

CHAPTER 11

Microscopy and *Prymnesium parvum*: Observations and Challenges

GREGORY M. SOUTHARD

Abstract

Prymnesium parvum is an algal species responsible for toxic fish kills in Texas reservoirs and two freshwater fish hatcheries. The current Texas Parks and Wildlife Department (TPWD) method for identifying and estimating densities of *P. parvum* in water samples uses a compound light microscope, hemacytometer, and trained personnel adept at identifying this particular species among mixed algal communities. Repeated observations of *P. parvum*-infested water samples using epifluorescence microscopy suggest that *P. parvum* may yield distinct fluorescence emission pattern(s) compared to the other types of algae in mixed samples. This observation led to an investigative effort regarding the feasibility of specialized microscopic techniques, flow cytometry, and other fluorescence-based applications that might facilitate *P. parvum* cell enumeration. The following report is an overview of that investigation.

Introduction

Toxin-producing algae have been an area of health and economic concerns and study for marine biologists for decades and recently have become an issue for freshwater systems in the United States. *Prymnesium parvum*, also called “golden alga”, is a chrysoomonad haptophyte, which was first implicated in minor fish kills along the Pecos River in Texas during the 1980s (Linam et al. 1991). In recent years, this species has caused catastrophic fish mortalities in several Texas river systems and reservoirs as well as being problematic at two Texas Parks and Wildlife Department (TPWD) freshwater fish hatcheries.

An important aspect of toxic algal management plans at TPWD fish hatcheries involves monitoring cell densities to assess the need to implement control methods. Currently, trained personnel monitor *P. parvum* in reservoirs and hatchery ponds using light microscopy at magnification up to 1000X to identify the organism and a hemacytometer to calculate cell concentration.

Description of P. parvum

Microscopically, *P. parvum* has often been described as having variations in size and shape depending upon the phase of the life cycle as well as nutrient availability. Examination is facilitated by using unpreserved and unfiltered water samples since *P. parvum* can pass through or be damaged by most plankton nets and the cells are distorted by fixatives. Additionally, living cells have a characteristic swimming motion that aids

in their identification. When samples must be preserved for long-term storage, buffered Lugol's solution at 4°C is recommended although there may be some deleterious effects upon the cells, including loss of the haptonema.

The following description of *P. parvum* is adapted from Green et al. (1982). Cells glide smoothly in a forward direction while spinning on their longitudinal axis. Cells are 8-15 µm long and 4-10 µm wide, sub-spherical to elongate in shape with a rounded posterior end and tapered anterior end that bears two equal or sub-equal flagella of 12-20 µm long or approximately 1.5 times the length of the cell. A short haptonema (3-5 µm) is directed forward and arises from the same groove as the two flagella. There are two large yellow-green chloroplasts situated laterally and parietally, often deeply lobed and each containing an immersed pyrenoid. The nucleus lies centrally between the two chloroplasts. A golgi body is parabasal and a contractile vacuole is sometimes present. The flagellar action is heterodynamic in swimming cells with the anterior flagellum beating with a rapid, almost "flicking" movement (Green et al. 1982) as the posterior flagellum undulates freely. In resting cells, the flagellar action is slower and more regular with the amplitude of waves increasing towards the distal end of the flagellum. Cells are covered by organic scales of two types and in two layers: scales of the outer layer have narrow inflexed rims, a pattern of radiating fibrils on the proximal face and concentrically arranged fibrils, sometimes observed only in the central area, on the distal face; scales of the inner layer have surface patterning similar to the outer scales but with a rim strongly inflexed over the distal face. These scales require electron microscopy to be observed.

Light Microscopy

The oldest and simplest method for identifying algal species is by visual observation using a light microscope and a glass slide. While this approach is commonplace, it has many limitations. It requires trained personnel with an aptitude to distinguish subtle differences among taxa or variations associated with life history or nutrient availability (Culverhouse 1995). Cell counts can be very time-consuming, especially when phytoplankters are numerous. Additionally, density estimates using the hemacytometer likely are unreliable at low cell concentrations. In cases where the alga is present in low numbers or as a part of a complex mixture, it may be undetectable leading to the conclusion that the alga is absent when it is actually present. Despite these drawbacks, TPWD Inland Fisheries staff currently use light microscopy and hemacytometer counting chambers to identify and quantify *P. parvum* cells because faster, more accurate and inexpensive methods are unavailable.

Epifluorescent Microscopy

Epifluorescent microscopy was explored as an option to improve *P. parvum* cell identification and enumeration methodology. When illuminated using epifluorescence, *P. parvum* cells fluoresce a gold color that contrasts with the green or red color of many other algal species (Figure 1). This observation prompted further investigation into use of fluorescent microscopy to identify and quantify algae samples. Also of note, in some

water samples we observed an organism that appeared to preferentially graze on *P. parvum* (Figures 2A, B, C, and D). The organism closely resembles the ciliate *Coleps* sp. (Fig. 2E and 2F), but it has not been positively identified. This protist could be useful as a biological control agent if it is a significant predator of *P. parvum*, but more research is needed to demonstrate feasibility of using this organism as a control method. *Coleps* spp. have occasionally been associated with morbidity and mortality among fish (Szekely and Berezky 1992; Wooster and Bowser 1994)

Pigment analysis via fluorescence excitation spectra has been studied and widely used for *in vivo* characterization of marine and freshwater phytoplankton communities. *In vivo* fluorometry (IVF) uses direct measurement of chlorophyll fluorescence in living cells and has been used by oceanographers and limnologists for locating and measuring algae for over 20 years. The IVF method is useful for gross estimates of algal growth patterns, but not very helpful to identify algal species.

Various attempts have been made to achieve taxonomic identification of phytoplankton based on *in vivo* bio-optical characteristics such as fluorescent excitation and absorption spectra (Hoepffner and Sathyendranath 1991). Oldham et al. (1985) were the first researchers to report identification of marine phytoplankton using total fluorescence “fingerprinting”. Neveux and Panouse (1987) reported fluorescence characteristics of the chlorophylls and phaeophytins and the patterns associated with different algal taxa. Currently, fluorescence “signatures” of four taxonomic groups can be readily distinguished: chromophytes (golden-brown algae), chlorophytes (green algae), cryptophytes and rhodophytes (red algae), and cyanobacteria (blue-green algae). Specifically, the chlorophyll accessory pigment (CAP) ratio is used to differentiate these taxa in marine waters.

Other techniques for monitoring algal communities include estimation of chlorophyll *a* concentration, algal biomass, and primary productivity (USEPA 446.0, 1997; Yentsch and Yentsch, 1979). Recently developed methods measure fluorescence based on fixed wavelength fluorometry using a filter system for excitation and detection, scanning spectro-fluorometry based upon the excitation or emission spectra of fluorescence, fast repetition rate fluorometry, and a pump and probe technique (Kolber and Falkowski 1993).

Although the new techniques are promising, there are some drawbacks of using fluorescent patterns to characterize algal species. A common feature among these techniques is pigment concentration is estimated through the correlation of *in vivo* fluorescence with the isolated photosynthetic pigment values. Attempts to quantify phytoplankton pigments *in situ* using fluorescence characteristics counter the fact that photosynthetic pigments are bound to protein in living cells (Cowles et al. 1993; Kolber and Falkowski 1993). Because of that, *in vivo* fluorescence of phytoplankton should also take into consideration these pigment-protein complexes, which have a variety of structures and different roles in photosynthesis. *In vivo* fluorescence excitation spectra of phytoplankton depend not only on the taxonomic position of algae, but also on the photoadaptation state. The cellular pigment content, the ratio of total chlorophyll *a* to

accessory pigments, and the efficacy of energy transfer to chlorophyll *a* are sensitive to the light conditions of culture growth (Wilhelm and Manns 1991). Vertical profiles of a species will vary in photoadaptive state due to the gradient of ambient light. The bulk of chlorophyll and carotenoid-protein complexes consists of various light harvesting pigment proteins that absorb light and then convert light energy into chemical energy. The characteristics of phytoplankton *in-vivo* fluorescence, including its fluorescence efficiency, depend upon various factors other than only taxonomy. For *P. parvum*, quantitative pigmentation has been found to be nearly independent of growth phase (Wilhelm and Manns 1991) and the pigment pattern is sensitive to nutrient limitations. Also of note, for *P. parvum*, the predominant fraction of the light harvesting protein gave absorption spectra rich in chlorophyll *c* (c_1 , c_2 , and c_3) as well as xanthophylls (Wilhelm and Wiedemann 1991).

Flow Cytometry

Fluorescence becomes a powerful tool in cell identification when the cells can be separated and analyzed individually, as with flow cytometry (Børsheim et al. 1989). With the aid of laser-based flow cytometry and cell sorting, pigment autofluorescence, stain-induced fluorescence, and light scatter are used as probes to quantify and sort subpopulations of phytoplankton cultures and natural populations (Yentsch et al. 1983). Once fluorescence signature coordinates are established, flow cytometry is a convenient way to count individual species of phytoplankton in mixed samples. Chlorophyll *a* fluorescence and forward light scatter are two parameters measured using flow cytometry and that produce discriminatory signature ranges, but they are highly variable depending upon life history, distribution, concentration, and other factors (Yentsch et al. 1983). Thus, as with the visual method, there also are disadvantages with using flow cytometry to identify and quantify algal cells. Flow rates typically make selection of individual cells nearly impossible or investigation of organisms in low-concentrations time-consuming or impossible. Also, there is natural variation in the form or biochemistry of the algae themselves, ranging from morphological features to amount of chlorophyll or associated pigments produced (Culverhouse 1995).

Challenges

The TPWD Inland Fisheries Division has the need for a quicker and more reliable method to identify and quantify *P. parvum* in water samples, whether it occurs in the hatchery setting or as part of management plan for affected water bodies. Fluorescence characteristics may be useful to distinguish this alga from other species, but techniques in the field of microscopy or cytometry need to be developed and current TPWD personnel lack the expertise to develop these methods. Additionally, the specialized equipments are expensive and bulky. Thus, cost-effective alternatives to these technologies that are suitable for hatchery or field operations are needed

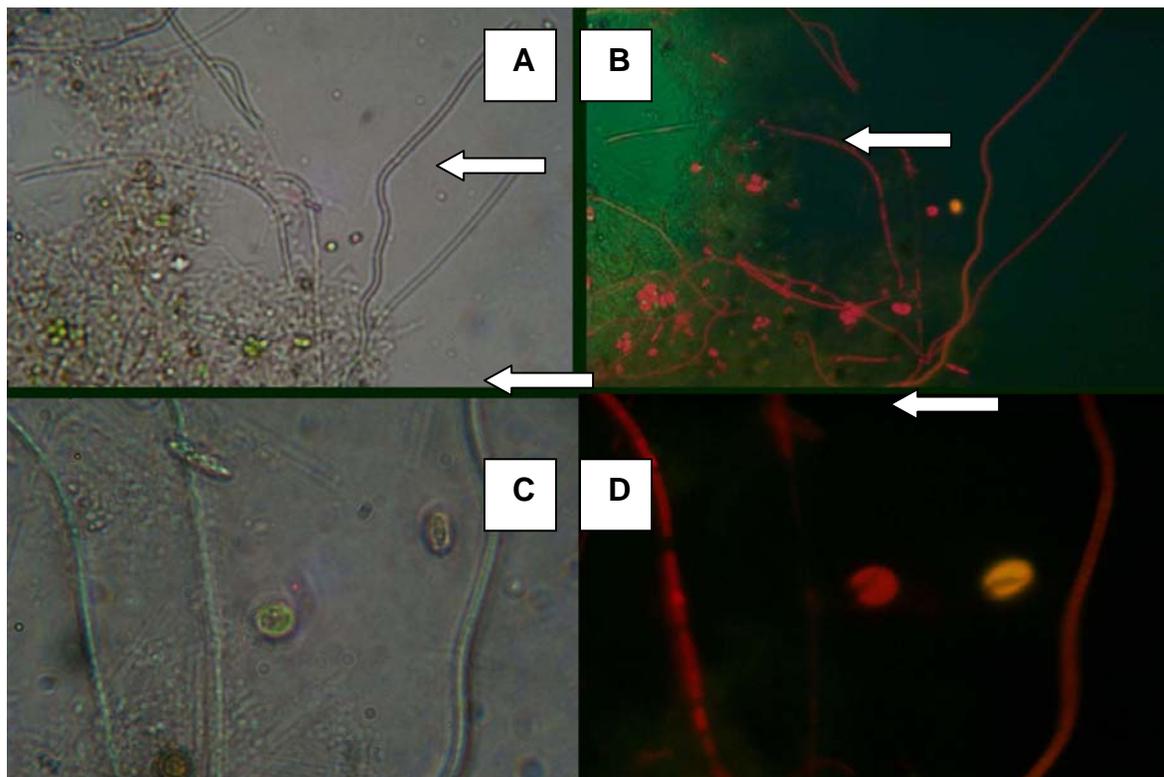


FIGURE 1.—Brightfield and epifluorescent microscopy images of mixed phytoplankton community containing *Prymnesium parvum* (arrows) cells: (A) brightfield (100X); (B) epifluorescence (100X); (C) brightfield (400X); (D) epifluorescence (400X).

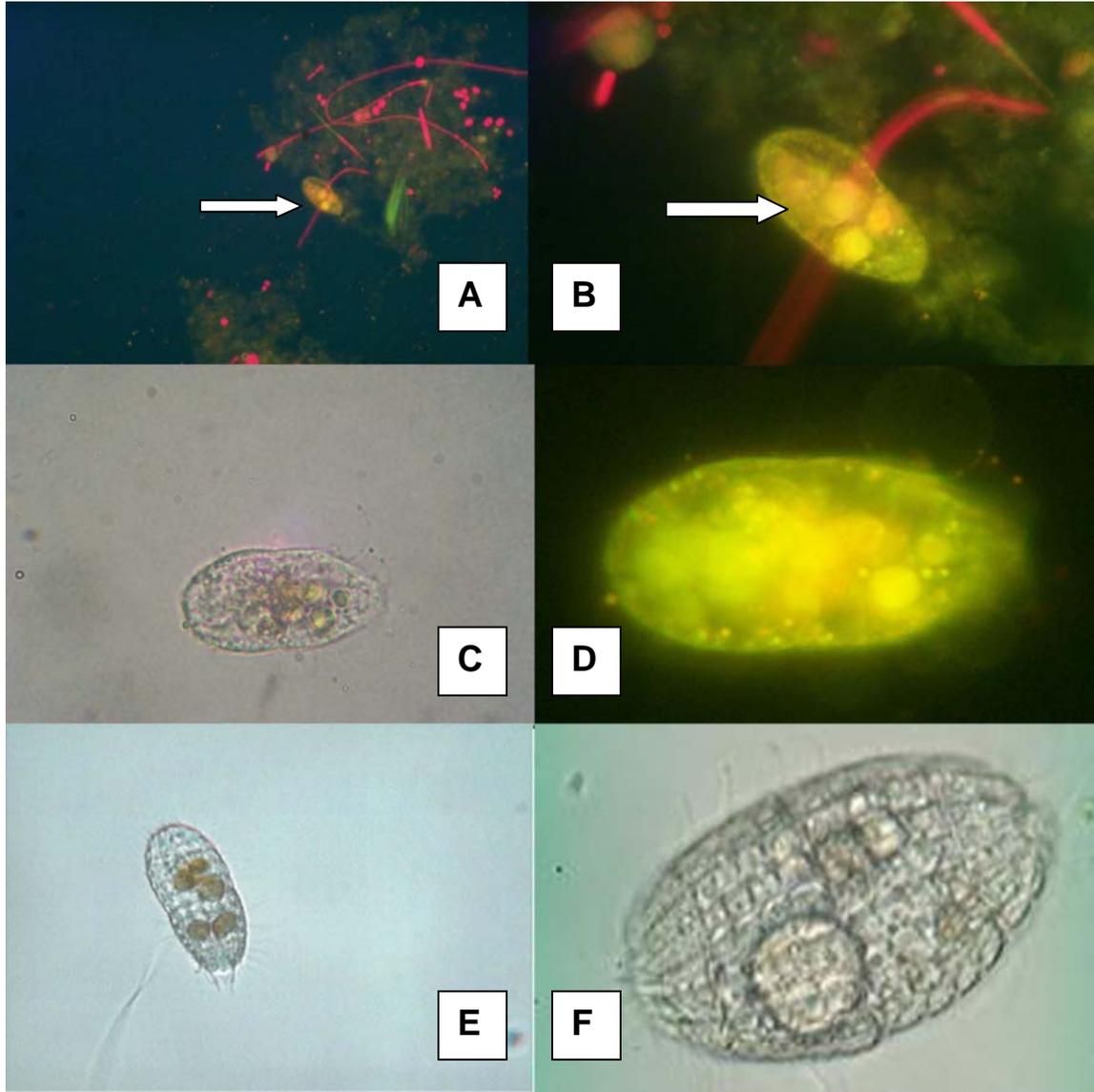


FIGURE 2.—Brightfield and epifluorescent images of mixed algal community, suspect *Prymnesium parvum* grazer (arrows), and the protist *Coleps* sp. (A) Mixed algal species (epifluorescence 100X); (B) Mixed algal species (epifluorescence 400X); (C) Suspect *P. parvum* grazer (brightfield 400X); (D) Suspect *P. parvum* grazer (epifluorescence 1000X); (E) *Coleps* species (brightfield 400X) [http://www.funsci.com/fun3_en/protists/prot_14.jpg]; (F) *Coleps* species (brightfield 1000X) [<http://www.ph-karlsruhe.de/NATUR/GARTEN/2001/kraeuterspirale/index.html>].