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Harmful Algae 4 (2005) 423–431
HARMFUL ALGAE
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Relative contribution of exotoxin and micropredation to icthyotoxicity of two strains of *Pfiesteria shumwayae* (Dinophyceae)

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Received 8 July 2004; received in revised form 22 July 2004; accepted 26 July 2004

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.

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

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.,

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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doi:10.1016/j.hal.2004.07.003

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 though the Gulf Coast as well as in northern European and New Zealand waters (Rublee 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 chomatographic resins (e.g. C18), and inhibit GH_4C1 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 icthyotoxicity 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