Demonstration of toxicity to fish and to mammalian cells by *Pfiesteria* species: Comparison of assay methods and strains

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Toxicity and its detection in the dinoflagellate fish predators *Pfiesteria piscicida* and *Pfiesteria shumwayae* depend on the strain and the use of reliable assays. Two assays, standardized fish bioassays (SFBs) with juvenile fish and fish microassays (FMAs) with larval fish, were compared for their utility to detect toxic *Pfiesteria*. The comparison included strains with confirmed toxicity, negative controls (noninducible *Pfiesteria* strains and a related nontoxic cryptoperidiniopsoid dinoflagellate), and *P. shumwayae* strain CCMP2089, which previously had been reported as nontoxic. SFBs, standardized by using toxic *Pfiesteria* (coupled with tests confirming *Pfiesteria* toxin) and conditions conducive to toxicity expression, reliably detected actively toxic *Pfiesteria*, but FMAs did not. *Pfiesteria* toxin was found in fish- and algae-fed clonal *Pfiesteria* cultures, including CCMP2089, but not in controls. In contrast, noninducible *Pfiesteria* and cryptoperidiniopsoids caused no juvenile fish mortality in SFBs even at high densities, and low larval fish mortality by physical attack in FMAs. Filtrate from toxic strains of *Pfiesteria* spp. in bacteria-free media was cytotoxic. Toxicity was enhanced by bacteria and other prey, especially live fish. Purified *Pfiesteria* toxin extract adversely affected mammalian cells as well as fish, and it caused fish death at environmentally relevant cell densities. These data show the importance of testing multiple strains when assessing the potential for toxicity at the genus or species level, using appropriate culturing techniques and assays.

toxigenic dinoflagellates | *Pfiesteria piscicida* | *Pfiesteria shumwayae*

Outbreaks of toxic *Pfiesteria piscicida* and *Pfiesteria shumwayae*, dinoflagellates that prey upon fish and other organisms, in the two largest estuaries on the U.S. mainland during the 1990s provide a compelling example of linkages between fish kills/disease and impacts on human health (1–3). Previous research has established that toxicity is highly variable among strains within a given toxic algal species (4), including *Pfiesteria* spp., ranging from strains with negligible toxicity to highly toxic strains (1, 5–7). *Pfiesteria* strains can cause larval fish death by physical attack (1). Their toxicity status was operationally defined by a multiagency/academic consensus group (8). Under conditions conducive to toxicity expression (9–11), actively toxic (TOX-A) strains, grown with live finfish, are capable of killing fish with toxin involvement at low to moderate cell densities ($\geq 4 \times 10^2$ to 10^3 cells per ml, minutes to several hours). Impacts are exacerbated by *Pfiesteria* physical attack (6, 7, 12). The same strains separated from live fish for days or longer can sometimes produce sufficient exotoxin to cause death of sensitive larval stages without physical contact (7). Noninducible (NON-IND) strains apparently are incapable of killing fish with toxin (1, 6, 8).

A hydrophilic *Pfiesteria* toxin (*Pf*Tx), isolated in 1997 (13) and consisting of a metallated organic complex (see the supporting information, which is published on the PNAS web site), has been shown to affect fish and mammals (1, 13–15). After 7 years, *Pf*Tx has been purified and a pharmacological mode of action has been described (16). The time course for analysis is within the range for other dinoflagellate toxins, limited by the quantity of available toxic culture: e.g., ciguatoxin and maitotoxin required 23 years (17, 18) and 17 years (19, 20), respectively, from isolation to purification and structural elucidation. Additional *Pf*Tx is being produced (chemical structure being published elsewhere) so that standards can be developed for routine use. In the interim, various fish assays have been used in attempts to detect toxic *Pfiesteria* strains (5, 6, 10–12, 21–25). Although some techniques have provided evidence of toxicity to fish (5, 10, 14, 24), researchers using other methods have concluded that *Pfiesteria* is not toxigenic at the species [*P. shumwayae* (21, 22)] or genus (23, 25) levels. A comparative technique analysis was warranted to resolve these divergent conclusions, which have important policy implications in coastal resource management (3, 26).

Here we compare the two techniques most frequently used to detect toxic *Pfiesteria*, standardized fish bioassays (SFBs) with juvenile fish (10) and fish microassays (FMAs) with larval finfish (1, 21) or shellfish (7), for utility in detecting actively toxic *Pfiesteria* strains. SFBs, often maintained for weeks, originally were calibrated by using *Pfiesteria* spp. strains capable of rapid fish-killing at low to moderate cell densities (4×10^2 to 10^3 cells per ml) and confirmed to produce hydrophilic *Pf*Tx (10, 14). SFBs were not developed to distinguish between toxicity and physical attack as factors in fish death; rather, they were designed to detect toxic *Pfiesteria* strains, known to routinely prey upon fish (1, 10). Small-scale assays were also used to test for toxicity of culture filtrate from SFBs. FMAs, maintained for hours to several days, have been proposed for use in ruling out the presence of toxic *Pfiesteria* (21, 23) based on lack of fish death in culture filtrate. The following hypotheses were tested: (*i*) *Pfiesteria* spp. strains toxic to fish vary in *Pf*Tx release and can produce toxin in bacteria-free media; and (*ii*) toxicity is significantly enhanced by bacteria and other prey, especially live fish.

Materials and Methods

Dinoflagellate Cultures and SFBs with Juvenile Fish. *Pfiesteria* strains [*P. piscicida* CAAE2200 isolated in 1996 from Beaufort Point, NC, cloned and tested in 1999–2001 by A.S.G. and H.G.M.; *P. shumwayae* CAAE1024C, referred to as 101272 in ref. 11, isolated in 2000

Abbreviations: CAAE, Center for Applied Aquatic Ecology; CCMP, Culture Center for Marine Phytoplankton; DGGE, denaturing gradient gel electrophoresis; FMA, fish microassay; LC_{50}, 50% lethal concentration; NON-IND, noninducible strain, unable to kill fish with toxin; *Pf*Tx, hydrophilic *Pfiesteria* toxin; SFB, standardized fish bioassay; TOX-A, actively toxic.

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from Marshall Creek, Chincoteague Bay, MD, and cloned (1); "CAAE" indicates the collection of the Center for Applied Aquatic Ecology] were grown with benign algal prey (*Cryptomonas* sp.) (ref. 1 and supporting information). Algae-fed *Pfiesteria* cultures were added to separate sets of SFBs (7 liters, $n = 3$–10, depending on the experiment, ≈70 flagellated cells per ml) in a biohazard level III containment system, following ref. 10. They were maintained for ≤14 weeks (2000–2004; toxicity to fish and *Pf*Tx verified as in refs. 10 and 14) with tilapia (*Oreochromis mossambica* or *Oreochromis nilauticus*, 3–10 juveniles per container depending on the experiment, total length 4–5 cm). *P. shumwayae* strain CCMP2089 [isolated from the Pamlico Estuary, NC, in 1999 (21); "CCMP" indicates the collection of the Culture Center for Marine Phytoplankton, Bigelow Laboratory, West Boothbay Harbor, ME], previously used by other researchers to negate toxigenicity in this species (21) and genus (23), was cultured and assayed as above (techniques in refs. 10 and 11).

To compare *Pfiesteria* toxicity when SFBs were inoculated with algae- versus fish-fed cultures, one of the SFBs that exhibited fish death (≥4 h as in ref. 11) involving toxic *Pfiesteria* was randomly selected and *Pfiesteria* isolates were inoculated from this SFB into a second set of SFBs ($n = 5$). SFBs were monitored for fish mortality (excluding occasional cannibalism), water quality, *Pfiesteria* abundance, and other microbes as in ref. 10. Rapid removal of dead fish maintained good water quality as in ref. 10 [i.e., total ammonia <1 mg/liter; pH 7.6–8.2 (supporting information)]. After repeated rapid fish death occurred, *Pfiesteria* strains were recloned and cultured with cryptomonads or with a fish cell line (27) in bacteria-free conditions to assess whether *Pfiesteria* can show toxicity in the absence of bacteria.

Negative controls included SFBs of juvenile tilapia treated identically but without dinoflagellates. Feeding controls (attempt to account for dinoflagellate physical attack of fish) included SFBs with cryptoperidiniopsoids [CAAE543A-AC1 (CCMP2302), a nontoxic *Pfiesteria*-like organism (1)], and SFBs with NON-IND *Pfiesteria* [*P. piscicida* clone CAAE1036C; clone from isolate CAAE2200, tested in 2003; and CCMP1832 (supporting information)]. Cryptoperidiniopsoids and NON-IND as well as toxic *Pfiesteria* strains prey upon fish by raptorial feeding (1, 28). Feeding control isolates were inoculated into SFBs as above. Dinoflagellates from fish- and algae-fed cultures were identified by plate tabulation as in ref. 1 and sequence-specific 18S ribosomal DNA-based PCR molecular probes (29, 30).

Filtrate from positive SFBs [fish death in ≤4 h with $\geq 3.0 \times 10^2$ to 9.28×10^3 cells per ml of *P. piscicida* (CAAE2200, in 2001) or *P. shumwayae* (CAAE1024C and CCMP2089, in 2001–2003)] and negative controls were tested for fish-killing activity by A.S.G. and H.G.M. within a biohazard level III containment system [10–20 liters gently filtered (0.2-μm porosity) and pooled; one juvenile tilapia per 100-ml container, total length ≈2 cm; gentle aeration; $n = 10$]. Whole cultures were tested for comparison. Toxic activity was inferred from mortality in ≤24 h. Fish were also examined for epidermal integrity ($n = 32$; supporting information).

Microbial Communities. PCR amplification of rDNA,[§§] followed by denaturing gradient gel electrophoresis (DGGE) (ref. 31 and supporting information), was used to evaluate microbial composition in the water column. Prokaryotes (≥0.2- 5.0-μm and ≥5-μm size fractions) in algae-fed cultures plus bacteria, control (minus dinoflagellates, and plus feeding controls), and positive SFBs were compared.

FMAs with Larval Fish. *P. shumwayae* strains CAAE1024C and CCMP2089 (algae- or fish-fed) were tested in FMAs (one fish per 10-ml well, $n = 6$, in triplicate; supporting information) by using *Cyprinodon variegatus* (age <25 days). Two sets of FMAs were used, with (7, 12, 21) versus without (1, 12) a filter partition (0.4-μm porosity, Costar 3450) to prevent physical contact of dinoflagellates and fish. A cell density gradient was imposed by adding filtered media (0.22-μm porosity, 15 practical salinity units) to effect initial *Pfiesteria* densities of 0 (controls), 0.25×10^2, 0.5×10^2, 0.8×10^2, 1×10^3, 2×10^3, 3×10^3, and 5×10^3 cells per ml. Values for the 50% lethal concentration (LC_{50}) were determined at 24, 48, and 72 h. NON-IND *Pfiesteria* and cryptoperidiniopsoids were also tested (density gradient up to 4×10^4 cells per ml).

Tests for *Pfiesteria* Culture Purity. A standard mix of antibiotics (Sigma P4083) was inoculated into one culture each of algae-fed *P. shumwayae* and *P. piscicida* [final concentrations 100 units of penicillin per ml, 0.10 mg of streptomycin per ml, 0.20 mg of neomycin per ml (ref. 32 and supporting information)]. After 48 h, the culture was aseptically transferred to sterile nutrient-enriched broth (1 ml, 23°C) and agar media (0.1 ml, 37°C), incubated in darkness, and checked daily (8 d) for bacterial and fungal growth. Bacteria-free *P. shumwayae* fed fish cells was prepared as described in ref. 27. Uninoculated broth tubes or agar plates served as controls. Cultures were also examined under light microscopy (phase-contrast, ×750) after 7–10 d (32). Growth was not detected; thus, cultures were considered bacteria- and fungus-free, defined as lacking demonstrable unwanted prokaryotes and eukaryotes (32–34), and culture filtrates were tested for cytotoxicity.

Toxicity, Toxin Production, and Environmental Relevance. Filtrate (0.45-μm porosity) from SFBs, algae-fed cultures, and negative controls were encoded and sent to P.D.M. and J.S.R. for blind analysis of *Pf*Tx according to ref. 14. The toxin(s) from *Pfiesteria* spp. cultures have yielded no diagnostic UV chromophore at concentrations typically present in culture extracts. High-performance liquid chromatography analysis was carried out to test for the presence/absence of *Pf*Tx by using bioassay-guided fractionation, i.e., retention time and associated cytotoxicity and/or fish assays. ^{13}C NMR spectra of the *Pf*Tx were also obtained (supporting information). Additional controls were equivalent or higher volumes (≥750 ml) of filtered seawater (0.22-μm porosity; 15 practical salinity units with deionized water), and algal culture (≈1.500×10^4 cells per ml). Samples were processed for toxin extraction, purification, cytotoxicity, and fish toxicity. For cytotoxicity assays, the rat pituitary GH_4C_1 cell line (ref. 13 and supporting information) and the colorimetric assay of ref. 14 were used. Also, filtrate from SFBs and algae- plus bacteria-fed *Pfiesteria* cultures (3-μm filter porosity, to remove *Pfiesteria* and cryptomonads) was refiltered (0.22-μm porosity), and retained bacteria were resuspended in fresh medium and tested for cytotoxic activity. An elutropic solvent scheme of increasing polarity was used to partition the soluble extract derived from *Pfiesteria* culture (supporting information). Data for *Pf*Tx concentrations are reported on a per-cell basis as dry residue weight taken up in standard volumes (dry weight of *Pf*Tx residue obtained from the total number of cells in a given sample, added to a known volume), to enable comparison of *Pfiesteria* isolates.

For fish assays with *Pf*Tx, 50 μl of *Pf*Tx extract in 100% methanol carrier was added to 2-ml FMAs (15 practical salinity units) with larval *C. variegatus* [age 7 d, one fish per FMA, 24 h, $n = 6$, two sets (supporting information)]. Extract from control cultures was also tested. We normalized fish mortality on a picograms of toxic extract per *Pfiesteria* cell basis, and calculated the cell density required for sufficient *Pf*Tx to kill test fish. The data were compared with *Pfiesteria* field densities based on semiquantitative PCR of water samples from two estuarine fish kills, and also compared with published data for other algal toxins (supporting information). For the standard curve in semiquantitative PCR, cell numbers of a serially diluted, mixed clonal *P. piscicida* culture were quantified in light microscopy, and the DNA was extracted and quantified. *P.*

[§§] *P. piscicida* CCMP1832 rDNA sequence is GenBank accession no. AF077055; *P. shumwayae* CCMP2089 rDNA sequence is GenBank accession no. AF218805.

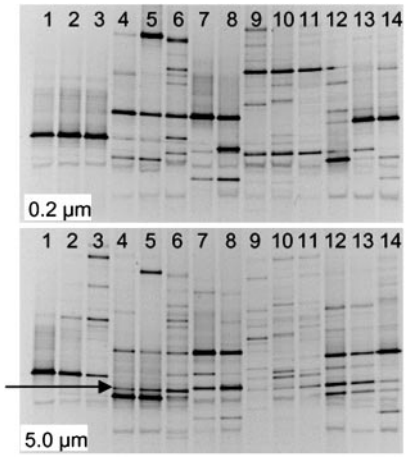

Fig. 1. DGGE images of PCR products, amplified for prokaryotic 16S rDNA by using eubacterial primers (31). DNA template was isolated from size-fractionated media from SFBs [0.2- to 5-μm fraction (*Upper*) and >5-μm fraction (*Lower*)]. Lanes 1–6, toxic *P. shumwayae* (two cultures evaluated, $n = 3$: CAAE1024C in lanes 1–3, CCMP2089 in lanes 4–6); lane 7, cryptoperidiniopsoid CAAE543A-AC1; lane 8, *P. piscicida* CAAE1332T-AC2 (CCMP2361); lanes 9–14, negative controls (media from fish cultures minus dinoflagellates). The presence of band 10 (arrow) in lanes 4–6 (toxic *P. shumwayae* CCMP2089) and lanes 9–14 (negative controls) suggests that this bacterium does not play a role in *Pfiesteria* toxicity.

piscicida cells were also quantified in a set of internal spikes [linear relationship between cell numbers and DNA, $R^2 = 0.9518$ (supporting information)].

Results

Environmental Conditions and Microbial Communities. Water quality in control and test SFBs with TOX-A *P. shumwayae* was comparable and followed recommended conditions for toxicity expression in *Pfiesteria* spp. (ref. 10 and supporting information). Presumptive *Vibrio* spp. generally were higher in negative control SFBs than in test SFBs plus toxic *Pfiesteria* strains (Student's t test, $P < 0.1$; supporting information). PCR/DGGE analyses indicated no significant pattern of specific bacterial growth in control versus test SFBs (Fig. 1) (supporting information). No bands were consistently, exclusively present in all lanes of the toxic *Pfiesteria* samples or in negative controls except for band 10, which was also present in several toxic samples.

SFBs with Juvenile Fish. There was 100% fish survival and fish appeared active and healthy in all controls (fish minus dinoflagellates, fish plus cryptoperidiniopsoids, and fish plus NON-IND *Pfiesteria*). Feeding controls did not cause juvenile fish death even at high densities (up to 2.00×10^4 cells per ml; supporting information). In contrast, at low to moderate densities ($\geq 4 \times 10^2$ to 10^3 cells per ml), there was 100% fish mortality in SFBs with *Pfiesteria* spp. strains verified as actively toxic (CAAE2200, CAAE1024C, and CCMP2089) (Table 1 and Figs. 2, 3, and 4). Fish death was usually preceded by lethargy alternating with hyperactivity as in refs. 1 and 12. Thus, when these strains were grown under appropriate conditions in SFBs [pH >7, total ammonia <1 ppm, etc. (10)], they expressed toxicity to fish. There was no significant correlation between initial or final *Pfiesteria* cell densities and time to first fish death in SFBs ($P > 0.1$). Fish-fed CAAE1024C ($P < 0.00005$), but not CCMP2089, killed additional fish more rapidly and had higher toxicity per cell than algae-fed subclones ($P < 0.01$) (Fig. 2). Whereas CCMP2089 killed fish more rapidly in SFBs ($P < 0.0008$), fish-acclimated CAAE1024C killed at lower cell densities than fish-acclimated CCMP2089 ($P < 0.02$) (Fig. 3).

Fish death occurred in ≤24 h in filtrates, and in supernatant media after centrifugation, from SFBs conducted in 2000–2001 with *P. piscicida* CAAE2200 and *P. shumwayae* CAAE1024C (e.g., Fig. 5*a*). However, mortality was markedly lower than in unfiltered water plus *Pfiesteria* cells. Toxic activity of filtrates from positive SFBs of these strains (fish death in ≥4 h) was similar. CCMP2089 was less toxic, indicated by no fish mortality or epidermal lesions (as in ref. 21) in culture filtrate. However, additional tests yielded low fish mortality in filtrate from SFBs with CCMP2089 (6%, $n = 13$ trials, 8 fish per trial; significantly different from controls with 0% fish death, $P < 0.05$) (24). Beginning at 3 h of exposure, fish exposed to CAAE1024C culture and filtrate had necrosis and loss of large areas of epidermis (Fig. 5 *c* and *d*). Mild to moderate erosions predominated, but scattered areas had a focal breach of the basement membrane (ulceration). Edges of eroded or ulcerated

Table 1. Toxic activity of purified *Pf*Tx extract from filtrate of *Pfiesteria* isolates in FMAs with larval *C. variegatus*, and cytotoxicity to mammalian cells

Culture and year tested	Culture: Volume, liters	Culture: Cells per ml	Toxic activity,* pg of toxin extract per cell	50 μl of *Pf*Tx extract yields time to fish death (cell equivalents)†	Cytotoxicity (GH_4C_1 rat pituitary cells)
Cultures from positive SFBs					
P. shumwayae CAAE1024C					
Trial 1 (algae-fed, then SFBs, 2003)	1	$1.5 \pm 0.3 \times 10^3$	58–202	<30 min (41 ce)	+
Trial 2 (fish-fed, SFBs, 2003)	1	$0.8 \pm 0.2 \times 10^2$	43–251	NA	+
Trial 3 (fish-fed, SFBs, 2004)	6.4	11.63×10^4	47	<28 min (817 ce)	+
Trial 4 (fish cell-fed − bacteria, 2004)	0.7	3.5×10^5	—	NA	+
P. shumwayae CCCMP2089					
Trial 1 (algae-fed, then SFBs, 2003)	1	$1.2 \pm 0.2 \times 10^3$	67–142	<28 min (64 ce)	+
Trial 2 (fish-fed, SFBs, 2003)	1	$1.4 \pm 1.1 \times 10^3$	150–284	NA	+
Trial 3 (fish-fed, SFBs, 2004)	6.4	1.1×10^4	0.5	<24 h (555 ce)	+
Trial 4 (algae-fed, 2002)	90	3.00×10^4	5	<40 min (1,500 ce)	+
Controls (no fish death, SFBs)					
P. piscicida (fish-fed, SFBs, CCMP1832, NON-IND, 2003)	300	2.50×10^4	6	<90 min (1,250 ce)	+
P. piscicida (fish-fed, SFBs, CAAE2200, NON-IND, 2003)	1	1.3×10^3	ND	No mortality	−
Cryptoperidiniopsoid (fish-fed, SFBs, CAAE543-AC1, 2003)	1	3×10^2	—	No mortality	−
Fish − dinoflagellates (2003)	7		—	No mortality	−

All control fish remained active with no signs of stress. NA, not available; ND, not detectable.
*Toxicity activity, based on mass of *Pf*Tx fraction purified from HW40F size-exclusion chromatography.
†Cell equivalents (ce) = μl of purified *Pf*Tx extract (derived from size-exclusion chromatography) × the cell density used to produce that purified extract.

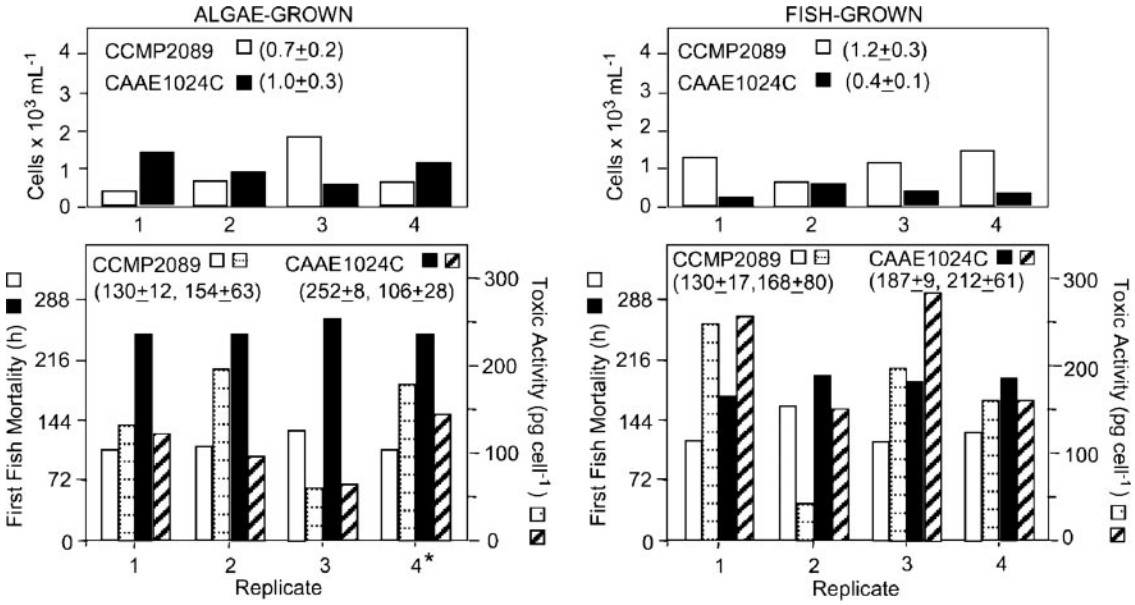

Fig. 2. *Pfiesteria* cell densities and cytotoxic activity in SFBs. (*Left*) SFBs of four subclones of algae-fed *P. shumwayae* CCMP2089 and CAAE1024C, showing water-column cell densities at the time of first fish mortality (*Upper*) and time of first fish mortality (left ordinate) and estimated cytotoxic activity per cell at time of second fish mortality (right ordinate) (*Lower*). (*Right*) Data from SFBs of four subclones previously fed fish (from subclone 4 at left).

areas showed moderate to severe swelling of Malpighian epithelial cells, with epithelial lifting/intraepidermal clefting, and occasional apoptotic cells. The underlying dermis was expanded multifocally by edema. After 4–12 h of exposure, fish had more severe skin erosions, but no time-dependent increase in severity was noted after 4 h. No remarkable gross lesions were discerned, and no remarkable microscopic abnormalities were found in controls. CAAE1024C had lost most toxicity by 2003 [14 ± 7% fish death in filtrate from SFBs (24)], compared with its toxicity in 2001 (80 ± 9% fish death in filtrate from SFBs with CAAE1024C) (Table 1 and Fig. 5*a*).

FMAs with Larval Fish. There was no larval fish death in negative controls, and only one feeding control strain tested (CAAE1036C) caused larval fish death in FMAs when allowed physical contact. At 72 h there was 83 ± 10% mortality at the highest cell density of this strain (3.881×10^4 cells per ml), 33 ± 10% mortality at 1.538×10^4 cells per ml, 22 ± 15% mortality at 8.74×10^3 cells per ml, 1 fish death (of 18 in total) at 3.64×10^3 cells per ml, and no fish mortality at lower cell densities. FMAs with toxic strains that were allowed physical contact with fish yielded variable data (Fig. 6). For *P. shumwayae* strain CAAE1024C, time to first fish death was more rapid at lower cell densities when subpopulations had been acclimated to algal prey before the FMAs; for CCMP2089, the reverse was observed. For both strains, higher cell densities were required for 24-h LC_{50}s than for 48- to 72-h LC_{50}s, and inoculum cell density was directly related to mortality (linear regression, $R^2 = 0.72$–0.98). Although each strain had been capable of killing juvenile tilapia in SFBs in ≤4 h (with *Pf*Tx presence and toxicity to fish confirmed),

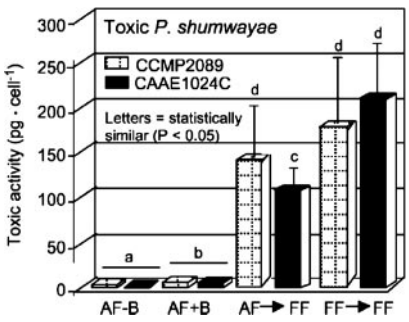

Fig. 3. Estimated cytotoxic activity per flagellated cell (examples, *P. shumwayae* CAAE1024C and CCMP2089), from testing purified *Pf*Tx extract residues with GH_4C_1 rat pituitary cells: AF-B, algae-fed, bacteria-free culture; AF+B, algae-fed culture plus bacteria (for both clones significantly higher than in bacteria-free culture; $P < 0.025$); AF→FF, previously algae-fed subclones taken into SFBs; and FF→FF, fish-fed subclones inoculated into additional SFBs.

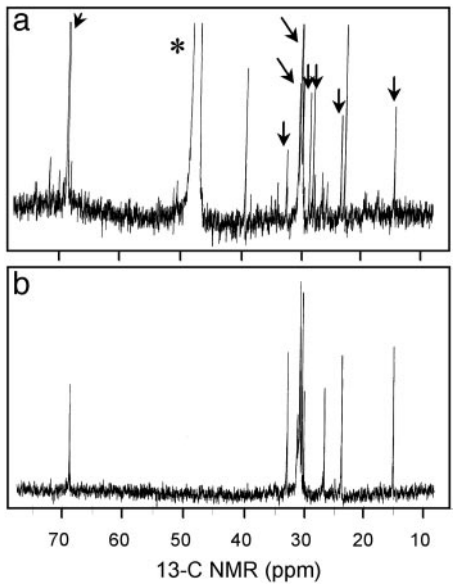

Fig. 4. ^{13}C NMR spectra of *Pf*Tx from *Pfiesteria*. (*a*) *Pf*Tx from *P. shumwayae* (CAAE1024C) after size exclusion (HW40F column) and subsequent passage through three sequential C_{18} columns using d4 methanol (*) as a solvent (supporting information. This material, from a fish-fed culture, shows multiple molecular species (congeners) compared with *b*; arrows indicate peaks in common with *b*. (*b*) *Pf*Tx from algae-fed *P. piscicida* (CCMP1832) after the same chromatographic steps used to generate *a*, followed by passage through a bidentate column using 100% HPLC-grade water as a solvent (supporting information).

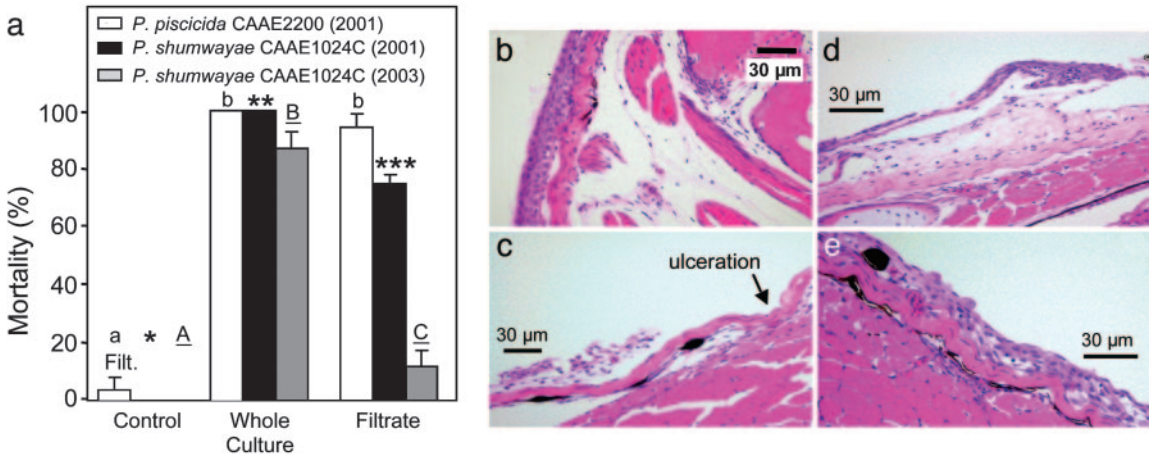

Fig. 5. Experiments with fish in whole cultures of *Pfiesteria* versus in culture filtrate. (*a*) Juvenile tilapia killed (percent) by whole cultures and filtrate of *P. shumwayae* CAAE1024C and *P. piscicida* CAAE2200 from positive SFBs (means ± 1 SE; $n = 10$ fish per treatment; repeated 6 time for whole cultures, 10 times for filtrates; letters and asterisks indicate significant differences ($P < 0.01$ to 0.05]; modified from ref. 11). *P. piscicida* and *P. shumwayae* 2000–2002 tests were compared with retests in 2003. The *P. piscicida* strain had lost toxicity (no fish death in whole-culture SFBs or filtrate; data not shown). The *P. shumwayae* strain had lower toxicity [caused some death in whole-culture SFBs and filtrate (24)]. (*b*) Skin and underlying musculature from an unexposed control fish (pectoral area, epithelial cells four to six cell layers thick). At the dermal–epidermal interface there was mild artifactual separation, accentuating the stratum spongiosum. Deep to the skin were normal subcutis, skeletal muscle bundles, and bone. (*c–e*) Eroded and ulcerated skin from fish exposed for 8 h to cell-free filtrate from toxic *P. shumwayae* CAAE1024C, showing necrosis and fragmentation of eroded epidermis (lifted from the basement membrane) (*c*); dermal–epidermal cleft (blister-like) with underlying area of severe dermal edema that expanded the stratum spongiosum (*d* and *e*). Necrosis of Malpighian epithelial cells in the epidermis and dermal edema suggest that these changes were not artifacts of handling, immersion fixation, or processing. (*e*) After 4 h, increased edema between Malpighian epithelial cells and spongiosis. Occasional apoptotic cells and intracellular edema are consistent with early epithelial injury.

and also killed some juvenile fish in filtrate from SFBs (this study and refs. 11 and 24), there was no larval fish death in FMAs unless these strains were allowed physical access (as in ref. 21).

Cytotoxicity. Cytotoxicity assays with GH_4C_1 cells (14), coupled with sequential chromatographic analyses, showed that strains of *Pfiesteria* spp. that tested positive for fish mortality and toxin in SFBs also were toxic in algae-fed cultures with or without extracellular bacteria, although bacteria and prey enhanced toxicity (e.g., Fig. 3). Cytotoxic activity per cell was significantly lower from algae-fed than fish-fed toxic *Pfiesteria* (Fig. 3; $P < 0.0001$). No cytotoxicity or fish toxicity was found from lipophilic fractions, or from media with or without bacteria without *Pfiesteria* (supporting information).

Fish Response to Purified *Pf*Tx Extract. *Pf*Tx extracts yielded similar ^{13}C NMR spectra for *P. piscicida* and *P. shumwayae*, and they consistently caused larval fish mortality, whereas there was no mortality of control fish exposed to carrier solvent alone (Table 1 and Fig. 4). Controls did not contain *Pf*Tx, except for feeding control NON-IND *Pfiesteria* CCMP1832 (Fig. 4). Although that strain did not kill fish in SFBs or FMAs, *Pf*Tx extracted from a large culture volume rapidly killed larval fish (Table 1). Thus, some NON-IND *Pfiesteria* strains produce this ichthyotoxin at very low levels, whereas others produce undetectable *Pf*Tx. The picograms of toxic extract per cell data for *Pfiesteria* were comparable to published data for other toxic algae (supporting information). Densities of *Pfiesteria*/*Pfiesteria*-like cells [generally $\geq 3 \times 10^2$ to 1.4×10^3 cells per ml (1)] or of *Pfiesteria* cells [estimated by semiquantitative PCR at ≈300–500 *P. piscicida* cells per ml (supporting information)] in fish kills involving toxic *Pfiesteria* were more than cell densities required to produce sufficient *Pf*Tx to kill fish in these assays (Table 1).

Discussion

This study demonstrates several points about *Pfiesteria* and, more generally, toxic dinoflagellates. Most importantly, it underscores the high variability in toxicity expression among strains within a given toxic algal species (reviewed in ref. 6). Also, *Pfiesteria* toxicity and its detection depended on the assay conditions and prey type. SFBs, originally standardized with actively toxic *Pfiesteria* strains (5, 10, 14), were reliable in detecting toxic *Pfiesteria*, based on confirmation of *Pf*Tx, but cytotoxicity assays and toxin assays were more sensitive for detecting *Pf*Tx than fish death in SFBs or FMAs. Because of the variability in toxin release shown by toxic strains of *Pfiesteria* spp. under different conditions, use of fish assays to form conclusions about presence versus absence of toxin production by a given strain should be accompanied by appropriate *Pf*Tx detection assays. Other important findings are confirmation of toxin production as indicated by cytotoxic activity by filtrate from *Pfiesteria* toxic strains in bacteria-free media, and of strain-dependent stimulation of *Pfiesteria* toxicity by live fish. In support of previous research (1, 11, 15), *Pf*Tx was also shown to be lethal to fish and toxic to mammals, and fish mortality from *Pfiesteria* was exacerbated by physical attack during predation (6). Physical attack by *Pfiesteria* was earlier hypothesized to facilitate *Pf*Tx entry into fish tissues and to generally weaken fish prey (6).

CCMP2089, like various other *Pfiesteria* spp. strains (1, 11, 14, 16), was toxic to fish and to mammalian cells, and at environmentally relevant cell densities. We suggest that previous conclusions that this strain did not produce exotoxin (21–23) were based on large-scale fish assay conditions that were not conducive to *Pfiesteria* toxicity expression [e.g., pH as low as 5.6, ammonia as high as 30 mg/liter (refs. 21 and 23 and methods ref. 35, versus methods for SFBs in ref. 10)], and/or tests for toxin/toxicity that were insufficient (21–23). Moreover, algae-fed CCMP2089, used as a culture source for FMAs in refs. 21 and 22, and an older strain used in ref. 25 (≈7 years in culture), would have had very low *Pf*Tx activity, based on the data from this study. The present findings indicate that lack of fish death in filtrate of FMAs (used in refs. 21 and 22) and SFBs (used in ref. 25) is not a sufficiently sensitive indicator of toxin presence/absence. Here, when filtrates from CCMP2089 were analyzed for *Pf*Tx by using cytotoxicity and ichthyotoxin assays, *Pf*Tx was confirmed. Thus, in FMAs, *P. shumwayae* strain CCMP2089 produced exotoxin that would have contributed to larval fish stress, but it did not produce sufficient exotoxin to kill fish

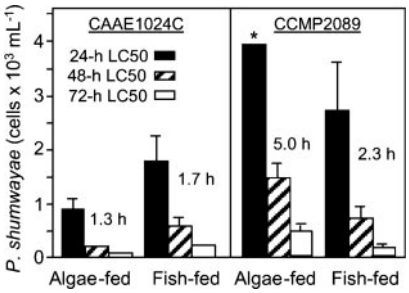

Fig. 6. LC_{50} values (24, 48, and 72 h) in FMAs of algae- and (juvenile) fish-fed *P. shumway*ae allowed physical contact with larval fish, comparing two toxic clones (means + 95% confidence intervals; h indicates time to first fish death; *, 24-h LC_{50} was greater than the highest density evaluated).

as a toxin effect alone (21–23) or, in some tests, only occasionally did so (24).

The assumption previously had also been made (23), without precedent (4), that toxicity in all dinoflagellates is inexorably linked to polyketide synthase (PKS) genes. An insensitive molecular assay was used (23), given the high heterogeneity among known PKS genes (36), and when a PKS gene in strain CCMP2089 was not detected, it was concluded that *Pfiesteria* spp. are not toxigenic (23). Yet, when a degenerate nonribosomal peptide synthase primer set (37) was used in the same study, a PKS-encoding gene accidentally *was* amplified in CCMP2089 (23). A related study (21) misstated that fish lesions had been attributed only to *Pfiesteria* in *Pfiesteria*-related fish kills (in refs. 1 and 38) and concluded (21), solely on the basis of strain CCMP2089, that *Pfiesteria* cannot cause fish lesions as a toxic effect. Here, however, filtrate from a toxic strain *did* cause epidermal lesions in fish with toxin involvement, without physical attack by *Pfiesteria* cells. It was also previously asserted (23) that the only bioactive substance found in *Pfiesteria* cultures was a lipophilic plasticizer artifact from synthetic salts (supporting information). Yet, the reference cited in support of that assertion (14) *had* described isolation of hydrophilic *Pf*Tx. As shown by comparison of this study with refs. 21–23 and 25, recognition of variability in toxicity expression and other traits among strains (6), use of culture conditions conducive to toxicity expression (5, 10, 11), and appropriate assays for toxin will remain critical in forming sound insights about toxicity of harmful algal species and genera.

Many questions remain about controls on toxicity expression in *Pfiesteria*, such as the role of bacteria. *Pfiesteria* is capable of producing toxin in bacteria-free media, yet *Pfiesteria* toxin production was much higher in the presence of extracellular bacteria plus algal or fish prey. Similar results have been found for other toxic algae (e.g., ref. 39). Work with *Pfiesteria* in other SFBs has indicated differences in the bacteria flora (K.J.C. and S.C.C., unpublished data). Dynamic interactions between *Pfiesteria* and bacteria, perhaps including endosymbiotic bacteria, may play a role in stimulating *Pfiesteria* toxin production and/or release. For example, bacterial metabolism may convert exudates from *Pfiesteria* cells into toxic compounds, or enzymes released from bacteria may facilitate release of toxin from *Pfiesteria* cells. Bacteria may also play a role in loss of toxicity in *Pfiesteria* toxic strains over time in culture, as observed for other toxic algae (reviewed in ref. 1). Determination of molecular and environmental controls on its toxin production and release versus retention will further advance scientific understanding about toxic *Pfiesteria*.

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