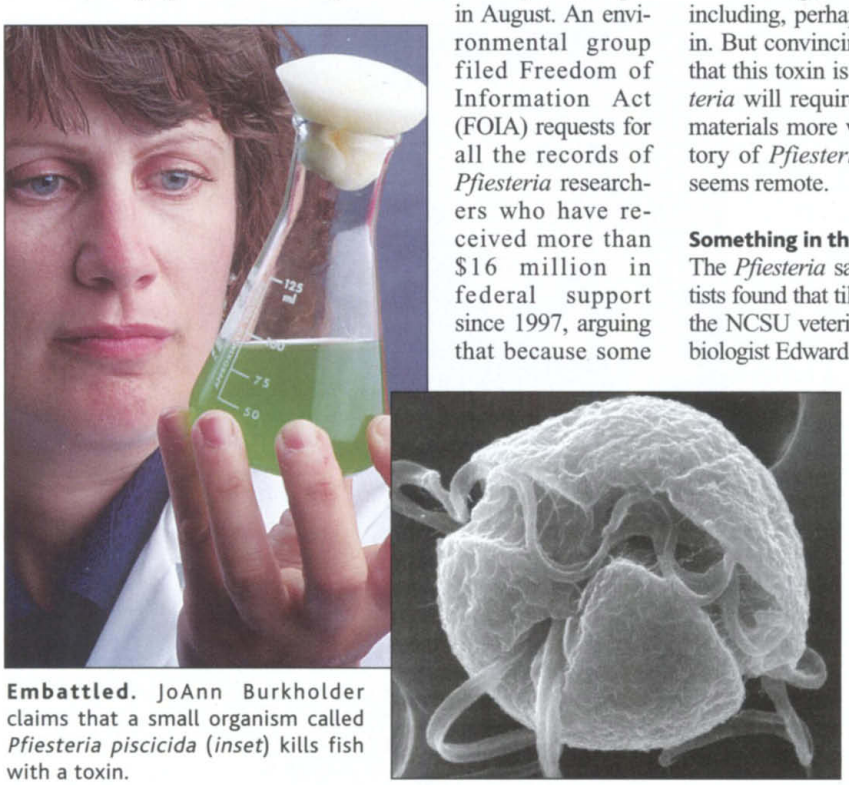
Ten years after ecologists in North Carolina found evidence that a toxic microbe caused mass fish die-offs, the toxin remains unidentified and the research is being challenged

The Science of *Pfiesteria*: Elusive, Subtle, and Toxic

Ever since it was blamed for massive fish kills in estuaries in the eastern United States a decade ago, a tiny one-celled organism known as *Pfiesteria piscicida* has fascinated and horrified the public. This dinoflagellate has been accused of slaying more than a billion fish and, in a superaggressive mode, releasing a potent neurotoxin that has sickened fishers and lab workers. It is also at the center of a raging scientific debate.

Pfiesteria has adorned journal covers, been discussed in Congress, and even inspired a popular scientific thriller about its co-discoverer JoAnn Burkholder, an aquatic ecologist at North Carolina State University (NCSU) in Raleigh. It was Burkholder and her assistant Howard Glasgow who first described the microbe's bizarre "phantomlike" behavior—how it lies dormant in sediments, then changes form and "ambushes" live fish with toxin. But almost every aspect of this research is now disputed.



Embattled. JoAnn Burkholder claims that a small organism called *Pfiesteria piscicida* (*inset*) kills fish with a toxin.

Criticism of Burkholder's work reached a crescendo this summer. The *Pfiesteria* toxin has yet to be identified. And in three papers, scientists attempting to replicate her results found no evidence that *Pfiesteria* has a complex, 24-stage life cycle—or that it produces a deadly toxin. They agree that *Pfiesteria* kills fish, but their strain killed merely by feeding on them. Burkholder says the recent skeptics have shown only that they don't know how to follow her published protocols for culturing toxic *Pfiesteria*. "[Critics are] saying it's a hoax based on these three papers, which all come from a few nontoxic strains," she says.

One reason the debate has grown contentious is that Burkholder has been reluctant to share her cultures, citing costs and other concerns. "We have had so many problems with discrediting efforts and industry threats," she says. Burkholder has churned the waters, too, by challenging grant awards and firing off long critiques of rivals when criticized at meetings and in the press.

One group that sympathizes with Burkholder gave the controversy a new spin in August. An environmental group filed Freedom of Information Act (FOIA) requests for all the records of *Pfiesteria* researchers who have received more than $16 million in federal support since 1997, arguing that because some groups promised but failed to produce a toxin, they misused the funds. Donald Anderson of the Woods Hole Oceanographic Institution in Massachusetts laments the "*National Enquirer*" tone of the debate: "It's been very damaging to our field."

Anderson is one of several prominent scientists who defend Burkholder and say some of her critics are drawing conclusions in the media that go beyond their data. Another is Donald Boesch of the University of Maryland (UMD) Center for Environmental Science, who says: "She's very controversial. She annoys some people. But her hypothesis really hasn't been significantly challenged by these papers." Oceanographer Ted Smayda of the University of Rhode Island, Narragansett, blames the "anti-Burkholder camp" for upping the ante.

Burkholder and her collaborators are confident that she will be vindicated. They suggest that key results will be presented at a harmful algae meeting later this month—including, perhaps, proof of the elusive toxin. But convincing the scientific community that this toxin is real and comes from *Pfiesteria* will require that Burkholder share her materials more widely. Given the bitter history of *Pfiesteria* research, that possibility seems remote.

Something in the water

The *Pfiesteria* saga began 1988, when scientists found that tilapia added to an aquarium at the NCSU veterinary school kept dying. Fish biologist Edward Noga and Burkholder, then a new professor, identified the cause as a new dinoflagellate from North Carolina estuarine water that could live on either algae or fish. They later learned that *Pfiesteria* blooms had appeared in sync with some fish kills in North Carolina. And under the microscope, Burkholder found, *Pfiesteria* was a fascinating shape-shifter, assuming many guises, including large amoeba-like blobs and various cysts, as well as dinospores.

The NCSU group published its findings in a 30 July 1992 letter to *Nature*, "New 'phantom' dinoflagellate is the causative agent of major estuarine fish kills." At the time, "we were discovering a lot of new and unique dinoflagellates and algal blooms," says Wayne Carmichael of Wright State University in Ohio, an algal toxin expert not involved in these studies. *Pfiesteria*, he adds, "was an interesting and intriguing possibility."

It was also controversial. Burkholder soon found herself battling state officials and scientists who questioned whether the microbe

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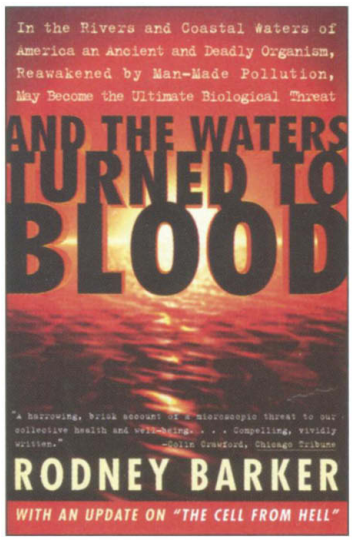
had any role in fish kills and whether, as Burkholder also claimed, manure runoff from a booming hog industry stimulated *Pfiesteria* blooms. Then in 1993, a dramatic incident gave new weight to Burkholder's studies. Glasgow, her research assistant, reported mood swings, difficulty in reading, and severe memory loss after working for months in the presence of aerosols from *Pfiesteria* fish tanks. Several other workers, including Burkholder, experienced problems they attributed to *Pfiesteria*. In response, NCSU and federal officials mandated that *Pfiesteria* could be studied only using biosafety level 3 (BSL-3) precautions—the same as for HIV—and the state closed a river where *Pfiesteria* was found at the site of fish kills. These events are described in a 1997 book, *And the Waters Turned to Blood* by Rodney Barker, which Burkholder considers generally accurate.

Pfiesteria fever

Pfiesteria hit the national radar screen in 1997. Maryland state scientists linked *Pfiesteria* to several fish kills in the Chesapeake Bay estuary that, because people stopped buying seafood, cost Maryland's seafood industry $50 million. Thirteen watermen working near the site of the kills reported memory loss, sores, and other symptoms.

Also that year, at a meeting at the National Institute of Environmental Health Sciences (NIEHS) in Research Triangle Park, North Carolina, Burkholder announced that her team had "isolated and purified" a water-soluble toxin, according to an NCSU press release. (Burkholder now says the term "purified" was "not correct.") At the same event, biochemist Daniel Baden of the University of Miami in Florida described a substance he had purified from Noga's fish tanks, a fat-soluble toxin. Congress subsequently appropriated new money for *Pfiesteria* research partly through ECOHAB, an interagency grants program, and NIEHS kicked in more. And Burkholder received the 1997 Scientific Freedom and Responsibility Award from the American Association for the Advancement of Science (publisher of *Science*).

As public concern escalated, the governor of North Carolina convened a 14-member panel of North American experts on toxic algae that identified gaps in *Pfiesteria* science. High on the to-do list, they said, was distributing *Pfiesteria* cultures through the Bigelow Laboratory, a repository in West Boothbay Harbor, Maine. The lab received $275,000 from ECOHAB but never got cultures from Burkholder's group.



Alarm. A 1997 book fanned concerns about *Pfiesteria*'s neurotoxicity.

Muddying the waters

The ECOHAB program funded only part of a large proposal submitted by Burkholder. But she received $1.5 million from the North Carolina legislature and a foundation to build a new lab and a large biosafe facility, where she began generating large quantities of *Pfiesteria*. A collaborating group led by chemists John Ramsdell and Peter Moeller of the National Oceanic and Atmospheric Administration's (NOAA's) lab in Charleston, South Carolina, was supposed to isolate the toxin from her cultures. Other labs set out to pursue various studies; because Burkholder's cultures were not available, they attempted to replicate her experiments following her protocols.

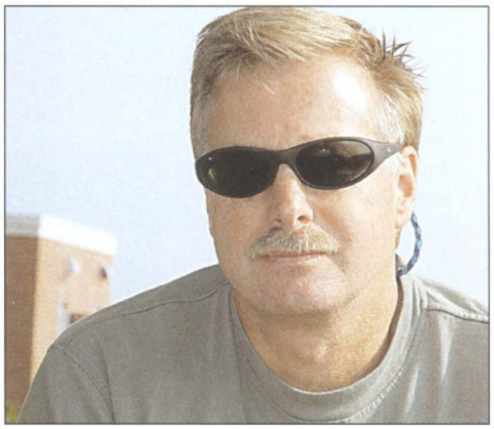
Researchers took heart from one technical advance at this time: UMD School of Medicine researcher David Oldach and Parke Rublee of the University of North Carolina (UNC), Greensboro, developed a molecular probe for *Pfiesteria*, which previously could be identified only by microscope. But in other ways the science got murkier. Scientists realized that *Pfiesteria*-laden waters contained other, similar-looking species. One of these, dubbed *Pfiesteria shumwayae*, was also sometimes toxic, Burkholder reported. Fish biologists suggested that low oxygen could explain many fish kills and that the deep sores typically found on affected fish could have many causes, such as a fungus that is usually found in the sores.

Efforts to isolate the toxin dragged on. Lacking any clear biomarkers for human exposure, the U.S. Centers for Disease Control and Prevention (CDC) gave *Pfiesteria*-suspected illness the nebulous title "possible estuary-associated syndrome" and launched a cohort study. At a CDC workshop in 2000, an expert panel found "firm evidence … that *Pfiesteria* is toxic to fish" but concluded that the "extent and nature of the hazard to human health … remain unknown."

Toxin-free?

This summer, several of Burkholder's critics launched a blitz: Accompanied by a flurry of press releases, they published three papers at odds with Burkholder's central claims about *Pfiesteria*. In the *Journal of Phycology*, a separate NOAA team using molecular techniques found that *P. piscicida* had a normal dinoflagellate life cycle (see sidebar on p. 348).

Another team, led by fish pathologist Wolfgang Vogelbein of the Virginia Institute of Marine Science (VIMS) in Gloucester Point, reported in the 5 August *Nature* that *P. shumwayae* can attack and kill fish apparently without making a toxin. Attempting to follow Burkholder's procedures, they kept dinos and 25 to 40 tilapia in a fish tank for several weeks until fish began dying, then added fish daily to replace dead ones. Then, to study fish death in a simpler system, they tested how quickly water from their fish-killing tanks could kill larval minnows in small dishes.



Challenger. Wolfgang Vogelbein has found that *Pfiesteria* can kill fish without producing a toxin.

The team reported that the fish larvae died at rates comparable to those in Burkholder's toxic cultures—in 24 to 48 hours. But water with dino cells removed didn't kill fish. In addition, when fish were separated from *Pfiesteria* cells with a membrane, they didn't die, suggesting that they succumbed only when *Pfiesteria* made physical contact. The VIMS group also produced videos of the dinos aggressively feeding on the tilapia.

Vogelbein thinks that *P. shumwayae* may be lethal to fish only in the lab. In a river, he says, the fish would presumably swim away. Says Vogelbein: "I don't know if anything like this occurs in the field." In another paper in the 5 August online *Proceedings of the National Academy of Sciences* (*PNAS*), an overlapping team led by chemist Robert Gawley of the University of Miami suggested that *Pfiesteria* lacks the genes to make the polyketide toxins that are typically produced by fish-killing dinoflagellates. This paper suggests that other factors caused the fish kills, such as a different toxic alga.

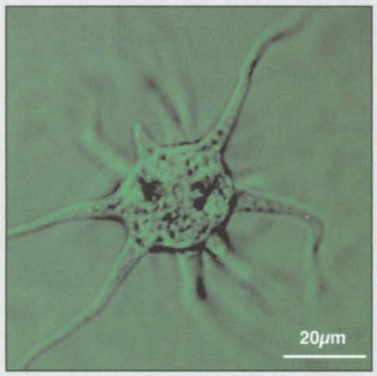
Burkholder says the VIMS researchers are

CREDITS: (TOP TO BOTTOM) VIRGINIA INSTITUTE OF MARINE SCIENCES; TOUCHSTONE/SIMON & SCHUSTER

Pfiesterian Lifestyle: Simple or Complex?

One amazing—and, to some observers, incredible—aspect of *Pfiesteria*'s biology is what JoAnn Burkholder describes as its 24-stage life cycle, including amoeboid forms. Now a recent study directly challenges this claim, concluding that *Pfiesteria*'s life cycle is much simpler. The new data are "pretty nice," says protistologist Wayne Coats of the Smithsonian Institution, who has not taken sides in the *Pfiesteria* fight (see main text), although he says there is "still room" for debate.

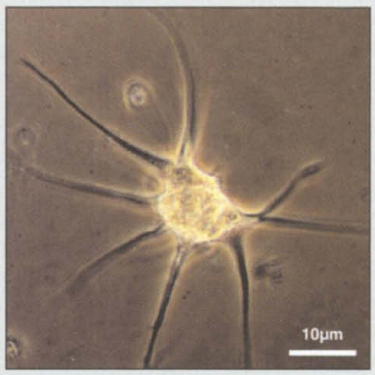
For 10 years, Burkholder, of North Carolina State University in Raleigh, has described how *Pfiesteria piscicida* can assume many shapes besides the expected flagellated dinospore and cysts—such as much larger, spiky amoebas. But Burkholder's group has never fully documented with still images or video a dinospore transforming into an amoeba, a "very difficult" task, as Coats describes. Burkholder's claim is problematic for another reason, too: The team induces some life-cycle stages by exposing *Pfiesteria* to fish—which inevitably carry other microbes and possible contaminants.



In the new study, a group led by molecular biologist Wayne Litaker of the National Oceanic and Atmospheric Administration used a fluorescent probe that binds to nucleic acid to track *P. piscicida*'s life cycle. The team reports in the 20 June *Journal of Phycology* that *Pfiesteria* fed on algae or fish have the regular asexual and sexual stages typical of dinoflagellates. "There's nothing unusual" about them, Litaker says.

Litaker's team did find amoebas in their fish tanks, but these turned out to be true amoebas. He suggests that what Burkholder's group perceived as dinospores transforming into amoebas were actually dying dinospores shedding their shells.

Burkholder rejects the findings, suggesting that Litaker's strain of *Pfiesteria* had lost toxicity. Nontoxic strains rarely produce amoebas, she says. She also points out that her own group used molecular techniques to verify the identity of *Pfiesteria* amoebas last year in a report in *Environmental Health Perspectives*. Litaker, however, notes that the probes were designed to detect *Pfiesteria* and would not have detected true amoebas. University of Maryland molecular biologist David Oldach, a collaborator of Burkholder, calls her analysis "pretty good" but adds, "additional controls could be done." He hopes to work with Burkholder to confirm her results.



Striking resemblance. A standard amoeba (genus *Korotnevella*) from a *Pfiesteria*-containing fish tank (*left*) looks similar to an amoeba that JoAnn Burkholder has identified as a stage of the dinoflagellate *Pfiesteria*.

One argument Burkholder has used to support *Pfiesteria*'s exotic life cycle is that the pattern has been seen before. For example, Lois Pfiester, the pioneering phycologist after whom *Pfiesteria* is named, described amoebas and more than 30 other stages of the dinoflagellate *Cystodinedria inermis* in the early 1980s. Coats says that some of these early reports "should be viewed cautiously," however, because they were done using field collections, not clonal cultures.

On the other hand, there are "other dinos that do strange things," says Coats—particularly parasitic dinoflagellates. One odd case is that of a stickleback fish parasite described by mollusc biologist John Buckland-Nicks of St. Francis Xavier University in Nova Scotia in the 1990s. Buckland-Nicks says he isolated individual cysts in distilled water, and 7 months later "various forms" emerged, including amoebas. "I do know this kind of complex life cycle is possible."

–J.K.

grasping at straws because they failed to culture toxic *Pfiesteria*. The time to fish death is the givewaway, she says: Vogelbein misread her papers, which say that in her studies, it takes "minutes to hours" rather than a day or two. (She also says, however, that only in the early 1990s did she have strains "hot" enough to kill tilapia in minutes.) Burkholder says the VIMS team ignored her protocols specifying limits for ammonia and pH levels in their fish tanks, and they didn't expose dino cultures to fish long enough to set up a toxic attack. She says her group has reported that *Pfiesteria* attaches to and feeds on fish. And in her published studies, she adds, fish separated from toxic cells by a membrane still die.

Burkholder is pleased that another group at Old Dominion University in Norfolk, Virginia, has recently confirmed that *Pfiesteria* produces a toxin. In the March issue of the new journal *Harmful Algae*, a team led by Andrew Gordon and Harold Marshall—who received cultures from Burkholder—report that extracts of water from tanks of *P. piscicida* and *P. shumwayae* do indeed kill fish, although more slowly than in direct contact. Gordon says it's important to follow Burkholder's protocol closely. It took his group a year to learn how to rapidly restock tanks with fish, making them "hotter" and more toxic. At first, "we were ready to give up and say there was no soluble toxin," he says.

Vogelbein says his group can get *Pfiesteria* to kill fish in 45 minutes if there are enough dinoflagellates in the tanks. What Burkholder calls "hot" tanks, he suggests, could be "entirely a cell density effect." But he adds that "It's entirely possible that there are strains that produce toxins." The only way to know for sure, he says, is for Burkholder to share her cultures.

An unsharing culture

But that's a touchy subject. Burkholder feels strongly that she cannot just give cultures away, mainly because of the cost. She insists on training to make sure other labs grow cultures the right way, safely, and don't wrongly discredit her. Generating culture plus "proper training" costs $40,000 per lab, she says. Nevertheless, using largely her own funding, she says she has given 41 researchers in 33 labs toxic cultures and training.

However, most of these groups received the dinos growing on algae or as buffered cells. Only one of these labs—Gordon and Marshall's group at Old Dominion—are replicating Burkholder's experiments by actively culturing "toxic" *Pfiesteria* in their own fish tanks. Like other Burkholder collaborators, they have agreed that they won't share cultures with other groups. "It puts me in a position that I'm kind of uncomfortable with," says Gordon, who notes that sharing is expected in his field, microbiology. But, he says, "the ethics seem to be different in the world of [toxic] marine plankton."

Plans to distribute cultures through the

CREDITS: (LEFT TO RIGHT) T. QIAN AND J. LEMASTERS/CELL AND MOLECULAR IMAGING FACILITY, UNC CHAPEL HILL; J. SPRINGER, NCSU CENTER FOR APPLIED AQUATIC ECOLOGY

Bigelow Lab in Maine fell apart for other reasons. Burkholder said she never sent cultures because Bigelow wasn't prepared to feed fish to the cultures to keep them toxic, and it didn't have a BSL-3 facility. Robert Andersen of Bigelow, however, says that at the time, "there was no consensus in the community" that the toxin existed. Burkholder cites another reason for her reluctance: She learned that she was on a "top 10 list of the worst scientists in the country according to some conservative group" and that chicken farm barons Frank and Jim Perdue were "protesting" her research. "I really pulled back because I feared people working on these cultures, coming out saying they're not toxic, [were] not culturing them correctly; I felt I was in a major Catch-22."

Burkholder disputes claims in a 6 August *New York Times* article—cited in a *Science* editorial—that she has refused to give cultures to various groups (*Science*, 23 August, p. 1237). Some of these requests Burkholder disqualified because they were verbal. She says NCSU considers only written requests. The University of Maryland Biotechnology Institute (UMBI) once offered several thousand dollars for cultures, but "it wasn't enough." An e-mail that microbiologist Pat Gillevet of George Mason University sent in 2001 requesting amoebas was never received, according to university logs, Burkholder says.

Her supporters say she's right to be cautious. "Some people have not dealt with her in a professional way," says marine biochemist Craig Cary of the University of Delaware, Newark and Lewes. "JoAnn often takes it personally, and rightfully so." Cary says Burkholder was initially reluctant to send his group toxic cultures. "We had to really gain her respect and her confidence. It was very, very frustrating. But I never held it against her," Cary says.

Burkholder doesn't hesitate to stand up for herself. Twice, grant award decisions have been reviewed because she felt she had not been treated fairly. And she is known for scathing, single-spaced, multipage letters she sends her critics. Once, she acknowledges, after a scientist made remarks at a Gordon Conference that she says she found "very denigrating" and "personally directed," she sent a letter to his supervisor, John Wells, director of the Institute of Marine Sciences at UNC Chapel Hill. Wells says he investigated, but "no action was taken," and the scientist "is in excellent standing at this institution."

In August, a group sympathetic to Burkholder launched a strike on her critics. The Waterkeeper Alliance, an environmental group led by Robert F. Kennedy Jr., filed FOIA requests demanding the records of every researcher who has received federal funds since 1997 for *Pfiesteria* research (Burkholder included). Some labs are negotiating with the group or sending copies of old grant proposals as a first response. But if they have to turn over e-mails and lab notebooks, it could mean hundreds of hours of work, Vogelbein says. "To me, it looks like an attack on the way science is done in this country." Burkholder says she's not directly involved in the filing but agrees that teams that couldn't grow toxic *Pfiesteria* should not have received funding.

One of the group's targets is Baden, who once collaborated with Burkholder but is now co-author on the *PNAS* paper that challenges her research. Baden, now at UNC Wilmington, received funding to study the toxin he unveiled in 1997, but has never published on it. Baden says by e-mail that, at the time, "I believed we had isolated a toxin from *Pfiesteria*," but now thinks "there is no way of telling" what organism produced the toxic effects he observed.

Closing in?

After the latest round of critical papers, some scientists are privately calling the hunt for *Pfiesteria* the "cold fusion" of biology—a wild goose chase whipped up by media coverage. "It's beyond science. It's a sickness," says protistologist Tom Nerad of the American Type Culture Collection in Manassas, Virginia. But Burkholder thinks that many of her critics are simply impatient. She points out that identifying algal toxins can be difficult: It took scientists 25 years for brevetoxin, 7 years for maitotoxin.

Ambiguous evidence. Sick and dying fish have been associated with blooms of *Pfiesteria* in eastern U.S. estuaries.

CREDIT: NC STATE UNIVERSITY/CENTER FOR APPLIED AQUATIC ECOLOGY

Even some of Burkholder's fiercest critics think there was a harmful substance of some kind in her *Pfiesteria* tanks. The strongest evidence, they say, is that lab workers got sick. Theories abound, however, on what this substance was.

The problem all along, says Allen Place of UMBI, is that "it was never just *Pfiesteria* in the fish tanks." Burkholder's gold standard for determining toxicity is to add *Pfiesteria* to fish tanks and see if the fish die. But this process adds an assemblage of organisms. UMBI researchers and Gillevet have identified more than 60 organisms—bacteria, eukaryotes, fungi—living in the fish tanks. "It's a milky mess," says Place, and any of these components could have produced a toxin.

Gawley, the Miami chemist, suggests that because the VIMS group took steps to remove contaminants, such as dosing the broth with antibiotics, they may have unwittingly removed the source of a toxin. Burkholder agrees that a bacterial "cofactor" might be involved. Still another theory being investigated by a group at the UMD School of Medicine and the VIMS team is that *Pfiesteria*-rich water contains a bioactive agent that affects the brain of mammals, but not fish.

Burkholder, meanwhile, insists she and her team are close to identifying the toxin, and Ramsdell says they've made a recent "breakthrough." After years of working with fractions separated from a gemisch of materials, keeping those that trigger a cell assay for toxicity, they have found a way to grow toxin-producing cells on algae free of contaminants. A compound isolated from these relatively clean cells yields the same nuclear magnetic resonance signature as the mixture originally found in fish tanks, Ramsdell says. "We have been tracking the same molecule the whole time," he says.

Ramsdell has a video that shows this compound killing a larval fish in 5 minutes, and NOAA's Moeller says they will describe partial structural data at the harmful algae meeting in St. Petersburg, Florida, later this month. But even if they have isolated a lethal molecule, "we're not making the claim that it's *the Pfiesteria* toxin," Ramsdell says; "we'll still need to prove it's what's killing fish."

Before skeptics are convinced, Burkholder's experiments will have to be reproduced by other groups, which may be hard to do. Several scientists have talked about trying to find a way to share Burkholder's cultures; Chris Zarba of the Environmental Protection Agency says that EPA is ready to put up $300,000 to fund Burkholder's lab to provide cultures if a "joint proposal" is submitted. Those involved hope an agreement might be worked out after the October meeting. "Within half a year, we could have the answer that we want," Rhode Island's Smayda says. It's possible, but a long shot.

—JOCELYN KAISER