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## Bioassay for ichthyocidal activity of *Pfiesteria piscicida*: Characterization of a culture flask assay format

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### Abstract

The description of the heterotrophic dinoflagellate *Pfiesteria piscicida* as the causative agent of fish lesions and deaths along the mid-Atlantic estuaries has revealed the need for bioassays to assess its potential toxigenicity. We designed a bioassay in which fish are exposed to clonal dinoflagellate strains or environmental consortia (e.g. environmental water or sediment samples) in 750-mL culture flasks, and examined the relationships among dinoflagellate proliferation profiles, the presence of *P. piscicida* and fish deaths. Assay development and characterization were accomplished with dinoflagellate clonal cultures (*P. piscicida*, *Karlodinium micrum*, *Prorocentrum minimum*, CCMP1828, CCMP1829, and CCMP1834) co-incubated with sets of two young adult sheepshead minnows (*Cyprinodon variegatus*). Variables characterized included water quality (pH, dissolved oxygen, ammonia, nitrate and nitrite concentrations), the effect of the presence of fish on the proliferation and compositions of protist (dinoflagellate, protozoa, diatom) and bacterial populations, and time of fish death. The presence of fish in experimental flasks induced proliferation of *P. piscicida* and *Cryptoperidiniopsis* sp. (but not *K. micrum* or *P. minimum*) with populations rising between days 6 and 10, and declining 4 to 5 days later. Some fish deaths occurred when or soon after *P. piscicida* cell numbers were maximal. We conclude that this assay enables the assessment of acute effects of ichthyocidal dinoflagellates on fish during the first 10 days (Stage A) of the experimental course. Fish deaths during the subsequent 10 to 20 days (Stage B) may be attributed to the proliferation of ichthyocidal dinoflagellates, pathogenic bacteria and/or deteriorating water quality, whereas those beyond a period of approximately three weeks (Stage C) can be most certainly attributed to deteriorated water quality. Application of the flask assay to environmental samples [n=53] yielded fish deaths in all three stages: A, 78%; B, 11%, and C, 2%, with fish still living in 9% of the sample waters tested at the conclusion of the experiment beyond three weeks. The majority of samples that resulted in fish death in stage A tested positive for *P. piscicida* by PCR. If implemented with cautious interpretation, this assay should prove useful in monitoring blooms for the presence of *P. piscicida* and other dinoflagellate species potentially harmful to fish.

**Abbreviations:** ASW – artificial seawater, CCMP – Provasoli-Guillard National Center for Culture of Marine Phytoplankton, CFU – colony-forming unit, DO – dissolved oxygen, NTS – non-transcribed spacer, PCR – polymerase chain reaction

## Introduction

During summer 1997, mass fish mortalities (ca.15,000 fish) occurred in Chesapeake Bay and its tributaries, requiring public waterway closures that touched off widespread media coverage and substantial economic losses in the local seafood industry, as chronicled by the Maryland Department of Natural Resources (<http://www.dnr.state.md.us/pfiesteria/sop.html>). State agencies responded to the crisis through a Technical Advisory Committee, which formulated a list of possible causes for further investigation: “physical irritation from microbial infections of stressed fish, harmful chemicals, secondary infections by bacteria, viruses, and fungi, *Pfiesteria piscicida*; and other microorganisms” ([http://www.dnr.state.md.us/bay/pfiesteria/98\\_lesion.html](http://www.dnr.state.md.us/bay/pfiesteria/98_lesion.html)).

An increase over the past decade or two in the episodic frequency of ocean disease events, usually affecting only marine life, has contributed to rising awareness of health issues in the marine environment (Harvell et al. 1999). The 1997 fish kills in the Chesapeake Bay were unusual, because human health effects among individuals exposed to the presumably contaminated waters were reported, leading to descriptions of a new toxic exposure syndrome characterized by unique neurological effects and memory loss (Grattan et al. 1998; Bever et al. 1998). The description of the heterotrophic dinoflagellate *P. piscicida* or its toxin(s) as the causative agent of the above-mentioned fish lesions and deaths, and deleterious effects on human health along the mid-Atlantic estuaries (Steidinger et al. 1996; Grattan et al. 1998; Bever et al. 1998; Kane et al. 1998; Matuszak et al. 1998) revealed the pressing need for bioassays that would enable the assessment of its potential toxigenicity. To investigate the effects of *P. piscicida* on fish we found it necessary to attempt to replicate its reported ichthyocidal activity under controlled laboratory conditions. This would enable us to establish causal links on which to base mechanistic investigations and potential production of the proposed bioactive/toxic agent(s) necessary for its characterization.

Although others have attempted to establish fish bioassays in aquaria (Burkholder et al. 1992), no consensus methodology has been published. The urgent need for such a consensus is documented in the proceedings of a workshop organized by the Centers for Disease Control and Prevention (CDC), focused on this need (“Inter-Laboratory Quality Assurance of Fish Bioassays for the Microorganism *Pfiesteria pis-*

*cicida*”, National Center of Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA, 25-26, January, 2000). The need for replicates of multiple experimental conditions with parallel treatments required a small scale, higher throughput assay. Thus, we designed a bioassay format in 750-mL vented culture flasks, in which complicated variables such as the mechanical action of air pumps and effects of biofilters were avoided. The assay protocol was optimized with clonal dinoflagellate cultures, including *P. piscicida*, and the indicator of the biological activity to be measured was fish mortality. In order to characterize the assay system we monitored population dynamics of selected bacteria and protista by assessing cell types and numbers, and measured multiple water quality parameters over the course of the experiments. This information also led to the definition of those conditions in which the possible causes of fish mortality may be unrelated to the biological activity of interest. The assay was tested with environmental sediment and water samples from sites in Maryland where fish-kill events had been reported earlier, and the presence of *P. piscicida* had been confirmed by PCR (Robledo et al. 2000b). The fish mortality bioassay described here may constitute a useful tool to investigate ichthyocidal activity of any suspected aquatic microorganism or consortium, and provide opportunities for the isolation and identification of the causative agent.

## Materials and methods

**Dinoflagellate cultures:** Clonal cultures of *P. piscicida*, *K. micrum*, and *P. minimum* were gifts of Drs K. A. Steidinger (Florida Department of Environmental Protection, St. Petersburg, FL), D. K. Stoecker (Center for Environmental Studies, University of Maryland, Cambridge, MD), and D. W. Coats (Smithsonian Environmental Research Center, Edgewater, MD), respectively. Dinoflagellate cultures CC-MP1828, CCMP1829, and CCMP1934, were purchased from CCMP (West Boothbay Harbor, ME). Mixed dinoflagellate cultures were established from environmental samples collected by the Core Facility for Culture of Toxic Dinoflagellates, Center of Marine Biotechnology (University of Maryland Biotechnology Institute, Baltimore, MD). Dinoflagellate cultures were maintained in *f/2* medium (Guillard 1975), enriched with soil and chicken manure extract, under alternating periods of 14 h light and 10 h dark (white-

fluorescent;  $150 \mu\text{mol photon m}^{-2}\text{s}^{-1}$ ) at  $23^\circ\text{C}$ , and fed with *Rhodomonas* sp. (CCMP768). Cell densities were assessed by fixing culture samples in an equal volume of buffered 2.5% glutaraldehyde solution, and counting dinoflagellate zoospores in a hemacytometer.

*Experimental fish:* Initial feasibility studies used a local variety of bullhead minnows (*Pimephales vigilax*), but all subsequent experiments were carried out with approximately 60-day old sheepshead minnows (*Cyprinodon variegatus*) purchased either from Chesapeake Culture (Hayes, VA) or Aquatic Biosystems Inc. (Fort Collins, CO), and gradually acclimated to 7 ppt salinity (pH 8.0) at  $23^\circ\text{C}$  for at least two weeks prior to use.

*Collection and processing of environmental samples.* Eight independent sites in Maryland, Kings Creek and St. Peter's Creek (Manokin River), Jenkins Creek (Tangier Sound), Pocomoke River, Nanticoke River, Chicamacomico River, and Middle River, were sampled for water and sediment from June to October, 1999 (Table 1). Water samples were filtered through  $20 \mu\text{m}$  nylon filters. Sediment samples were resuspended into a slurry with artificial sea water (ASW; salinity 7 ppt, pH 8.0, aerated overnight and filter-sterilized ( $0.2 \mu\text{m}$ ); Instant Ocean, (Aquarium Systems Inc., Mentor, OH) and sequentially sieved through a coarse metal mesh and a  $60 \mu\text{m}$  nylon filter.

*Fish bioassays:* All bioassays were carried out in triplicate in upright 750-mL culture flasks fitted with vented caps (Corning Inc., Acton, MA). For experiments with clonal dinoflagellate cultures, flasks were inoculated with  $50 \text{ mL}$  of a  $9000 \pm 90 \text{ cells mL}^{-1}$  dinoflagellate clonal cultures, and ASW added to a final volume of  $500 \text{ mL}$ , for a final cell density of  $900 \pm 9 \text{ cells mL}^{-1}$ . For experiments where environmental samples were tested, either a sieved ( $20 \mu\text{m}$ ) water sample in ASW ( $250 \text{ mL}$  water sample:  $250 \text{ mL}$  ASW) or a suspension of sieved ( $60 \mu\text{m}$ ) sediment slurry in ASW ( $50 \text{ mL}$  slurry:  $450 \text{ mL}$  ASW) were placed in the culture flasks, and the pH adjusted to 8.0 by addition of  $\text{CaCO}_3$ . Control flasks contained either  $500 \text{ mL}$  ASW, autoclave-sterilized sediment slurry in ASW ( $50 \text{ mL}$ :  $450 \text{ mL}$ ), or f/2 medium in ASW ( $50 \text{ mL}$ :  $450 \text{ mL}$ ). Two fish were placed in each experimental or control flask, and maintained at  $23^\circ\text{C}$  under a 14 h light/10 h dark light cycle (white-fluorescent;  $150 \mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ). Fish were fed with Tetra Min (Tetra Sales, Blacksburg, VA), approximately 1 g flake/20 g fish every other day. The

headspace in the assay flasks was purged daily with pure oxygen for approximately 30 sec. Fish were monitored for deaths three times a day, and water samples collected every other day and processed for dinoflagellate counts, and microbial and water quality analysis. In the event both fish died, these were replaced by another set of two, which were likewise monitored.

*PCR-based detection of P. piscicida:* Environmental water and sediment samples, and experimental and control flasks were monitored for the presence of *P. piscicida* with a PCR-based detection assay (Robledo et al. 2000a). Briefly, DNA from cultured *P. piscicida* was extracted using the DNeasy 96 Tissue kit (QIAGEN, Chatworth, California) following Robledo et al. (2000b). DNA concentration and quality was estimated by optical density at 260 and 260/280 nm, respectively. Water samples from the environment ( $50 \text{ mL}$ ) and fish bioassays ( $1\text{--}2 \text{ mL}$ ) were centrifuged ( $2,000 \times g$ , 10 min), the pellet resuspended in  $100 \mu\text{L}$  of TE (Tris-EDTA buffer, pH 7.4), boiled for 10 min, clarified by centrifugation, and the supernatant ( $10 \mu\text{L}$  aliquots) used for PCR detection of *P. piscicida*.

PCR primers which amplify a 429 bp target sequence in the non-transcribed spacer (NTS) and small subunit (SSU) of the *P. piscicida* rRNA gene, were used for the detection of the dinoflagellate as reported elsewhere (Robledo et al. 2000a). Controls for the integrity of the DNA templates consisted of reaction mixtures containing "universal" actin primers (provided by Dr. G. W. Warr, Medical University of South Carolina, Charleston, SC).

*Bacteriological analyses:* Total culturable bacteria were enumerated using half-strength Marine Agar ( $18.7 \text{ g}$  Difco Marine broth 2216,  $15 \text{ g}$  Difco Bacto-agar, and  $1000 \text{ mL}$   $\text{dH}_2\text{O}$ ) as described by Alavi et al. (2001). Four selective/differential nutrient media were prepared according to the instructions of the manufacturer (Oxoid Limited, Hampshire, England) to isolate and identify potential bacterial pathogens. *Aeromonas* Medium, Desoxycholate Citrate Agar for pathogenic enteric bacteria, *Pseudomonas* Agar Base plus C-F-C selective supplement (cetrimide, fucidin, and cephaloridine), *Cholera* Medium TCBS for *Vibrio* spp. were dispensed into sterile petri dishes for subsequent inoculation.

Water samples ( $10 \text{ mL}$ ) for bacteriological analysis were collected aseptically from each flask that was sampled every 7 days during the course of the experiment. Each water sample was serially diluted with 15 ppt sterile ASW and a  $100 \mu\text{L}$  inoculum spread in

Table 1. Environmental samples tested.

| Sample    | Sampling Location                | Fish Mortality<br>A/B/C/nd | PCR for<br><i>P. piscicida</i> | pH  | Ammonia<br>(mg L <sup>-1</sup><br>N) |
|-----------|----------------------------------|----------------------------|--------------------------------|-----|--------------------------------------|
| Water:    |                                  |                            |                                |     |                                      |
|           | Kings Creek, Manokin River       | A                          | +                              | 5.9 | 9                                    |
|           | Jenkins Creek, Tangier Sound     | A                          | -                              | 5.7 | 3                                    |
|           |                                  | A                          | -                              | 9.2 | 14                                   |
|           |                                  | A                          | -                              | 6.7 | 14                                   |
|           |                                  | A                          | -                              | 8.6 | 20                                   |
|           |                                  | B                          | -                              | 7.4 | 5                                    |
|           | Pocomoke River                   | C                          | -                              | 6.3 | 2                                    |
|           | Pocomoke Sound                   | A                          | -                              | 5.6 | 2                                    |
|           |                                  | A                          | -                              | 8.8 | 8                                    |
|           | St. Peter's Creek, Manokin River | B                          | +                              | 7.6 | 14                                   |
|           | Nanticoke River                  | nd                         | -                              | 7.9 | 3                                    |
|           | Chicamacomico River              | A                          | -                              | 7.9 | 15                                   |
|           |                                  | A                          | -                              | 5.6 | 12                                   |
|           |                                  | A                          | -                              | 8.5 | 4                                    |
|           |                                  | A                          | -                              | 8.9 | 5                                    |
|           | Middle River                     | A                          | +                              | 8.7 | 17                                   |
|           |                                  | A                          | +                              | 8.9 | 10                                   |
|           | Chesapeake Bay Area (MD DNR)     | B                          | -                              | 6.2 | 4                                    |
|           |                                  | A                          | -                              | 6.2 | 17                                   |
|           |                                  | A                          | -                              | 6.1 | 8                                    |
|           |                                  | A                          | -                              | 6.4 | 5                                    |
|           |                                  | B                          | -                              | 6.0 | 8                                    |
|           |                                  | A                          | -                              | 7.8 | 7                                    |
|           |                                  | A                          | -                              | 7.8 | 12                                   |
|           |                                  | A                          | +                              | 7.8 | 6                                    |
|           |                                  | B                          | -                              | 7.6 | 3                                    |
|           |                                  | A                          | -                              | 8.5 | 14                                   |
|           |                                  | A                          | -                              | 7.9 | 14                                   |
|           |                                  | A                          | -                              | 7.9 | 5                                    |
|           |                                  | A                          | -                              | 8.0 | 3                                    |
|           |                                  | A                          | -                              | 7.4 | 3                                    |
|           |                                  | A                          | +                              | 7.1 | 3                                    |
|           |                                  | A                          | -                              | 7.0 | 2                                    |
|           |                                  | A                          | -                              | 7.1 | 2                                    |
|           |                                  | no death                   | +                              | ND  | ND                                   |
|           |                                  | no death                   | -                              | ND  | ND                                   |
| Sediment: |                                  |                            |                                |     |                                      |
|           | Kings Creek, Manokin River       | B                          | +                              | 5.1 | 15                                   |
|           |                                  | A                          | +                              | 6.2 | 10                                   |
|           | Jenkins Creek, Tangier Sound     | B                          | ND                             | 6.6 | 15                                   |
|           |                                  | A                          | ND                             | 6.5 | 9                                    |
|           | Pocomoke River                   | A                          | ND                             | 5.1 | 3                                    |
|           |                                  | A                          | ND                             | 5.9 | 10                                   |
|           |                                  | A                          | ND                             | 5.5 | 9                                    |
|           |                                  | B                          | ND                             | 6.4 | 9                                    |
|           | Pocomoke Sound                   | A                          | -                              | 6.7 | 2                                    |
|           | St. Peter's Creek, Manokin River | A                          | ND                             | 5.9 | 3                                    |
|           |                                  | A                          | ND                             | 6.5 | 11                                   |
|           |                                  | nd                         | ND                             | 5.2 | 9                                    |
|           | Nanticoke River                  | A                          | ND                             | 7.9 | 20                                   |
|           |                                  | A                          | +                              | 7.2 | 14                                   |
|           | Chicamacomico River              | B                          | ND                             | 7.8 | 6                                    |
|           |                                  | A                          | ND                             | 6.0 | 20                                   |
|           | Fish Farm, Manokin River         | B                          | +                              | 8.0 | 13                                   |

ND: not determined

MD DNR: Maryland department of natural resources

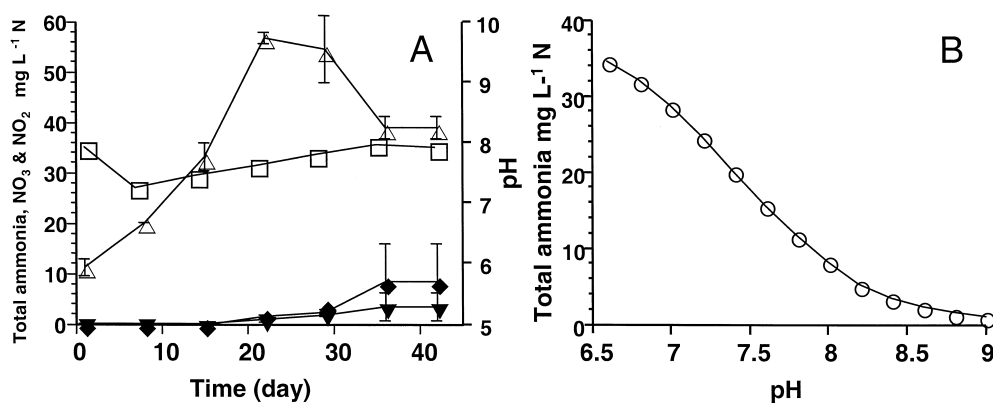


Figure 1. Changes in water quality in culture flasks with unexposed fish during a 6-week period. Panel A: Ammonia ( $\Delta$ ), nitrate ( $\blacktriangledown$ ), nitrite ( $\blacklozenge$ ), and pH ( $\square$ ) in 500 mL ASW with 2 fish in a 750-mL culture flask. Panel B: Acute toxicity of total ammonia concentration as a function of pH [Adapted from “Acute Criteria for Total Ammonia: General Warm-Water Fishery”, from a publication (Clean Water Commission, Department of Natural Resources (2001); <http://mosl.sos.state.mo.us/csr/10csr/10c20-7b.pdf>). Acute toxicity is defined as “conditions producing adverse effects or lethality on aquatic life following short-term exposure”].

duplicate on each of the five bacteriological media. The bacteria were incubated at 35 °C for 24 h. Following incubation, colony forming units (CFUs) mL<sup>-1</sup> of sample were determined. Bacteria identification was based on colony growth on selective media as well as coloration following identification by established methods (<http://www.cfsan.fda.gov/~ebam/bam-mm.html>).

**Enumeration of protozoa and diatoms:** Water samples (1 mL) for enumeration of protozoa and diatoms were collected from each flask every other day, and cell densities were assessed by fixation with Lugol’s solution (Lányi 1987), and counting under the light microscope.

**Water quality analyses:** Concentrations of ammonia, nitrate, and nitrite in experimental flasks were measured by HACH colorimetric assay kits following the manufacturer’s instructions (HACH Co., Loveland, CO). In a separate experiment, dissolved oxygen levels were monitored for 28 days in a single tissue culture flask containing 500 mL sterile ASW and two sheepshead minnows where the headspace was routinely purged with oxygen as described above. Oxygen concentrations were recorded continuously using a Sable Systems ReadOx-4H oxygen analyzer (Sable Systems, Henderson NV) with an oxygen microelectrode that did not require stirring (Model E101, Cameron Instrument Co., Port Aransas TX). Oxygen data were acquired and analyzed using a Sable Systems DATASCAN V software package (Sable Systems, Henderson NV). All other experimental conditions were the same as described for the previous experiments.

## Results

### *Development and characterization of the fish bioassay*

#### *Fish maintenance in flasks*

Prior to exposing fish to cultured dinoflagellates or environmental samples, we characterized the system by maintaining fish in 750-mL flasks either in ASW (500 mL) or ASW (450 mL) with *f/2* medium (50 mL), and assessing changes in water quality [pH, total ammonia, nitrite, and nitrate] throughout the experiment (Figure 1A). Ammonia concentrations increased gradually to peak in the fourth week, followed by a steady decline. Both nitrate and nitrite reached detectable levels of 0.5–1 mg L<sup>-1</sup> N in the third week, rising to approximately 8 mg L<sup>-1</sup> N by week six. Fish deaths were observed at these high concentrations of nitrogen species, although at much higher ammonia levels than those compatible with fish survival, as indicated by water quality guidelines (Figure 1B).

#### *Proliferation of clonal dinoflagellates in presence or absence of fish*

Upright flasks were inoculated with clonal cultures of *P. piscicida*, *P. minimum*, and *K. micrum*, and incubated either with or without fish. Proliferation of dinoflagellates was assessed by triplicate counts every other day. Proliferation of *P. piscicida* and CC-MP1834, a recent *P. piscicida* isolate, was reproducibly enhanced in the presence of the fish (Figure 2A). Cell counts revealed a peak in cell density from day

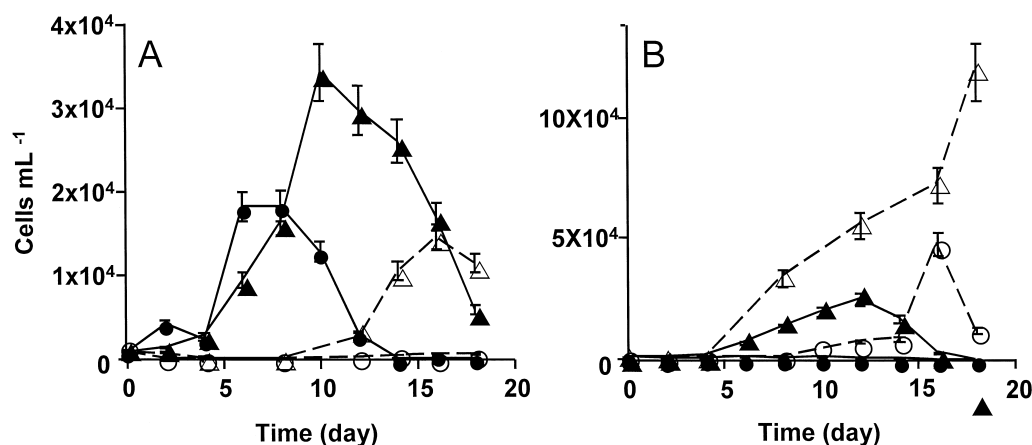


Figure 2. Effects of the presence of fish on dinoflagellate proliferation profiles. Clonal cultures of *P. piscicida*, CCMP1834, *K. micrum*, and *P. minimum* were incubated in ASW in the presence or absence of fish. Panel A: *P. piscicida* (circles) and CCMP 1834 (triangles) with fish (●, ▲) and without fish (○, △). Panel B: *K. micrum* (circles) and *P. minimum* (triangles) with fish (●, ▲) and without fish (○, △).

8 to 10 followed by a rapid decline. For CCMP1834, however, a smaller peak centered at about 16 days was apparent in the absence of fish. As independent isolates of the same species, it is noteworthy that there is greater variability in cell densities obtained in this system with the more recently established isolate CCMP1834 in the presence of fish. Furthermore, in the absence of fish *P. piscicida* growth profiles showed a broader peak of lower density (up to 500 cells mL<sup>-1</sup>), also centered around 16 days.

A culture consisting primarily of *Cryptoperidiniopsis* sp., CCMP1828 which contains *P. piscicida*, showed a cell growth profile that remains unaffected by the presence of fish, although a modest increase in cell density appeared to coincide with that of *P. piscicida* (data not shown). In contrast, both *K. micrum* and *P. minimum*, grew at higher cell densities in the absence of fish (Figure 2B).

#### Reproducibility of *P. piscicida* growth profiles

It was important to establish whether *P. piscicida* cultures in the presence of fish conserved the same growth characteristics from flask to flask in order to make valid experimental comparisons. Proliferation of *P. piscicida* from clonal cultures, environmental water and sediment revealed reproducible profiles (Figure 3). Dinoflagellate zoospore densities in flasks containing clonal culture (Figure 3A) and environmental water samples (Figure 3B) peaked around day 6, at about  $2 \times 10^4$  cells mL<sup>-1</sup>. In contrast, the emergence and proliferation of zoospores from sediment samples, while reproducible, follows a profile different from that observed with clonal culture and water

samples, with cell density peaks at day 8 (approx.  $4 \times 10^2$  cells mL<sup>-1</sup>) and 16 (approximately  $1.4 \times 10^3$  cells mL<sup>-1</sup>) (Figure 3C).

#### Assessment of microbial populations introduced by the experimental fish in the bioassay

Because it was predictable that in addition to dinoflagellates, the environmental water and sediment samples under examination would introduce into the bioassay system a diverse array of microorganisms of which one or more components could be responsible for the ichthyocidal effects observed, it was important to assess the potential contribution of the experimental fish to this complex microbial consortium. Accordingly, in selected experimental and control flasks we examined the composition and progression of the microbial flora throughout the experiment. Water samples from experimental flasks in which fish were exposed to either clonal dinoflagellate cultures (*P. piscicida*, *G. galatheanum*, *P. minimum*, CCMP1828, CCMP1829, CCMP1834), or held (unexposed) in a culture medium/ASW mixture (50 mL f/2: 450 mL ASW) were analyzed. Control flasks contained either the above dinoflagellate cultures or ASW, in the absence of fish. Results of the enumeration of dinoflagellates, protozoa and bacteria in flasks containing either fish exposed to *P. piscicida* or unexposed fish, during a 4-week experiment, are illustrated in Figure 4. Results for all flasks examined are described below.

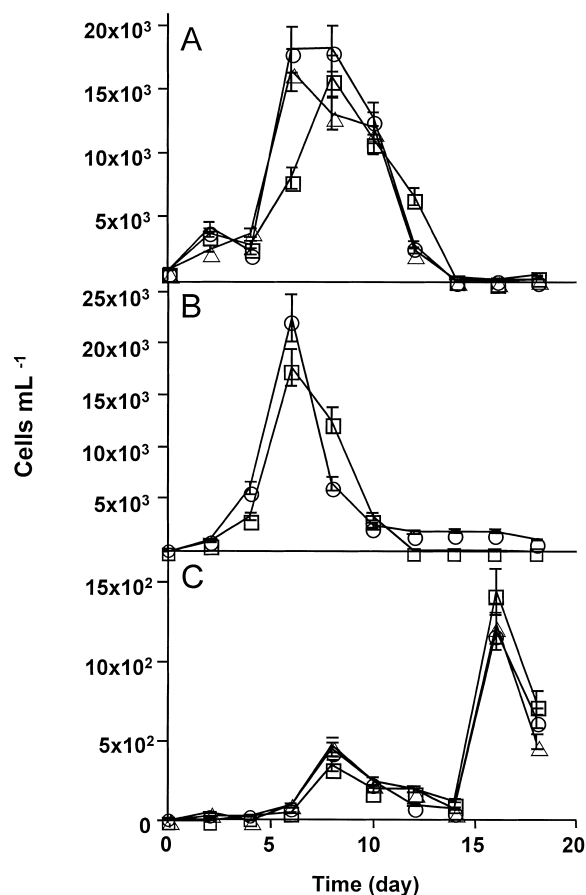


Figure 3. Reproducibility of dinoflagellate proliferation profiles. Panel A, *P. piscicida* clonal culture; Panel B, environmental water samples; Panel C, environmental sediment samples.

#### Bacteria

In most flasks examined, the initial total culturable bacteria density began on day 1 at  $10^5$  CFU mL<sup>-1</sup>, increased to  $10^7$  CFU mL<sup>-1</sup> between days 7 and 21, and declined to ca.  $10^5$  CFU mL<sup>-1</sup> thereafter. Between 6 and 12 readily distinguishable colony types were found initially, while overall species diversity decreased slightly over the course of the experiment. No pathogenic *Pseudomonas* spp. were found in flasks with or without fish. However, the selective medium used to detect *Pseudomonas* spp. is specific for *Burkholderia cepacia*, *P. aeruginosa*, *P. putida* and *P. fluorescens*, and is unlikely to permit the growth of most other aquatic or marine *Pseudomonas* spp. By contrast, *Vibrio* spp. increased from densities of  $< 10^1$  CFU mL<sup>-1</sup> to between  $10^4$  to  $10^5$  CFU mL<sup>-1</sup> by day 28. Although a rigorous taxonomic identification was not attempted, *V. parahaemolyticus*, *V. alginolyticus* and *V. fluvialis* may have been present based

on colony morphology when grown on *Cholera* Medium TCBS. *Aeromonas* spp., as enumerated with Oxoid *Aeromonas* medium, were ubiquitous at high densities from the initial hour of inoculation ( $10^3$  to  $10^4$  CFU mL<sup>-1</sup>), possibly by introduction from a "point" source, and generally increased in population with time. Surprisingly, coliform bacteria (identified as lactose-positive colonies on Desoxycholate Citrate Agar) were abundant in all flasks containing fish tested by day 14 (or earlier). Both observations suggest either the fish or the fish fecal waste as the most probable source.

#### Protozoa and diatoms

In flasks where fish were exposed to *P. piscicida*, protozoa and diatoms (undetermined species) were counted and compared to *P. piscicida* numbers, in the context of the water quality parameters, pH and ammonia, nitrate and nitrite concentrations. In general, the peak cell densities of protozoa were observed around days 5–15, that is between the *P. piscicida* cell density peak and the general elevation of the nitrogen species examined (Figures 4 and 5). In flasks where no dinoflagellates had been inoculated, cell density profiles of protozoa were similar to those in flasks with exposed fish. Although the ciliate and diatom populations were not differentially counted, these were readily observable throughout the course of the experiments. Ciliates, diatoms and protozoa were absent in those flasks where no fish were added to the dinoflagellate clonal cultures.

#### Water quality (Nitrogen species and pH)

Issues of water quality during prolonged maintenance of fish in a small volume, closed system, impose limitations on the time during which fish deaths may be attributed to dinoflagellate activity. The accumulations of metabolic products in flasks where fish are held unexposed to dinoflagellates (Figure 1), as well as enumeration of bacteria and other microorganisms (Figure 4), establish that even this relatively simple system can be quite complex. Accordingly, it was important to determine whether the interaction of an additional system component, the dinoflagellate culture, may alter metabolite production by the fish. There was no apparent impact of the presence of clonal *P. piscicida* on the generation of nitrogen species by fish, but rather a steady decline of dinoflagellate cell numbers when the concentration of ammonia in the flasks rose above 5–10 mg L<sup>-1</sup> N, with pH around 8 (Figure 5). Flasks with environmental water and sedi-



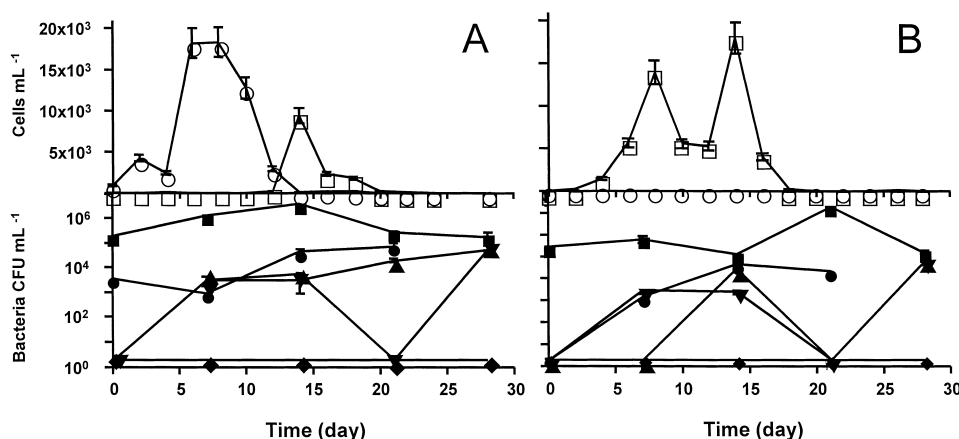


Figure 4. Microbial populations in bioassay flasks. Panels A: fish exposed to clonal *P. piscicida*. Panels B: unexposed fish (control). Top panels: cell density of dinoflagellates (○) and protozoa (□). Bottom panels: total culturable bacteria (■), *Aeromonas* spp. (●), *Vibrio* spp. (▲), *Pseudomonas* spp. (◆), and enteric bacteria (▼).

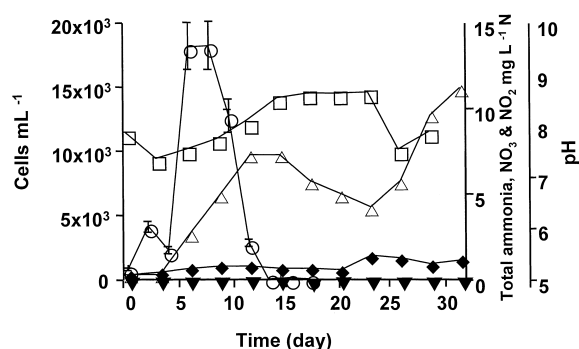


Figure 5. Changes in water quality in bioassay flasks with during a 4-week period. Ammonia (▲), nitrate (▼), nitrite (◆), and pH (□) for a bioassay flask containing fish exposed to proliferating *P. piscicida* (○).

ment samples, which contained complex bacterial communities, attained lower ammonia concentrations relative to those in the control and clonal dinoflagellate culture flasks (data not shown). Differences in water chemistry were observed in flasks within 12 hours of a fish death, compared to parallel flasks in which no fish death had occurred (Figure 6). The observed trend in these flasks was for total ammonia (ionized and un-ionized) to increase, while both nitrite and nitrate decreased, with a clear increase of the toxic un-ionized ammonia.

#### Dissolved oxygen

Except for brief periods immediately following the daily purging of the flask headspace with pure oxygen, dissolved oxygen concentration remained at or below  $1.5 \text{ mg L}^{-1}$  for the entire 28 days of the experiment (Figure 7). During this time, fish showed no

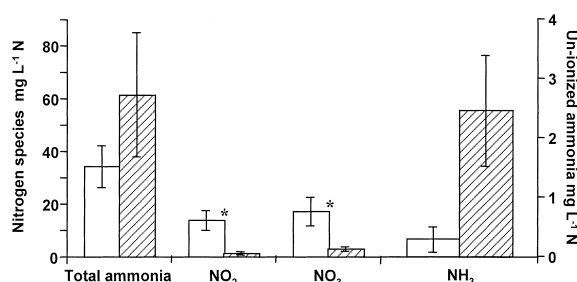


Figure 6. Changes in water chemistry upon fish death. From left to right, nitrogen species of total ammonia, nitrite and nitrate in replicate control flasks (fish unexposed to dinoflagellates) with (striped bars) and without (open bars) fish death that represent one standard deviation ( $n=3$ ). Total ammonia and un-ionized ammonia in replicate control flasks with (striped bar) and without (open bar) fish death that values represent average and range ( $n=2$ ).

obvious signs of disease or stress, but were occasionally found at the water surface. Fish continued to feed throughout the experiment.

#### Summary of the fish bioassay

Proliferation of 11 clonal dinoflagellates cultures inoculated to fish bioassay flask and observed fish death were shown in Figure 8. In order to facilitate the interpretation of bioassay results, we have divided the bioassay period into three stages: “stage A” (first 10 days), “stage B” (10–20 days), and “stage C” (beyond 20 days). The definition of the initial 10-day period (Stage A) is critical to the experiment, because it is sufficiently brief that fish deaths which occur in this time period may not be attributable to pathogenic bacteria which colonize the flask (Figure 4). Likewise, in most cases the accumulation of potentially toxic me-

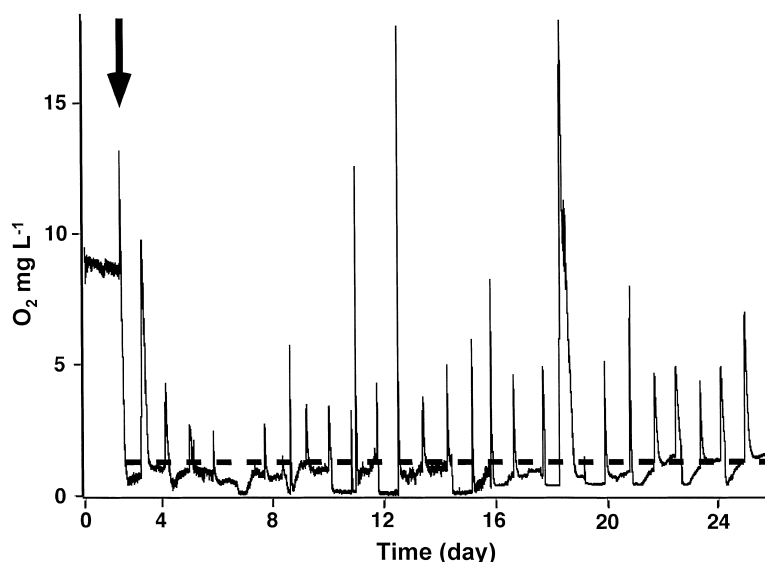


Figure 7. Dissolved oxygen levels in bioassay flask. Culture flasks with vented cap containing 500 mL of ASW and 2 sheepshead minnows (2.0–3.5 cm, *C. variegatus*). On days 12 and 18, oxygen was administered for 10 min, to verify the calibration of the measurements. Arrow represents addition of fish. Dashed line represents average oxygen concentration after addition of fish [ $1.2 (1.7 \text{ mg L}^{-1} \text{ (SD); } n = 58,309]$ ].

tabolites will not reach levels high enough to contribute to fish death during this first period (Figures 1 and 5), although ammonia concentrations and pH changes should be closely monitored throughout the experiment, including this initial stage. Further justification for the close monitoring of water chemistry comes from the observation that water quality rapidly changes upon fish death (Figure 6).

The apparent association of *P. piscicida* cell densities and fish deaths is noteworthy. A rapid proliferation of *P. piscicida* reaching a density of  $4 \times 10^3$  cells  $\text{mL}^{-1}$  in the experiment shown in Figure 8a, was concurrent with a fish death. In Figures 8b, 8c, and 8d, showing clonal *P. piscicida* growth curves in flasks where no fish deaths were observed, differences in proliferation profile (shape, time of the peak, and maximal cell densities) with that of Figure 8a, suggests that the “bloom intensity” could be associated with fish deaths. However, this conclusion must be approached with caution, in light of additional results shown in Figure 8e. This shows a fish death at a similar early time point, which is not associated with a rapid rise in cell numbers to such a high threshold, although there are indications of proliferation beginning at that time, followed by a peak of around  $4 \times 10^4$  cells  $\text{mL}^{-1}$  which is closely associated with a second “stage A” fish death in this flask. Similarly, Figure 8f shows an example of an experiment in which a fish death occurs, but the peak comes slightly

after that shown in panel (a). The latter panels, (e) and (f), reveal higher cell densities than those in panels (b), (c), and (d), suggestive of a threshold phenomenon for lethality. Similar studies with the more recent *P. piscicida* isolate, CCMP1834, which shows slower proliferation and greater variability in growth profiles than the 1997 isolate Figure 8a, 8b, 8c, 8d, 8e, 8f, are shown in Figure 8g, 8h and 8i. Although occurring later, the cell density peak is relatively “sharp” and shaped more like those seen in panels a, e, and f, and closely associated with fish mortality (although at the end of stage A), whereas in the other instances there is either no death or a stage C death due most likely to deteriorated water quality. Interestingly, CCMP1828, a mixed culture of *Cryptoperidiniopsis* spp. and *P. piscicida*, shows a small rise in cell counts on day 5 (Figure 8j and 8k), followed by a major peak occurring between days 16 (panel k) and 18 (panel j). Adding further to the circumstantial evidence for threshold and intensity requirements to obtain fish mortalities measured in this assay, no fish deaths were observed in these flasks. Thus, our preliminary characterization of the flask assay format using clonal cultures of *P. piscicida* indicate an early peak of dinoflagellate proliferation sometimes associated with a fish death, suggesting that the *P. piscicida* high cell numbers must be a major contributor to fish death. Although this may be a reasonable working hypothesis, the observation that in some flasks where

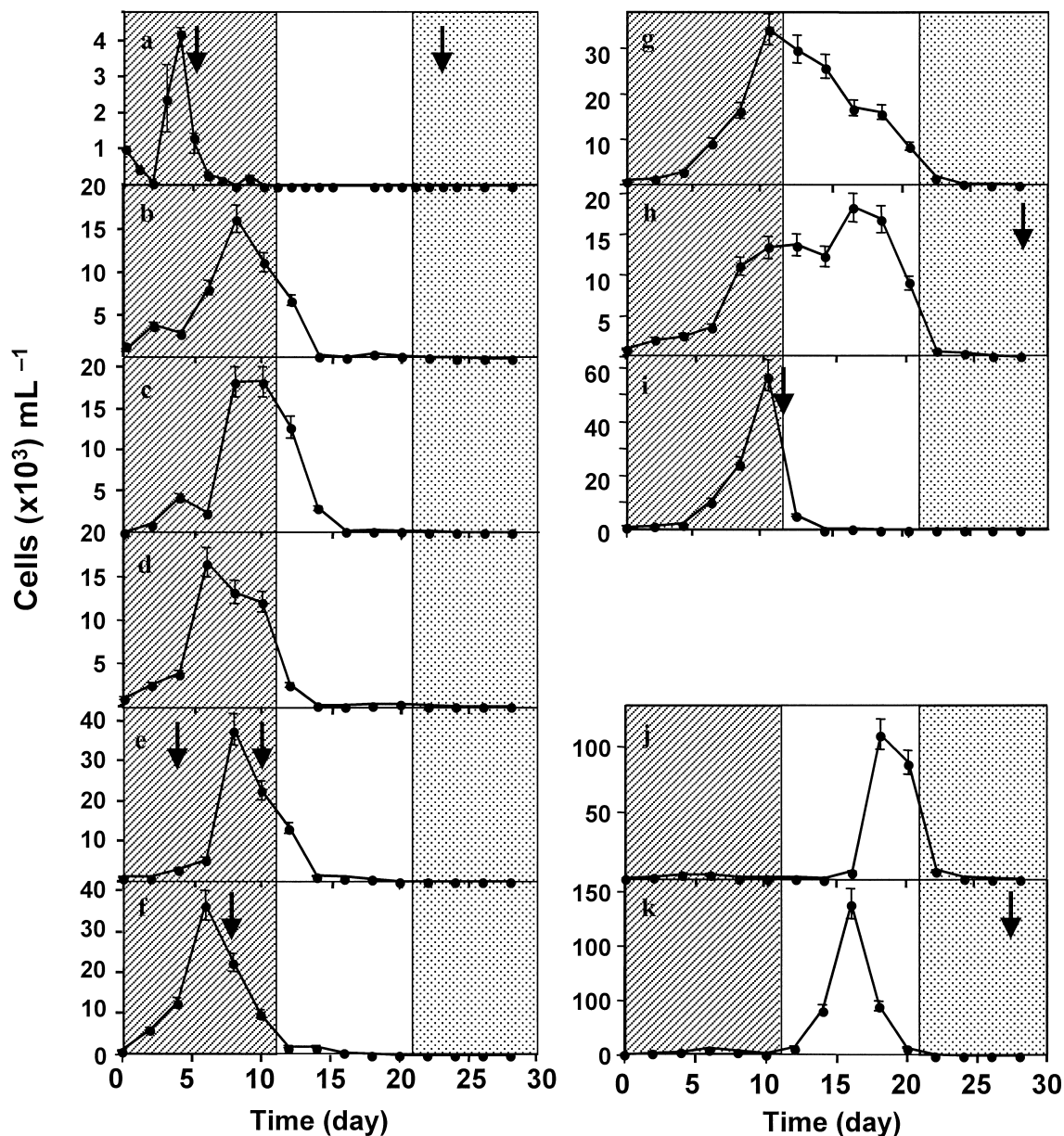


Figure 8. Definition of stages A, B, and C for *P. piscicida* bioassay: Proliferation profiles of *P. piscicida* (a-f), CCMP1834 (g-i), and CCMP1828 (j,k) in bioassay culture flask format. Fish death is indicated by an arrow. For each panel, stage A (0–10 d; striped area), stage B (10–20 d; open area), and stage C (20–30 d; dotted area).

fish appear unaffected during active proliferation of *P. piscicida*, albeit with distinguishable growth profiles, indicates that further work is needed to elucidate the apparent association between *P. piscicida* high cell numbers and fish death.

#### Implementation of the bioassay for water and sediment samples

Upon development and characterization of the flask bioassay system by exposing fish to clonal dinoflagellate cultures, we tested its application to water and sediment environmental samples collected in Chesapeake Bay tributaries, where fish kills attributed to *P.*

*piscicida* took place in the late summer of 1997. The sites of sample collection are indicated in Table 1, together with the results from their testing by the flask bioassay, classified by stage of fish mortality. This information is accompanied by data from PCR-based detection for *P. piscicida*, pH and ammonia measurements at the time of the initial fish death (or at the conclusion of the experiment in the case no fish death was observed). A comparative analysis of fish mortality by *P. piscicida* clonal cultures and environmental samples is presented graphically in Figure 9.

Testing ichthyocidal activity of environmental samples using the bioassay confirmed that *P. piscicida*-PCR positive samples resulted in acute fish deaths (Stage A) for both sets of fish added. Nevertheless, an equal or greater proportion of the environmental samples, which tested negative for the presence of *P. piscicida* by PCR, also yielded acute fish deaths in the bioassay stage A. This fact can be appreciated by comparison of the inner circle stage A section of the pie chart in panels II and III (environmental water and sediment, respectively, Figure 9) with the outer ring stage A. Both of these sectors exceed stage A % mortality measured in clonal *P. piscicida* (panel I, Figure 9), for which case there is by definition no PCR-negative instance. Although the cause of death in PCR-negative environmental samples remains to be determined, *Pfiesteria*-like dinoflagellates were observed in these samples when examined under the microscope. It is noteworthy, that no skin lesions were observed during the course of the bioassay in any of the fish exposed either to the clonal dinoflagellate cultures or the environmental samples.

## Discussion

The design and detailed characterization of a culture flask bioassay was motivated by the need for a rapid and relatively simple method for assessing the potential ichthyocidal activity of *P. piscicida*. This was the assay format of choice, because it would emphasize simplicity, small scale and high throughput. The first aspect examined was the reproducibility of dinoflagellate proliferation profiles in the assay format selected. Clonal dinoflagellate cultures, including well-established *P. piscicida*, exhibited reproducible growth curves in the culture flasks. The fact that *P. piscicida* is abundant in estuarine/marine environments has resulted in repeated recovery of fresh isolates. In our assay system, *P. piscicida* cultures from

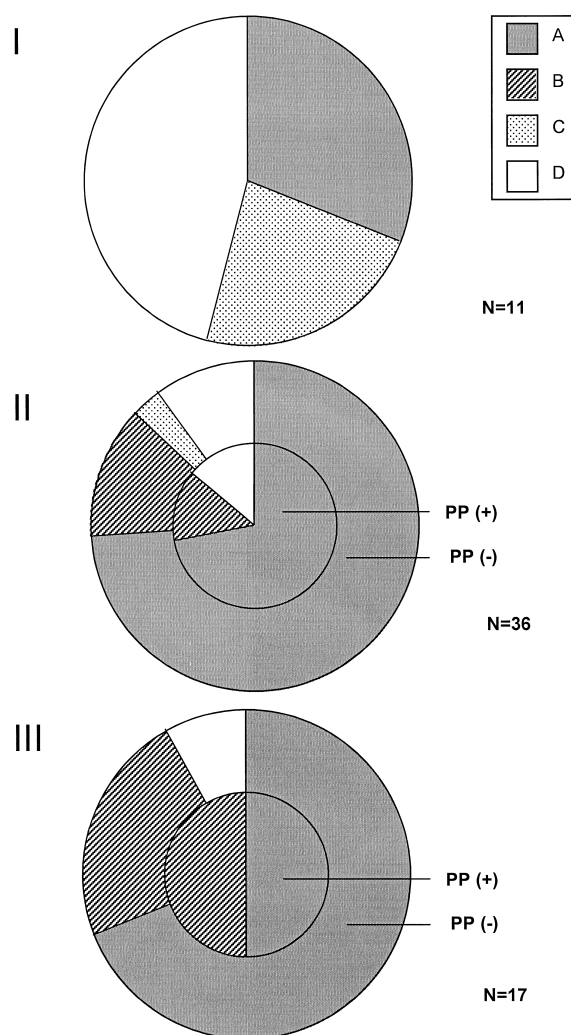


Figure 9. Implementation of *P. piscicida* bioassay to environmental samples: Panel A (solid area): fish death recorded in bioassay stage A; Panel B (striped area): fish death recorded in bioassay stage B; Panel C (dotted area): fish death recorded in bioassay stage C; Panel D (open area): no fish death recorded. Top chart (I): Clonal *P. piscicida* culture; middle chart (II): environmental water samples; bottom chart (III): environmental sediment samples. On middle and bottom charts, the inner circle indicates samples PCR-positive for *P. piscicida* and the outer circle indicates samples PCR-negative for *P. piscicida*.

these fresh environmental isolates showed detectable differences in proliferation curves, with the most recent isolates displaying greater variability, and a slightly longer lag overall prior to log phase growth. This highlights the sensitivity of this bioassay. It is also apparent from the data shown here that there exists a cell density threshold associated with ichthyocidal activity. Clearly, we see several instances of a

rapid rise in *P. piscicida* to high cell densities associated closely in time with fish death. It is these events on which we currently focus mechanistic investigations. The presence of fish selectively modulated the proliferation of some dinoflagellate species, with a growth enhancing effect on *P. piscicida* and *Cryptoperidiniopsis* sp. The fish-derived factor(s) that stimulate or inhibit growth of dinoflagellates in flasks have not been identified yet, but their effects on dinoflagellate proliferation were also highly reproducible in this assay format.

Once the dinoflagellate growth profiles in the flask format had been characterized, it was critical to assess those water quality parameters, such as dissolved oxygen, ammonia, nitrate and nitrite levels, and pH, that are known to impact fish health (Wedemeyer 1996). Although dissolved oxygen was not measured in all experimental flasks, results in a pilot experiment over eight weeks suggests that the daily purging with oxygen of the flask headspace (approximately 250 mL) provides sufficient oxygen for fish survival. Thus, attention was focused on pH and concentrations of ammonia, nitrate and nitrite, nitrogen species that are expected to accumulate during normal fish metabolism in a closed assay system. Although the pH varied from flask to flask during the experiments, at the value ASW was initially buffered (pH 8.0), the level of ammonia required for acute toxicity would be approximately 8 mg L<sup>-1</sup> N. To our surprise, fish survived in this system well past this limit, into the range of ammonia 60 mg L<sup>-1</sup> N, perhaps due to a slow acclimatization to the ammonia. Once the initial set of fish perished, however, the death of the replacements was very rapid, usually in a few hours, suggesting that the initial set of fish present from the outset had acclimated during the course of the experiment to high ammonia concentrations that are toxic to naïve fish. The toxic effects of nitrogen metabolites have been reported for several fish species (Miller et al. 1990). Their effects vary with salinity and ionic state, with nitrite constituting the greatest health threat. The equilibrium between ionized ammonia (non-toxic) and un-ionized ammonia (toxic) is governed by the relationship % un-ionized ammonia = 100/[1 + anti-log (pK<sub>a</sub> - pH)], where the pK<sub>a</sub> is temperature-dependent (Wedemeyer 1996). Interconversion by sequential oxidation of un-ionized ammonia to nitrate through the intermediate nitrite occurs in free equilibrium, leading to methemoglobinemia (brown blood disease) when nitrite acts on hemoglobin (Wedemeyer 1996). Nitrifying bacteria, such as those present in

aquarium biofilters, will metabolize some of the nitrogen species produced, contributing to a healthy environment in large-scale bioassays, such as the aquarium tank format. In order to simplify the design, however, we did not attempt biofiltration in the flask format.

One important observation to come out of this study, which should be considered in any experimental format aimed at examining bioactive effects of phytoplankton on fish, is the fact that in addition to normal metabolites, fish introduce their own biotic microenvironment in the flasks. The presence of ciliates, diatoms, protozoa, and bacteria, such as *Vibrio*, *Aeromonas*, and coliforms, in flasks containing fish, which are not detectable in the dinoflagellate clonal cultures (Alavi et al. 2001), supports this conclusion. Although this may not be surprising or novel, it was critical to document this phenomenon in detail in order to define the conditions necessary for a valid bioassay, because in a closed system some of the microbial species identified may reach numbers potentially harmful to fish. The exogenously introduced microbial flora showed distinct profiles from flask to flask. We did not observe uniformity even while using fish from the same stock, as evidenced, for example, by substantial variability of the bacterial populations between the flasks. Because these did not appear to constitute a significant threat to fish health until the second week, we set a conservative 10 day cutoff to consider fish death as possibly due to *P. piscicida* or other dinoflagellates activity when associated with a cell density peak occurring during that time period. Proliferation of diatoms or protozoa appeared to lag behind that of *P. piscicida* or *Pfiesteria*-like dinoflagellates in the flask assay format. Ciliates have been reported as proliferating following a dinoflagellate bloom and decline in a profile that suggests grazing on dinoflagellates (Stoecker et al. 2000). The sieving procedures for sample preparation we implemented were designed to eliminate ciliates and other organisms larger than the dinoflagellates of interest, and avoid predation. Despite these measures, a variable number of ciliates were present in the flasks in which fish were added, but not in those flasks where no fish was added to the clonal dinoflagellate cultures. This observation further suggests that the experimental fish may introduce an array of microbial species, including ciliates, in the flask environment. Formalin-treatment of the experimental fish (Speare and MacNair 1996), a possible alternative for circumventing the problem, has several drawbacks, includ-

ing (1) additional stress to the fish which would constitute an additional burden to the immune system, and for which the overall impact on fish health would be difficult to account; (2) the intrinsic variability of the treatment which would be difficult to measure reliably; and (3) the likelihood that the treatment may be effective for the external microbial community associated with fish skin and mucous, but not for the gut microbial flora. Under the assumption that bacteria and other microflora will colonize the flasks regardless of the treatment, we chose to measure and classify the microbial populations rather than attempt to eliminate them. Potentially pathogenic bacterial populations, for example, rose from very low but immediately detectable numbers present at the introduction of all components into the flask, to significantly high densities at about day 10, coincident with the rise in nitrogen species to threshold toxic values.

In summary, our studies clearly indicate that although the flask bioassay may constitute the simplest format to assess fish mortality related to harmful dinoflagellates, it is still a complex and dynamic system. Nevertheless, the development and characterization of the flask bioassay has permitted the following observations: (a) the presence of fish stimulates proliferation of *P. piscicida* but this is not a general phenomenon; (b) the levels of ammonia, nitrate and nitrite fluctuate but gradually increase to concentrations beyond limits for fish survival at approximately 21 days; (c) the experimental fish may carry a diverse microbial flora, including some recognized fish pathogens, protozoa, and dinoflagellates, which actively proliferate in the flasks. From these observations we have empirically determined the operational parameters under which a fish death may be attributable to factors associated with a peak in dinoflagellate proliferation.

Based on these observations, we defined three assay stages that are relevant to the interpretation of the data. The critical threshold of the first 10 days constitutes "stage A", during which dinoflagellate proliferation may be the cause of fish death. This is followed by the intermediate "stage B", from 10 to 20 days, during which the combined impact of rising nitrogen species and proliferation of bacteria and protozoa render a fish death uninterpretable for our purposes. Finally, a fish death past 20 days, "stage C", would be most likely caused by accumulation of toxic nitrogen species (Figures 8 and 9).

Although the fish deaths were not reproducible for all *P. piscicida* isolates (Figure 8), when these take

place in less than 10 days and are synchronous with the dinoflagellate proliferation peak, then a preliminary causative connection may be established and further investigated. The advantages of the system are that many samples may be screened simultaneously to maximize sample throughput. To our knowledge, we have described here a flask format bioassay for *P. piscicida* to one of the higher degrees of detail than we are able to find published elsewhere in the literature, and should be widely applicable for the identification and further investigation of aquatic microorganisms that exhibit ichthyocidal activity.

The implementation of the bioassay using environmental samples showed a higher propensity for acute fish deaths (Stage A) in *P. piscicida*-PCR positive samples. However, interpretation of this observation may be obscured by the fact that an equal or greater proportion of the environmental samples which tested negative for the presence of *P. piscicida* by PCR also yielded acute fish deaths, for which no particular cause has been identified. However, these samples mostly contain *Pfiesteria*-like organisms, and the highly specific PCR assay we implemented will detect only *P. piscicida* (Robledo et al. 2000b). Therefore, we cannot rule out the possibility that the high ratio of acute fish deaths observed in the environmental samples may be due to other *Pfiesteria* species which have been described as ichthyocidal, such as the recently reported *P. shumwayae* (Glasgow et al. 2000). Although this may limit the interpretation of the results for complex environmental samples, it would broaden the applicability of the bioassay.

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