

A Rat Model of the Cognitive Impairment from *Pfiesteria piscicida* Exposure

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Pfiesteria piscicida Steidinger & Burkholder, an estuarine dinoflagellate known to kill fish, has also been associated with neurocognitive deficits in humans. We have developed a rat model to determine the cause-and-effect relationship between exposure to *Pfiesteria*-containing water and cognitive impairment and to determine the neurobehavioral mechanisms underlying the *Pfiesteria* effect. The rat model of *Pfiesteria* toxicity can also provide important information concerning the toxin or toxins responsible for neurocognitive deficits resulting from *Pfiesteria* exposure. With the rat model we have repeatedly documented a *Pfiesteria*-induced choice accuracy impairment during radial-arm maze learning. The *Pfiesteria*-induced impairment was relatively specific to the acquisition phase of training. When rats were pretrained, *Pfiesteria* treatment did not affect performance. However, when these same rats were retrained on another task, the *Pfiesteria*-induced impairment became evident. *Pfiesteria*-induced effects were also seen in a locomotor activity test in the figure-8 apparatus and selected components of the functional observational battery. *Pfiesteria* effects on choice accuracy in the radial-arm maze in rats constitute a critical component of the model of *Pfiesteria* toxicity, as the hallmark of *Pfiesteria* toxicity in humans is cognitive dysfunction. Our finding that analysis of the first six sessions of radial-arm maze testing is sufficient for determining the effect means that this test will be useful as a rapid screen for identifying the critical neurotoxin(s) of *Pfiesteria* in future studies. **Key words:** cognition, functional observational battery, learning, locomotor activity, *Pfiesteria piscicida*, radial-arm maze, rat model. — *Environ Health Perspect* 109(suppl 5):757–763 (2001). <http://ehpnet1.niehs.nih.gov/docs/2001/suppl-5/757-763levin/abstract.html>

Pfiesteria piscicida Steidinger & Burkholder is a toxic estuarine dinoflagellate that causes lethal toxic effects in fish (1,2). Adverse health effects have been found in individuals after accidental *Pfiesteria* exposure in the laboratory (3). The health effects in these individuals were characterized by cognitive disturbance, fatigue, mood lability, dermal lesions, and respiratory impairment. Similar associations have been seen in a cohort of individuals exposed in field conditions in Maryland (4). In order to rigorously determine the cause-and-effect relationship between *Pfiesteria* exposure and neurobehavioral impairment, an experimental animal model is necessary. We have developed a rat model of *Pfiesteria*-induced neurocognitive impairment to demonstrate and characterize the causative relationship and to help identify the critical toxin(s) underlying *Pfiesteria*-induced impairment.

We conducted a series of studies to characterize *Pfiesteria* effects on cognitive performance in the radial-arm maze (5–9). These studies laid the groundwork for the rat model of *Pfiesteria*-induced cognitive impairment. The route of exposure for humans is not certain. It is likely to be inhalation of the aerosol with some possibility of ingestion or transdermal absorption. The subcutaneous (sc) route was chosen for the current studies to ensure delivery of known amounts of *Pfiesteria* into the body in a reliable fashion. Because the field is at an early stage in which the chemical

identity of the toxin(s) is not known, our intention was to determine the nature of the neurobehavioral toxicity in a reliable manner, then proceed with the more environmentally relevant but more difficult to control routes such as aerosol inhalation when chemical analysis could be used to verify the dose delivered. We documented a consistent *Pfiesteria*-induced learning impairment when *Pfiesteria* was administered before training was initiated (6). However, when *Pfiesteria* was administered after rats were trained on the radial-arm maze, no deficit in retention was observed. Nevertheless, when these *Pfiesteria*-treated rats were subsequently given a new task to learn, they showed a significant deficit. The learning impairment was evident at least 10 weeks after a single exposure. With this series of studies we developed a reliable model of the neurocognitive deficits caused by *Pfiesteria* exposure. The detailed results of these studies are presented in this article. They have brought us to the point where we can use the animal model to investigate both the character of the *Pfiesteria*-induced cognitive deficit and the mechanisms of its action, including the search for the critical toxin or toxins.

Methods

Pfiesteria samples for this series of studies were collected from aquaria at the North Carolina State University laboratory of J. Burkholder, in which *Pfiesteria* were killing fish. The water in the aquaria had ammonia

levels less than 200 µg/L and nitrate levels less than 500 µg/L. The aquarium water was injected with no additives into sealed glass test tubes. The tubes were then frozen on dry ice for at least 1 hr. The samples were frozen as a precaution to attenuate possible degradation of the toxin(s). In all the studies, the tubes holding the samples were warmed until no ice crystals remained before sc injection into the rats. In studies 1–4, controls were injected sc with a volume that was the average of the dosed groups. In studies 5–7 all rats in control and dose groups received the same volume of injection—3 mg/kg.

Subjects were adult female Sprague-Dawley rats (Zivic-Miller, Allison Park, PA, USA) except for those in the juvenile experiment, which used 24-day-old male and female Sprague-Dawley rats. The rats were housed in groups of 2–4 in plastic cages with wood shavings. All rats in a cage received the same *Pfiesteria* exposure. They were in an approved vivarium immediately adjacent to the behavioral test facility. They were on a reverse 12:12 light:dark cycle with testing during the behaviorally active, dark phase. All rats had *ad libitum* access to water, and except for experiment 1, they were on scheduled feeding after testing. These studies were conducted under an approved protocol of the Animal Care and Use Committee of Duke University in an AAALAC-approved facility.

Experiment 1: The Pilot Study

This was an initial evaluation of the acute and persisting behavioral effects of *Pfiesteria*. Six rats were injected sc with *Pfiesteria* in doses from 35,600 to 961,200 *Pfiesteria* cells per kg of rat body weight. Six control rats were not injected. Two days after exposure we began

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testing rats in the win-shift radial-arm maze task (below) for 18 sessions over 6 weeks. The rats were on an *ad libitum* feeding schedule to ensure that the *Pfiesteria* exposure did not adversely affect free-feeding body weight. In later studies, the usual procedure of daily scheduled feeding after testing to maintain body weight at approximately 85% of *ad libitum* levels was used so that the rats were more motivated to run the maze for food reinforcement.

Experiment 2: The Repeat Study

This study was a more focused evaluation of the 106,800 cells/kg *Pfiesteria* dose. The same *Pfiesteria* sample used in experiment 1 was used in this experiment. It had been stored sealed and frozen at -4°C for 7 weeks. Ten rats were injected sc with *Pfiesteria* and 10 with saline. Win-shift radial maze training began 2 days after exposure.

Experiment 3: The Fresh Sample Study

In this study we evaluated the effects of a fresh sample of *Pfiesteria* collected from aquaria at North Carolina State University in Raleigh, North Carolina. The sample was frozen at -4°C overnight. Ten rats were injected with *Pfiesteria* at the benchmark dose of 106,800 cells/kg and 10 controls were injected with aquarium water collected from tanks that did not contain *Pfiesteria*. The rats began training in the win-shift radial maze task for 18 sessions over 6 weeks starting 2 days after exposure. After testing, the rats were sacrificed and the brain, blood, lungs, liver, kidneys, and spleen were collected for pathological assessment. Gross and microscopic examination of hematoxylin and eosin (H & E) stained sections were made in search of lesions or signs of pathology. Glial fibrillary acidic protein (GFAP) immunoreactivity was determined to check for more subtle signs of toxicity in *Pfiesteria*-exposed animals

Experiment 4: The Pretraining Study

Here we determined if the deficits observed in radial-arm maze performance were due to impairments in learning or memory. Rats were pretrained for 18 sessions on a radial-arm maze win-shift task before *Pfiesteria* administration. Then they were administered *Pfiesteria* samples at doses of 0, 35,600, or 106,800 cells/kg. As in experiment 3, a fresh sample of *Pfiesteria* collected from aquaria at North Carolina State University, which had been frozen at -4°C overnight, was used and the vehicle control was aquarium water without *Pfiesteria*. Two days after exposure, testing on the radial-arm maze win-shift task resumed. The rats were tested for the following 6 weeks for an additional 18 sessions. Then to assess persistent *Pfiesteria* effects on learning, we changed the rules of the task

from win-shift in which there was one reward at the end of each maze arm to repeated acquisition in which there were rewards at the ends of only three of the eight arms (radial-arm maze methods are discussed below). Rats were tested for six sessions over 4 weeks, using a repeated acquisition task in the same 8-arm radial maze with the same environmental cues. The rats were also assessed at 1 hr, 1 week, 4 weeks, and 9 weeks postexposure using the Functional Observational Battery (FOB) (below).

Experiment 5: The Cue Structure Study

In this study, we examined the importance of testing environment on the *Pfiesteria* effects on radial-arm maze choice accuracy. Two similar radial 8-arm mazes were used in this experiment. One maze was located in a standard test room with dimensions of 4.57×3.43 m and a ceiling 2.90 m high. The other maze was in a sound-attenuating chamber 3.35×3.05 m and a ceiling 1.98 m high. Half the rats in each condition were tested in each room. After 18 sessions of training, the testing room of the rats was switched from one room to the other for six sessions. For a final three sessions the testing room was switched back to the original training room.

The studies were vehicle-controlled experiments using two fresh *Pfiesteria* samples. The samples were collected directly from an aquarium at the Burkholder laboratory in which *P. piscicida* cultures were actively killing fish. The *Pfiesteria* cell concentration was determined by counting identified cells per unit volume under light microscopy (10). Taxonomy was confirmed by scanning electron microscopy. The aquarium water was injected with no additives into sealed glass test tubes and frozen overnight before injection into the rats. Doses of 35,600, 106,800, and 320,400 *Pfiesteria* cells/kg of rat body weight were injected sc. In addition one group was injected with the equivalent amount of water from the *Pfiesteria* culture as the 106,800 cells/kg dose from which the cells were removed by filtration (0.02-micron filter size). The 106,800 filtered group was included to test the necessity of administering the *Pfiesteria* cells for the toxic effects seen. There were two control groups, one injected with saline and one with water from aquaria without *Pfiesteria*.

Experiment 6: The Sample-Type Study

This provided a screening analysis of the neurobehavioral potency of three *Pfiesteria* cultures: B-113-3 (Pf-113), B-7-28-B (Pf-728), and B-Vandermere (Pf-Van), which were gathered at three different sites. There were six dose groups. Ninety-six (12/group) adult female Sprague-Dawley rats were injected (sc) with a single dose of *Pfiesteria* taken from

aquarium-cultured *Pfiesteria* (35,600 or 106,800 *Pfiesteria* cells/kg of rat body weight). The *Pfiesteria*-treated rats were compared with groups of rats injected with either saline (saline controls) or aquarium water without *Pfiesteria* (tank-water controls). The three sample types had the same average concentration (23,700–24,300 cells/mL). The potency of the samples in killing fish per day of exposure varied from 49% lethality for Pf-113 to 93% for Pf-Van and 100% for Pf-728. One control group ($n = 12$) was injected with saline and one ($n = 12$) with aquarium water not containing *Pfiesteria*. We used a neurobehavioral screen consisting of a short six-session sequence of radial-arm maze testing, two 1-hr sessions in the figure-8 activity maze, and two sessions of the FOB.

Experiment 7: The Juvenile Study

We examined the doses of the *Pfiesteria* sample type Pf-728, which was most effective in experiment 7 in juvenile rats. Seventy-two male and female Sprague-Dawley rats 24 days of age were injected (sc) with a single dose of *Pfiesteria* sample type Pf-728 from the Burkholder laboratory. Each of the following conditions was tested with 12 male and 12 female rats: tank-water controls, 35,600 cells/kg, and 106,800 cells/kg. The behavioral assessment began 2 days after injection.

Radial-arm maze training. A radial 8-arm maze, constructed of wood and painted black, was used in this study. The maze consisted of a central arena 50 cm in diameter with eight 10-cm \times 60-cm arms extending radially. Food cups were located 2 cm from the end of each arm. The maze was 30 cm above the floor and was located in a testing room that contained many extra-maze visual cues. The cues were kept in the same position throughout training. The rats in each *Pfiesteria* treatment condition were tested on the win-shift radial-arm maze procedure 3 days per week. Before each session, all the arms of the maze were baited with 1/3–1/2 piece of sugar-coated cereal (Kellogg's Froot Loops; Kellogg Company, Battlecreek, MI, USA). A radial-arm maze test session was started when the rat was placed in a circular plastic ring in the central platform. After 10 sec the ring was lifted and the rat was allowed to freely explore the maze. Arm choices were recorded when the rat had placed all of its paws beyond the threshold of the arm. The reinforcers (Froot Loops) were not replaced during the session. The radial-arm maze test session continued until the rat had entered all eight arms or 5 min had elapsed. Because it has been determined to be a sensitive and reliable index through more than 15 years of study, the number of entries until an error was made (entries to repeat) was used as the choice

accuracy measure. Entries to repeat is a better measure than the number of errors to finish the maze in that it indexes when the first error occurs. Because the radial-arm maze becomes more difficult as the session progresses and fewer reinforcements remain, it is important to determine at what stage of the session the first error occurs. During the early stages of training in the radial-arm maze rats often do not finish the maze during the time allotted. The entries to repeat score is a way to index choice accuracy even when the maze is not completed. Random chance performance for the entries to repeat measure on an 8-arm maze as determined by computer simulation is 3.25 (11). If the rat did not repeat an entry or enter at least five arms within 5 min, no choice accuracy score was taken for the session and the average value for the other sessions was entered in the session block. The response latency measure was the total session duration divided by the total number of entries (seconds per entry). In the repeated acquisition procedure, three of eight of the arms were baited. On a particular day the same arms were baited for five consecutive trials. Each day the arms baited were changed in a pseudorandom order. The trial was continued until either the rat entered all three baited arms or 3 min elapsed. The dependent measure for repeated acquisition was errors per trial. The testers were blind to the treatment conditions of the rats.

The Functional Observational Battery. The FOB is a series of observations and tests used to evaluate the overall neurological integrity of the rat. Testing was conducted at 7 and 13 days after *Pfiesteria* exposure. Detailed descriptions of the procedures and scoring criteria have been published elsewhere (12,13). Home-cage observations included any abnormal motor movements as well as activity level. Lacrimation, salivation,

piloerection, ease of removal, and handling reactivity were ranked, according to the defined criteria, as the rat was removed from the cage and held in the observer's hand. The rat was then placed on the top of a laboratory cart (60 × 90 cm) and allowed to freely explore for 3 min. During that time, the observer ranked and/or described any gait abnormalities, arousal, activity level, abnormal motor movements, and excretion level (urination, defecation). The number of rearing responses was also counted. Next, the rat's reactions to the sound of a metal clicker, a pinch near the end of the tail, approach of a pen, and touch on the rump were rated. The aerial righting reflex and pupillary response to light were also tested. Finally, forelimb and hindlimb grip strength, landing foot splay, rectal temperature, and body weight were measured. The same observer conducted all portions of the study and was blind to the treatment condition of each rat. All rats from each cohort were tested in one day.

Locomotor activity in the figure-8 apparatus. The rats were tested for locomotor activity in the figure-8 apparatus in a quiet test room. The figure-8 locomotor activity test has been widely used in behavioral toxicology studies (14). Each figure-8 locomotor apparatus consisted of a continuous enclosed alley 10 cm × 10 cm in the shape of an 8, which was 70 cm long and 42 cm wide. There was a central arena 21 cm × 16 cm with a ceiling 20 cm high and two blind alleys extending 20 cm from either side. Eight photobeams crossed the alleys to index locomotor activity; one was located on each of the two blind alleys and three on each of the two loops of the figure-8. The number of photobeam breaks in each 5-min block in a 1-hr session were tallied by a microcomputer.

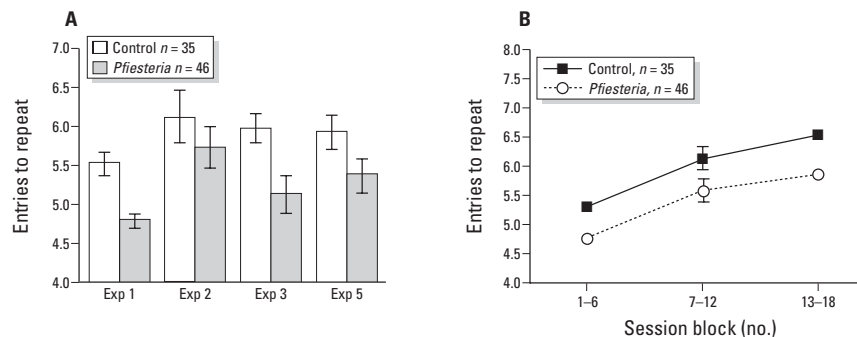


Figure 1. (A) *Pfiesteria* effects averaged over the first 18 sessions of testing in the radial-arm maze. Combined data from all experiments (mean ± SEM) (6,8). Experiment 1: Scores for both groups were lower because food restriction was not used in this study. Experiment 2: The *Pfiesteria* effect was attenuated because the toxin used was aged. In experiment 4, the initial 18 sessions of radial-arm maze training preceded *Pfiesteria* exposure and thus was not included. Experiment 5: Data are from rats trained in the standard test room. Main effect of *Pfiesteria*: * $p < 0.005$. (B) Experiments 1, 2, 3, and 5: Acquisition in the win-shift radial-arm maze task (mean ± SEM) (6,8). Main effects of *Pfiesteria*: all sessions, $p < 0.005$; sessions 1–6, $p < 0.01$; sessions 7–12, $p < 0.05$; sessions 13–18, $p < 0.01$.

Results

Experiment 1: Pilot Study

The *Pfiesteria*-treated rats had significantly lower average entries to repeat scores ($p < 0.005$) than controls averaged over 18 sessions of testing (6). The controls averaged 5.5 ± 0.2 entries to repeat, whereas the *Pfiesteria*-treated rats averaged 4.8 ± 0.1 . Latency was not significantly affected by *Pfiesteria* exposure. This effect was replicated in later studies (below) in which we tested the effects of *Pfiesteria* exposure on radial-arm maze acquisition over 18 sessions (Figure 1A,B).

Experiment 2: Repeat Study

Significant learning took place, but there was no significant effect of *Pfiesteria* exposure (6). Over 18 sessions of training, controls averaged 6.1 ± 0.3 entries to repeat; *Pfiesteria*-exposed rats averaged a slightly lower 5.7 ± 0.3 . We hypothesized that the 7-week storage time of the sample attenuated its potency. J. Burkholder and her group have also found that the toxic effects of *Pfiesteria* on fish decline rapidly over 48 hr after removal of the cells from the aquarium (15), which is consistent with this interpretation. In subsequent experiments only samples stored frozen overnight were used.

Experiment 3: Fresh Sample Study

There was a significant effect of *Pfiesteria* exposure on choice accuracy in the radial-arm maze (Figure 2) (6). The main effect of *Pfiesteria* exposure was significant ($p < 0.025$), with the controls averaging 6.2 ± 0.2 entries to repeat and the *Pfiesteria*-exposed rats averaging 5.4 ± 0.2 entries to repeat over the 24 sessions of testing. There was a significant effect of session block ($p < 0.0001$) and a significant session block × *Pfiesteria* interaction ($p < 0.025$). Analyses of the simple main effects of *Pfiesteria* at each of the session blocks showed significant *Pfiesteria*-induced deficits during sessions 10–12 ($p < 0.05$), 13–15 ($p < 0.005$) and 16–18 ($p < 0.005$). After we had trained the rats for our standard 18 sessions for acquisition, we

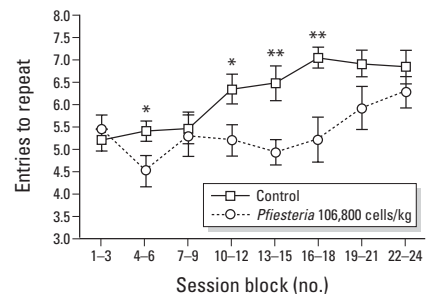


Figure 2. Experiment 3: Acquisition in the win-shift radial-arm maze (mean ± SEM). Main effect of *Pfiesteria*: $p < 0.025$ (6). * $p < 0.05$; ** $p < 0.005$.

added an additional phase of testing, for a total of 24 sessions because the *Pfiesteria*-treated rats had not shown any learning during the standard 18-session training period; the shorter training period was sufficient for control rats. The *Pfiesteria*-treated rats improved during the additional phase of training such that they overcame the significant deficits seen earlier. This improvement shows that *Pfiesteria*-treated rats were not incapable of learning the maze, but just retarded in their acquisition. The delayed acquisition is not a demonstration of recovery since additional training was required. With true recovery, training on a new task would proceed at the same rate as that for controls. This was tested in experiment 4 (below). No significant effects of *Pfiesteria* exposure were seen in terms of response latency. No significant effects of *Pfiesteria* exposure were seen in the complete blood count assessment and white blood cell differential counts. Gross and microscopic examination of H & E-stained sections did not reveal any obvious lesions or signs of pathology. GFAP immunoactivity was not increased in the brains of *Pfiesteria*-exposed animals. However, these pathological tests were performed approximately 8 weeks after dosing.

Experiment 4: Pretraining Study

With the postacquisition win-shift testing, there were no significant *Pfiesteria*-induced deficits (8). Averaged over the 18 sessions of testing after dosing, the controls averaged 6.1 ± 0.2 entries to repeat, whereas the low-dose group averaged 6.6 ± 0.2 and the high-dose group averaged 6.6 ± 0.2 entries to repeat. This showed that *Pfiesteria* treatment at a dose that significantly impaired win-shift radial-arm maze performance during the acquisition phase did not impair performance during the postacquisition phase. Since the same sensorimotor, motivational and working memory functions are required, this result supported the existence of a relatively selective *Pfiesteria* effect on learning. *Pfiesteria* was

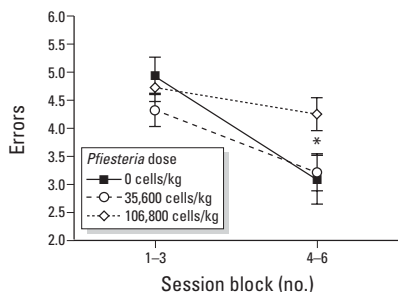


Figure 3. Experiment 4: repeated acquisition training on the radial-arm maze ($n = 12$ per group), $*p < 0.05$, according to Dunnett's test, in control group vs the group administered the *Pfiesteria* dose of 106,800 cells/kg (6).

not without effect during this phase of testing. There was a significant *Pfiesteria* effect on response latency ($p < 0.05$). The 106,800-cells/kg dose caused a significant decrease in latency relative to either the controls ($p < 0.25$) or the 35,600 cells/kg dose group. Controls averaged 25.9 ± 3.3 sec per entry, the 35,600-cells/kg group averaged 24.2 ± 2.9 sec per entry, whereas the 106,800-cells/kg dose group averaged 16.4 sec per entry.

To further assess the persistent effects of *Pfiesteria* on learning, the rats were switched to the repeated acquisition procedure in the radial-arm maze. The three groups performed equally poorly during the first phase of training just after the switch (Figure 3). However, there was a significant *Pfiesteria*-induced learning deficit caused by the higher dose during the second training block ($p < 0.05$). There were significant *Pfiesteria* decreases in latency in both session block 13 ($p < 0.025$) and session block 46 ($p < 0.01$). Before switching to repeated acquisition training, the rats had a total of 36 sessions of training on the same maze with the same cues. Thus, the *Pfiesteria*-induced deficit did not seem to be due to problems associated with familiarization with handling, the maze, or environmental cues. The *Pfiesteria*-induced learning deficit seen in the repeated acquisition task was 10 weeks after the time of *Pfiesteria* exposure, providing evidence for persistent effects. Future studies will carry out the testing for more extensive periods to determine if the *Pfiesteria*-induced repeated acquisition deficit is related to the shift from prior win-shift radial-arm maze testing.

The FOB was also used to assess the effects of *Pfiesteria* exposure in experiment 4. The rats were tested on the FOB 1 hr,

1 week, 4 weeks, and 9 weeks after *Pfiesteria* exposure. The 1-week and 4-week time points were at the same time as win-shift radial-arm maze retesting, and the 9-week time point was at the same time as repeated acquisition radial-arm maze testing. There were no significant differences on the measures of sensorimotor function, no abnormal motor behaviors, and no changes in physiological parameters (e.g., body temperature). The only significant *Pfiesteria*-induced changes were differences in habituation across repeated testing sessions. There was significant habituation in the controls and low *Pfiesteria* groups ($p < 0.005$) on measures of arousal and rearing (Figure 4). Rats receiving the high dose of *Pfiesteria*, however, showed significantly less habituation ($p < 0.05$). The lack of habituation in *Pfiesteria*-treated rats in the FOB could be a representation of the cognitive deficits also seen in these rats, i.e., a learning impairment. However, there are possible noncognitive explanations as well. Future studies will help to determine the relationship of learning deficits to other neurobehavioral changes caused by *Pfiesteria* exposure.

Experiment 5: Cue Structure Study

During the initial phase of training there was a *Pfiesteria*-induced impairment in radial maze acquisition in the standard test room. The improvement in choice accuracy (entries to repeat) from the first session block (session 13) to the second session block (session 46) was analyzed. Analysis of entries to repeat during the early phase of acquisition showed there was a significant treatment \times session block interaction ($p < 0.05$) (Figure 5A). Follow-up analysis of the improvement showed there was a significant main effect of

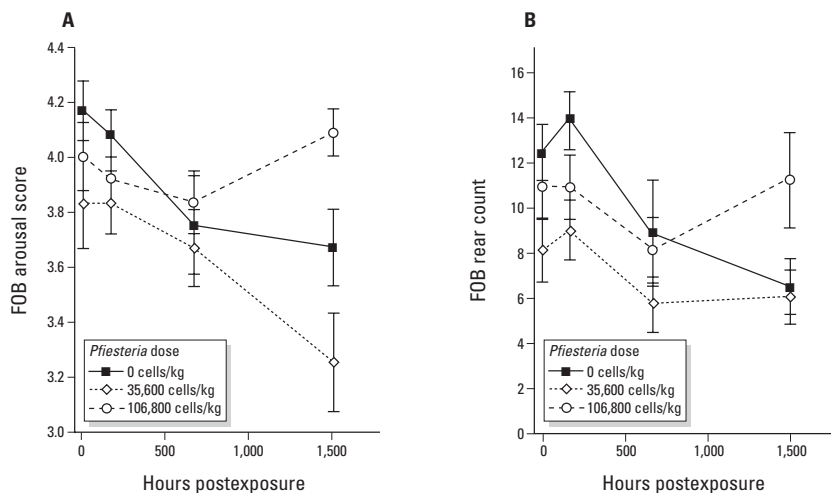


Figure 4. Experiment 4: Functional Observational Battery—arousal and rearing measures. Linear trend over time, Dunnett's test comparing *Pfiesteria* 0 vs 106,800 cells/kg. $p < 0.05$ for both arousal and rearing (6). Measurements were taken at 1 hr, 1 week, 4 weeks, and 9 weeks post-*Pfiesteria* exposure.

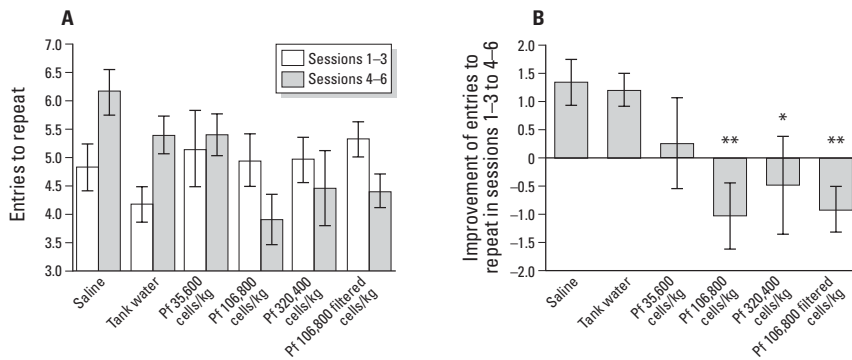


Figure 5. *Pfiesteria* effects during initial learning (experiment 5) in the radial-arm maze in the standard test room (mean \pm SEM) (β). (A) Average choice-accuracy (entries to repeat) during sessions 1–3 and 4–6. (B) Improvement with training. * $p < 0.05$; ** $p < 0.025$ vs saline- or tank-water control group.

Pfiesteria treatment ($p < 0.05$). The planned comparisons of the control groups versus the *Pfiesteria*-treated groups showed that the 106,800-cells/kg, the 320,400-cells/kg, and 106,800-cells/kg filtered groups each had significantly ($p < 0.05$) less improvement than either the saline and tank-water-treated control groups. In fact, each of these groups had, on average, a decline in choice accuracy from the first to the second training session block (Figure 5B). This result is similar to that seen in experiment 3 in this series (Figure 2). The lowest dose group (35,600 cells/kg) was not significantly different from either control group. This shows the same threshold for effect as was seen in experiment 4 (Figure 3). In contrast, the rats tested on the same task in the sound-attenuating chamber did not show a deficit. The controls showed similar rates of acquisition in the two rooms ($p = 0.38$). The differential effect was seen only with the *Pfiesteria*-exposed rats ($p < 0.005$). With the maze in the sound-attenuating chamber the controls showed an improvement of 0.72 ± 0.55 entries to repeat, whereas the *Pfiesteria*-treated rats showed a similar improvement of 0.93 ± 0.29 entries to repeat ($p = 0.68$). In contrast, with the maze in the standard open test environment, the controls showed an improvement of 1.26 ± 0.23 entries to repeat, whereas the *Pfiesteria*-treated rats actually showed a worsening of performance with -0.54 ± 0.32 ($p < 0.005$). During the 18-session acquisition period, the rats trained in the standard test room showed a magnitude of *Pfiesteria*-induced deficit similar to that seen in previous studies (Figure 6).

When the rats were shifted from testing in one room to the other, the *Pfiesteria*-treated rats shifted from the sound-attenuating chamber to the standard test room had a significantly greater decrement in performance than the controls ($p < 0.05$). As shown in Figure 7, the controls averaged a net loss of -0.87 ± 0.38 entries to repeat from sessions 16–18 to 19–21, whereas the *Pfiesteria*-treated rats

averaged a net loss of -1.71 ± 0.27 entries to repeat over the same period. In contrast, there was no difference between the *Pfiesteria*-treated and control rats switched from testing in the standard test room to the sound-attenuating chamber. The change from the standard test room to the chamber did cause a significant overall decrease in accuracy ($p < 0.05$) simply because the deficit with this shift was substantially less than the deficit when animals were shifted from the chamber to the standard test room.

The hypothesis that the test environment was important in the expression of the *Pfiesteria*-induced effect was tested over the course of sessions 16–27. For the first block of sessions (16–18) during this period, the rats continued to be tested in the test room in which they had previously been trained. During the next two session blocks (sessions 19–21 and sessions 22–24) they were switched to the test room opposite the one in which they had initially been trained. Then, during the final session block (sessions 25–27), the rats were switched back to their original test room. Thus, all the rats were tested in both test rooms A and B on either an ABBA or a BAAB schedule over the final four blocks of testing. The results from the initial training phase showed that the *Pfiesteria*-induced deficit was present in the standard test room (A) but not in the sound-attenuating chamber (B), so the difference in choice accuracy performance in the two rooms was analyzed. This analysis showed that the groups given 35,600 cells/kg ($p < 0.05$) and 106,800 cells/kg ($p < 0.025$) were significantly worse than the tank-water controls when they were tested in the standard test room versus the sound-attenuating room. Restriction of possible distracting cues in the sound-attenuating chamber may have reduced expression of the *Pfiesteria* effect on learning. The attentional explanation is a hypothesis that will be tested explicitly in the operant attentional testing in future studies.

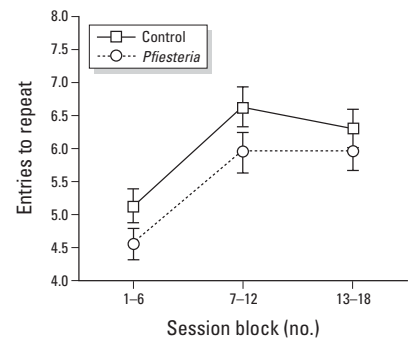


Figure 6. Acquisition performance on the radial-arm maze (mean \pm SEM) in the standard test room (β).

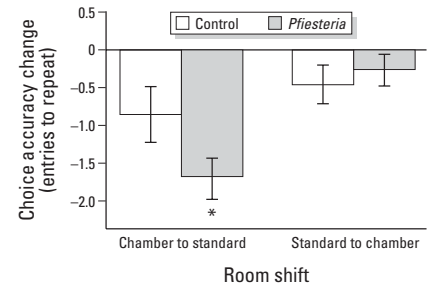


Figure 7. *Pfiesteria* effects during the room shift in testing on the radial-arm maze (mean \pm SEM). Difference in entries to repeat scores sessions 19–21 (postshift) and sessions 16–18 (preshift) (β). * $p < 0.05$ vs control groups.

There were no overt signs of generalized debilitation in the animals during the period of maze testing. No significant *Pfiesteria* treatment effects were seen in terms of average response latency. The FOB measurements taken during this period did not detect neurological deficits or overt toxicity.

Locomotor activity measurements were taken in the figure-8 maze 11 weeks after exposure. Although no substantial *Pfiesteria*-induced alterations were observed in average locomotor activity, a significant *Pfiesteria*-induced change was observed in habituation. The middle-dose and high-dose *Pfiesteria* groups had significantly greater rates of habituation than the tank-water controls (Figure 8). No difference was seen between the tank-water-treated and the saline-treated controls or the low-dose *Pfiesteria* group. There was a nearly significant greater habituation in the filtered middle-dose *Pfiesteria* group. The greater linear trend in the *Pfiesteria* groups comprised slightly greater activity counts during the early time blocks and slightly lower during the later time blocks.

Experiment 6: Sample-Type Study

There was a significant effect of all three *Pfiesteria* samples ($p < 0.05$), impairing choice accuracy over the first six sessions of radial-arm

maze testing (Figure 9) (9). No differences were seen between the different doses of *Pfiesteria* or between the two control groups. One of the three *Pfiesteria* samples caused a significant latency increase in the radial-arm maze, but the interpretation of this effect was clouded by the finding of significant differences in the latency of the saline- and tank-water-treated control groups.

At the time of the radial-arm maze choice accuracy impairment, no overt *Pfiesteria*-related effects were seen using the FOB, indicating that the *Pfiesteria*-induced choice accuracy deficit was not due to generalized debilitation. In the figure-8 maze, the same *Pfiesteria* treatment, which increased latency in the radial-arm maze, caused a significant mean decrease ($p < 0.05$) in activity over the 1-hr locomotor test (Figure 10). The rate of habituation (linear trend of decreased locomotion) was not significantly affected. *Pfiesteria* effects on choice accuracy in the radial-arm maze and activity in the figure-8 maze and in early radial-arm maze training may be useful in a rapid screen for identifying the critical toxin(s) of *Pfiesteria* in future studies.

Experiment 7: The Juvenile Study

Rats given the higher dose of *Pfiesteria* showed a significant ($p < 0.05$) impairment in radial-arm maze choice accuracy relative to that of the control group averaged over the six sessions of testing (Figure 11) (9). No sex-related differences in response to *Pfiesteria* were seen. Unlike the adults given the same *Pfiesteria* cultures, the juveniles did not show significant *Pfiesteria*-induced increased response latency in the radial-arm maze or decreased activity in the figure-8 activity apparatus.

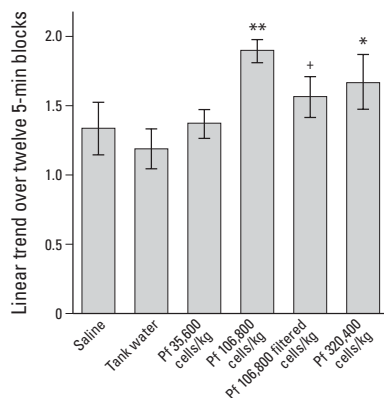


Figure 8. *Pfiesteria* effects the linear trend (slope) of decreasing activity in a 1-hr session in the figure-8 maze (8). Paired comparisons of tank-water controls vs Pf 106,800 cells/kg ($p < 0.005$); vs Pf 106,800 filtered cells/kg ($p < 0.10$); vs Pf 320,400 cells/kg ($p < 0.005$).

Discussion

We have documented a persistent learning deficit in rats after exposure to water containing *Pfiesteria piscicida*. The effect is reproducible and robust (6,8,9). The radial-arm maze learning deficit is now very well established, with a total of 36 control and 48 *Pfiesteria*-treated rats being run and a significant *Pfiesteria*-induced deficit of $p < 0.005$. The impairment did not appear to be secondary to generalized health impairment of the animals. Clinical measures of health, blood cell counts and initial histopathological screens did not detect any effects. The FOB did not detect abnormal unconditioned behavior or reflexes, which would explain the deficits in choice accuracy. The roles of memory and nonassociative factors in the *Pfiesteria*-induced deficit were evaluated. When the rats were administered *Pfiesteria* after pretraining, they remembered the previous learning and performed just as well as controls. The acute *Pfiesteria* exposure did not appear to produce persistent sensory, motor, motivational, or memory deficits sufficient to impair radial-arm maze choice accuracy once the task had been learned. Only when the rats were later trained on a different task (repeated acquisition) in the radial maze did they show a significant *Pfiesteria*-induced deficit. Chronic exposure may well have more pervasive effects on both learning and memory, as seen in chronically exposed humans (3).

In experiment 6, the findings were extended to show that the deficit occurs in both adult and juvenile rats and in both male and female juveniles. The effect in the current study of a significant *Pfiesteria*-induced choice accuracy deficit during the average of the entire first six sessions was more pervasive

than in one of our previous studies (8). There was a significant interaction of *Pfiesteria* treatment \times session block. Follow-up tests of *Pfiesteria* effects on the rate of acquisition, showed a significant *Pfiesteria*-induced deficit in the difference between the first and second blocks of three sessions. However, an analysis including all of the data for the previous four studies (6,8) shows a significant ($p < 0.025$) *Pfiesteria*-induced deficit for the average choice accuracy over the first six sessions of training in the radial-arm maze.

Although all *Pfiesteria* samples tested in the neurocognitive studies caused a significant impairment in choice accuracy, only the Pf-728 sample induced a significant increase in response latency in the radial-arm maze. The robustness of this effect was evidenced by the finding that the same *Pfiesteria* sample was the only one that caused significant hypoactivity in the figure-8 apparatus. The Pf-728 sample may have had a different array of toxins, which caused the additional effect of hypoactivity. Juvenile rats appear to be resistant to the hypoactivity caused by the Pf-728 *Pfiesteria* in the adults. In contrast to adult rats, no effect of *Pfiesteria* was seen on locomotor activity in either the radial-arm maze or the figure-8 apparatus in the juveniles.

These data again provide evidence for the specificity of the *Pfiesteria*-induced impairment on learning. A prominent symptom of *Pfiesteria* intoxication in humans is cognitive disturbance (3,16). The current results provide additional support for this observation. However, more neurobehavioral studies are needed to determine the critical mechanisms of action of *Pfiesteria*.

Experiments 5 and 6 provided evidence for neurobehavioral tests that could be used

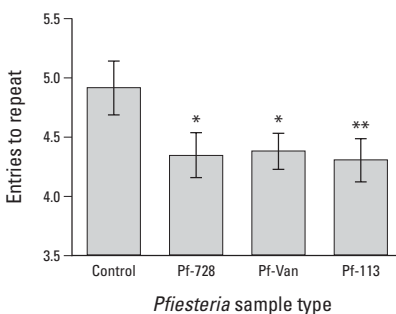


Figure 9. *Pfiesteria*-induced deficit of initial learning in the radial-arm maze (mean \pm SEM) in adult rats (9). Control, $n = 12$ treated with saline and $n = 12$ treated with tank water; Pf-728, $n = 12$ treated with 35,600 cells/kg and $n = 12$ treated with 106,800 cells/kg; Pf-Van, $n = 12$ treated with 35,600 cells/kg and $n = 12$ treated with 106,800 cells/kg; Pf-113, $n = 12$ treated with 35,600 cells/kg and $n = 12$ treated with 106,800 cells/kg. Paired comparisons of control vs Pf-728 ($p < 0.05$); vs Pf-Van ($p < 0.05$); vs Pf-113 ($p < 0.025$).

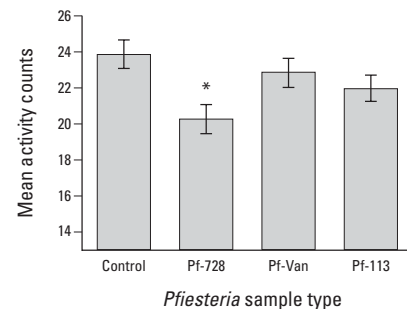


Figure 10. Significant *Pfiesteria*-induced activity reduction in the group exposed to the Pf-728 sample (mean \pm SEM) across the 1-hr session in the figure-8 apparatus in adult rats. Mean \pm SEM per 5-min block (9). Control, $n = 12$ treated with saline and $n = 12$ treated with tank water; Pf-728, $n = 12$ treated with 35,600 cells/kg and $n = 12$ treated with 106,800 cells/kg; Pf-Van, $n = 12$ treated with 35,600 cells/kg and $n = 12$ treated with 106,800 cells/kg; Pf-113, 12 treated with 35,600 cells/kg and 12 treated with 106,800 cells/kg. * $p < 0.005$ vs control group.

for rapid screening of different *Pfiesteria* extracts. Radial-arm maze choice accuracy was the most sensitive of the behavioral assessments used. This measure detected significant *Pfiesteria*-induced deficits with all three of the *Pfiesteria* samples tested in adult female rats and with male and female juvenile rats tested with the Pf-728 sample. In contrast, no effects were seen with the FOB and only Pf-728 affected radial-arm maze response latency or figure-8 apparatus locomotor activity in adult animals.

There are a variety of negative effects of *Pfiesteria* intoxication in humans, but the hallmark is cognitive impairment (3,14). It is therefore essential that models of *Pfiesteria* intoxication include cognitive impairment as a component. The *Pfiesteria*-induced radial-arm maze choice accuracy deficit seen in the current and previous studies appears to fulfill this requirement. We have found that the

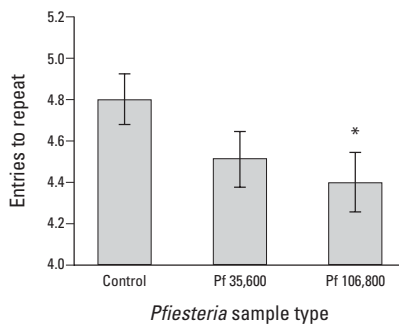


Figure 11. *Pfiesteria*-induced deficit of initial learning in the radial-arm maze (mean ± SEM) in juvenile rats. $n = 23$ – 24 rats/treatment group (9). * $p < 0.05$ vs control group.

first six sessions of radial-arm maze testing are sufficient for detecting the *Pfiesteria*-induced cognitive impairment. This test can be of great use in the determination of the specific toxin or toxins responsible for *Pfiesteria*-induced cognitive impairment.

In vivo neurobehavioral tests are needed to determine the functional toxic effects of *Pfiesteria* exposure. Other assays are important but cannot demonstrate the cause-and-effect relationship between *Pfiesteria* exposure and functional impairment. *In vitro* cell assays are useful for examining intracellular mechanisms of action but cannot define the functional consequences of *Pfiesteria* exposure. The potency of *Pfiesteria* in fish lethality is important for ecotoxicological assessment but does not predict the extent of neurobehavioral effect in mammals. Human studies are important for monitoring possible health effects but often the exposure information is scanty or missing and the causal link cannot be determined. The radial-arm maze choice accuracy measure gathered in six test sessions over a period of two weeks after *Pfiesteria* exposure has been shown in the current studies as well as previous studies (6,8) to be a sensitive indicator of *Pfiesteria*-induced cognitive impairment. This test can serve as a sensitive and relatively efficient indicator of neurotoxicity in the search for the critical neurotoxin(s) of *Pfiesteria*.

REFERENCES AND NOTES

- Burkholder JM, Noga EJ, Hobbs CW, Glasgow HB Jr, Smith SA. New "phantom" dinoflagellate is the causative agent of major estuarine fish kills. *Nature* 358:407–410 (1992).
- Burkholder JM, Glasgow HB Jr. *Pfiesteria piscicida* and

Pfiesteria-like dinoflagellates: behavioral impacts and environmental controls. *Limnol Oceanogr* 42:1052–1075 (1997).

- Glasgow HB Jr, Burkholder JM, Schmechel DE, Tester PA, Rublee PA. Insidious effects of a toxic estuarine dinoflagellate on fish survival and human health. *J Toxicol Environ Health* 46:501–522 (1995).
- Grattan LM, Oldach D, Tracy JK, Greenberg DR. Neurobehavioral complaints of symptomatic persons exposed to *Pfiesteria piscicida* or morphologically related organisms. *Md Med J* 47:127–129 (1998).
- Levin ED, Schmechel DE, Glasgow HB Jr, Burkholder JM, Deamer-Melia NM. *Pfiesteria piscicida* effects on cognitive performance in rats. Presented at the Southeastern Estuarine Research Society. Atlantic Beach, North Carolina, 1996.
- Levin ED, Schmechel DE, Burkholder JM, Glasgow HB Jr, Deamer-Melia N, Moser VC, Harry JG. Persisting learning deficits in rats after exposure to *Pfiesteria piscicida*. *Environ Health Perspect* 105:1320–1325 (1997).
- Levin E, Simon B, Schmechel D, Glasgow HB Jr, Deamer-Melia N, Burkholder J, Moser V, Jensen K, Harry G. Learning deficits in rats after *Pfiesteria* exposure [Abstract]. *Toxicol Sci* 42:37 (1998).
- Levin ED, Simon BB, Schmechel DE, Glasgow HB Jr, Deamer-Melia NJ, Burkholder JM, Moser VC, Jensen K, Harry GJ. *Pfiesteria* toxin and learning performance. *Neurotoxicol Teratol* 21:215–221 (1999).
- Levin ED, Rezvani AH, Christopher NC, Glasgow HB Jr, Deamer-Melia NJ, Burkholder JM, Moser VC, Jensen K. Rapid neurobehavioral analysis of *Pfiesteria piscicida* effects in juvenile and adult rats. *Neurotoxicol Teratol* 22:533–540 (2000).
- Burkholder JM, Glasgow HB Jr. Interactions of a toxic estuarine dinoflagellate with microbial predators and prey. *Arch Protistenkd* 145:177–188 (1995).
- Levin ED. Unpublished computer program.
- Moser VC, McCormick JP, Creason JP, MacPhail RC. Comparison of chlordimeform and carbaryl using a functional observational battery. *Fundam Appl Toxicol* 11:189–206 (1988).
- McDaniel KL, Moser VC. Utility of a neurobehavioral screening battery for differentiating the effects of two pyrethroids, permethrin and cypermethrin. *Neurotoxicol Teratol* 15:71–83 (1993).
- Crofton KM, Howard JL, Moser VC, Gill MW, Reiter LW, Tilson HA, MacPhail RC. Interlaboratory comparisons of motor activity experiments: implications for neurotoxicological assessments. *Neurotoxicol Teratol* 13:599–609 (1991).
- Burkholder JM, Glasgow HB Jr. Personal communication.
- Grattan L, Oldach D, Perl T, Lowitt M, Matuszak D, Dickson C, Parrott C, Shoemaker R, Kauffman C, Wasserman M, et al. Learning and memory difficulties after environmental exposure to waterways containing toxin-producing *Pfiesteria* or *Pfiesteria*-like dinoflagellates. *Lancet* 352:532–539 (1998).