



## Relative contribution of exotoxin and micropredation to ichthyotoxicity of two strains of *Pfiesteria shumwayae* (Dinophyceae)

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### Abstract

The mechanism by which *Pfiesteria shumwayae* (Glasgow and Burkholder) kills fish is controversial. Several studies have implicated a *Pfiesteria*-associated exotoxin in fish mortality while other studies indicate that physical attack of dinoflagellates on fish (micropredation) and not exotoxin is responsible. We examined the ichthyotoxicity of two strains of *P. shumwayae* (CAAE 101272 and CCMP 2089) in a bioassay system that exposed test fish to the dinoflagellates both with and without direct contact in the same aquarium at the same time. Dinoflagellate-free supernatants from both strains were also tested for toxicity. The results showed that direct contact between *P. shumwayae* and fish significantly enhanced fish mortality with both strains ( $P < 0.001$ ). About 87.5% and 100% of fish died when exposed directly to CAAE 101272 and CCMP 2089, respectively. When protected from direct contact with *Pfiesteria* cells, 19% of the fish exposed to CAAE 101272 and 6% of those exposed to CCMP 2089 died. No deaths were observed in controls. Supernatant killed fish when obtained from cultures of CAAE 101272 but not when obtained from CCMP 2089.

Analysis of variance showed that, for both strains, fish mortality in *Pfiesteria*-inoculated bioassays was significantly higher than control bioassays both with and without direct contact ( $P < 0.001$ ). Differences between strains were not significant ( $P = 0.3$ ). These results indicate that both strains are associated with exotoxin production. However, the dominant and most consistent mechanism of fish mortality observed in this study required physical contact between fish and *Pfiesteria* cells.

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**Keywords:** CCMP 2089; CAAE 101272; Exotoxin; *Pfiesteria shumwayae*; Toxicity

**Abbreviations:** CCMP, Center for Culture of Marine Phytoplankton; CAAE, Center for Applied Aquatic Ecology; BSL3, biosafety level 3; SFB, standardized fish bioassay; PC, protective container; TPC, toxic pfiesteria complex

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### 1. Introduction

The toxic *Pfiesteria* complex (TPC; thus far including *Pfiesteria piscicida* and *P. shumwayae*) consists of estuarine dinoflagellates that have been associated with production of toxins that kill fish and are implicated in human illness (Steidinger et al.,

1996; Burkholder et al., 1992, 2001a; Glasgow, 2000; Glasgow et al., 1995, 2001a; Grattan et al., 1998). Fish deaths attributed to toxic *Pfiesteria* outbreaks in natural waters have occurred extensively in North Carolina's Albemarle-Pamlico estuarine system (Burkholder et al., 1995). Occasional fish mortality and identification of TPC organisms in samples have occurred in tributaries of the Eastern Shore of Chesapeake Bay (Lewitus et al., 1995; Burkholder et al., 1999). Potentially toxic *Pfiesteria* spp. are apparently cosmopolitan having also been found in waters from New York through the Gulf Coast as well as in northern European and New Zealand waters (Ruble et al., 1999; Glasgow et al., 2001b; Jakobsen et al., 2002; Rhodes et al., 2002).

Like many so-called "toxic algae" (Gentien and Arzul, 1990; Anderson, 1991; Bates et al., 1998; Edvardsen and Paasche, 1998; Twiner et al., 2004), *Pfiesteria* spp. include both toxic and apparently benign (non-inducible) strains (Burkholder et al., 2001b). Some toxic *Pfiesteria* spp. have maintained ichthyotoxic activity for several years in the laboratory while others have been reported to lose toxicity after >6–8 months when grown with live fish, and more rapidly (weeks) when cultured with algal prey in the absence of live fish (Burkholder et al., 2001a).

A water-soluble toxin from *Pfiesteria*-containing cultures has been isolated and partially purified (Burkholder et al., 2001b; Glasgow et al., 2001b; Burkholder and Glasgow, 2001; Moeller et al., 2001). The activity and a mode of action for this bioactive substance have been examined using a reporter gene assay (Fairey et al., 1999; Kimm-Brinson et al., 2001; Melo et al., 2001). Partial structural information has been obtained on this compound but the complete structure is still unsolved (Moeller et al., 2003). This *Pfiesteria*-associated toxin has been demonstrated to be present in extracts from *P. piscicida* and *P. shumwayae* strains, including CCMP 2089 (Burkholder et al., 2002), a strain of *P. shumwayae* that has been reported to be non-toxicogenic (Vogelbein et al., 2002; Berry et al., 2002). Toxic components contained in filtrates derived from *Pfiesteria*-containing cultures in our laboratory (Gordon et al., 2002) display some apparently different characteristics from the compounds isolated by Moeller et al. (2001, 2003). Notably, stability of the toxic activities appears to differ. Thus *Pfiesteria* may be associated with

production of multiple toxic compounds or a parent compound may be transformed into more stable toxic derivatives. Toxins isolated by both laboratories are similar in that they are ichthyotoxic, bind to hydrophobic chromatographic resins (e.g. C18), and inhibit GH<sub>4</sub>C1 rat pituitary cells in culture (Gordon and Gordon, unpublished; Kimm-Brinson et al., 2001).

A criticism of the standardized fish bioassay (SFB; Burkholder et al., 2001c) utilized for evaluation of the toxicity of *Pfiesteria* isolates is that, since these systems are complex and contain many microorganisms in addition to *Pfiesteria*, they do not demonstrate that *Pfiesteria* is responsible for production of toxins that may be observed. While it is true that fish-containing aquaria used in SFB harbor a veritable microbial zoo, the control tanks contain fish at the same density as the *Pfiesteria*-inoculated tanks and have total bacterial counts that do not significantly differ from experimental tanks (Marshall et al., 2000). While total bacterial numbers do not differ significantly in experimental and control SFB, denaturing gradient gel electrophoresis (DGGE) analyses indicate that microbial communities vary between fish-containing bioassay aquaria that have been maintained under apparently identical conditions and that certain phylogenetic groups of bacteria appear to be common to fish-killing bioassay aquaria (Coyne et al., 2002). Although microbial communities may differ in control and experimental aquaria, *Pfiesteria* must be either directly or indirectly involved in exotoxin production since it is only observed when *Pfiesteria* is present.

Another issue with SFB is that they do not discriminate between exotoxin and physical attack by *Pfiesteria* as a mechanism of ichthyotoxicity. An alternative assay has been developed (Lovko et al., 2003) that includes physical separation of *Pfiesteria* and larval sheepshead minnows (*Cyprinodon variegatus*). This concept is an improvement in that it allows distinction between ichthyotoxicity mediated by physical attack and exotoxin. However that assay has not, to date, detected *Pfiesteria*-associated exotoxin. Plausible explanations for this are that assay conditions may inhibit exotoxin production or that *Cyprinodon* larvae are less sensitive to effects of the exotoxin than tilapia used in SFB.

Several recent publications (Berry et al., 2002; Vogelbein et al., 2002; Litaker et al., 2002) have questioned previously published descriptions of the

life cycle and toxicity of *Pfiesteria* spp. Berry et al. found no toxicity in centrifuged samples from a fish-killing culture of *P. shumwayae* CCMP 2089. Similarly Vogelbein et al. found that while CCMP 2089 killed fish, no detectable toxic activity diffused across a 3  $\mu\text{m}$  pore size membrane to kill larval sheepshead minnows in the micro-assay they developed to test for *Pfiesteria* toxin (Lovko et al., 2003). The broad conclusion of these authors was that *Pfiesteria* spp. do not produce exotoxin that kills fish. At about the same time as these papers were published, Gordon et al. (2002) published data showing that toxic activity could be demonstrated in cell-free (0.2  $\mu\text{m}$ ) filtrates from highly toxic SFB of both *P. piscicida* and *P. shumwayae*, and Springer et al. (2002) demonstrated toxicity to bivalve larvae that diffused across dialysis membrane (14 kDa). Subsequently Burkholder et al. (2002) reported that toxin purified from CCMP 2089 killed GH<sub>4</sub>C1 cells and had identical chromatographic properties to previously described *Pfiesteria*-associated toxins. Thus we are left with the question of whether strain variability, culturing methods, toxin detection methods, or some combination of these factors led to conflicting results and conclusions by different research groups.

In the present study we compared ichthyotoxicity and *Pfiesteria*-associated exotoxin production in SFB containing two strains of *P. shumwayae* under identical conditions. One strain, CCMP 2089, has been reported to require physical contact to kill fish and to be non-toxicogenic (Vogelbein et al., 2002; Berry et al., 2002) while the second strain, CAAE 101272, has been reported to be associated with production of soluble toxin (Gordon et al., 2002).

## 2. Materials and methods

### 2.1. *P. shumwayae* strains

*P. shumwayae* (CAAE #101272 Center for Applied Aquatic Ecology [CAAE], North Carolina State University, Raleigh, NC, USA) and CCMP 2089 (Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP)) were used for this study. These strains were maintained under identical conditions and were either placed directly into SFB, or grown with cryptomonad algal prey as previously

described (Burkholder and Glasgow, 1995; Marshall et al., 2000; Burkholder et al., 2001a). Species identifications were re-confirmed in our laboratory using molecular techniques (Ruble et al., 1999; Bowers et al., 2000).

### 2.2. Bioassays with live fish

*Pfiesteria* strains were grown in the presence of live fish following the standardized fish bioassays (SFB) of Burkholder et al. (2001a,c), Marshall et al. (2000), *Pfiesteria* Interagency Coordination Working Group [PICWG] (2001). All SFB were maintained under BSL3 containment. In addition, laboratory personnel wore portable respirators (3M Airstream Breath Easy with 456-03-01 organic vapor cartridge). In each SFB one *Pfiesteria* strain was grown in the presence of fish in triplicate 9.5 L aquaria containing water and Instant Ocean salts (salinity (S) = 15; Aquarium Systems, Mentor OH, USA; pH 7.5; 25–27 °C). Corresponding control aquaria were also established in triplicate. Bioassay organisms were tilapia (*Oreochromis niloticus*, total length 2–4 cm, Aqua Safra, Bradenton, FL). Eight fish were added to each aquarium. Water quality (ammonia, nitrite, salinity and temperature) and dinoflagellate numbers were monitored periodically in the SFB as previously described (Marshall et al., 2000). Bioassays were considered to contain actively toxic *Pfiesteria* when added fish died overnight or more rapidly and new fish placed into the aquaria died in 24 h or less (Burkholder et al., 2001a). SFB utilized in this study were maintained for a period of 6 or 7 months for CCMP 2089 and CAAE 101272, respectively.

In experiments testing for the presence of exotoxin, protective containers (PC) constructed from tissue culture flasks (Corning, Acton, MA, #430641 250 mL vent-cap) and polycarbonate membrane filter sheets (Millipore Bedford, MA, isopore TTTP, 2  $\mu\text{m}$ ) were used to protect some fish from direct contact with *Pfiesteria* cells. Both sides of the tissue culture flask were cut out (ca. 7 cm  $\times$  8 cm on each side) and replaced with filter membrane. The filter membrane was secured with silicone aquarium sealant (Perfecto Manufacturing, Nobelsville, IN). Each container was aerated by gently bubbling air through capillary tubing inserted through the vented cap. In each trial one fish was placed into each of four PC held within one SFB

aquarium. Four additional fish were placed directly into the same aquarium allowing direct contact with *Pfiesteria* cells. Fish were exposed for 48 h. Trials were performed in selected SFB aquaria on different days. Aquaria were selected for trials only if they were actively toxic. Dead fish were tallied and removed after 24 and 48 h. After 48 h zoospore counts on samples from inside and outside of the containers were obtained (Marshall et al., 2000). PC were cleaned between trials by immersion in bleach (6.15% hypochlorite) for 18–24 h followed by a thorough rinse in tap water. PC were then rinsed in 5% HCl, rinsed in tap water and air-dried. A separate set of PC was used for each *Pfiesteria* strain and for control SFB.

Data from trials using PC were analyzed by split-plot analysis of variance (ANOVA) after rank transforming the data. Data from all trials with each *Pfiesteria* strain were combined for statistical comparison of strains. Significance of differences among treatments was determined by Tukey's post-hoc analysis.

The possibility that the presence of dead fish in SFB was responsible for exotoxin was also tested. In this experiment four fish were placed into PC in one control SFB aquarium. Four additional fish were sacrificed by holding them at 4 °C for 45–60 min in a plastic bag. These fish were placed into the SFB aquarium with the four fish inside PC and left there for 48 h. Fish in PC were checked after 24 and 48 h. This experiment was repeated 10 times over a period of 6 weeks.

Diffusion rate into PC was tested using phenol red (0.05%; MW = 354.4) added to aquarium water. Dye concentration inside and outside the container was measured by absorbance (560 nm, Shimadzu UV160U) after 0, 4 and 22 h.

Bioassays to assess toxic activity in supernatants from centrifuged sub-samples of fish-killing and control SFB were performed as follows. Water (ca. 900 mL) from highly toxic SFB (killing tilapia in ca. 4 h) and corresponding control SFB was centrifuged ( $9635 \times g$  for 20 min IEC PR-7000). Supernatants were either assayed immediately for toxicity or stored at  $-20$  °C. Bioassays on supernatants were performed in a custom-built glove box within the BSL3 laboratory. In each trial 80–100 mL supernatant was added to plastic cups (Solo ultra clear 266 mL, Solo cup Co. Wheeling, IL, USA) and one small ( $\sim 2$  cm) tilapia was added to each cup ( $n = 10$  cups each for control and experimental supernatants). The cups were covered with plastic screen to prevent fish from escaping and each cup was aerated gently using a manifold system. Toxic activity was measured by percent mortality after 24 h.

### 3. Results

Water quality parameters in all SFB remained well within tolerance limits for tilapia with no notable

Table 1

Mortality of tilapia exposed directly to *P. shumwayae* (CCMP 2089) in aquaria and within protective containers placed into the same aquaria that excluded *Pfiesteria* cells

Trial	Mortality in aquarium	Mortality in container	Zoospores ( $\text{mL}^{-1}$ )
1	4/4	0/4	3460
2	4/4	0/4	6560
3	4/4	0/4	5000
4	4/4	0/4	3340
5	4/4	0/4	3240
6	4/4	0/4	3440
7	4/4	0/4	2460
8	4/4	1/4	5560
9	4/4	0/4	5820
10	4/4	0/4	4360
11	4/4	1/4	3920
12	4/4	0/4	940
13	4/4	1/4	1940
Total	52/52 (100%)	3/52 (6%)	$3849 \pm 1600$

No dinoflagellates were detected in the containers after the 48 h exposure period. No deaths were observed inside or outside of containers in corresponding control aquaria.

deviations between control and experimental SFB aquaria. Ammonia was  $0.3 \pm 0.2$  ppm, nitrite was  $0.4 \pm 0.2$  ppm, and salinity was  $18.5 \pm 1.5$ . Zoospore numbers in SFB of both strains were dynamic and were, on average, significantly higher ( $P < 0.001$ ; *t*-test) in CCMP 2089 than in CAAE 101272 SFB when trials utilizing PC were performed (Tables 1 and 2).

### 3.1. Bioassays with PC

SFB containing each strain began killing fish within several weeks after inoculation as is typical for toxic *Pfiesteria* spp. (Burkholder et al., 2001a). No fish died in control SFB inside or outside of PC. No fish (0/40) died inside PC when dead fish were intentionally added to control SFB outside the PC. A total of 29 experimental trials in which fish inside and outside of PC were exposed to actively toxic *P. shumwayae* in SFB were performed. Of these, 13 trials utilized CCMP 2089 and 16 used CAAE 101272.

About 100% (52/52) of fish exposed directly to *P. shumwayae* CCMP 2089 died within 24 h. Only three fish of 52 (6%) died when exposed to CCMP 2089 within PC (Table 1). CAAE 101272 killed 87.5% of directly exposed fish, and 12 fish (19%) died within PC (Table 2). Fish deaths inside of the PC were

observed in two of three individual SFB aquaria in which trials were performed with CCMP 2089 and three of eight aquaria containing CAAE 101272. Outside of the PC, fish mortality from both strains was consistently 100% after 24 h when zoospore density was  $1000 \text{ mL}^{-1}$  or higher (Fig. 1A). Ichthyotoxicity inside of the PC was not related to zoospore density ( $r^2 = 0.03$ ; Fig. 1B). No zoospores were detected inside of the PC in any of the trials. The tracer dye concentration was equilibrated across the container membranes within 22 h.

Split-plot ANOVA showed that fish death both inside and outside of the PC in SFB was significantly greater ( $P < 0.001$ ) in the *Pfiesteria*-containing SFB than in corresponding controls. Mortality of fish outside of PC (exposed to direct physical attack by *Pfiesteria*) was significantly higher than mortality of fish inside of PC ( $P < 0.001$ ). Although more mortality was observed inside PC in SFB containing CAAE 101272, differences between strains were not significant ( $P = 0.3$ ).

### 3.2. Bioassays with supernatants

During the study period the SFB containing CCMP 2089 became sufficiently toxic to kill fish in 4 h only

Table 2  
Mortality of tilapia exposed to a culture of *P. shumwayae* (CAAE 101272) inside and outside of protective containers

Trial	Mortality in aquarium	Mortality in container	Zoospores ( $\text{mL}^{-1}$ )
1	4/4	0/4	2018
2	4/4	0/4	1460
3	4/4	4/4	3100
4	4/4	3/4	380
5	2/4	2/4	760
6	3/4	1/4	120
7	2/4	0/4	100
8	4/4	0/4	700
9	3/4	0/4	260
10	4/4	0/4	2360
11	4/4	0/4	1140
12	2/4	0/4	na
13	4/4	0/4	4980
14	4/4	2/4	900
15	4/4	0/4	2160
16	4/4	0/4	280
Total	56/64 (87.5%)	12/64 (19%)	$1381 \pm 1355$

No dinoflagellates were detected in the containers after the 48 h exposure period. No deaths were observed inside or outside of containers in corresponding control aquaria. na—not available.

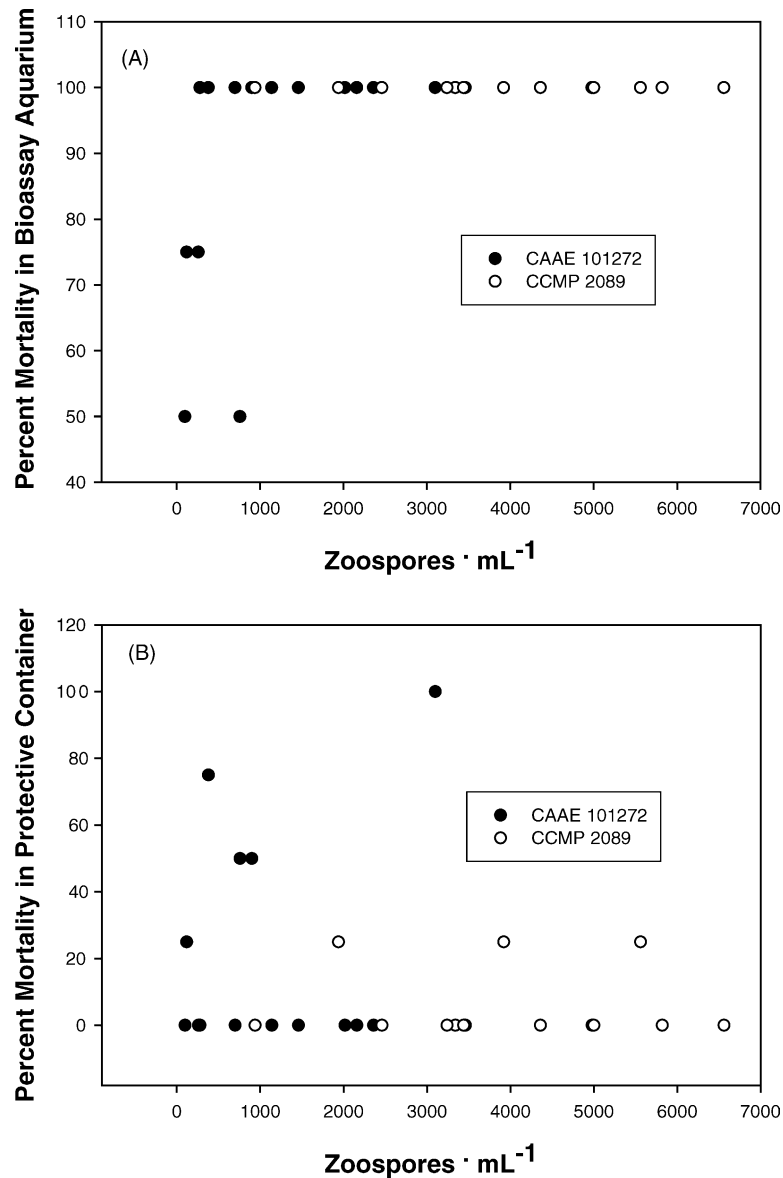


Fig. 1. Relationship between zoospore density and fish mortality in (A) SFB allowing physical contact between fish and *Pfiesteria* and (B) in protective containers within SFB that prevented physical contact.

once. CAAE 101272 killed fish within 4 h on five occasions. Supernatants obtained from these highly toxic SFB killed test fish in four of five supernatants obtained from CAAE 101272 SFB but did not kill fish when obtained from a similarly lethal SFB containing CCMP 2089 (Table 3). In supernatants obtained from CAAE 101272 SFB, toxin concentration, as measured

by percent fish mortality, was variable and ranged from 0% to 70%. No notable difference in toxicity was observed between supernatants that were tested immediately and those that were stored frozen. This is consistent with the stability of toxic activity observed in cell-free filtrates that were stored frozen for up to 6 months (Gordon et al., 2002).

Table 3

Toxicity of dinoflagellate-free supernatants derived from highly toxic (fish death in ca. 4 h) SFB aquaria containing *P. shumwayae* CCMP 2089 or CAAE 101272

Strain	Trial	Mortality in supernatant	Zoospores (mL <sup>-1</sup> ) in the culture
CAAE 101272	1	5/9	na
CAAE 101272	2	0/10	9280
CAAE 101272	3	1/10	3360
CAAE 101272	4	7/10	na
CAAE 101272	5	5/10	na
CCMP 2089	1	0/10	4480
CCMP 2089	2	0/10	4480

Both trials using strain CCMP 2089 were collected from one SFB on the same date. This was the only occasion during the study that CCMP 2089 killed fish in 4 h. Trials with CAAE 101272 utilized sub-samples from SFB collected on different dates. No fish deaths were observed in supernatants derived from control aquaria. na—not available.

#### 4. Discussion

Various studies addressing the toxigenicity of *Pfiesteria* spp. have utilized different culture methods, different bioassays for toxin detection, and different strains of *Pfiesteria*. In this study we compared two strains of *P. shumwayae* using identical culture and bioassay methods. One of these strains has been reported to be nontoxic and the other has been associated with toxin production. In this study, while physical contact significantly enhanced ichthyotoxicity, exotoxin (as measured by mortality inside PC) was detected in some trials with both strains. More fish mortality in SFB containing CAAE 101272 was attributable to exotoxin than in SFB containing CCMP 2089, however the difference was not statistically significant. These results suggest that the primary factor leading to different conclusions regarding exotoxin production associated with *P. shumwayae*, is culturing methods and/or bioassays used to detect exotoxin rather than significant differences between these strains.

Bioassays in dinoflagellate-free supernatants yielded somewhat different results than those utilizing PC. We have previously reported that cell-free filtrates of toxic *Pfiesteria* SFB had measurable exotoxin when obtained from SFB that killed fish in ca. 4 h (Gordon et al., 2002). Filtrates from less toxic SFB generally do not kill fish (Gordon et al., unpublished data). Accordingly, in this study, supernatants were prepared only when SFB aquaria were sufficiently toxic to kill fish within 4 h. Lack of detectable toxicity in the supernatant derived from

CCMP 2089 and toxicity in four of five supernatants from CAAE 101272, like the data derived from PC, suggest that CCMP 2089 is more weakly toxigenic than CAAE 101272. Absence of measurable ichthyotoxicity in supernatants derived from CCMP 2089 is also consistent with results of Berry et al. (2002). However sufficient replicates for statistical comparison are not available for supernatants since CCMP 2089 only killed fish within 4 h on one occasion in the present study and, as a result, only one supernatant was tested.

Whether the exotoxin observed in this study is a direct product of *P. shumwayae* or a result of interactive processes within the complex microbial community in SFB cannot be determined from these results. A role of *P. shumwayae* in production of exotoxin in SFB is apparent since toxin was not detected in SFB without *P. shumwayae* or when dead fish were added to control SFB.

In this study exotoxin was detected only occasionally and was not related to zoospore abundance. This is consistent with the observations of Burkholder et al. (2001b) indicating that toxigenicity varies among clones, changes over time, and may be irreversibly lost. Thus other clones may be found to be associated more consistently with exotoxin production than the strains examined in the present study. These results demonstrate that, with these two strains of *P. shumwayae*, exotoxin levels that are detectable by fish bioassay are not consistently present in actively toxic (Burkholder et al., 2001a) SFB. The inconsistent appearance of detectable levels of exotoxin and the lack of correlation between exotoxin and zoospore

density may indicate a role of other microorganisms present in SFB in production of *Pfiesteria*-associated exotoxin. Another possible explanation is that the observed exotoxin is principally an endotoxin that is released sporadically, possibly due to cellular death and lysis.

In summary we found that the most consistent and dominant mechanism mediating fish mortality in SFB containing *P. shumwayae* required direct contact between fish and *Pfiesteria*. Soluble toxin reached high enough concentrations to kill fish when contact with *Pfiesteria* cells was prevented in both strains we studied at some times. Thus both contact-mediated mechanism(s) (micropredation) and *Pfiesteria*-associated exotoxin can contribute to fish mortality caused by *P. shumwayae* in SFB.

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### References

- Anderson, D.M., 1991. Toxin variability in Alexandrium. In: Graneli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Proceedings of the Fourth International Conference on Toxic Marine Phytoplankton, Elsevier, New York, pp. 41–51.
- Bates, S.S., Garrison, D.L., Horner, R.A., 1998. Bloom dynamics and physiology of domoic-acid-producing *Pseudo-nitzschia* species. In: Anderson, D.M., Cembella, A., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algae. NATO ASI Series G: Ecological Sciences, vol. 41. Springer-Verlag, Berlin, pp. 267–292.
- Berry, J.P., Reece, K.S., Rein, K.S., Baden, D.G., Haas, L.W., Ribeiro, W.L., Shields, J.D., Vogelbein, W.K., Gawley, R.E., 2002. Are *Pfiesteria* species toxicogenic? Evidence against production of ichthyotoxins by *Pfiesteria shumwayae*. PNAS 99, 10970–10975.
- Bowers, H.A., Tengs, T., Glasgow, H.B., Burkholder, J.M., Oldach, D.W., 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol. 66, 4641–4648.
- Burkholder, J., Glasgow, H., 1995. Interactions of a toxic estuarine dinoflagellate with microbial predators and prey. Arch. Protistenk. 145, 177–188.
- Burkholder, J.M., Glasgow, H., 2001. Toxic *Pfiesteria* in North Carolina estuaries from 1991 to the present. BioScience 51, 827–841.
- Burkholder, J., Noga, E., Hobbs, C., Glasgow, H., Smith, S., 1992. New “Phantom” dinoflagellate is the causative agent of major estuarine fish kills. Nature 358, 407–410.
- Burkholder, J., Glasgow, H., Hobbs, C., 1995. Fish kills linked to a toxic ambush-predator dinoflagellate: distribution and environmental conditions. Mar. Ecol. Prog. Ser. 124, 43–61.
- Burkholder, J., Mallin, M., Glasgow, H., 1999. Fish kills, bottom-water hypoxia, and the toxic *Pfiesteria* complex in the Neuse river and estuary. Mar. Ecol. Prog. Ser. 179, 301–310.
- Burkholder, J.M., Glasgow, H.B., Deamer-Melia, N., 2001a. Overview and present status of the toxic *Pfiesteria* complex (*dinophyceae*). Phycologia 40 (3), 186–214.
- Burkholder, J.M., Glasgow, H.B., Deamer-Melia, N.J., Springer, J., Parrow, M.W., Zheng, C., Cancellieri, P., 2001b. Species of the toxic *Pfiesteria* complex, and the importance of functional type in data interpretations. Environ. Health Persp. 109 (Suppl. 5), 667–679.
- Burkholder, J.M., Marshall, H.G., Glasgow, H.B., Seaborn, D.W., Deamer-Melia, N.J., 2001c. The standardized fish bioassay process for detecting and culturing actively toxic *Pfiesteria*, used by the two reference laboratories for Atlantic and south-eastern states. Environ. Health Persp. 109 (Suppl. 5), 745–756.
- Burkholder, J.M., Glasgow, H.B., Ramsdell, J.S., Moeller, P.D., Marshall, H.G., Gordon, A., Morton, S.L., Law, J.M., Shumway, S.E., Levin, E., Glibert, P.M., Parrow, M.W., Deamer, N.J., Springer, J.J., Melia, G.M., Reed, R., Zhang, C., 2002. Recent findings about cell cycles, life cycles, ecology and toxicity of *Pfiesteria*. In: Proceedings of the Xth International Conference on Harmful Algae, St. Pete Beach, FL, October 21–25.
- Coyne, K., Gordon, A., Burkholder, J., Glasgow, H., Marshall, H., Cary, C., 2002. Denaturing gradient gel electrophoresis (DGGE) analysis of eukaryotic and bacterial communities associated with cultures of *Pfiesteria piscicida*. In: Proceedings of the Xth International Conference on Harmful Algae, St. Pete Beach, FL, October 21–25.
- Edvardsen, B., Paasche, E., Anderson, J., 1998. Bloom dynamics and physiology of *Prymnesium* and *Chysochomulina*. In: Cembella, D.M., Hallegraeff, A., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algae. NATO ASI Series G: Ecological Sciences, vol. 41. Springer-Verlag, Berlin, pp. 193–208.
- Fairey, E., Edmunds, S., Deamer-Melia, N., Glasgow, H., Johnson, F., Moeller, P., Burkholder, J., Ramsdell, J., 1999. Reporter gene assay for fish killing activity produced by *Pfiesteria piscicida*. Environ. Health Persp. 107, 711–714.
- Gentien, P., Arzul, G., 1990. Exotoxin production by *Gyrodinium cf. aureolum* (Dinophyceae). J. Mar. Biol. Assoc. UK 70, 571–581.
- Glasgow, H., 2000. The biology and impacts of toxic *Pfiesteria* complex species. Ph.D. Dissertation. Department of Marine,

- Earth, Atmospheric Sciences, North Carolina State University, Raleigh, NC, p. 175.
- Glasgow, H., Burkholder, J., Schmeche, D., Tester, P., Rublee, P., 1995. Insidious effects of a toxic estuarine dinoflagellate on fish survival and human health. *J. Toxicol. Environ. Health* 46, 501–522.
- Glasgow, H.B., Burkholder, J.M., Morton, S.L., Springer, J., 2001a. A second species of ichthyotoxic *Pfiesteria* (Dinamoebales, Dinophyceae). *Phycologia* 40, 234–245.
- Glasgow, H.B., Burkholder, J.M., Mallin, M.A., Deamer-Melia, N.J., Reed, R.E., 2001b. Field ecology of toxic *Pfiesteria* complex species, and a conservative analysis of their role in estuarine fish kills. *Environ. Health Persp.* 109 (Suppl. 5), 715–730.
- Gordon, A.S., Dyer, B.J., Seaborn, D., Marshall, H.G., 2002. Comparative toxicity of *Pfiesteria* spp., prolonging toxicity of *P. piscicida* in culture and evaluation of toxin(s) stability. *Harmful Algae* 1, 85–94.
- Grattan, L., Oldach, D., Perl, T., Lowitt, M., Matuszak, D., Dickson, C., Parrott, C., Shoemaker, R., Wasserman, M., Hebel, J., Charache, P., Morris, J., 1998. Problems in learning and memory occur in persons with environmental exposure to waterways containing toxin-producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. *Lancet* 352, 532–539.
- Jakobsen, K.S., Tengs, T., Vatne, A., Bowers, H.A., Oldach, D.W., Burkholder, J.M., Glasgow, H.B., Rublee, P.A., Klaveness, D., 2002. Discovery of the toxic dinoflagellate, *Pfiesteria*, from northern European waters. *Proc. R. Soc. London B* 269, 211–214.
- Kimm-Brinson, K.L., Moeller, P.D.R., Barbier, M., Glasgow, H.B., Burkholder, J.M., Ramsdell, J.S., 2001. Identification of a P2X<sub>7</sub> receptor in GH4C1 rat pituitary cells: A target for a bioactive substance produced by *Pfiesteria piscicida*. *Environ. Health Persp.* 109, 457–462.
- Lewitus, A., Jesien, R., Kana, T., Burkholder, J., Glasgow, H., May, E., 1995. Discovery of the “Phantom” dinoflagellate in Chesapeake Bay. *Estuaries* 18, 373–378.
- Litaker, R.W., Vandersea, M.W., Kibler, S.R., Madden, V.J., Noga, E.J., Tester, P.A., 2002. Life cycle of the heterotrophic dinoflagellate *Pfiesteria piscicida* (Dinophyceae). *J. Phycol.* 38, 442–463.
- Lovko, V.J., Vogelbein, W.K., Shields, J.D., Haas, L.W., Reece, K.S., 2003. A new larval bioassay for testing the pathogenicity of *Pfiesteria* spp. (Dinophyceae). *J. Phycol.* 39, 600–609.
- Marshall, H.G., Gordon, A.S., Seaborn, D.W., Dyer, B., Dunstan, W.M., Seaborn, A.M., 2000. Comparative culture and toxicity studies between the toxic dinoflagellate *Pfiesteria piscicida* and a morphologically similar cryptoperidiniopsisoid dinoflagellate. *J. Exp. Marine Biol. Ecol.* 255, 51–74.
- Melo, A.C., Moeller, P.D.R., Glasgow, H.B., Burkholder, J.M., Ramsdell, J.S., 2001. Microfluorimetric analysis of a purinergic receptor (P2X<sub>7</sub>) in GH<sub>4</sub>C<sub>1</sub> rat pituitary cells: effects of a bioactive substance produced by *Pfiesteria piscicida*. *Environ. Health Persp.* 109 (Suppl. 5), 731–738.
- Moeller, P.D., Morton, S.L., Mitchell, B.A., Silversten, S.K., Fairey, E.R., Mikulski, T.M., Glasgow, H., Deamer-Melia, N.J., Burkholder, J.M., Ramsdell, J.S., 2001. Current progress in isolation and characterization of toxins isolated from *Pfiesteria piscicida*. *Environ. Health Persp.* 109 (Suppl. 5), 739–743.
- Moeller, P.D., Ramsdell, J.S., Morton, S.L., Eaker, S., Burkholder, J.M., Glasgow, H.B., Deamer-Melia, N.J., 2003. Isolation and structural information on a water soluble toxin derived from *Pfiesteria piscicida*. In: Proceedings of the Second Symposium on Harmful Algae in the US, Woods Hole, MS, December 9–13.
- Pfiesteria Interagency Coordination [PICWG] 2001. Glossary of *Pfiesteria*-related terms. Consensus document involving NOAA, US EPA, CDC, representatives from environmental and health agencies in multiple Atlantic and Gulf Coast states, and academics. US EPA, Baltimore, 9 pp.
- Rhodes, L.L., Burkholder, J.M., Glasgow, H., Rublee, P.A., Allen, C., Adamson, J.E., 2002. *Pfiesteria shumwayae* (Pfiesteriaceae) in New Zealand. *NZ J. Freshwater Mar. Sci.* 36, 621–630.
- Rublee, P., Kempton, J., Schaefer, E., Burkholder, J., Glasgow, H., Oldach, D., 1999. PCR and FISH detection extends the range of *Pfiesteria piscicida* in estuarine waters. *Virginia J. Sci.* 50 (4), 325–336.
- Springer, J., Shumway, S.E., Burkholder, J.M., Glasgow, H.B., 2002. Interactions between two commercially important species of bivalves and the toxic estuarine dinoflagellate, *Pfiesteria piscicida*. *Mar. Ecol. Prog. Series* 245, 1–10.
- Steidinger, K., Burkholder, J., Glasgow, H., Hobbs, H., Garrett, J., Truby, E., Noga, E., Smith, S., 1996. *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae Fam. nov.), a new toxic dinoflagellate with a complex life cycle and behaviour. *J. Phycol.* 32, 157–164.
- Twiner, M.J., Dixon, S.J., Trick, G.C., 2004. Extracellular organics from specific cultures of *Heterosigma akashiwo* (Rapidophyceae) irreversibly alter respiratory activity in mammalian cells. *Harmful Algae* 3, 173–182.
- Vogelbein, W.K., Lovko, V.J., Shields, J.D., Reece, K.S., Mason, P.L., Haas, L.W., Walker, C.C., 2002. *Pfiesteria shumwayae* kills fish by micropredation not exotoxin secretion. *Nature* 418, 967–970.