Hydrobiologia **401:** 215–227, 1999.
J.P. Zehr & M.A. Voytek (eds), Molecular Ecology of Aquatic Communities.
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Protistan community structure: molecular approaches for answering ecological questions

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Key words: 18S rRNA, protistan biogeography, protists, RFLP, oligonucleotide probe, protistan community structure, *in-situ* hybridization, microbial ecology, molecular ecology

Introduction

Protists are essential to the function of most aquatic food webs. Photosynthetic protists ranging in size from a few micrometers to more than 1 mm produce much of the organic material available in these ecosystems, particularly in pelagic ecosystems. In addition, protistan predators ranging up to more than 1 cm in size are major consumers of bacterial, cyanobacterial and protistan populations (Sherr & Sherr, 1994).

Our knowledge of the structure and function of protistan assemblages has advanced considerably in recent years, particularly with regard to planktonic communities. Estimates of abundance and biomass for a variety of protistan groups have been reported from diverse environments (Fenchel, 1967; Davis et al., 1985; Porter et al., 1985; Garrison et al., 1986; Caron & Swanberg, 1990; Stoecker et al., 1994). Major trophic modes for many of these species have been examined, and a number of generalized models of organic carbon production and utilization have been proposed (Figure 1). These depictions typically involve grouping species by trophic mode (i.e. phototrophy, heterotrophy, mixed nutrition) and size-dependent grazing by consumers.

It can be argued that protistan community structure among the larger protists is considerably better characterized than for the smaller protists. For example, extensive species lists of phototrophic protists such as diatoms and dinoflagellates exist, as well as distributional information. Likewise, the distributions and abundances of large heterotrophic protists in freshwater and marine ecosystems have been fairly well established. Probably the most thoroughly documented distributions of marine protists exist for the large, shelled species of Foraminifera and some Actinopoda which are used in paleoclimatological studies (Bé, 1959; Bé, 1960; Anderson, 1980; Dworetzky & Morley, 1987; Hemleben et al., 1988; Caron & Swanberg, 1990). Likewise, the use of the Utermöhl settling method, and the recent development and application of methods such as the quantitative protargol stain for the identification and enumeration of ciliates, have enabled studies of the distributions of these latter species (Utermöhl, 1958; Stoecker et al., 1989; Montagnes & Lynn, 1993).

Our knowledge concerning the diversity, abundance and distribution of large protists is more complete than for small species in part because of the presence of morphological features that are taxonomically useful, visible by light and/or electron microscopy, and which remain intact throughout sampling, preservation and examination procedures. These features may include cell size and shape, skeletal structures (loricae, frustules, tests) patterns of ciliature, thecal plates and body scales. Distinguishing attributes typically are more numerous and/or more apparent at low magnification for large protists, thus facilitating the acquisition of accurate identifications and quantitative information on their abundances.

In contrast, species diversity within assemblages of small protists (<20 μm in size) is not well understood, and the distributions of most of these species are poorly described. It is clear from studies of small protists in laboratory cultures, however, that these assemblages are composed of species that differ markedly in their rates of growth and trophic activity. A logical next step in improving our understanding of microbial trophodynamics, and the biogeochemical significance of small protists in aquatic ecosystems, is to begin to understand the biodiversity of these assemblages and the factors controlling their distributions and activities. Characterization of the biodiversity of small protistan assemblages, and the func-

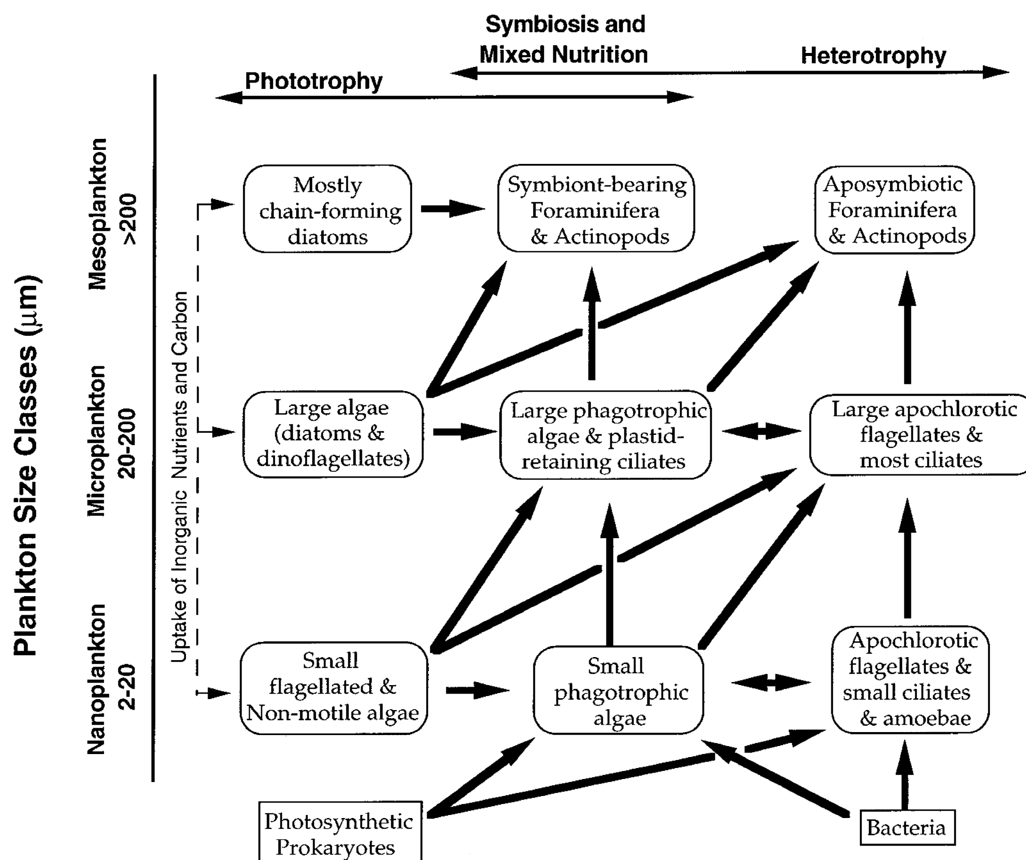

Figure 1. Diagrammatic representation of a microbial marine food web, with generalized roles for various protistan taxa. Taxa are separated by trophic mode (phototrophy, mixotrophy or photosymbiosis, heterotrophy) and size. Solid lines and arrows between the compartments indicate probable predator-prey relationships. This model assumes size-dependent trophic relationships (i.e. grazers eat prey smaller than themselves).

tional diversity that they represent, will provide new insights into how microbial food webs are structured and how they function in nature.

In this manuscript, we briefly describe some of the issues involved in the identification of small protists in natural mixed assemblages, explain the limitations of extant methods, and discuss several molecular approaches that have been applied, or are being developed, to address these issues. Much of this work is ground-breaking. Therefore, most of the impact of these approaches on our understanding of the biodiversity and biogeography of small protists is only beginning to be realized.

HNAN species identification: inherent problems

Techniques for examining assemblages of small protists in natural waters have included a variety of light microscopical techniques, electron microscopy and culture-dependent methods (Kopylov & Sazhin, 1989; Wehr, 1990; Rogerson & Laybourn-Parry, 1992; Vørs, 1993; Stoecker et al., 1994; Vørs et al., 1995). Generally, these methods strike a compromise between the ability to accurately enumerate individuals in a sample, and to identify species within the sample. For example, culture-dependent methods and electron microscopy provide information which aid in the identification of small protists, but these methods generally underestimate their abundances in natural samples (Caron et al., 1989). The use of light microscopy (most notably epifluorescence microscopical techniques) with preserved samples can provide more accurate abundance estimates, but these methods provide limited taxonomic information.

The most commonly employed methods in use today for counting natural assemblages of small planktonic protists entail the use of fluorochrome staining

and epifluorescence microscopy to visualize these microorganisms, and the autofluorescence of chlorophyll to differentiate phototrophic individuals. Typically, 2–20 μm algae are counted as a group (the phototrophic nanoplankton, usually abbreviated PNAN), while the heterotrophic nanoplankton also are grouped into a single entity (the HNAN, also sometimes called HNF = heterotrophic nanoflagellates). Epifluorescence microscopical techniques have been used widely to demonstrate abundances of small protists ranging from several hundred ml^{-1} to tens of thousands ml^{-1} in freshwater and marine planktonic ecosystems, on suspended detrital particles and in benthic communities (Davis et al., 1985; Caron et al., 1986; Pick & Caron, 1987; Sanders et al., 1989; Alongi, 1991; Bak et al., 1991; Sherr et al., 1993).

These latter methods for enumerating protists <20 μm in size provide relatively quick and accurate estimates of abundance, but they also act to aggregate species into a few "black boxes" that are then each represented by a single behavior. Species and even generic distinctions often cannot be made because of the lack of distinguishing morphological features apparent at this level of examination. Within these broad HNAN/PNAN characterizations, however, there is considerable variability among species with respect to light preferences and nutrient uptake capabilities (for PNAN), food preferences and ingestion rates (for HNAN), or even basic nutritional modes (e.g. numerous PNAN species possess some degree of phagotrophic ability). These differing abilities result in considerable variation in the activities and growth rates of nanoplanktonic protists, and thus their relative contribution to community metabolism and other aspects of ecosystem function. A considerable amount of research continues to be directed at documenting and understanding this diversity among small protists (Fenchel, 1986; Sanders, 1991b). Incorporation of these varied behaviors into descriptions of microbial food webs would considerably increase our accuracy of predicting and explaining energy production and flow among microbial species in nature.

There are several impediments in reaching this goal. Two key problems are the accurate determination of the species diversity (i.e. number of species) within natural communities of small protists, and the related problem of identifying and quantifying the abundances of individual species within natural, mixed assemblages. Small protists usually have few morphological features that are distinguishable using light microscopy. It is therefore difficult, and often impossible, to identify species or even genera of PNAN and HNAN using epifluorescence microscopical techniques that have become common for quantifying these assemblages. Electron microscopy and direct microscopical examination of living specimens are often sufficient for identification (Vørs, 1993; Vørs et al., 1995), but this approach is cost- and labor-intensive and does not easily provide quantitative information about these species.

The accurate identification of minute protists can be problematic even in pure culture because of the scarcity (or variability) of distinctive morphological features. Therefore, uncertainties persist regarding the taxonomy and systematics of a number of these species (Patterson & Larsen, 1991; Knauber et al., 1996). For example, the identification of species within the chrysomonad genera *Ochromonas* and *Spumella* is very difficult. These genera contain scaleless phototrophic and heterotrophic species, respectively, that are identified based largely on features of size, shape and details of the flagella and chloroplasts (when present).

Some of these characters can vary with physiological condition of the species. For example, cell size varies considerably with growth state in some phagotrophic chrysomonads (Fenchel, 1982). Average cell volume of *Paraphysomonas imperforata* varied by a factor of approximately six between different growth phases and also as a consequence of prey type (Caron et al., 1985). Chloroplast size and activity can change in some mixotrophic chrysophytes. The chloroplast size in *Poterioochromonas malhamensis* was greatly reduced and chlorophyll autofluorescence was not detectable when high abundances of bacteria were present as food (Caron et al., 1990). Interestingly, this 'phototrophic' chrysomonad was able to grow phagotrophically in continuous darkness.

Even those species that possess 'reliable' morphological features can produce aberrant forms of diagnostic characters under physiological stress. For example, species identifications in the scale-bearing colorless chrysomonad genus *Paraphysomonas* are based on the morphology of the siliceous scales produced by these microorganisms (Preisig & Hibberd, 1982). Nevertheless, scaleless forms of these individuals have been observed under severe silica limitation (Leadbeater & Barker, 1995). This behavior could hamper the identification of individual cells of *Paraphysomonas* in natural water samples where physiological condition is unknown.

HNAN species identification: a case study with a pedinellid flagellate

We have been enriching, isolating and culturing species of heterotrophic flagellates from marine and freshwater environments for several years. Our goal in this work has been to establish a collection of HNAN species for ecological and molecular biological investigations that represents the diversity of free-living, heterotrophic flagellates. Our culture collection now includes approximately 80 clonal cultures of phagotrophic flagellates spanning several major taxa (chrysomonads, dinoflagellates, choanoflagellates, heliozoa, pedinellids, prymnesiophytes, bodonids and bicosoecids).

Enrichment cultures in this study have been initiated using standard methods (Caron, 1993), and clonal cultures of dominant species have been established by micropipetting individual cells from these enrichments. Live and preserved specimens have been examined initially by light microscopy and identified where possible or grouped into major taxonomic categories (e.g. chrysomonads). Negatively stained (1% uranyl acetate) specimens have been examined by transmission electron microscopy to aid identifications where applicable. For example, electron microscopy allows visualization of the body scales of species of *Paraphysomonas*. These scales allow differentiation of the genus *Paraphysomonas* from *Spumella* (whose species do not produce scales) and form the basis for species identifications within the genus *Paraphysomonas* as described by Preisig & Hibberd (1982).

Species examinations conducted in this manner have provided sufficient detail for the identification of many of the clones that we have established. We have been unable, however, to easily resolve the identification of a number of isolates through the use of light and electron microscopy. For example, we obtained several clones of a flagellate that possessed a single emergent flagellum and lacked chloroplasts. Cursory light microscopy of free-swimming and preserved specimens, and transmission electron microscopy, revealed no additional useful details except for the presence of mastigonemes on the flagellum.

Nucleic acids of one of these isolates were extracted and small subunit rDNA (ssu rDNA) was amplified and sequenced (Caron et al., In press). Phylogenetic analysis of full length sequence of this gene revealed that this species was, in fact, most closely related to the pedinellid species *Pteridomonas danica* (Figure 2). Subsequent microscopical examination of our cultures in different physiological states revealed the presence of short tentacles near the flagellum and some cells attached by a stalk to particles in the cultures (Figure 3A,B). These features are characteristic of *Pteridomonas* species (Larsen & Patterson, 1990). Tentacles were not present on the specimens originally examined because they are often retracted in free-swimming or preserved cells (Figure 3C). To date, we have been unable to obtain electron micrographs of specimens with intact tentacles. Some pedinellid species are characterized by the long flagellum extending into a pronounced ‘wing’. This feature also would have aided identification by electron microscopy, but it is absent in species of *Pteridomonas* (Moestrup, 1995).

The situation described above is not meant to indicate that the identification of cultures of *Pteridomonas* is difficult by morphological criteria. Nor do we suggest at this time that molecular identification of HNAN is easier or more straightforward than morphological identification. However, our experience with this isolate exemplifies the problematical nature of identifying HNAN species, even in pure culture. Clearly, the accurate identification of species in natural assemblages of small protists using light microscopical examination of preserved samples presents an extremely difficult task for some species. We obtained complete sequence information for the ssu rDNA of our *Pteridomonas* isolate in order to identify this clonal culture. This degree of thoroughness may not be necessary in the future for making distinctions at the generic (or perhaps even species) level. Partial sequence identity (established, for example, via partial sequencing or hybridization with species- or group-specific oligonucleotides) may be sufficient for ‘matching’ new isolates with species in sequence data bases. These latter approaches are already possible for some heterotrophic protists that parasitize humans (Uliana et al., 1991; Benavides et al., 1993; Gast & Byers, 1995), and they are widely employed for prokaryotes.

Alternatively, recent studies using restriction fragment length polymorphisms (RFLP) have proven useful for differentiating cultured isolates of some free-living protists. We have employed this approach with our collection of small (<10 μm), free-living heterotrophic flagellates as a means of grouping clonal cultures of these species (Lim 1997). Groupings based on RFLP patterns of the ssu rRNA gene were able to differentiate between genera and even many species of these protists. While this type of approach may not

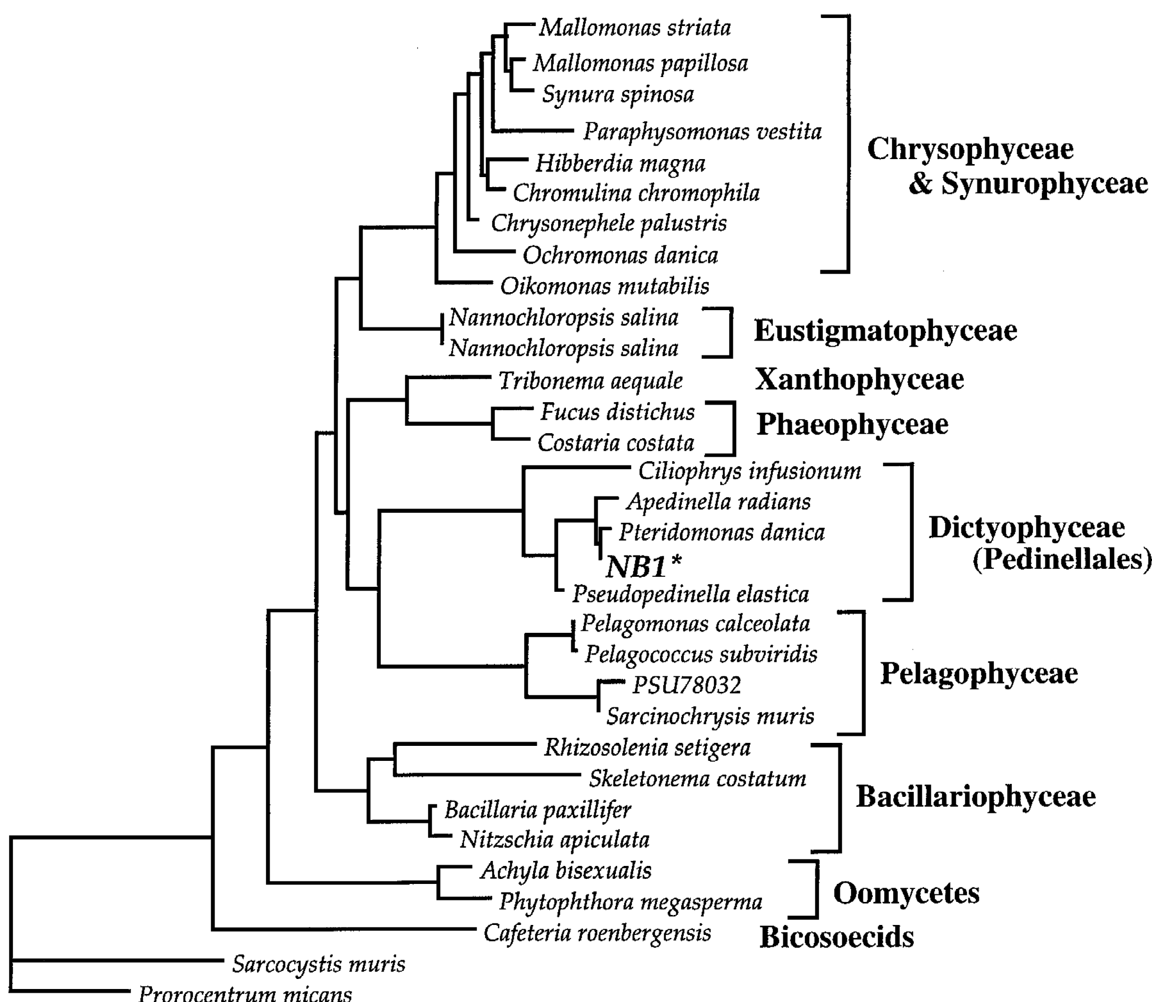

Figure 2. Phylogenetic analysis of the heterotrophic nanoflagellate clone NB1. This tree is the result of a maximum likelihood phylogenetic reconstruction using 1394 characters of which 288 were informative. This analysis places clone NB1 well within the pedinellids. The sequence for NB1 was only 0.66% different than *Pteridomonas danica* (12 out of 1814 sites) and 3.53% different than *Apedinella radians* (64 out of 1814 sites) in an alignment of these three species.

provide unequivocal species identifications, it is a very useful ‘first cut’ for separating isolates into related groups.

Molecular characterization of HNAN species undoubtedly will proceed hand-in-hand with morphological descriptions and identifications in the future. Ultimately, this collective approach will help to derive new identification schemes that will be useful to ecologists studying natural microbial assemblages.

HNAN species identification: detection in natural assemblages

The correct identification of cultured species of HNAN is essential for accurately cataloging autecological data for these species. In addition, the extrapolation of this information to the ecological and biogeochemical significance of small protists in nature relies on the accurate identification and enumeration of these organisms in natural assemblages. A mechanistic understanding of the occurrence and abundance of these species (i.e. biogeographical information) can be obtained only through an ability to quantitatively determine the abundance of species in natural

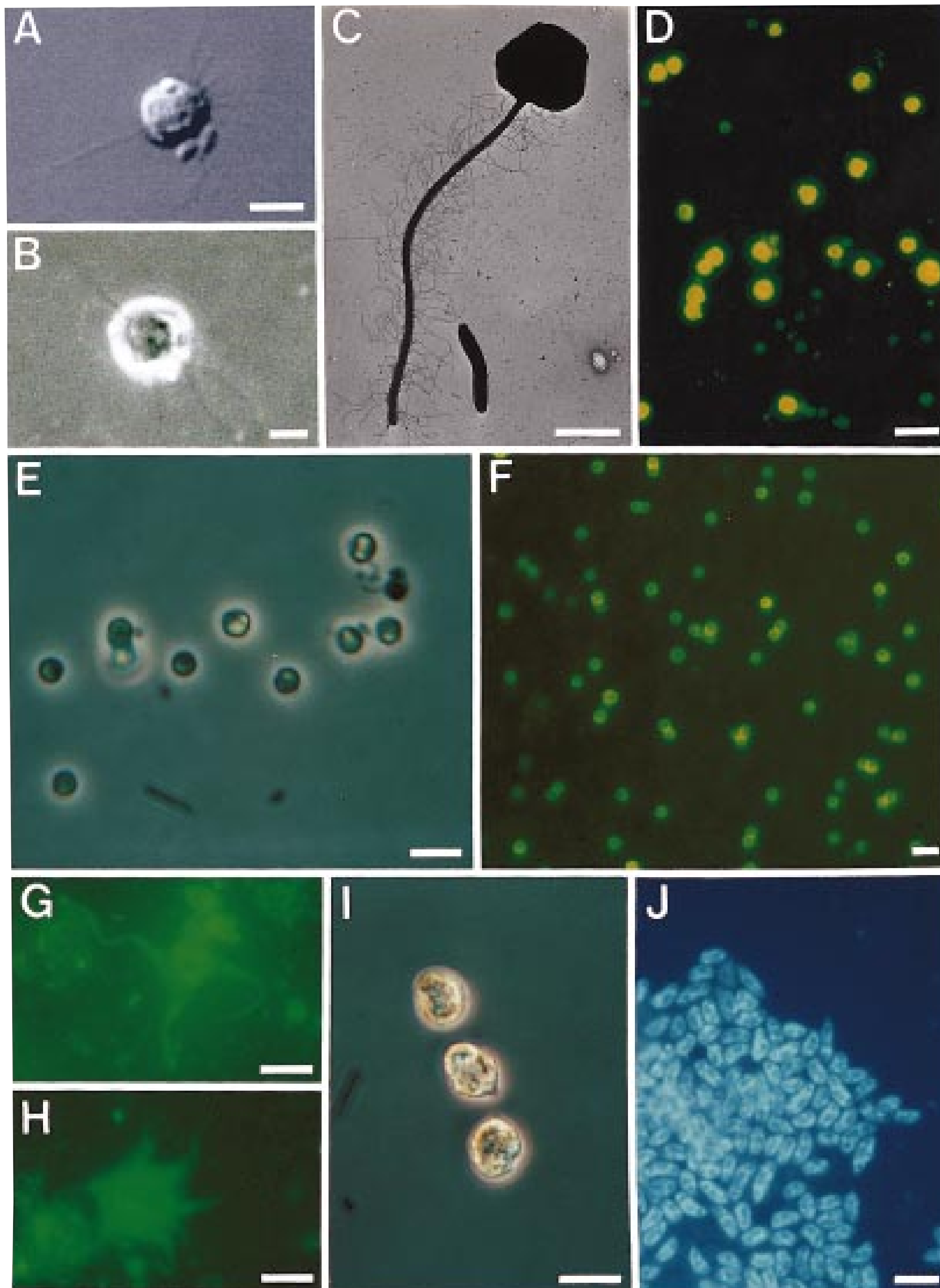

Figure 3.

assemblages. As detailed above, these latter determinations are exceedingly difficult using light microscopical methods that are used routinely for enumerating these populations. The inability of extant methods of routine sample analysis to provide *quantitative* data on the occurrence of ecologically-relevant species of small protists remains a fundamental hindrance to the study of these microorganisms.

We have adapted the use of fluorescently labeled oligonucleotide probes targeted against ssu rRNA for enumerating species of small (5–10 μm) heterotrophic flagellates in natural samples. The overall scheme involves the establishment and identification of clonal cultures of the target species (and closely related species), probe design and testing, and then application in a quantitative *in-situ* hybridization technique (Figure 4). We have taken an approach that we hope can serve as a template for probe design and application for a wide variety of small protists.

Our initial work was directed at testing the feasibility of using oligonucleotide probes in *in-situ* hybridizations using cultured species of heterotrophic flagellates. Primary considerations included signal strength and permeability of the protistan cells (Lim et al., 1993). Signal amplification using a biotin-avidin system proved necessary in order to visualize 3–10 μm cells in late stationary growth phase when ribosomal number was low.

Another major step in the development of our technique for applying oligonucleotide probes was the development of a *quantitative* method that could be employed with natural samples (i.e. preventing loss of sample during dehydration, hybridization and rinsing). Centrifugation and immobilization in gelatin have been employed, but these methods can result in significant losses of preserved cells from samples during the probing process. Our quantitative *in-situ* hybridization method employs Transwell® cell culture inserts for Corning Costar tissue culture dishes that allow solution exchange through a polycarbonate filter while retaining the target population in the Transwell chamber (Lim et al., 1996). This method is essentially an adaptation of the fluorochrome staining methods that are used routinely for counting nanoplankton assemblages (Sherr et al., 1993).

The development of species-specific oligonucleotide probes for identifying and counting small protists in natural water samples focused on the heterotrophic chrysomonad species *Paraphysomonas imperforata* (Caron, et al., In press). This phagotrophic protist is nanoplanktonic in size, its distribution is cosmopolitan, it is easily enriched and cultured in the laboratory (there is considerable physiological information for this species), and it can be readily identified by electron microscopy because of the species-specific, siliceous body scales that it produces. Oligonucleotide probes targeting the ssu rRNA of this species were designed based on sequence comparisons for this species, several congeners and a variety of other related species of protists. Testing performed in slot blots and *in-situ* hybridizations indicated specificity for *P. imperforata* even in comparison to closely related congeners (Figure 3D).

Field applications of oligonucleotide probes targeting *P. imperforata* have already altered our perception of heterotrophic flagellate assemblages of natural plankton communities (Lim et al., 1999). Although this cosmopolitan species was often the numerical dominant of our enrichment cultures initiated from a wide variety of aquatic ecosystems, thus far we have not observed situations in which *P. imperforata* constituted more than ≈1% of the total nanoplankton of unenriched, natural samples. Based on our study using this molecular approach, we conclude that this species dominates enrichment cultures because it is strongly favored by the cultures conditions provided in

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Figure 3. (A–C) Differential interference contrast (A), phase (B) and transmission electron (C) micrographs of the heterotrophic nanoflagellate *Pteridomonas* sp. The stalk and some of the tentacles were visible on the live specimens in (A) and (B), and the beating flagellum was visible between the tentacles in (B). Only the flagellum was visible in preserved specimens, the stalk and tentacles were absent (C). (D) Epifluorescence micrograph of a mixture of the two heterotrophic nanoflagellates *Paraphysomonas imperforata* and *P. bandaiensis*. *P. imperforata* cells have been probed with a species-specific oligonucleotide probe that causes these cells to be highly fluorescent. Unprobed *P. bandaiensis* cells (about 1/2 the size of the *P. imperforata* cells) are faintly visible due to background fluorescence. (E,F) Light micrograph (E) and epifluorescence micrograph of immunofluorescently-labelled cells of the 'brown tide' alga *Aureococcus anophagefferens*. (G,H) Epifluorescence micrographs of two naked amoebae (star-like shapes in center of micrographs) probed with eukaroyote-specific oligonucleotide probes. (I) Light micrograph of the free-living form of the photosynthetic dinoflagellate *Scrippsiella nitricula* that occurs as an intracellular symbiont in the polycystine radiolarian *Thalassicolla nucleata*. (J) Epifluorescence micrograph of the symbiotic dinoflagellate *Gymnodinium beii* in a squashed foraminiferal specimen. The intracellular symbionts lack typical dinoflagellate morphology which is present during the free-living stage (compare to specimens in (I)). Fluorescent signal in these specimens is a result of probing with a species-specific oligonucleotide probe designed for *G. beii*. Marker bars = 5 μm (A–F), 10 μm (I,J) or 15 μm (G,H).

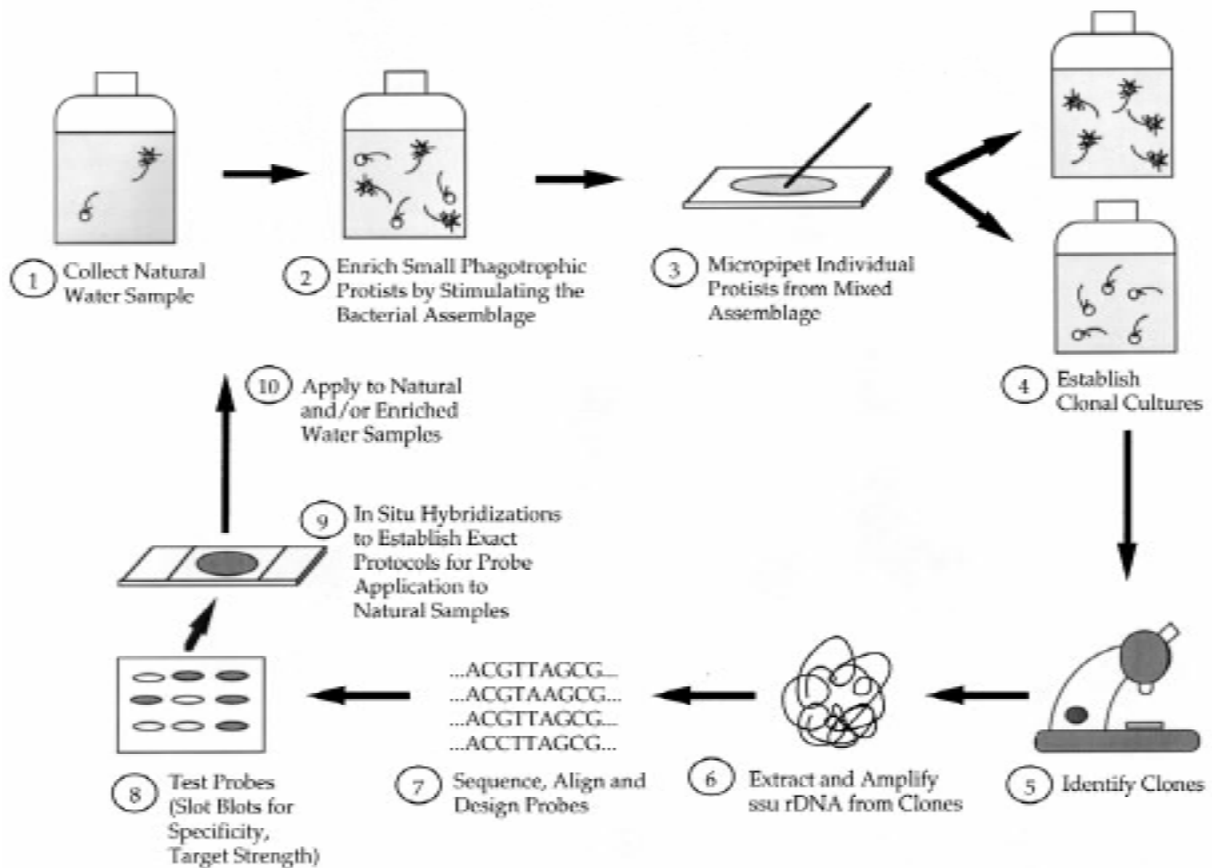

Figure 4. Generalized protocol for the design, testing and application of ssu rRNA-targeted oligonucleotide probes for heterotrophic nanoflagellates (or other small protists); (1–5) establishment of clonal cultures and identification by morphological criteria; (6 – 7) extraction, sequencing and comparison of ssu rDNA from closely- and distantly-related species for the design of oligonucleotide probes; (8 – 9) testing specificity of probes against extracted nucleid acids and with whole cell preparations of target and non-target species; (10) application of probes to natural samples.

our enrichment technique. Moreover, we postulate that natural assemblages of heterotrophic flagellates may be far more diverse than we have suspected previously. Our experiences are indicative of the poor understanding we presently have of the ecology of small protists, and the new insights that can be gained from the application of molecular biological methods to the study of these microorganisms.

PNAN and Yeasts

Species identifications among microscopic algae and fungi present much the same difficulties that are associated with the identification of HNAN. Many of the minute algae possess few morphological characters that are useful as taxonomic criteria. Pigment composition has provided a means of characterizing the major phototrophic taxa present within planktonic as well as benthic environments (Riaux-Gobin & Klein, 1993; Bidigare & Ondrusek, 1996), but further taxonomic characterization is not possible by this approach. Identification of marine fungi has been based on morphological and physiological characteristics determined in the laboratory using pure cultures. These features are of limited value for identifying species in natural microbial assemblages.

Recent applications of DNA sequence analysis to the characterization of species of marine yeasts is an excellent example of the exploitation of molecular biological approaches for studies of the biodiversity of small eukaryotes (Fell et al., 1992; Fell, 1993). The use of ssu rDNA primers has revealed a diverse assemblage of microscopic fungi, and a community

complexity that far exceeds what had been demonstrated using culture-dependent methods up to that time. Extension of this approach to analyses of natural microbial communities should provide new insights into the abundances and distributions of these poorly documented microorganisms.

Molecular identification based on DNA sequence data has been reported recently as an ecological tool for species of PNAN (Knauber et al., 1996; Simon et al., 1997), and also for some larger algae of interest to human health (Miller & Scholin, 1996; Scholin & Anderson, 1996; Scholin et al., 1996). The application of this approach for small algae, however, has not yet become common. Instead, the use of immunological methods has received more attention for these species (Shapiro et al., 1989; Campbell et al., 1994). Such methods have allowed, for example, the enumeration in natural samples of *Aureococcus anophagefferens*, a 2–3 μm pelagophyte which does not possess flagella and has few distinctive morphological features (Sieburth et al., 1988; Anderson et al., 1989) (Figure 3E,F). Immunoprobes also have been used effectively to determine the occurrence of different strains of chroococcoid cyanobacteria in mixed natural cyanobacterial assemblages (Campbell & Iturriaga, 1988).

The reason for the more widespread use of antibody techniques for small algae rather than DNA-based methods is not clear, but may relate to the ease of obtaining antibodies commercially, and thus the smaller investment necessary to conduct this work (relative to equipping a lab for molecular biology). A potential advantage of the DNA-based methods, however, is that sequence information is unlikely to change over ecologically-relevant time scales, while antigenic characters of some protists can change rapidly due to physiological condition and/or life stage.

Amoeboid protists

Perhaps the most recalcitrant problems of species identification of small protists exist for the non-testate ('naked') amoebae. At present, it is not commonplace to identify or enumerate these protists from preserved specimens. This situation is due to the difficulty of distinguishing these amorphous-shaped cells by light microscopy (especially in preserved samples), and this problem is further complicated by the attachment of amoebae to particles which may obscure vision or absorb fluorochromes that are used to stain protists.

Abundances of amoebae in natural ecosystems generally are determined using dilution-enrichment approaches which are dependent upon the growth of these species in the enrichments (Davis et al., 1978; Rogerson & Laybourn-Parry, 1992; Anderson & Rogerson, 1995). Identifications (which are primarily based on features of the living cells) can be performed on amoebae growing from these enrichments but, as with some flagellates, 'species' identifications often are hampered by the morphological variability of these specimens. Some species are even capable of transformation into flagellated life stages. The prevalence of these latter forms in natural samples is unknown.

The use of culture-dependent methods for enumerating amoebae in natural samples also raises questions as to the accuracy of these abundance estimates. Some species (or individuals) of amoebae may not grow in enrichment cultures. Lack of growth would result in an underestimation of their abundances in natural samples. Conversely, excystment of encysted amoebae could overestimate the abundance of individuals that are trophically active. This latter problem is also a concern for estimating the abundances of other protistan taxa that produce cysts.

Biogeographical data for amoebae are meager relative to our knowledge of other free-living protists because of our present inability to obtain quantitative information on the abundances of these species from preserved samples. As a result, their ecological/biogeochemical roles in most aquatic ecosystems are poorly understood and probably underestimated (Arndt, 1993). Detection of amoebae using DNA-based protocols could enable new approaches for their identification and enumeration in natural samples. These methods would obviate the need to base enumeration and identification on the ability of individuals to grow in culture. The design and application of species- or group-specific oligonucleotide probes could provide a routine means of obtaining quantitative information for amoebae in preserved samples, and potentially also recognize other life stages of these species (e.g. cysts, flagellates). In addition, the use of oligonucleotide probes does not result in staining of particulate material (Lim et al., 1996). This feature has considerable advantage for visualizing protists such as amoebae that are typically associated with particles in the plankton (Figure 3G,H).

Difficulties associated with the identification of amoeboid protists are not constrained only to small species. Although the identification of larger, free-living 'amoeboid' or sarcodine protists (Foraminifera

and Actinopoda) has not been considered particularly problematic, specific aspects of their biology still present unique difficulties. Some of these issues relate to the complex life cycles of these species. For example, many of the Foraminifera, Radiolaria and Acantharea form tests or central capsules as adult specimens that can attain diameters greater than 1 mm (pseudopodial networks can increase the effective size of some of these specimens to > 1 cm). The identification of the shell-bearing stages of these specimens is based largely on the skeletal structures. In contrast, these species reproduce by the release of swarmers that are flagellated cells several micrometers in size (Anderson, 1983; Bé et al., 1983). The identification of sarcodine swarmer cells presents difficulties that are similar to the identification of HNAN species; few morphological details are apparent by light microscopy that enable their accurate discrimination in natural samples. As a consequence, there is no quantitative information on the occurrence or depth distributions of these swarmer cells in oceanic plankton communities.

Other problems concerning the identification of sarcodine protists relate to variable morphology during the adult life stages of some species. Taxonomically-relevant skeletal structures vary considerably for some species of Radiolaria and benthic Foraminifera (Bjørklund & Swanberg, 1987; Bowser et al., 1995; Gooday et al., 1996). At one extreme, some species of Foraminifera are capable of complete loss of the test (Cedhagen & Tendal, 1989). The ecological significance of this variability is intriguing and may be a consequence of environmental influence, but it can result in erroneous identifications or even the description of synonyms. On the other hand, such morphological variability may mask the presence of cryptic species, as has been postulated for the solitary Radiolarian *Thalassicolla nucleata* (Amaral Zettler et al., 1998). DNA sequence information provides additional insight to classical morphological approaches for resolving these issues.

Dinoflagellates

Dinoflagellates are a highly diverse taxon of protists. These species constitute a significant fraction of both the phototrophic and heterotrophic protistan assemblages of plankton communities, and thus have tremendous ecological significance as primary producers and consumers. Heterotrophic dinoflagellates exhibit a variety of interesting and unique feeding behaviors that significantly impact phytoplankton assemblages (Jacobson & Anderson, 1986; Gaines & Elbrächter, 1987; Hansen, 1991; Lessard, 1991; Jeong, 1994), and mixotrophic (phagotrophic) activity among phototrophic dinoflagellates is common (Sanders, 1991a). In addition, some species cause harmful algal blooms (Anderson & Garrison, 1997), or occur as symbionts or parasites in a variety of protistan and metazoan hosts (Cachon & Cachon, 1987; Trench, 1987).

Dinoflagellates have received ample attention from protistan taxonomists, and morphology-based schemes for their identification are well established. An important consideration for ecological studies of dinoflagellates, however, is that many of these species possess relatively complex life cycles that pose significant problems for identification *in situ*. An extreme example of this behavior exists for the ambush-predator dinoflagellate, *Pfiesteria piscicida*, for which more than two dozen life stages have been described (Burkholder et al., 1995a,b; Burkholder & Glasgow, 1997). At present, the existence of multiple life stages makes the identification of this species in natural assemblages very difficult (if not impossible) because different methodologies must be used to establish the presence of the various life stages. In general, the distributions in nature of dinoflagellates with multiple life stages will remain enigmatic until tools are developed that will allow detection and enumeration of all of the various life stages in a single process.

Morphological variations (or life stage transformations) of dinoflagellates can be induced by environmental conditions, but they can also be the result of physical association of dinoflagellates with other organisms. The morphologies of dinoflagellate parasites and symbionts in their free-living states are sometimes radically different than their morphologies in a host. Dinoflagellate symbionts of the planktonic Foraminifera and Actinopoda, for example, do not produce thecal plates or flagella when they occur as intracellular symbionts (Anderson & Bé, 1976; Anderson, 1980). The development of DNA-based protocols for species detection of these symbionts from DNA sequence information (Rowan & Powers, 1992; Gast & Caron, 1996) will enable ecological studies of these dinoflagellates in both the free-living thecate stages and as symbionts within various sarcodine species (Figure 3I,J).

Similarly, heterotrophic dinoflagellates that cause parasitic infections typically cannot be identified when

they occur in association with their hosts (Cachon & Cachon, 1987; Coats et al., 1996). The agents of these infections usually are identified only after lysis of the host cell or tissue and release of a free-living stage of the dinoflagellates. At present, there is little information concerning the impact of these parasitic infections on populations of their hosts in nature, in part because of the difficulties of identifying and counting these parasites. DNA-based protocols for the detection of dinoflagellate parasites in the cytoplasm or tissues of infected hosts, and in their free-living forms, will greatly enhance ecological studies of these relationships.

Perspectives on the Future

Traditional methodological approaches cannot easily address many unresolved issues and unanswered questions concerning the biodiversity and biogeography of small protists in aquatic food webs. As a consequence, many aquatic biologists have looked to molecular biology within the last decade as a panacea for rapidly solving many of these problems. This highly optimistic attitude has been somewhat tempered in recent years, as limitations and/or problems have arisen when trying to adapt these methods for use on natural assemblages of microorganisms. These setbacks have inspired more care in incorporating molecular methods into ecological research programs, but they have not diminished the potential for these approaches to answer long-standing ecological questions. As evidenced by this volume, molecular techniques and conceptual approaches clearly are providing new insights into the ecology of marine bacteria, protists and fungi. Expectations concerning these approaches are just beginning to be realized. The application of these new powerful molecular approaches, in concert with traditional methods of study, will continue to enlarge our base of knowledge concerning the ecology of free-living microorganisms.

Acknowledgements

The authors are grateful to Dawn M. Moran and Katie Rose Cellineri for some of the micrographs used in Figure 3. This study was supported by NSF grant OCE-9216270. Woods Hole Oceanographic Institution Contribution Number 9776.

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