

Current Progress in Isolation and Characterization of Toxins Isolated from *Pfiesteria piscicida*

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The isolation and partial purification of toxic substances derived from *Pfiesteria piscicida* Steidinger & Burkholder extracts is described. Four distinct bioassay systems were used to monitor bioactivity of the *P. piscicida* extracts, including a high throughput cell cytotoxicity assay and a reporter gene assay as well as assays using brine shrimp and fish. Using these bioassays to guide fractionation, we have isolated two distinct, active fractions from *Pfiesteria* culture medium and cell mass extracts on the basis of their solubility characteristics. We have identified and characterized a bioactive lipophilic substance from *Pfiesteria*-derived extracts as di(2-ethylhexyl)phthalate, a commonly used plasticizer. The source of this typically man-made substance has been identified as originating from Instant Ocean (Aquarium Systems, Mentor, OH, USA), a commercially available seawater salt mixture used to prepare our mass culture growth medium. We have developed chromatographic methodology to isolate a bioactive polar compound isolated from extracts of *Pfiesteria* culture and presently report the characterization of the activity of this substance. The molecular structural analysis of the polar active component(s) using mass spectrometry and nuclear magnetic resonance spectroscopy is currently under way. **Key words:** assay, chromatography, GH₄C₁, *Pfiesteria piscicida*, toxin bioassay. — *Environ Health Perspect* 109(suppl 5):739–743 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/739-743moeller/abstract.html>

Pfiesteria piscicida Steidinger & Burkholder is a dinoflagellate that has been the cause of serious concerns regarding both environmental and human health since its discovery in the Albemarle–Pamlico estuarine ecosystem in North Carolina and in the Chesapeake Bay in Maryland (1). Subsequent discovery and positive identification of this organism in other eastern seaboard states has only heightened these concerns (2). Information on the organism and its growth patterns and life cycle has been published previously (3). Still, there remains very little chemical information regarding the biologically active compounds produced by or associated with the organism. There is evidence of neurotoxicity as well as causality for severe skin damage (1,4–6). However, a direct link of *Pfiesteria*-produced metabolites to these symptoms has been hampered by the inability of investigators to isolate and chemically characterize such compounds from cultured algae.

Chemical isolation from *Pfiesteria* cultures has proven to be a difficult challenge for several reasons. First, in any natural product isolation scheme, there is a paucity of literature upon which to base an experimental protocol. Research scientists are constrained to use anecdotal reports, secondary literature, and even personal observations as their guide. Second, in the case of *Pfiesteria*, the matters are complicated further because biological activity is often lost over short periods of time, presumably because oxidative degradation, assay misfunction, or perhaps permanent binding to

chromatographic solid support media. This behavior appears to be a departure from the typical behavior of toxins derived from other dinoflagellates. Many of these toxins, such as okadaic acid, saxitoxin, and the brevetoxins, can often be stabilized or are stable enough of their own accord to isolate, purify, and market commercially (7). Third, *P. piscicida* appears to secrete or release (8) the majority of its active substance(s) rather than sequestering them internally, as is more usual in dinoflagellates. This external release of metabolites exacerbates the isolation and purification problem. Extraction processes designed to isolate highly polar organic compounds from natural seawater (SW) or highly ionic culture medium often have poor reproducibility because of the multiple and constantly varying matrices present. These matrices can be in the form of natural oils, agricultural runoff, petroleum wastes, and so forth. Many organic compounds can be found in SW at any given time. In the case of laboratory *Pfiesteria* culture, purification of active metabolites is just as complicated, as currently used conditions require the presence of fish to induce toxicity. Extraction processes dealing with *P. piscicida* must deal with everything associated with the fish, including microorganisms and the secondary metabolites they may produce. Such a complexity of varying matrix with potential of a short-lived biological activity can prove quite a challenge to a natural products isolation program.

Adding to the difficulty of isolating and purifying toxins from *Pfiesteria* are the

divergent leads and implications found in the literature on isolation of biologically active metabolites from this alga. In one of the earliest abstracts from the Society of Toxicology on *P. piscicida* metabolites, the major toxic activity was described as hydrophobic (lipophilic) (9). This report described highly lipophilic active components with molecular weights of 16,000–20,000 atomic mass units (amu) being excreted into the water. This information would suggest that the target molecule(s) are proteinaceous, requiring very different purification schemes from those used for smaller secondary metabolites. In both cases, the collective results would target the lipophilic portions of *Pfiesteria* extract as the source of activity and in turn would indicate a purification protocol very different from that used for larger proteins or polar metabolites. In his article in *Science News*, Mlot recorded Baden and Rein's support for a lipophilic active substance (10). They reported that this crystalline lipophilic material demonstrated the same symptoms, including notable skin damage, as those found in wild *Pfiesteria* events.

In contrast, our own research has yielded *Pfiesteria*-produced biological activity from only the polar extracts as opposed to the lipophilic-soluble active metabolites. This

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active polar component adds another dimension to the purification scheme, making the situation appear even more challenging. Mass spectrometric analyses of partially purified active extracts have yielded no mass data over 500 amu, disallowing any proteins in our findings. The divergent paths listed above describe a situation that covers the entire range of polarity as well as molecular size and type (proteins to small secondary metabolites). This divergence highlights the critical need to ensure that all involved laboratories are using similar extracts derived from the same organism. The current situation may lead one to believe that different strains or perhaps completely different organisms are being studied under the name *P. piscicida*. The reported data and findings also highlight the great complexity surrounding basic research on *P. piscicida* and its putative toxins.

The intent of this article is to provide current information on putative toxins on the basis of our own research findings related to the toxic metabolites derived from *Pfiesteria* extracts.

Materials and Methods

Cultures

Culture vessels were filled with 15 psu salinity water (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The water was continually filtered and aerated, and each culture vessel was maintained for 14 days before adding three tilapia. Fish were acclimated for 7 days to ensure viability and were fed daily with several flakes of Tetra Marin (Tetra, purchased at a local Pet Warehouse store) fish food. To this was added the *Pfiesteria* inoculum. After being tested as repeatedly lethal to tilapia, the species identification of the dinoflagellates was again verified from the fish-killing cultures using scanning electron microscopy of suture-swollen cells (11). The ammonia and pH levels were monitored carefully throughout the culturing process.

Mass Culture Harvest

Culture water (300 L) containing *Pfiesteria* cells was removed from the culturing tanks at North Carolina State University in Raleigh, North Carolina, and sent frozen to the Marine Biotoxins Center in Charleston, South Carolina. This culture was passed through a Whatman GF/B glass fiber filter (Whatman International Ltd., Maidstone, UK) using vacuum filtration to effectively separate the unlysed *Pfiesteria* cell mass from the culture medium (SW/Instant Ocean). Both the cell mass (CM) as well as the filtered SW medium were then processed separately for subsequent extraction workup and testing.

Extraction of Mass Cultures

Figure 1 demonstrates the basic fractionation scheme for the initial partitioning of *Pfiesteria* cultures. Cells collected on the GF/B glass-fiber filters were placed into a beaker and lysed in 500 mL cold (0°C) methanol (MeOH) using a Branson model 450 sonicator (Branson Ultrasonics Corp., Danbury, CT, USA) fitted with a microprobe. Sonication was carried out for 3–5 min at full power to ensure complete cell lysis. The resultant methanolic solution was then decanted, centrifuged, and filtered through a 0.22- μ m filter (GHP Acrodisc; Gelman Sciences, Ann Arbor, MI, USA) to remove any remaining cell debris and other particulates. The filtered extract was concentrated on a rotary-evaporator (Büchi RE121, Büchi Labortechnik AG, Flawil, Switzerland) and then fractionated via glass long-column chromatography over 100-Å pore size silica gel. Five sequential fractions were eluted using an elutropic solvent gradient series of increasing polarity. This elutropic series used ethyl acetate (EtOAc), MeOH, and water to effectively partition the extract over the full range of solute polarity. Fractionation was assisted by positive nitrogen gas pressure applied to the top of the glass column. Each fraction (CM 1–5) was then dried first using rotary evaporation (35°C), then under high vacuum overnight to remove any residual water. The fractions were subsequently submitted for bioassay analysis (below) as well as for chemical analysis using mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR).

Extraction of the Seawater Medium

The spent SW culture medium was extracted using a large glass column (6" diameter, 12" length, 4-L capacity) charged with C18 (100 Å) reverse-phase solid support. The C18 was conditioned with MeOH and rinsed with deionized water prior to operation. The SW media was passed through the C18 column followed by a deionized water rinse to remove all excess sea salts. The organic compounds adsorbed to the C18 solid support were eluted first with MeOH, then EtOAc. Both organic fractions tested positive in the battery of assays (below). The organic washes were combined and concentrated using rotary evaporation (35°C) followed by subsequent high-vacuum drying overnight to remove any remaining water. This dried fraction was taken up in MeOH and passed through glass-column chromatography using silica as the solid phase in a fashion identical to that described above for the CM extract. Five fractions were generated paralleling those obtained from the cell mass extract. The sample fractionation scheme for both CM extracts and the SW medium is summarized in Figure 1.

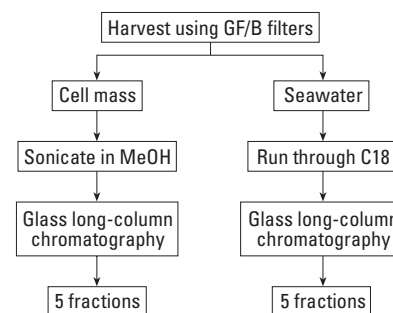


Figure 1. Fractionation scheme used for both CM and SW medium extracts of *P. piscicida*.

As a safety precaution, after the initial extraction processes, both the remaining cell debris and water were tested for any residual activity using the battery of assays described in the next section. In all cases after the described extraction, they were found to be negative and at this point were deemed safe for disposal.

Assays

Determination of toxicity/biological activity was performed on the 10 extracts (CM 1–5, SW 1–5). This screening included a brine shrimp bioassay, a cytotoxicity assay (12) modified for GH₄C₁ rat pituitary cells, a fish bioassay, and a reporter gene assay (13,14). The two cell-based assays were conducted as published, whereas the fish and shrimp bioassays were developed in house solely for activity screening purposes. It is important to note that active extracts are in very limited supply and that the purpose of this work is to isolate enough purified material for structural analysis, not to evaluate assays used to guide fractionation. It is the information gathered from the entire battery of assays used that provides bioassay-guided fractionation control. Assays were chosen on the basis of observed activity. These assays are described as follows:

Brine shrimp bioassay. The brine shrimp bioassay was set up in the following fashion. To reduce variability because of age of *Artemia*, all assays were performed using *Artemia* that were 16–20 hr old. The bioassay was performed in 12-well plastic plates (Corning Costar Corp., Cambridge, MA, USA). Each well contained 1 mL 35 psu artificial SW (Instant Ocean) in which 20 *Artemia* were transferred from the hatching chamber. Twenty microliters of each methanolic *Pfiesteria* SW or CM extract was added for each fraction obtained. The assay plate was examined over a time course of 48 hr to determine both the number of dead and time of death of the *Artemia*. Limited supply of active extract prohibited any form of replication.

Fish bioassay. The fish bioassays were performed in 24-well plastic plates (Corning Costar). Each well contained 2 mL 15 psu

seawater in which 1 juvenile sheepshead minnow (*Cyprinodon variegatus*) 7–10 days of age was placed. Twenty microliters of methanolic *Pfiesteria* SW or CM extract was added for each fraction tested. The assay plate was examined over a time course of 48 hr at room temperature to monitor fish behavior as well as to record time of death.

Cytotoxicity assay (12). Cytotoxicity assays (GH₄C₁ assay) are used as a first-tier screen for toxic activity. Cytotoxicity was measured by using a microtiter plate assay using the mitochondrial indicator 3-(4,5-dimethylthiazol-2,5-diphenyl) tetrazolium bromide (MTT) for an end point measurement. GH₄C₁ cells (30,000 per well) were plated in 0.1 mL of appropriate media in 96-well tissue culture plates (Corning Costar). The GH₄C₁ cell line is derived from rat pituitary cells. Test samples (2 μ L methanolic extract) were added to each well and incubated for approximately 24 hr. Each fraction was analyzed in duplicate with a 2- μ L MeOH negative control used to test the sample vehicle. After incubation, 15 μ L MTT (5 mg/mL in phosphate-buffered saline) was added to each well and incubated for 4 hr at 37°C. Mitochondrial dehydrogenases in live cells convert the MTT to an insoluble formazan crystal, resulting in a purple color. The crystals were solubilized by the addition of 1% sodium dodecyl sulfate in 0.1 N HCl and absorbance read at 570 nm with a Titer Tek 96-well plate reader (EFLAB, Helsinki, Finland). The plate reader subtracts nonspecific absorbance by media and nonconverted MTT to yield a corrected absorbance value.

GH₄C₁ reporter gene assay (13,14). The luciferase reporter gene assay is currently used as a second-tier screen for biological activity. GH₄C₁ *c-fos-luc* transfected cells are seeded in a 96-well clear-bottom white plate (Corning Costar) at a density of 30,000 cells per well and are allowed to incubate overnight to ensure attachment. Cells were treated with samples for 4 hr at 37°C. Experimental media were then removed from wells and 20 μ L of cell lysis buffer was added. Lysis proceeded at room temperature for 20 min, then the plates were read in the luminometer (Lumistar, BMG, Durham, NC, USA). Within the luminometer, each well was injected with 20 μ L luciferase and ATP (Luciferase assay reagent; Promega, Madison, WI, USA), and the luminescence generated from each well was read over a 10-sec interval.

Gas chromatography–mass spectrometry, liquid chromatography–mass spectrometry, and nuclear magnetic resonance spectroscopy. All semipurified and purified compounds derived in our isolation schemes were submitted for structural analysis and chemical characterization using gas chromatography–MS (GC–MS) (Finnigan Magnum Series;

Thermo Finnigan, San Jose, CA, USA); liquid chromatography–MS (LC–MS) (Perkin-Elmer Sciex Triple Quad Biomolecular Mass Analyzer; Applied Biosystems, Foster City, CA, USA) and nuclear magnetic resonance (Bruker DMX 500; Bruker, Billerica, MA, USA) as appropriate. Preparative purification of polar active compound(s) is currently under way in efforts to generate enough material for complete structural analysis of the active compound(s).

Results

The main focus of the work described is the use of a battery of bioassays to guide fractionation in an effort to isolate, purify, and structurally characterize the toxic components of *P. piscicida* extracts. The extracts used at the time this report was prepared were not in a state of high purity. Multiple assays were required to monitor for both known and unknown activities (14) to guide the fractionation process. Results determined at this crude stage of the process are often in error, as extracts contain many compounds having multiple activities that often interfere with a given assay. It must also be noted that the very limited supply of active extract made multiple testing in any given assay prohibitive. In this light, each assay performed maintained negative controls only for internal assessment of the assay's function. Because the toxin(s) have yet to be structurally characterized and purified to an analytically pure state (without potential interfering agents) and because *Pfiesteria* represents a newly described organism, good negative control extracts have been difficult to define. It must again be noted that negative tests may be due simply to a poor choice of assay, or that the concentration of the active component is below detection limits. The testing of proper controls with well-defined samples will ultimately be done once we have a definitive handle on the bioactive substance(s). To counter these difficulties, we use a battery of assays and evaluate activity based on a compilation of all the results. The bioassay results reported in this article should be evaluated with this in mind. They have been generated on the basis of a simple positive/negative “hot or not” response. As multiple replicates were not advisable because of the limited supply of extract, error bars could not be generated at this time.

GH₄C₁ Cytotoxicity Assay

The results of our general cytotoxicity assay are summarized in Figure 2. In this graph, fractions (F1–F5) are represented for both the CM extracts and the SW culture medium. Activity data in this assay clearly demonstrated two distinct active fractions isolated from both CM and SW. The lipophilic activity found in

fractions F1 and F2 was generally weak for both sets (SW and CM) of fractions, though it was usually stronger in the CM extracts than the SW extracts. The polar fractions demonstrating activity (F3, F4) were consistently active in both sets but with a reversal of relative strength with respect to the lipophilic activity. That is, F3 SW and F4 SW fractions were much more active than the corresponding F3 and F4 obtained from the CM extract. This result, in addition to the need to sonicate *Pfiesteria* cells to cause cell lysis, gives credence to the claim that the dinoflagellate may, in some fashion, excrete or secrete its active metabolites into the water rather than the more common dinoflagellate mode of sequestering of toxins within the cell (8). We have found when using small 1-L batch cultures that biological activity varies greatly from culture to culture of the same *Pfiesteria* strain. Because of this, and because we are interested only in isolating a toxic molecule from these cultures, only relative hot or not data are reported in the bioassay guided fractionation steps. All data are relative to an SW vehicle negative control. It is noteworthy that even though activity was reduced or eliminated in time, the F1–F5 fractions in both sets of data remained relatively consistent in their activity profiles both over time and from culture batch to batch.

The combined active CM lipophilic fractions, F1 and F2, were further purified over silica gel normal-phase high-performance liquid chromatography (HPLC). This yielded an analytically pure oily substance (determined by MS and NMR). The structure of this compound was determined by GC–MS (Finnigan) and NMR (Bruker DMX 500 MHz, Bruker). It was identified as di(2-ethylhexyl)phthalate (DEHP), a common plasticizer and known ichthyotoxin and endocrine disrupter (15). Other common phthalates have also been observed and identified from the lipophilic fractions, but to date these have represented insignificant quantities compared with DEHP. Phthalic esters are very commonly found in significant amounts in solvents and are used as plasticizers in plastic containers. They can be found virtually anywhere as contaminants (15). However, finding them in our *Pfiesteria*

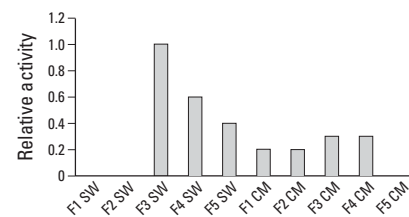


Figure 2. Results of the cytotoxicity assay on CM and SW extracts. Activity reported is relative to negative MeOH controls.

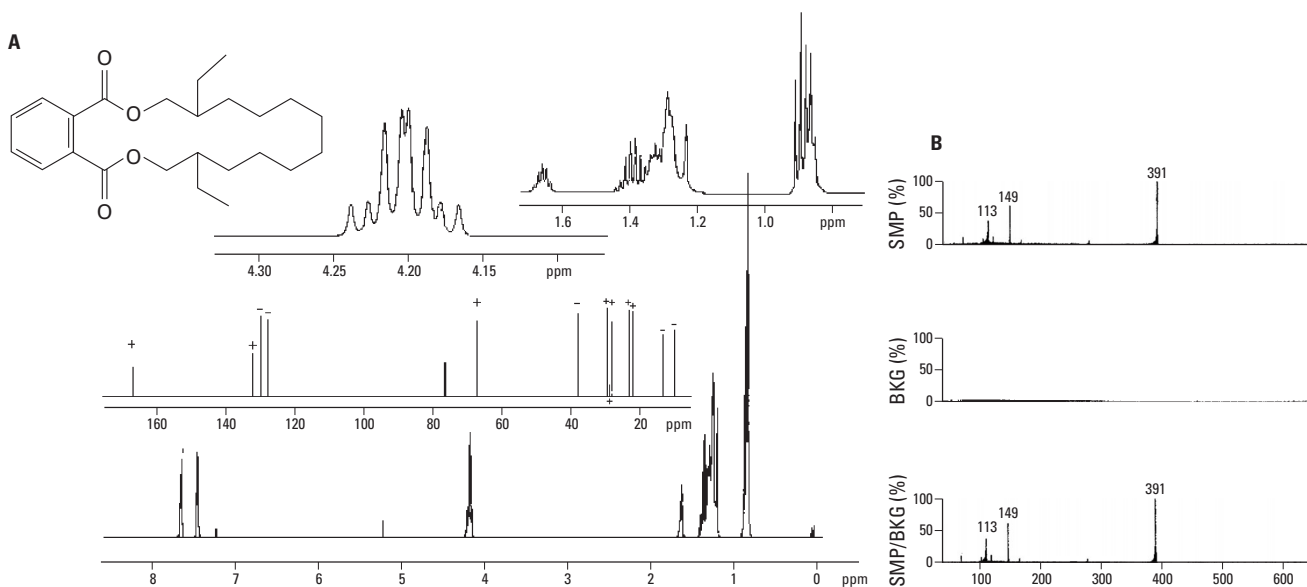


Figure 3. (A) Confirmatory ^1H - and ^{13}C -NMR spectra of CM-/SW-derived DEHP. Spectra are identical to those obtained with commercially available DEHP. (B) GC-MS spectra of purified DEHP from the lipophilic fractions F1 CM and F2 CM. The M+1 parent ion at 390 and the characteristic phthalate fragment ion at 149 are characteristic of DEHP.

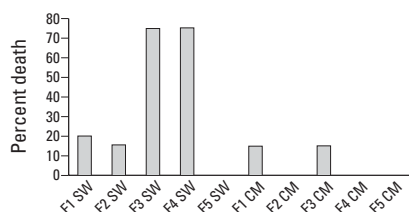


Figure 4. Brine shrimp assay results for CM and SW F1–F5 obtained from *P. piscicida* extracts.

extracts in such large quantities was quite surprising. We use only glass columns and precleaned support materials, as well as redistilled solvents free of contamination (GC grade). As such, we felt that our extraction methodology simply could not account for up to 10–20 mg of extracted DEHP from just 15 L of culture. This necessitated a search for the source of DEHP. We feel certain that we have identified the major source for this contaminant and determined it as the artificial seawater (Instant Ocean) used in preparing the culture medium. It is likely that the process used in making or packaging Instant Ocean uses many plastic components, which in turn could provide the source of the phthalate ester. To demonstrate this, 45 g Instant Ocean salts were extracted with 150 mL of highly pure chloroform (redistillation followed by GC-MS analysis to demonstrate phthalate-free conditions). After removing the solvent, the residual oil, approximately 35 mg, was analyzed with GC-MS and NMR. Analysis confirmed large amounts of DEHP present in these salts. (Conformational spectral data shown in Figure 3A, B.) We feel that

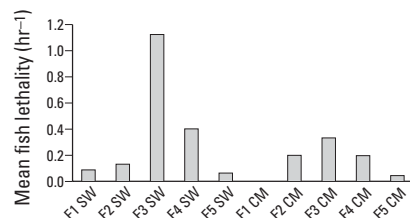


Figure 5. Sheephead minnow fish bioassay on the same SW and CM fractions used in the brine shrimp and cell cytotoxicity assays.

this finding is very important and should be noted by all laboratories when testing fractions isolated from any matrix associated with Instant Ocean. Because of the variability of the DEHP concentration found in Instant Ocean, the culturing process we used has reverted from culturing with Instant Ocean to culturing with filtered Gulf Stream water obtained off the coast of North Carolina.

Brine Shrimp Bioassay

This assay also demonstrated two active areas, based on chromatographic partitioning (Figure 4). It should be noted that there was a great deal of variability in the levels of activity from batch to batch. This could be observed in activity profiles of the brine shrimp assay; in some cases low activity could be seen in F1 and F2 fractions and in other cases there would be no activity. The activity found in F1 and F2 in both CM and SW appears to be due predominantly to DEHP, as we have observed no killing behavior in this assay when testing these extracts after removal of the phthalate esters by silica gel HPLC. The brine shrimp

demonstrated a high degree of sensitivity to DEHP. However, the susceptibility of the brine shrimp to the phthalic ester was considerably weaker compared to the activity found in the more polar F3 and F4. These later fractions obtained from both CM and SW were consistently lethal to *Artemia* with every culture batch tested.

Fish Bioassay

Fish time-of-death data are summarized in Figure 5. The fractions containing DEHP have indicated low-level activity in this assay, as the literature has suggested (13). As in the brine shrimp bioassay, variability in activity could be seen from culture batch to batch. However, we observed very strong ichthyotoxicity with fractions F3–F5, with F3 often causing fish death in less than 1 hr. In this assay the SW extracts F3–F5 were much more potent than the corresponding CM extracts, although relative activity profiles in parallel fractions was similar.

An interesting response of the F3 and F4 fractions was observed upon dilution. In non-lethal doses of extract or in diluted F3 and F4 fractions, the fish would invert and appear dead, only to revive within 12 hr to an apparent normal state. This phenomenon is also quite common from extracts derived from weakly active culture. No explanation for this behavior has been determined at this time.

GH₄C₁ Reporter Gene Assay

For both sets of fractions (CM and SW), F3, F4, and in some cases F5, were active on the reporter gene assay (Figure 6). Variability in the levels of activity is easily accounted for by the crude fractionation (i.e., only five large

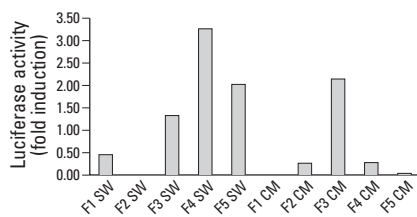


Figure 6. Reporter gene assay results for the same SW and CM fractions used for the brine shrimp and fish bioassays.

fractionation samples). Only F3 consistently demonstrated strong activity from CM extracts. This is most likely due to lower concentrations of the polar activity associated with the cell. The small induction of fractions F1 and F2 has generally been attributed to phthalate esters, particularly DEHP.

Discussion

We determined that the extracts of mass-cultured *P. piscicida* can yield two distinctive biological activities on the basis of polarity of the metabolite isolated. The nonpolar (lipophilic) fractions F1 and F2 consistently contained high concentrations of DEHP, a man-made phthalate ester commonly used in plastics as a plasticizer. Often the levels of phthalates in these fractions were able to kill shrimp and fish as well as give positive responses in the cytotoxicity assay and reporter gene assay. We have determined that the principal source of DEHP (and other phthalates) in these extracts is from the Instant Ocean used to make up the seawater medium. The compound DEHP was identified and confirmed through the use of GC-MS and NMR. After DEHP and other minor phthalates have been removed from these fractions via silica chromatography, we observed no other lipophilic biological activity from either set of extracts (CM, SW) in our battery of assays. We have also been unable to generate any lipophilic crystalline materials described in other reports or to isolate any excreted biologically active lipophilic proteinaceous compounds from cultured *Pfiesteria* extracts as reported by others.

The more polar (hydrophilic) fractions, derived from the SW extracts (F3, F4, and sometimes F5), contained a compound(s) that induced the GH_4C_1 reporter gene assay, demonstrated cytotoxicity in the GH_4C_1 assay, and killed both brine shrimp and fish. The F3 and F4 fractions obtained from the CM extracts of this strain induced the reporter gene assay, but only fraction F3 demonstrated any lethality to brine shrimp and occasionally fish. These fractions demonstrated activity in the GH_4C_1 cytotoxicity assay as well, although generally the responses were weak. We believe that the variability in assay response between CM and SW extracts could be due simply to a concentration effect, demonstrating that the active substance(s) is preferentially released from the cell rather than sequestered in any significant amounts. We believe that the polar fractions F3 and F4 from CM and SW probably represent the same compound or family of compounds. Preliminary LC-MS, NMR, and photo diode array-LC data (not shown) have yet to show any significant differences in the makeup of F3 and F4. This certainly does not rule out distinct toxic substances in each, however.

Currently, large-scale mass culturing is under way to provide sufficient quantities of active fractions F3 and F4 for further chemical and structural analysis.

Summary

Our research has yielded only polar, water-soluble components as the active substances contained in toxic *P. piscicida* cultures. We have observed no biologically active protein production and the active compound(s) we do observe exhibit molecular weights below 1,000 amu (16). Furthermore, after many trials, we have been able to isolate no lipophilic or hydrophilic chemical extract that will cause the formation of lesions in a fish bioassay. In fact, we can produce only minor lipophilic activity at all, and can produce no nonpolar crystalline materials, even from very large culture volumes. These

apparent incongruities highlight the need to replicate extraction procedures in multiple laboratories to verify results. They also emphasize the need for care in identifying the alga species used in toxin production.

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