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Metal Complexes and Free Radical Toxins Produced by *Pfiesteria piscicida*

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Metal-containing organic toxins produced by *Pfiesteria piscicida* were characterized, for the first time, by corroborating data obtained from five distinct instrumental methods: nuclear magnetic resonance spectroscopy (NMR), inductively coupled plasma mass spectrometry (ICP−MS), liquid chromatography particle beam glow discharge mass spectrometry (LC/PB-GDMS), electron paramagnetic resonance spectroscopy (EPR), and X-ray absorption spectroscopy (XAS). The high toxicity of the metal-containing toxins is due to metal-mediated free radical production. This mode of activity explains the toxicity of *Pfiesteria*, as well as previously reported difficulty in observing the molecular target, due to the ephemeral nature of radical species. The toxins are highly labile in purified form, maintaining activity for only 2−5 days before all activity is lost. The multiple toxin congeners in active extracts are also susceptible to decomposition in the presence of white light, pH variations, and prolonged heat. These findings represent the first formal isolation and characterization of a radical forming toxic organic-ligated metal complex isolated from estuarine/marine dinoflagellates. These findings add to an increased understanding regarding the active role of metals interacting with biological systems in the estuarine environment, as well as their links and implications to human health.

Introduction

Association of the heterotrophic estuarine dinoflagellate *Pfiesteria* (Dinophyceae) with large-scale toxic effects such as major fish kills and impacts on mammalian health has led to intensive and sometimes controversial research (*1*−*4*). Sporadic fish kills (*5*), human memory loss (*6*), and other environmental and human health-related phenomena associated with *Pfiesteria* blooms have resulted in large economic consequences in tourism, commercial fishing, and trophic impacts (*7*, *8*). A hydrophilic *Pfiesteria* toxin(s), isolated in 1997 but uncharacterized (*9*, *10*), was shown to impact fish and mammalian health (*4*, *8*−*11*).

Understanding the regulation of toxin production in *Pfiesteria* has been hindered because the environmental/molecular factors that influence toxin production are poorly understood, as is the case for many toxigenic microalgae. For example, although bacteria-free *Pfiesteria* can produce small amounts of toxin (*4*), bacteria and other organisms used as prey by *Pfiesteria* significantly enhance toxin production through unknown mechanisms (*4*) as in certain other toxigenic algae (*12*−*14*). *Pfiesteria* toxicity varies substantially both in nature and in culture (*4*, *5*, *8*). Major intraspecific variability in toxin production has been demonstrated in toxigenic microalgal species including *Pfiesteria* spp., as well as enhanced expression of toxicity under certain environmental conditions (*12*, *15*−*17*). Thus, microalgal toxicity may not be a fixed component of metabolism, providing rationale for the previously reported "on/off" nature of toxin production (*12*, *18*−*20*). Blooms of *Pfiesteria* have occurred in turbid eutrophic areas where nitrogen, phosphorus, trace metal concentrations/species, organic matter, and light can vary substantially (*5*, *8*, *21*). Nutrient availability, composition, or form, and interactions of these factors (e.g., nutrient ratios, light) can significantly affect algal growth and toxin production (*8*, *22*, *23*).

In addition to these complexities, unique challenges have been confronted in characterizing *Pfiesteria* toxins, especially the marked instability of the toxins in purified form (*10*; and Supporting Information for this manuscript). Here, using five distinct analytical techniques, we identified and characterized metal-containing organic toxins produced by *Pfiesteria piscicida*. We report the nature and a structure of this novel class of marine toxins.

Materials and Methods

Establishment of a non-axenic, algal-fed clonal culture (*P. piscicida* CCMP1921), growth conditions, harvest of the organisms, and methods of toxin isolation were followed as previously described (*4, 10,* and Supporting Information). The cytotoxicity bioassay with rat pituitary cells (GH_4C_1 cell line; *10*) was chosen for screening because of its observed reactivity in parallel with toxicity to sheepshead minnows (*Cyprinodon variegatus*) (*4*, *10*). As a blank control, 90 L of the same filtered natural seawater (salinity 37 ppt) used as culture media were prepared and analyzed (*4*, *10*).

Due to molecular instability (Supporting Information), toxin purification from *Pfiesteria* cultures was conducted rapidly (2−3 days) to enable molecular structural work prior to degradation. Decomposition and subsequent loss of bioactivity were clearly evident in the toxicity assays, MS, and NMR spectra used to screen fractionated samples (*4*, and Supporting Information). Preliminary data suggested that natural white light (presumably short wavelengths) caused rapid decomposition of the toxins and loss of activity, as known for the toxins of certain other microalgae (e.g., *Prymnesium parvum*; *33*−*35*). Therefore, all chromatography was completed under red light to enhance molecular stability.

The following five analytical techniques were used to identify and characterize *Pfiesteria* toxins: (*i*) ^{13}C NMR

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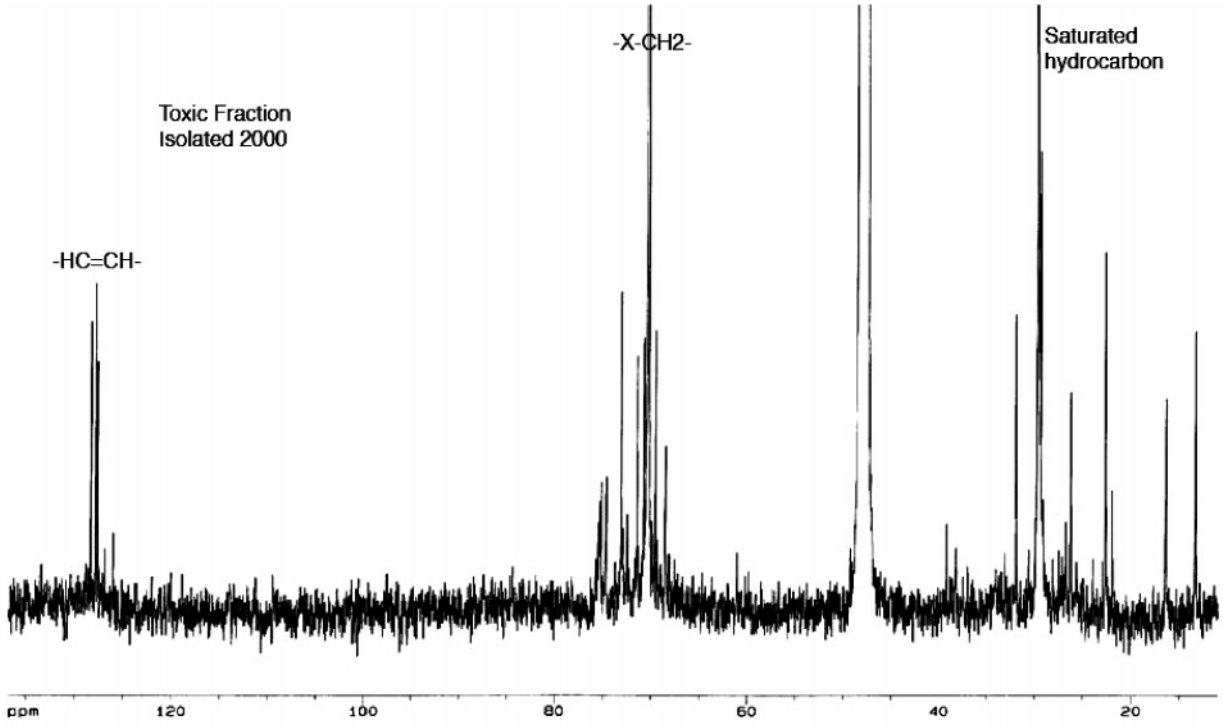

FIGURE 1. Characteristic ^{1}H decoupled ^{13}C NMR spectrum of an active fraction isolated and purified from 90 L of *P. piscicida* culture (d_4-MeOH).

spectral analysis of active fractions provided clues on the basic molecular skeleton of the observed suite of toxins (*24*). Electrospray ionization−mass spectrometry (ESI−MS) generated data for the mass range of the toxic substances (*25*) including information about molecular symmetry, since the mass indicated by the MS data was more than 2-fold higher than that predicted by the NMR experiments. (*ii*) Inductively coupled plasma mass spectrometry (ICP−MS) was used to analyze purified toxin fractions for total metal content (*26*). (*iii*) Toxin samples were analyzed by liquid chromatography particle beam glow discharge mass spectrometry (LC/PB−GDMS; *27*) to determine the presence of organic ligands bound to a metal core. (*iv*) A duplicate set of freshly prepared active toxin samples was analyzed using electron paramagnetic resonance (EPR) spectroscopy/spin trapping in an effort to demonstrate and identify a free radical species that was metal-mediated in its formation. The spin trap 2-ethoxycarbonyl-2-methyl-pyrroline-*N*-oxide (EMPO) has been used successfully to trap and identify oxygen, carbon, sulfur-based, and other short-lived radical species (*28*−*31*). Samples of the active toxins were treated with 20 mM EMPO immediately after re-suspending the dry, chromatographically purified samples in Chelex-treated water. The mixture of toxin and spin trap was transferred to an EPR flat cell sample holder and placed in the spectrometer cavity. The initial spectrum was measured within 3 min of the addition of the spin trap and every 30 min thereafter. Samples were either exposed to white light or protected from direct light following re-suspension and throughout data collection. (*v*) Finally, freshly prepared toxin samples were subjected to X-ray absorption spectroscopy (XAS) (*32*). This technique provided information about the local (<4 Å) ligand environment and the oxidation state of metal centers in inorganic cofactors or compounds.

Results

Nuclear Magnetic Resonance. NMR analysis of purified *Pfiesteria* toxins demonstrated general characteristics of hydrocarbons bound to an undefined heteroatom(s) (Figure 1). The NMR spectra of other active fractions (see Supporting Information) defined relatively simple molecular skeletons. In addition, they showed that active extracts contain multiple toxin congeners, demonstrated by multiple sets of similar NMR signals. This phenomenon is not surprising, as congeneric metabolite production is known in dinoflagellate biochemistry (*12*, *22*). This finding has important consequences, however, as it highlights how local environmental conditions may influence the toxin biosynthesis, profile, and extracellular stability.

Initially these compounds were dismissed as artifacts of isolation, yet isolation of the same compounds repeated over a 6-year period could not be ignored (see Supporting Information). To confirm that the toxic compounds seen in the NMR spectra were not simply a result of large-scale culture workup, controls (seawater, algal food source) were taken through identical chromatographic processes and determined to be nonactive. Furthermore, characteristic ^{13}C NMR resonances of toxins similar to those shown in Figure 1 were not observed without *Pfiesteria* culture. These experimental data confirmed that the toxins observed were indeed produced by *Pfiesteria piscicida* and not a natural seawater or process artifact. NMR and mass spectra demonstrated significant molecular decomposition of the active molecules over short periods of instrument time, highlighting our previous observations regarding toxin lability (Supporting Information).

The ^{13}C NMR spectra fit structures that would be expected to exhibit a highly lipophilic nature, rather than the observed water or methanol solubility. Figure 2 shows the ^{13}C NMR spectrum of a toxic fraction after several chromatographic purification steps. This congener originated from the parent fraction shown in Figure 1. There was a loss of double bond resonances (Figure 2 versus Figure 1), but the molecule retained the typical heteroatom (X) bound methylene carbon ($-CH_2-$, @70 ppm) as well as the typical highly saturated portion of the toxic molecules.

Metal Determination/Identification: ICP−MS and LC/PB−GDMS Analyses. High concentrations of both Fe and Cu were measured in active fractions using ICP−MS, with

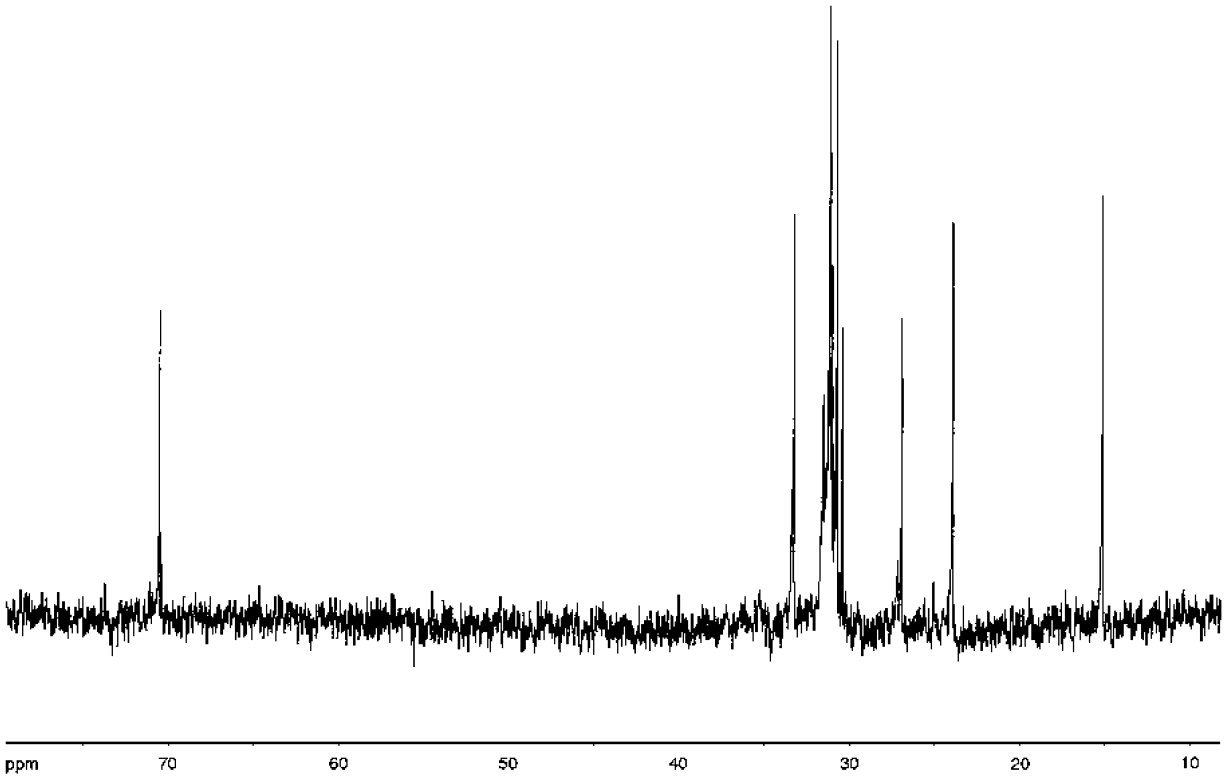

FIGURE 2. ^{1}H decoupled ^{13}C NMR spectra of a highly purified toxic fraction from *P. piscicida*, after several chromatographic purification steps. This spectrum defines the heteroatom bound carbon and remaining saturated hydrocarbon nature of the toxic molecule.

Cu typically the predominant metal (*Pfiesteria* toxin (*Pftx*): [Cu], 17.0 μg/g; [Fe], 1.5 μg/g). ICP−MS analysis of the blank control sample demonstrated significantly reduced concentrations of Cu compared to the purified toxic fractions (*control*: [Cu], 2.5 μg/g; [Fe], 0.5 μg/g), see Supporting Information). In addition, the ICP−MS data demonstrated that sulfur was present in the active fractions, providing data support that sulfur represented the heteroatom predicted in the ^{13}C NMR spectra. Our attention turned to the significant concentrations of Cu present in these active samples, as a likely metal cofactor involved in toxicity. The importance, presence, and role of iron are not yet understood nor addressed in this report, although the intimate association between Cu and Fe with respect to the biogeochemical processes affecting trace metals in estuarine environments is a topic of considerable interest and research (*42*, *43*).

LC/PB−GDMS confirmed that the *Pftx* had organic ligands that contain a series of methylene ($-CH_2-$) groups and that the molecule was rapidly decomposing. Analysis of toxin samples over a 4-h experimental period demonstrated several shifts in peak intensities. In addition, mass spectral analysis of the active compound using this technique revealed several fragmentation peaks demonstrating the loss of CH_2. These results confirmed both the presence and decomposition (see Supporting Information) of a saturated hydrocarbon species.

XAS. X-ray absorption spectroscopy confirmed the presence of Cu in the *Pfiesteria*-derived toxin samples. The near-edge spectrum indicated the presence of both Cu(II) and Cu(I). The edge energy of an illuminated sample was slightly lower than the edge energy of an identical sample maintained in darkness, suggesting a possible role for Cu in the light-induced formation of free radicals (see below). Copper extended X−ray absorption fine structure (EXAFS) was also carried out on an illuminated toxin sample. In addition to first shell interactions at 2.0 and 2.4 Å from low Z (primarily oxygen- and nitrogen-containing) ligands, the Fourier transform (FT) of these data indicated that a large scatterer was present at ~2.6 Å. This interaction can be modeled with either a Cu−S interaction or a Cu−Cu interaction, with the Cu−S interaction providing a slightly better fit to the experimental data (Supporting Information). These data are consistent with the ICP−MS, NMR, and EPR (below), suggesting the presence of sulfur as the heteroatom linking the carbon ligands to the metal core. The presence of long-range interactions observed is a clear indication that the Cu is bound by complex ligand(s), and eliminated the possibility that copper was simply present as aqua-copper in the toxin samples.

Radical Toxicity. The observed photoreactivity suggested that the metal-containing toxins are responsible for generating toxic free radical species, likely via redox cycling of the metal center. A metal−ligand organic-radical would explain the high toxicity and provide insights into many of the controversial findings regarding toxin isolation. Precedence exists for similar toxins isolated from terrestrial sources, dependent on metal ions for their activity (*44*−*46*).

EPR. Similarly shaped spectra developed for light-exposed and light-protected samples over the first 4 h of exposure to EMPO (Figure 3A and B), persisting up to 20 h. The light-protected spectrum was significantly reduced in observed intensity, however, indicating much less radical formation in the absence of light. A chromatographically purified seawater control sample was also exposed to light after the addition of EMPO, and demonstrated none of the spectral features of the toxin-containing samples (Figure 3C). This is an important observation because ICP−MS and EPR spectroscopy of the samples (EPR data not shown) indicated the presence of copper in both the toxin samples and, to a much lesser extent, in the control sample. If the EPR signal had been due exclusively to a metal/oxygen reaction, then the control seawater sample would have had the same (although diminished) spectrum as that of the active samples.

The signal observed in both the light-exposed and light-protected toxin samples was due to the presence of an organic

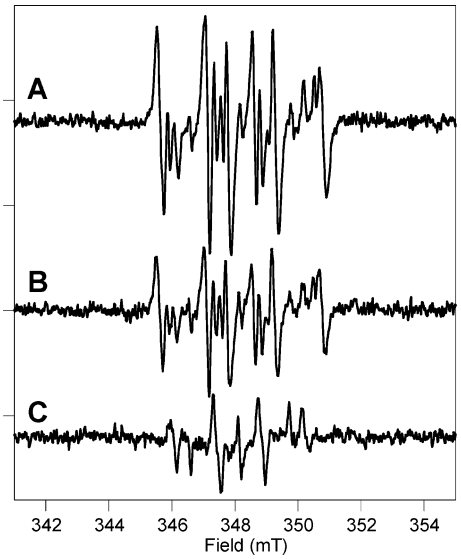

FIGURE 3. Radical signal as monitored by EPR spin-trapping after 4 h for (A) a light-exposed sample, (B) a light-protected sample, and (C) the light-exposed seawater control. Samples were re-suspended in 300 μL of chelex-treated water, and the spin trap EMPO was added to a final concentration of 20 mM, prior to being transferred to a EPR flat cell, either under room fluorescent light (A and C) or in nearly complete darkness (B). Initial spectra were collected 3 min after adding trap; subsequent spectra were collected every 30 min. The light-exposed samples were continually exposed to white light while in the EPR cavity, whereas the light-protected samples did not receive light exposure.

radical (carbon-centered) species. This radical species was identified through simulation of both a 4-h sample and a 16-h sample (*47*). While simulation of these spectra indicated a complex mixture of species, major constituents of the signal are a carbon-centered radical ($A_N = 1.49$ mT, $A_H^\beta = 2.14$ mT) and potentially either a carbonate- or thiol-based radical species ($A_N = 1.47$ mT, $A_H^\beta = 1.70$ mT and $A_N = 1.41$ mT, $A_H^\beta = 1.28$ mT). A thiol-based radical would be consistent with a sulfur heteroatom present in the ligand to the copper in a copper−toxin complex. A copper complex of this toxin likely results in 1-electron chemistry, either directly on the ligand or with oxygen, resulting in a C-centered radical species that can then undergo the rapid decomposition observed in other spectral techniques. It is also likely that the copper center undergoes redox cycling, thus providing a continuous source of radical species measured over the course of the EPR experiment. The role of the radical species formation in the resulting decomposition of the toxins, and therefore their rapid but short-lived efficacy, was monitored by time- and light-dependent EPR spectroscopy (Figure 4). The initial spectra of both the light-exposed and light-protected toxin samples are similar, but very different from the spectra that formed after 1 h. After 4 h the spectrum of the light-exposed toxin sample had the shape that it maintained throughout the remainder of the experiment. The intensity of the signal for the light-exposed sample continued to increase to a maximum at 8 h, and then declined. Similarly, the shape of the dark sample was stable after ~6 h.

By integrating the signal intensity at each time point, the relative signal intensity of each species and the seawater control were monitored over a 20-h experiment (Figure 5). The light-exposed sample significantly increased intensity over the first 8 h and then declined to its initial intensity, while the dark sample and the seawater control steadily increased at a reduced rate. While the radical signals were normalized to the integrated values of the seawater controls at $t = 0$ h, importantly, the controls had a spectral signature (shape) distinct from that derived from toxin samples, as shown in Figure 5. During the time course of the experiment, the intensity (not the shape) of the control spectrum increased by less than 40%. The dramatically different shape of the curve of the radical signal for the light-exposed toxin sample is interesting in view of the apparent photodegradation of *Pfiesteria* toxins. The rapid increase and decline of the trapped radical species would be consistent with the sometimes-rapid activity/toxicity of *Pfiesteria* (*8*), likely mediated by metal redox cycling, and with the reported rapid decomposition of the toxin (*8*) as observed by NMR and MS analysis.

Discussion

In this study, toxins produced by *Pfiesteria piscicida* were chemically characterized by corroborating data obtained from several distinct instrumental methods. The toxins examined from *P. piscicida* are ligated copper compounds. Corroborative evidence of the toxin's general structure was determined by combining analysis with the spectral data

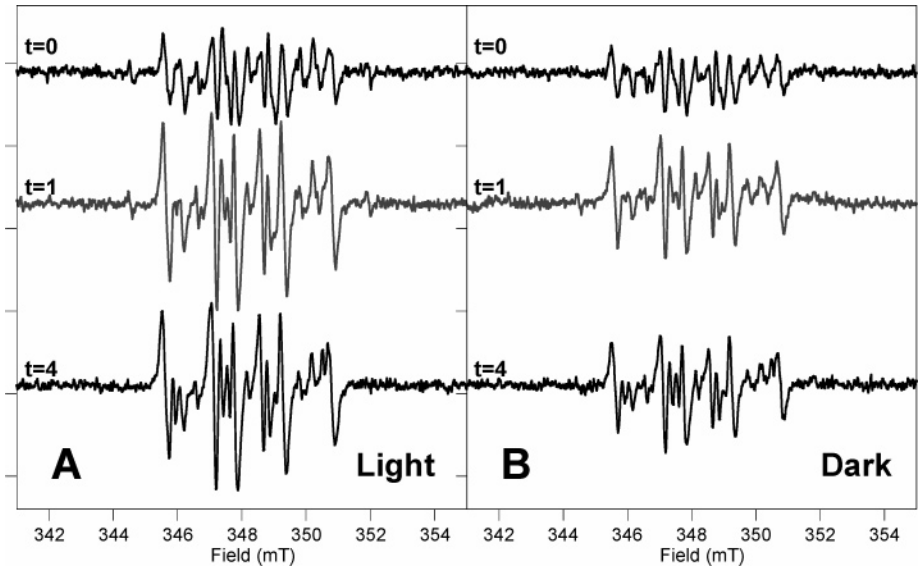

FIGURE 4. Light-dependent change in radical signal, monitored by EPR spin-trapping over 4 h. (A) Set of spectra showing an increase of the EPR signal upon exposure of the toxin sample to 20 mM EMPO and white light. (B) When protected from light, a similar change in signal occurred, but much less of the radical was formed and at a slower rate.

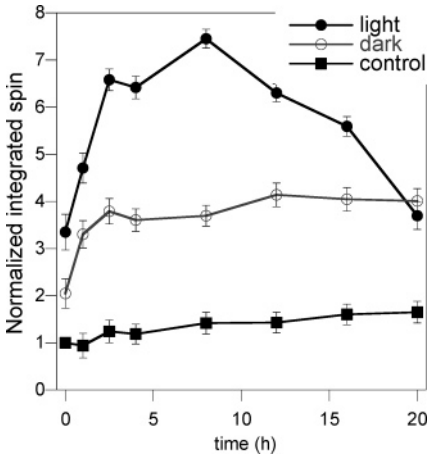

FIGURE 5. Normalized spin quantification of the light-exposed (●) and light-protected (○) toxin samples and the light-exposed seawater control (■) over time. Initial ($t = 0$) spectra were collected 3 min after adding the trap; subsequent spectra were collected every 30 min. Integrated areas were normalized to the $t = 0$ control samples (means; error bars indicate ranges of integration measurements at each time point; $n = 3$).

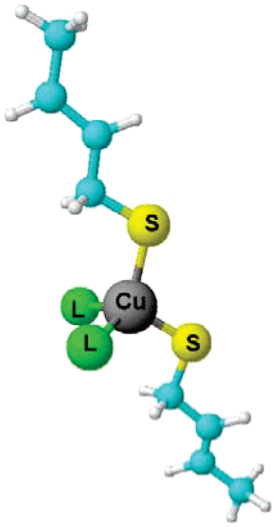

FIGURE 6. Model of the *Pfiesteria* toxins, showing the key features observed in this study. The drawing shows a Cu ion (black) in a distorted tetrahedral environment (suitable for a Cu(I) or Cu(II) ion) complexed by at least two thiol-containing organic ligands (sulfur, yellow; carbon, blue). The green spheres represent either terminal ligands to the Cu ion, additional symmetry related organic ligands, or bridging atoms in a coupled dinuclear complex. The thiol-containing ligand represents the minimum structure as determined by ^{13}C NMR spectroscopy. The extent of higher symmetry coordination (or H_2O coordination) or coordination geometry was not addressed in this study.

obtained by NMR, ICP−MS, AA, EPR, and XAS. A generic structure for this class of compounds based on our data is shown in Figure 6, where the copper ion is complexed by two sulfur atoms from the organic ligands (shown in blue).

The ligand set described incorporates: (*i*) the ^{13}C NMR data, (*ii*) the necessity for Cu with sulfur ligation, and (*iii*) a geometry that would be suitable for a Cu(I) ion. The oxidation state of the copper ion(s) in the toxin cannot be definitively assigned. EPR and XANES indicate some Cu(II) present in the toxic sample. This is either a result of the redox cycling of a mononuclear Cu(I) form of the active toxin, or an active species that contains antiferromagnetically coupled dinuclear Cu(II) ions. One of these examples is likely to explain the measured diamagnetic NMR spectrum of the complex.

The variation of the ligands surrounding the metal ion would certainly depend on the organic matter present in the surrounding environment. The toxicity of these metal-containing organic compounds is due to carbon−sulfur−metal based radical production. This mode of activity explains the high toxicity as well as the difficulty in observing the parent toxin molecules due to the short-lived, reactive nature of the free radicals. The complex estuarine speciation and biogeochemistry of trace metals coupled with alterations in salinity, mineralization, the presence and nature of organic material, and seasonal variations of temperature, light intensity, and nutrients all would be expected to influence the formation and concentration of these metal-containing toxins (*43*). Free radical production has been implicated in an array of biologically damaging functions spanning from DNA damage and protein oxidation to lipid peroxidation (*48*, *49*). Over-production of free-radical species can activate various signal transduction pathways, including apoptoic pathways leading to cell death. We hypothesize that the rapid, free-radical-mediated toxicity of *Pfiesteria* toxins may occur via production of a redox-cycling metal center and free radical(s) that can lead to specific reactions with "pro-toxins" which, in turn, can produce more active toxic species.

Similar inferences have been made for other toxins (*36*−*38*, and Supporting Information). For example, metal-mediated toxic activity involving *Prymnesium parvum* has been proposed (*39*, *40*), wherein a synergistic relationship between the ichthyotoxin and various trace metals reduces the amount of toxin needed to cause mortality. Our results with *Pfiesteria* toxins parallel these findings. Such a molecular system would explain many of the variant findings from researchers working on *Pfiesteria* toxins (*4*, *10*, *41*). It would also explain inconsistencies in reports of toxin production by *Pfiesteria*, especially difficulty in obtaining toxin and/or toxic material (e.g., *41*) when appropriate environmental and handling conditions were not met to isolate or stabilize short-lived free radical species.

The *Pfiesteria* toxins examined in this study are susceptible to decomposition in the presence of white light, pH variations, and prolonged heat. It is likely that sunlight and metal exposure are the two primary environmental factors that combine to initiate *P. piscicida* toxicity during toxic algal blooms. Light exposure could initiate redox cycling of the metal ion(s) resulting in radical formation and release of the toxin species. It is this photochemistry that appears to be responsible for both generation of the radical toxic species as well as the eventual toxin disappearance. The presence of multiple toxin congeners (Supporting Information) in the active extracts seriously complicated chromatographic purifications. Different findings reported on *Pfiesteria* toxicity (*4*, *10*, *41*) likely have their basis in these problematic areas and may, in part, simply be echoing historical research on other algal toxins (*8*, *15*−*18*, *33*, *50*−55). Some analogous difficulties have been reported, for example, in toxigenic cyanobacteria and diatoms (*56*, *57*). *Pfiesteria* spp. have been shown to produce exotoxin(s) that are excreted or secreted into the surrounding marine environment (*4*). Small concentrations can be found intracellularly, but toxic activity is predominantly isolated from culture media (*4*). Although relatively uncommon, exotoxins have also been reported from other microalgae such as phytoflagellates *Prymesium parvum* (Haptophyceae) (*39*, *40*, *50*) and *Heterosigma akashiwo* (Raphidophyceae) (*36*). Several mechanistic similarities are evident among these unrelated species, including toxins that are bioactive against mammalian cell lines testing positive in their ability to alter cytosolic free calcium (*10*, *12*, *18*, *36*, *39*, *40*, *54*).

This study represents, to our knowledge, the first formal isolation and characterization of labile toxic copper complexes isolated from cultures of an estuarine microalga. Solving the complex nature of *Pfiesteria* toxins required an interdisciplinary approach using a battery of sophisticated chemical and physical techniques. These data highlight the involvement of naturally occurring coordinated metals and associated free radical production in the toxicity of *P. piscicida*. The speciation of Cu in estuarine water acts as a significant cofactor influencing the production of toxin by *P. piscicida*. However, the metal speciation would be expected to depend upon many environmental factors characteristic of estuarine waters. Considering reports of other estuarine microorganisms with toxins similar to those reported for *Pfiesteria*, we suggest that the findings from this research may represent a general phenomenon common to metal-rich estuaries, and a new paradigm for toxin research involving metal mediated bio-toxicity within brackish and marine environments.

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Supporting Information Available

Information regarding the techniques and data generated in this report, and further instrumental descriptions and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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