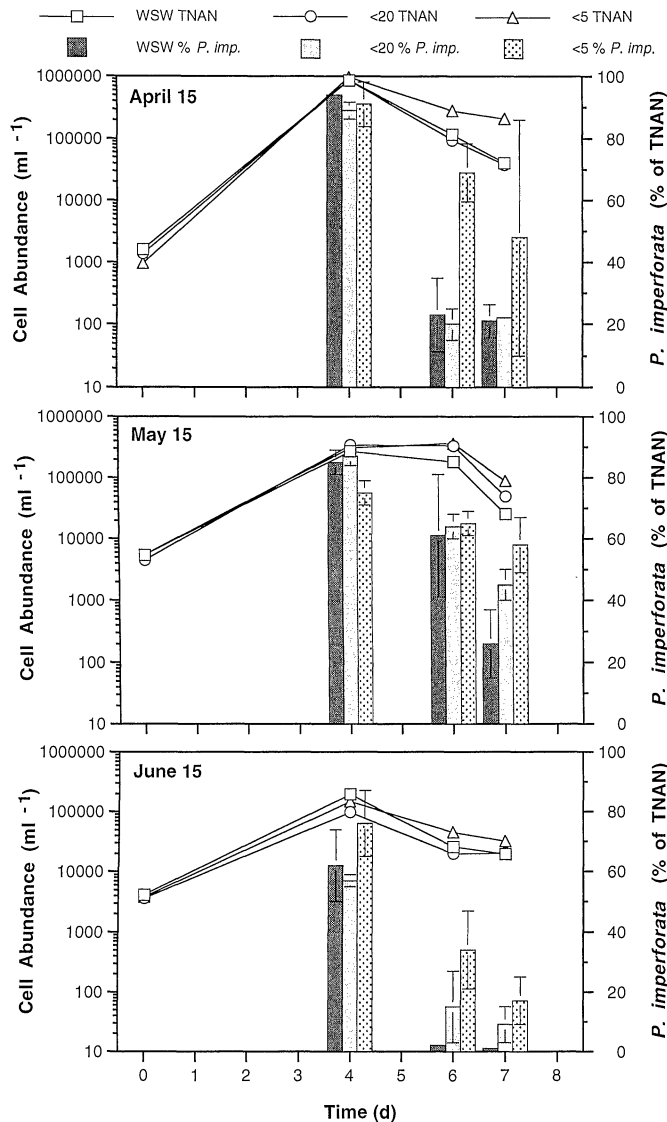


Fig. 7. Changes in the abundance of TNAN and *P. imperforata* in whole (unfiltered) seawater (WSW), <20- $\mu\text{m}$  filtrate, and <5- $\mu\text{m}$  filtrate of grazing experiments conducted with enrichment cultures started on 15 April, 15 May, and 15 June 1996. Error bars represent the standard error of the mean.

August and 1 September 1996 ( $3.1 \text{ d}^{-1}$ ) to estimate the abundance of *P. imperforata* initially present in the samples ( $N_o$ ). We used the growth equation,  $N_o = N_t e^{-\mu t}$  to estimate the initial abundance of *P. imperforata* ( $N_t$  is *P. imperforata* abundance in enrichment cultures after time  $t = 3 \text{ d}$ ;  $\mu$  is the growth rate of *P. imperforata* Clone VS1).

Estimated abundances of *P. imperforata* throughout the seasonal study were well within the upper and lower limits (dotted lines) of *P. imperforata* abundances obtained by direct counts of probed samples (Fig. 9). This analysis provided only a crude validation of our probe counts in natural samples because it is highly probable that *P. imperforata* growth rates were not the same in all experiments. Also, we could not confirm our predictions for some samples because initial abundances of the protists were below the limit of

detection for the method. Nevertheless, the relatively good agreement between the estimated initial *P. imperforata* abundances and probe counts throughout the seasonal study support our assertion that we obtained accurate counts of the protist in natural samples. The results of this analysis also demonstrated that the very low initial abundances of *P. imperforata* obtained by in situ hybridizations were capable of yielding the peak abundances of this species observed in many of the enrichment cultures from the seasonal study.

There are no published reports with which to compare our estimates of the natural abundances of *P. imperforata*. Past studies that have identified this species in natural water samples have relied on nonquantitative methods such as electron microscopy and/or enrichment culture to demonstrate its presence. However, a study by Fenchel (1982c) provided data on the quantitative occurrence of major groups of heterotrophic flagellates in a coastal marine environment based on identifications of broad, group-specific diagnostic features apparent using fluorochrome staining and epifluorescence microscopy. Estimates of *Paraphysomonas* spp. abundance by Fenchel during a 30-d period ranged from <10 to 200 cells  $\text{ml}^{-1}$ , and a most probable number (MPN) estimate of *Paraphysomonas* spp. on one of the sampling days yielded a density of 40 cells  $\text{ml}^{-1}$ . These estimates fall near the upper limit of the range of *P. imperforata* abundances enumerated by hybridization probes in the present study. The abundances of *Paraphysomonas* spp. reported by Fenchel, however, must be considered upper limit estimates of the abundances of *P. imperforata* because other chrysomonad genera such as *Spu-mella*, as well as different species of *Paraphysomonas*, cannot be properly distinguished by epifluorescence microscopy. In any case, the results of this previous field study are in agreement with our judgment that *P. imperforata* abundances were low in coastal waters.

The low abundances of *P. imperforata* that we observed in coastal water samples were in stark contrast to the ability of this species to dominate the nanoplankton in the same water samples enriched with bacteria (Fig. 2). *P. imperforata* grew rapidly and numerically dominated the nanoplankton in most of the enrichment cultures once bacteria became available. The prevalence of *P. imperforata* in enrichment cultures observed in the present study is congruent with observations from a number of taxonomic studies, which have documented that *P. imperforata* is a common species in a wide variety of marine habitats (Lucas 1967; Larsen and Patterson 1990; Vørs 1993a,b). Water samples in those studies typically were subjected to some form of enrichment cultivation or concentration before they were prepared for observation by electron microscopy.

Seasonal differences in the abundance of *P. imperforata* and the extent to which they dominated enrichment cultures were observed in this study. *P. imperforata* tended to dominate enrichment cultures established in the winter/spring months but not in the summer/fall months (Fig. 4). This finding was contrary to our original expectations because the growth rate of this protist has been shown to be directly related to temperature (Caron et al. 1986). Maximal growth rate of *P. imperforata* in that study was approximately  $4.0 \text{ d}^{-1}$  at  $26^\circ\text{C}$ . Therefore, we expected that *P. imperforata*

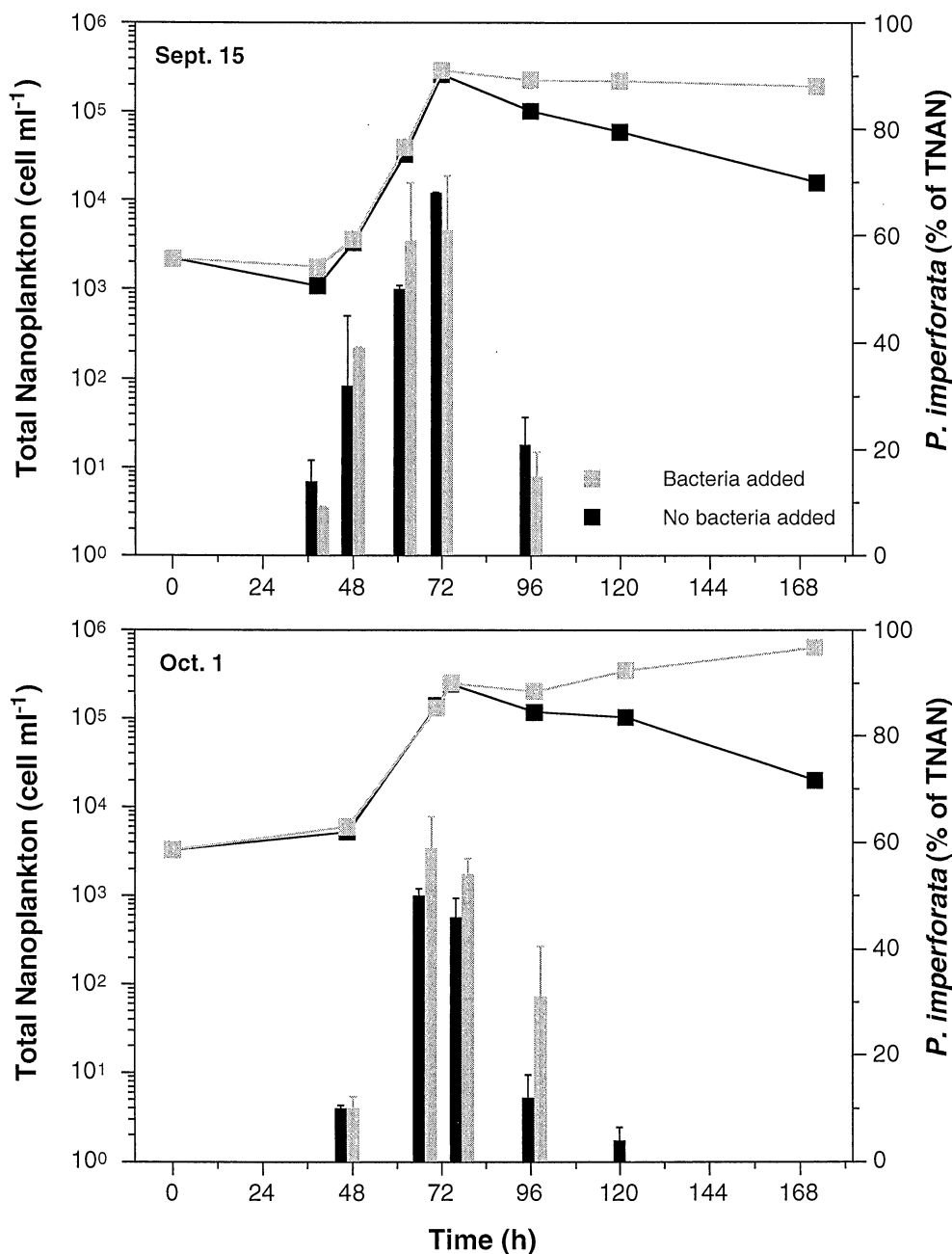


Fig. 8. Changes in the abundance of TNAN and *P. imperforata* in enrichment cultures started on 15 September and 1 October 1996, with and without bacteria added on subsequent days. Bacteria were added to the treatments on days 3, 4, 5, and 6 on both sampling dates.

might dominate enrichment cultures established during warm water periods.

Given the results of previous laboratory studies with *P. imperforata* and the method with which we conducted our experiments in the present study, our finding that this protist dominated enrichment cultures during cold water periods but not warm water periods implies several interesting features concerning the biogeography of this protist. First, enrichment temperature and the tolerance of *P. imperforata* to rapid changes in temperature appear to have played a role in determining the dominance of *P. imperforata* in winter/

spring enrichment cultures. All the enrichment cultures in the seasonal study were incubated at 20°C. Consequently, protists in water samples collected during the winter/spring months (average water temperature = 5°C) were subjected to greater temperature changes than assemblages in samples collected during the summer/fall (when water temperature averaged 18–20°C). The success of *P. imperforata* in enrichment cultures started during the winter/spring months therefore not only indicates an ability by this flagellate to exist (albeit at low abundances) in nanoplankton assemblages at temperatures that are well below its optimal growth

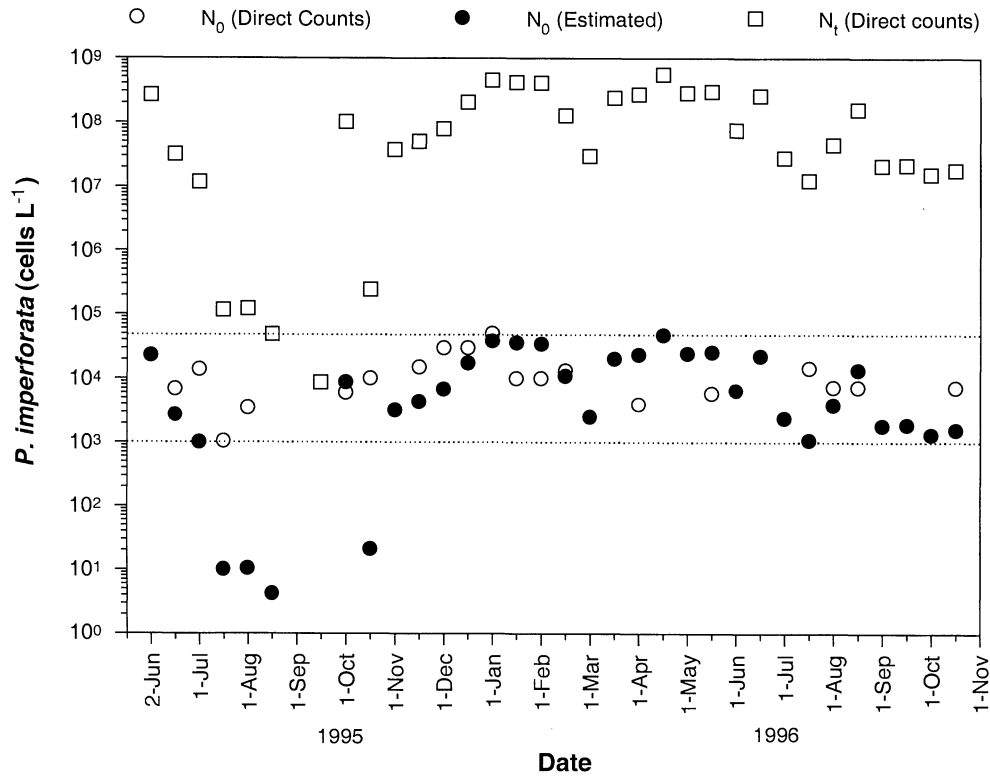


Fig. 9. Comparison of the seasonal abundance of *P. imperforata* in Vineyard Sound seawater obtained from direct microscopical counts, with abundances estimated using the growth constant equation,  $N_o = N_t e^{-\mu t}$  and the peak abundances of *P. imperforata* observed in the enrichment cultures.  $N_o$  is the estimated natural abundance of *P. imperforata*,  $N_t$  is *P. imperforata* abundance in enrichment cultures after time  $t$ , and  $\mu$  is the growth constant of *P. imperforata*. A value of  $\mu = 3.12 \text{ d}^{-1}$  was used for the calculation of  $N_o$  based on the average growth rate of *P. imperforata* Clone VS1 in enrichment cultures (Fig. 6). Time ( $t$ ) was 3 d based on the observation that *P. imperforata*, on average, attained peak abundances in the enrichments by day 3 (Fig. 5). The dotted lines represent the upper and lower limits of *P. imperforata* abundance obtained by direct counts of probed cells. Direct count values that are not plotted were below the limit of detection of the method.

temperature, but also an ability to dramatically increase its net growth rate (relative to other bacterivores) when growth conditions are optimal.

Another interesting feature of the biogeography of *P. imperforata* is our result that, somewhat surprisingly, this protist typically did not dominate the enrichment cultures initiated with water samples that had in situ temperatures above 20°C, even though growth rates of this species are higher at these warmer temperatures (Caron et al. 1986). This finding appears to imply that *P. imperforata* was outcompeted by other bacterivorous nanoplankton that grew more rapidly and dominated the enrichment cultures before *P. imperforata* could do so, or that *P. imperforata* was removed more rapidly than other nanoplankton species by consumers of nanoplankton in the enrichment cultures, or some combination of these two scenarios. The significance of direct competition of *P. imperforata* with other bacterivores is difficult to assess. *P. imperforata* grows rapidly, but other nanoplanktonic species exist that possess comparable growth rates (Fenchel 1986). We did not determine the identity of co-occurring species of nanoplankton in the enrichment cultures

that were not dominated by *P. imperforata*, but analyses of these species in the future may provide insights into the importance of competition for determining the species composition of our enrichment cultures.

Nano- to macrozooplankton-sized grazers did not appear to have a major role in whether or not *P. imperforata* dominated the enrichment cultures during the first 4 d of incubation. TNAN abundances and the percentage of TNAN that was comprised by *P. imperforata* were essentially the same in the <5- $\mu\text{m}$  filtrates, <20- $\mu\text{m}$  filtrates, and unfiltered seawater samples up to 4 d of growth, indicating that grazing by plankton larger than 5  $\mu\text{m}$  did not control the relative abundance of *P. imperforata* during the initial period of enrichment. Based on the results of these experiments, we conclude that the presence of other bacterivorous species of nanoplankton with initial densities and/or growth rates that exceeded those of *P. imperforata* was important in determining the species composition of enrichment cultures during the first 3–4 d of incubation.

High prey abundance as a consequence of organic enrichment presumably was a major factor that allowed *P. imperforata*

*forata* to grow rapidly and dominate many of the enrichment cultures, but it apparently was not instrumental in determining the outcome of the enrichments (i.e., whether or not *P. imperforata* dominated). High bacterial abundance also was not able to maintain *P. imperforata* dominance after the enrichment assemblages reached maximal abundances (Fig. 8). *P. imperforata* in the enrichment cultures dominated by this species responded quickly to increased prey concentrations and depleted the bacteria by the third or fourth day of incubation. Subsequent additions of bacteria, however, did not prevent *P. imperforata* abundances from decreasing dramatically during the next several days, demonstrating that food limitation alone could not account for selective losses of *P. imperforata* from the enrichment cultures after day 4 (Fig. 8).

Larger grazers, however, did affect *P. imperforata* and other nanoplankton populations in enrichments after day 4. We assumed that decreases in TNAN and *P. imperforata* abundances in the  $<5\text{-}\mu\text{m}$  treatments represented cell mortality due to food limitation for all the treatments. Decreases in TNAN and *P. imperforata* abundances in the whole seawater treatment were greater than changes in the  $<5\text{-}\mu\text{m}$  treatment (Fig. 7). This result indicates that micro- and macrozooplankton-sized grazers of *P. imperforata* were present in the water samples and affected TNAN and *P. imperforata* abundances in the later stages of the enrichment cultures, probably as nanoplankton growth rates slowed due to food limitation. Interestingly, selective grazing by these predators on *P. imperforata* may have occurred because abundances of this species decreased more rapidly than decreases in TNAN. The larger grazers present in unfiltered seawater appeared to be more important as grazers of *P. imperforata* because *P. imperforata* decreased more dramatically in the unfiltered seawater than in the  $<20\text{-}\mu\text{m}$  treatments that excluded the micro- and macrozooplankton grazers.

Cannibalism by *P. imperforata* also could have caused the selective disappearance of *P. imperforata* in the enrichment cultures. *P. imperforata* is capable of cannibalism, but it seems unlikely that *P. imperforata* had resorted to cannibalism because it appears to be a behavior in response to starvation (Goldman and Caron 1985). However, *P. imperforata* in enrichment cultures that were supplemented with bacteria also decreased in relative abundance after day 4 (Fig. 8). This result indicates that selective grazing on *P. imperforata* by consumers of nanoplankton may be a more likely explanation for decreases in the relative abundance of this protist in the enrichments after day 4. Alternatively, it is possible that *P. imperforata* resorted to cannibalism in response to cell-to-cell contact when it reached peak abundances, although the ecological advantage of such behavior to *P. imperforata* in the presence of abundant bacterial prey is not obvious.

Finally, the timing of sampling the enrichment cultures was also likely to have caused some of the variability observed in *P. imperforata* dominance in the seasonal study. Enrichment cultures from the seasonal study were sampled after 4 d of incubation, which was typically the day on which TNAN and *P. imperforata* reached stationary growth phase and when *P. imperforata* constituted the maximum percentage of TNAN (Figs. 5, 6). However, we observed instances

when *P. imperforata* abundances decreased dramatically between the third and fourth day of incubation so that abundances (absolute numbers and percentage of TNAN) on the fourth day did not reflect their maximal contributions (Fig. 5: 15 October; Fig. 6: 1 September + *P. imperforata*; Fig. 8). It is not possible to determine which sampling dates other than those listed above were affected by this problem. However, our observation that *P. imperforata* did not dominate enrichment cultures initiated during warm water periods cannot all be attributed to this situation (see 1 September data in Figs. 5, 6).

In contrast, enrichment cultures supplemented with *P. imperforata* cells demonstrated that initial cell densities affected the ability of *P. imperforata* to dominate enrichment cultures (Fig. 6). Cultures started with higher densities of *P. imperforata* were consistently dominated by *P. imperforata* to a greater extent than those with lower initial *P. imperforata* densities. In the 1 September enrichment culture, the initial *P. imperforata* density was  $<5\text{ cells ml}^{-1}$ , and *P. imperforata* never constituted  $>20\%$  of the TNAN. However, increasing the density of *P. imperforata* (by adding cultured *P. imperforata* Clone VS1) to  $15\text{ cells ml}^{-1}$  allowed *P. imperforata* to dominate the enrichment culture, constituting almost 80% of TNAN. Based on these results, it appears that small differences in the absolute abundance of *P. imperforata* at the beginning of the enrichment cultures were sufficient to allow *P. imperforata* to outgrow flagellate competitors and eventually dominate the enrichment cultures or vice versa.

An important finding of this study is that our bacterivorous protistan enrichment cultures selected for a species that was never numerically dominant in the natural assemblage. *P. imperforata* frequently dominated the enrichment cultures from the seasonal study despite never being present in any of our natural samples at abundances of  $>1\%$  of TNAN. This species and other *Paraphysomonas* species have been used repeatedly in laboratory studies of protozoan physiology and are assumed to be representative of ecologically important species of bacterivorous protozoa (Fenchel 1982b; Caron et al. 1985; Goldman et al. 1985; Caron et al. 1990; Landry et al. 1991; Choi and Peters 1992). Based on the results of our study, however, the enrichment culture method commonly employed to obtain cultures of heterotrophic protists may favor species that are more amenable to culture conditions rather than representative of numerically dominant cell types. Culture bias is a well-known phenomenon in bacteriology (see Amann et al. [1995] and references therein), but its prevalence among aquatic protists is not well documented. The present study draws attention to the possibility that many of our existing protistan cultures, and the ecological paradigms based on them, must be considered tentative until the biodiversity of natural protistan assemblages has been more thoroughly characterized.

Our present knowledge of how physical and biological factors affect the abundance, distribution, and diversity of protists in aquatic environments is still very limited. In situ hybridization with oligonucleotide probes enables direct measurements of the abundance of flagellate species in natural water samples in order to address fundamental ecological questions that are technically difficult to study using

extant methodology. The data obtained in this study are a step toward elucidating the factors that control the abundance and distribution of one of these species. In the future, the use of oligonucleotide probes to target a variety of species or species groups will provide a more complete picture of protistan diversity and the role that these microorganisms play in marine biogeochemical processes.

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