Articles

Persisting Learning Deficits in Rats after Exposure to *Pfiesteria piscicida*

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Pfiesteria piscicida and other toxic Pfiesteria-like dinoflagellates have been implicated as a cause of fish kills in North Carolina estuaries and elsewhere. Accidental laboratory exposure of humans to *P. piscicida* has been reported to cause a complex syndrome including cognitive impairment. The current project was conducted to experimentally assess the possibility of cognitive effects of *P. piscicida* exposure in rats. Samples of water from aquaria in which *P. piscicida* zoospores were killing fish were frozen, a procedure that has been found to induce encystment. Thawed samples were injected into albino Sprague-Dawley rats. A significant learning impairment was documented in rats administered samples of *P. piscicida* that were recently frozen. Prolonged storage of *Pfiesteria* samples diminished the effect. No effect was seen in the recall of a previously learned task, but when the rats were called upon to learn a new task, the *Pfiesteria*-treated animals showed a significant learning deficit. This effect persisted up to at least 10 weeks after a single injection of *Pfiesteria*. The *Pfiesteria*-induced learning deficit did not seem to be associated with any obvious debilitation or health impairment of the exposed rats. Deficits in habituation of arousal and rearing behavior were detected using a functional observational battery. No *Pfiesteria*-induced effects on blood count and white cell differential or in a standard pathological screening of brain, liver, lung, kidney, and spleen tissue were seen at 2 months after exposure. These studies document a persistent learning impairment in rats after exposure to the dinoflagellate *P. piscicida* in otherwise physically well-appearing rats. This effect may partially model the symptoms of cognitive impairments that humans have shown after *Pfiesteria* exposure. *Key words*: dinoflagellates, learning, memory, persisting, *Pfiesteria*, radial-arm maze, toxic. *Environ Health Perspect* 105:1320–1325 (1997). *http//ehis.niehs.nih.gov*

Pfiesteria piscicida Steidinger and Burkholder is a newly recognized species of toxic dinoflagellates. This species inhabits estuarine and coastal waters of the Mid-Atlantic and Southeastern United States and was first discovered swarming in a major fish kill in May 1991 (*1,2*). *P. piscicida* has a complex life cycle that includes at least 24 distinct life stages (*3*). The small flagellated vegetative form has been associated with the most lethal toxic effects (*1*). *P. piscicida* and other *Pfiesteria*-like species have been implicated as major causative agents of many recent massive fish kills (>1,000 fish) in North Carolina estuaries (*2,3*). Fish appear to be narcotized and show poor fright response after exposure to *P. piscicida* (*4*). The chemical identity of the toxin(s) in *P. piscicida* has not been determined. Preliminary *in vitro* studies have shown neural cells to be more sensitive than endothelial cells to toxic damage as measured by leakage of lactate dehydrogenase from the cells and lowered ATP levels (*5*). Adverse health effects in three medically examined people as well as seven other laboratory staff have been reported after accidental laboratory exposure (*6*). The health effects in the small heavily exposed cohort of three people were characterized by a complex syndrome including cognitive disturbance, fatigue,

mood lability, and dermal lesions. The purpose of the current experimental studies was to determine whether toxins from *P. piscicida*, like other marine toxins such as domoic acid, would cause cognitive deficits in a rodent model (*7,8*). These experimental neurobehavioral studies with *P. piscicida* would help lay the groundwork for future studies to identify critical toxins produced by *Pfiesteria*-like dinoflagellates and to characterize their impacts.

The radial-arm maze was chosen to assess spatial learning and memory in rats after *P. piscicida* exposure. Radial-arm maze performance is sensitive to the adverse effects of a variety of neural lesions, drug treatments, and toxicant exposures. Lesions, particularly of the hippocampus and related structures, impair radial-arm maze choice accuracy (*9*). Receptor antagonists of cholinergic and catecholaminergic neurotransmitters reliably impair radial-arm maze choice accuracy (*10*). Exposure to lead, tin, and pesticides also impairs choice accuracy in the radial-arm maze (*11*). A common way of running the radial-arm maze is to bait all eight arms of the maze and record the efficiency with which the subject retrieves the baits. This win-shift procedure was used in all four of the studies in this

project. Another procedure, repeated acquisition, entails repeatedly baiting a subset of arms in the maze for several trials within a session. Repeated acquisition on the radial-arm maze, although not as widely used as the win-shift procedure, has been found (like the win-shift procedure) to be sensitive to the adverse effects of muscarinic cholinergic receptor blockade with scopolamine (*12*) and exposure to organic tin (*13*). In addition to the cognitive assessment in the radial-arm maze, the effects of *P. piscicida* exposure were assessed using a functional observational battery (FOB), a broad screen used to identify potential neurobehavioral effects of chemicals, as well as changes in general health (*14*). Behavioral tests such as these are meant to be the first of a tiered approach to identify potential neurotoxic effects and to provide the basis for more detailed testing. In this study, we examined the rats using the FOB screen to identify any nonassociative processes that may be affected by *Pfiesteria* exposure. Furthermore, tissues taken from exposed rats were evaluated for associated pathology using a standard histopathology

screen. The goal of these studies was to document in an animal model the possible effects of *Pfiesteria* exposure on cognitive processes and overall health.

Materials and Methods

Subjects. Young adult female Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) were housed in groups of two to four in plastic cages with wood shavings. They had *ad libitum* access to water. In Study 1, the rats had *ad libitum* access to food, and in the other studies, they were fed daily after testing such that their weights were kept at 80–85% of free-feeding levels. The treatment and care of the rats was under an approved protocol of the Animal Care and Use Committee of Duke University in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved facility.

Win-shift radial-arm maze training. Behavioral testing was conducted on a radial eight-arm maze constructed of wood and painted black. The central arena was 50 cm in diameter, and eight 10 × 60 cm arms extended radially; food cups were located 2 cm from the distal end of each arm. The maze was positioned 30 cm above the floor in a testing room that contained many extra-maze visual cues such as a door, a shelf, a table, posters on the walls, and the tester who always sat in the same position during training. The rats were tested approximately 3 days/week. Before each session, all the arms of the maze were baited with one-third to one-half of a piece of sugar-coated cereal. At the beginning of the session, 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 at the proximal end of the arm. Because the reinforcements were not replaced during the session, only the first entry in each arm was rewarded. Subsequent reentries were scored as errors. The session continued until the rat had entered all eight arms or 5 min had elapsed. The choice accuracy measure was the number of entries until an error was made (entries to repeat). The response latency measure was the total session duration divided by the number of arms entered (seconds per entry).

Repeated acquisition radial-arm maze testing. Three of the arms of the eight-arm radial maze described above were baited before each trial. Five trials were run each session separated by 1 min intertrial intervals. The same arms were baited for all of the trials of any single session, but the arms baited were changed each session. The rats

were allowed up to 180 sec to finish each trial. Total errors to select the three baited arms were counted for each trial. If only two of the three baited arms were selected before the 180-sec time limit and there were more than eight arm entries in a trial, the number of errors of entries into unbaited arms (errors of commission) plus the error of omission of not selecting the last baited arm were calculated.

The functional observational battery (FOB). The FOB is a series of observations and tests used to evaluate the sensorimotor integrity of the rat. Detailed descriptions of the procedures and scoring criteria have been published elsewhere (*15,16*). 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 described gait abnormalities, arousal, activity level, abnormal motor movements, and excretion level (urination, defecation). The number of rearing responses were 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, and the aerial righting reflex and pupillary response to light were tested. Finally, forelimb and hindlimb grip strength, landing foot splay, rectal temperature, and weight were measured. The same observer conducted all portions of the study and was blind to the treatment conditions of each rat. All rats were tested on the same day.

Pathology. Eight weeks after *Pfiesteria* exposure, the rats in Study 3 were sacrificed under deep barbiturate anesthesia. Blood samples were taken from the aorta. Automated complete blood counts were made by a Serono-Baker Diagnostic Model 9000 (Serono-Baker, Allentown, PA) and microscopic determination of white cell differential counts were made of samples from control and exposed rats. The animals were then perfused via cardiac puncture with saline followed by 10% formalin and post-fixed overnight at 40°C. The brain, liver, lungs, kidney, and spleen were excised and placed in 10% formalin. Sections of each organ, as well as the brains bisected in the midsagittal plane, were dehydrated in ethanol, embedded in paraffin, and cut into 6 μm sections. Tissue cellularity was visualized with hematoxylin and eosin (H&E). In sections from the same paraffin-embedded brain sample, astrocytes were identified by immunohistochemistry

using polyclonal anti-GFAP (glial fibrillary acidic protein) antibodies (DAKO, Carpinteria, CA). Briefly, rehydrated sections were treated with 3% H_2O_2 for 10 min to remove endogenous peroxidase activity; sections were then rinsed for 20 min in phosphate-buffered saline (PBS) and incubated with non-immune goat serum in 1% bovine serum albumin (BSA)/PBS for 20 min prior to a 60 min incubation with rabbit anti-rat GFAP (diluted 1:2,000 in 1% BSA/PBS). A secondary anti-rabbit IgG antibody was added for 30 min, washed in PBS, incubated in avidin–biotin complex (ABC) reagent (Vectastain TM Elite Kit; Vector Laboratories, Burlingame, CA) for 30 min, rinsed with PBS, and stained with a 3,3′-diaminobenzidine (DAB) substrate.

Studies. We conducted four behavioral studies to assess *P. piscicida* effects on performance in the radial-arm maze. The first two studies were initial pilot studies to provide primary characterization of a single *Pfiesteria* sample, and the third and fourth studies were vehicle-controlled experiments using two fresh *Pfiesteria* samples. The *Pfiesteria* samples were collected directly from a North Carolina State University laboratory aquarium in which *P. piscicida* cultures were actively killing fish. Typically, the water in the aquarium had ammonia levels below 200 μg/l and nitrate levels below 500 μg/l. The samples for all of the experiments were documented to be from *Pfiesteria* cultures that caused fish lethality. The *Pfiesteria* cell concentration was determined by counting identified cells per unit volume under light microscopy (*2*). Taxonomy was confirmed by scanning electron microscopy. The aquarium water was injected with no additives into sealed glass test tubes. These tubes were frozen at -80° for at least 1 hr, a procedure that has been found to induce encystment of the *P. piscicida* cells (Burkholder et al., unpublished data). The freezing procedure was performed to examine the effects of the putative toxin without the potential added effects of injecting zoospores. In all of the studies, the tubes containing the samples were warmed at room temperature until no ice crystals remained before injection. Subcutaneous injection was chosen as a route of exposure because, in this way, we could be certain of delivery of the *Pfiesteria* into systemic circulation. The likely critical route of exposure in humans is oral, inhalation, and skin absorption (*6*). The current studies were conducted to help provide the basis for determining the toxin(s) critical for the neurobehavioral effects in mammals. Once the chemical identity of the toxin(s) is known, further studies with aerosol inhalation will be more feasible. The purpose of these studies was to identify and characterize the effects of

P. piscicida toxins in a controlled setting to help design subsequent studies more closely related to the route of exposure to *Pfiesteria*-like dinoflagellates in the field.

Study 1 (pilot study) was an initial pilot evaluation of the acute and persisting behavioral effects of *P. piscicida* exposure. Because there were no previous data concerning the neurobehavioral effects of *Pfiesteria* in rodents, we assessed the effects on a limited number of rats in this study. Six rats were subcutaneously (sc) administered *Pfiesteria* samples that contained 35,600–961,200 *Pfiesteria* cells/kg rat body weight (bw) [35,600 cells/kg (*n* = 1; 106,800 cells/kg bw (*n* = 3); 320,400 cells/kg bw (*n* = 1); and 961,200 cells/kg bw (*n* = 1)]. The *Pfiesteria* samples for Study 1 were kept frozen (-4°C) between 23 and 43 days as the rats were administered *Pfiesteria* on a staggered schedule. The samples were thawed and refrozen four times. Six control·rats were not injected. Observations of behavior were made for 6 hr after acute injection. Beginning 2 days after exposure, the rats began testing in the win-shift radial-arm maze task involving 18 sessions over the next 6 weeks. In Study 1, the rats were on an *ad libitum* feeding schedule to ensure that the *Pfiesteria* exposure did not adversely affect free-feeding body weight. In the following 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 explore the maze for food reinforcement.

Study 2 (repeat study) was a more focused evaluation of the *P. piscicida* samples that contained 106,800 cells/kg bw using the sample employed in Study 1. This sample of *Pfiesteria* had been stored sealed and frozen at -4°C for 7 weeks before use in this study. For this study, it was thawed for the fifth time. Ten rats were injected with *Pfiesteria* and 10 were injected with saline (sc injection). They were observed every 20 min for the 6 hr after administration for clinical signs of abnormal behavior and acute health impairment. Win-shift radial-arm maze training began 2 days after administration.

Study 3 (fresh sample study) evaluated the effects of a fresh sample of *P. piscicida* collected from the culture aquarium at North Carolina State University. The sample was only frozen at -4°C overnight and thawed only once just before injection. Ten rats were injected with *Pfiesteria* samples that contained 106,800 cells of *Pfiesteria*/kg rat body weight compared to 10 controls injected with control aquarium water collected by the same method except that the tanks did not contain *Pfiesteria*. The rats were observed for behavioral effects for the 6 hr after injection and began training in the win-shift radial-arm maze task 2 days after *Pfiesteria* exposure. After behavioral testing, the rats were sacrificed and the brain, lungs, liver, kidneys, and spleen were collected for pathological assessment.

Study 4 (pretraining study) determined if the deficits seen in radial-arm maze performance in the previous studies were due to impairments in learning or memory. Rats were pretrained for 18 sessions on a radial-arm maze win-shift task before *P. piscicida* administration. They were then administered *Pfiesteria* samples that contained 0, 35,600, or 106,800 cells/kg bw. As in Study 3, a fresh sample of *Pfiesteria* was used, which had been collected from aquaria at North Carolina State University and frozen at -4°C only overnight; the control dose was aquarium water without *Pfiesteria*. The samples were thawed only once just before exposure. Two days after exposure, testing on the radial-arm maze win-shift task resumed. The rats were tested for the following 6 weeks for 18 sessions. The rats were then tested for 6 sessions over 4 weeks using a repeated acquisition task in the same eight-arm radial maze. The rats in Study 4 were assessed at time points of 1 hr, 1 week, 4 weeks, and 9 weeks postexposure using a standardized FOB (*15,16*).

Data analysis. The choice accuracy (entries to repeat) and response latency (seconds per entry) measures were assessed by a within-subjects design analysis of variance. In Study 4, a linear trend analysis was conducted across doses, and Dunnett's test was used to compare each dose to control (*17*). A *p*-value less than 0.05 (two-tailed) was considered significant. For the FOB, individual neurobehavioral measures were analyzed as described by Creason (*18*). Two-way analyses of variance (ANOVAs) were conducted, with treatment as a grouping factor and repeated testing a within-subject factor. Continuous data were analyzed by a linear model (GLM; SAS Institute, Cary, NC) (*19*), while rank data were subjected to categorical analysis procedures (CATMOD; SAS Institute) (*19*). If the overall dose-by-time interaction in the ANOVA was significant at *p*<0.05, the data from each time point were analyzed using a one-way ANOVA followed by post hoc tests to determine which dose groups were significantly different from control. A significant overall dose factor effect prompted further analyses with the data collapsed across time to determine significant dose groups.

Results

Study 1. There was a significant effect of *P. piscicida* treatment on radial-arm maze choice accuracy. The *Pfiesteria*-treated rats had significantly lower average entries to repeat scores [$F(1,10)$ = 14.92; p<0.005] than controls averaged over 18 sessions of testing. The controls averaged 5.5 ± 0.2 entries to repeat and the *Pfiesteria*-treated rats averaged 4.8 ± 0.1 entries to repeat. There was no significant effect of session block or *Pfiesteria* × session block interaction on choice accuracy. Latency was not significantly affected by *Pfiesteria* exposure.

Study 2. In this study, 10 rats were injected with the *P. piscicida* solution used in Study 1, which had been frozen at -4C° for 7 weeks. Significant learning took place [$F(5,90)$ = 13.59; p<0.001], but there was no significant effect of *Pfiesteria* exposure. Over all 18 sessions of training, the controls averaged 6.1 ± 0.3 entries to repeat and the *Pfiesteria*-exposed rats averaged 5.7 ± 0.3 entries to repeat.

Study 3. A fresh solution of *P. piscicida* was used in Study 3. There was a significant effect of *Pfiesteria* exposure on choice accuracy in the radial-arm maze (Fig. 1). The main effect of *Pfiesteria* exposure was significant [$F(1,18)$ = 7.34; 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 [$F(7,126)$ = 7.14; p<0.0001] and a significant session block × *Pfiesteria* interaction [$F(7,126)$ = 2.36; 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). The *Pfiesteria*-treated rats improved during the later phase of training such that they overcame the significant deficits seen earlier. No significant effects of *Pfiesteria* exposure were seen in terms of response latency.
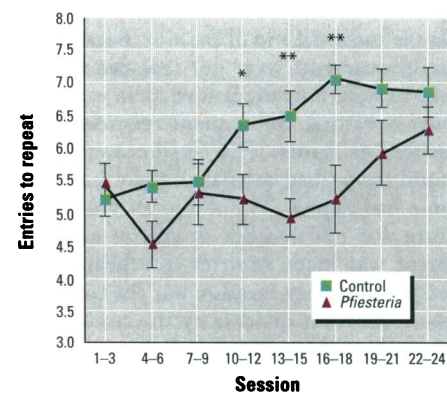
No significant effects of *P. piscicida*

Figure 1. Acquisition training in the win-shift radial-arm maze task (mean ± standard error) in Study 3 after exposure of rats to fresh *Pfiesteria* samples (106,800 cells/kg body weight). *Pfiesteria* main effect p<0.05.
*p<005.
**p<0.005.

exposure were seen in the complete blood count assessment and white blood cell differential counts (Table 1). Gross and microscopic examination of H & E-stained sections revealed no clearly observable lesions or signs of pathology. GFAP immunoreactivity was not increased in the brains of *Pfiesteria*-exposed animals.

Study 4. To differentiate the effects of *P. piscicida* on learning and memory, rats were pretrained on the radial-arm maze win-shift procedure for 18 sessions prior to *Pfiesteria* administration. There were no significant differences in performance between groups prior to dosing ($p = 0.74$). During the last 6-session block of training, the controls averaged 5.6 ± 0.2 entries to repeat while the animals selected for the low dose of *Pfiesteria* (samples that contained 35,600 cells/kg bw) and the high dose of *Pfiesteria* (samples that contained 106,800 cells/kg bw) averaged 5.4 ± 0.2 and 5.7 ± 0.4 entries to repeat, respectively. Then, beginning 2 days after dosing, the rats were tested for maintenance of working memory choice accuracy for an additional 18 sessions. There were no significant deficits caused by *Pfiesteria* administration on retention of win-shift radial-arm maze choice accuracy. *Pfiesteria* exposure did not significantly impair neurobehavioral function required for performance of the radial-arm maze task after the rats were pretrained. Averaged over the 18 sessions of testing after dosing, the controls averaged 6.1 ± 0.2 entries to repeat, the low-dose group averaged 6.6 ± 0.2, and the high-dose group averaged 6.6 ± 0.2 entries to repeat. There was, however, a significant *Pfiesteria* effect on response latency [$F_{(2,33)} = 3.66$; $p<0.05$]. Averaged over the 18 sessions of testing after exposure, the controls averaged 25.9 ± 3.3 sec/entry, the low-dose group averaged 24.2 ± 2.9 sec/entry, and the high-dose group averaged 16.4 ± 1.3 sec/entry. Post hoc Dunnett's tests showed a significant ($p<0.05$) difference between controls and the high dose group.

To assess the effects on learning, the rats were switched to the repeated acquisition procedure in the radial-arm maze. The three groups performed in a similar manner during the first phase of training (Fig. 2). There was no significant group effect during the first block of training sessions; however, there was a significant overall effect of *Pfiesteria* dose during the second training block [$F_{(2,30)} = 3.51$; $p<0.05$]. Dunnett's post hoc test comparing the treated groups to controls showed a significant deficit in the higher dose ($p<0.05$), but not in the lower dose group, relative to controls. There were significant *Pfiesteria* effects on response latency in both sessions 1–3 [$F_{(2,33)} = 4.30$; $p<0.025$] and sessions 4–6 [$F_{(2,33)} = 6.14$; $p<0.01$]. Dunnett's comparisons showed significant differences between controls and the high-dose group during both sessions 1–3 ($p<0.05$) and sessions 4–6 ($p<0.01$) and a significant difference between controls and the low-dose group only during sessions 4–6 ($p<0.05$) (Table 2).

The FOB was also sensitive to the effects of *P. piscicida* exposure. Rats in Study 4 were tested on the FOB 1 hr, 1 week, 4 weeks, and 9 weeks after exposure. The 1-week and 4-week FOB time points corresponded to the periods of win-shift radial-arm maze retesting. The 9-week FOB time point corresponded to the period of repeated acquisition radial-arm maze testing. Across repeated testing sessions, there was a significant habituation seen in the controls ($p<0.005$) with regard to the arousal and rearing measures (Fig. 3). Animals receiving the high dose of *Pfiesteria*, however, showed significantly less habituation than the control group. The linear trends analysis across test sessions showed a significant *Pfiesteria*-induced difference in arousal ($p<0.05$) and rearing ($p<0.05$). Post hoc Dunnett's tests showed significant differences between controls and the high-dose but not the low-dose group for both arousal and rearing ($p<0.05$). Controls ($p<0.005$) and the low-dose *Pfiesteria* group ($p<0.025$) showed significant downward linear trend for both

arousal and rearing. In contrast, the high-dose *Pfiesteria* group showed no significant downward trend for either arousal or rearing. There were no observed differences with the other measures of sensorimotor function, no abnormal motor movements, and no changes in physiological parameters (e.g., body temperature). Increased body tone approached statistical significance, with an overall main effect of dose of $p<0.06$. Collapsed across time, the analyses showed a trend ($p<0.10$) toward increased tone in both treatment groups. At all time points except the last, there were two to four more rats in either group that showed an apparent increased tone. Defecation also showed a significant dose effect ($p<0.05$) due to slightly lower defecation in the high-dose group which was most apparent at the 9-week test.

Discussion

The present set of studies detected a significant *Pfiesteria*-induced impairment in learning as assessed by choice accuracy in the radial-arm maze. *Pfiesteria*-induced learning deficits were seen in two different
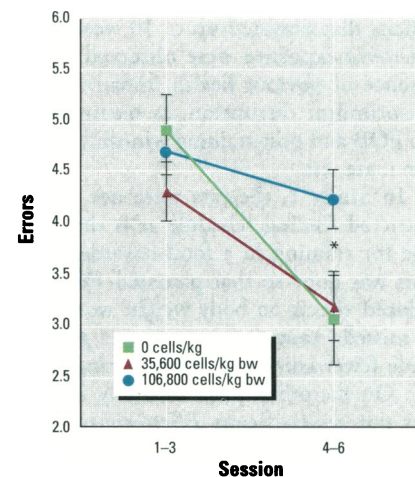

Figure 2. Repeated acquisition training on the radial-arm maze ($n = 12$/group) in Study 4 after exposure of pretrained rats to fresh *Pfiesteria* samples (35,600 or 106,800 cells/kg body weight).
*$p<0.05$ Dunnet's test of control versus the group administered *Pfiesteria* samples at 106,800 cells/kg bw.

Table 1. Blood analysis for Study 3 after exposure of rats to fresh *Pfiesteria* samples (mean ± standard error)

	Control	*Pfiesteria* (106,800 cells/kg bw)
White blood cells ($\times 10^3$/mm^3)	2.45 ± 0.35	2.64 ± 0.17
Red blood cells ($\times 10^6$/mm^3)	6.65 ± 0.14	6.67 ± 0.18
Hemoglobin (g/dl)	13.5 ± 0.3	13.3 ± 0.3
Hematocrit (%)	36.5 ± 0.9	36.1 ± 1.0
Mean corpuscular volume (μ^3)	54.9 ± 0.4	54.2 ± 0.3
Mean corpuscular hemoglobin (%)	20.2 ± 0.2	20.0 ± 0.2
Platelets ($\times 10^3$/mm^3)	815 ± 28	878 ± 38
Differential white blood cell count		
Segmented neutrophils (%)	11.9 ± 1.4	11.3 ± 2.3
Banded neutrophils (%)	0.5 ± 1.4	0.0 ± 0.0
Lymphocytes (%)	86.3 ± 1.5	85.7 ± 3.4
Monocytes (%)	0.9 ± 0.3	2.2 ± 1.2
Eosinophils (%)	0.4 ± 0.4	0.8 ± 0.04

Table 2. Response latency for repeated acquisition in Study 4 after exposure of pretrained rats to fresh *Pfiesteria* samples (mean ± standard error)

Dose (cells/kg body weight)	Sessions	
	1–3	4–6
0	53.2 ± 9.6 sec/entry	53.4 ± 11.1 sec/entry
35,600	33.0 ± 7.2 sec/entry	25.2 ± 5.3 sec/entry*
106,800	23.4 ± 4.0 sec/entry*	19.1 ± 3.4 sec/entry**

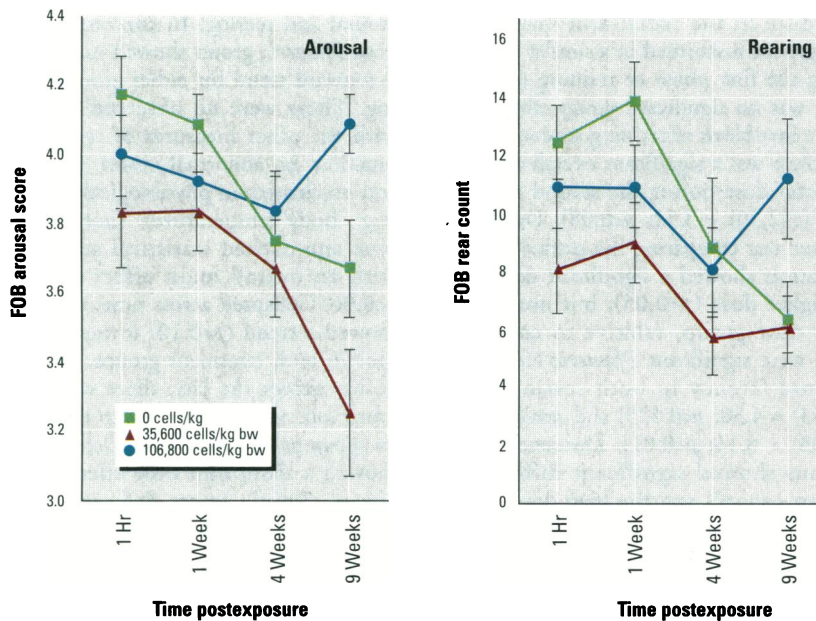*$p<0.05$ vs. control.
**$p<0.01$ vs. control.

Figure 3. Functional observational battery (FOB) arousal and rearing measures in Study 4 after exposure of pretrained rats to fresh *Pfiesteria* samples (35,600 or 106,800 cells/kg body weight). The linear trend of *Pfiesteria* effect is $p<0.05$, by Dunnett's test for both arousal and rearing in control vs. the group administered *Pfiesteria* samples at 106,800 cells/kg bw.

radial-arm maze tasks. The cognitive deficits that persisted up to 10 weeks after *Pfiesteria* exposure were observed in the absence of obvious health impairment or sensorimotor disruption, as measured by the FOB and post-training win-shift radial-arm maze test.

In Study 1, the rats were not on the restricted feeding schedule as is the usual case for training in a food-rewarded task. This was done so that potential *Pfiesteria*-induced effects on body weight would not be missed. Animals exposed to *P. piscicida* made fewer entries to repeat during training. On the other hand, in Study 2, there was not a significant *Pfiesteria*-induced deficit in radial-arm maze behavior. We hypothesize that the storage time of the sample (7 weeks) may have attenuated its potency. This is supported by the fact that toxic effects of *Pfiesteria* on fish decline rapidly and are not seen 48 hr after removal of *Pfiesteria* cells from the aquarium (Burkholder et al., unpublished observations). The attenuated effect of aged *Pfiesteria* samples in terms of both learning impairment in the rat model and toxicity to fish suggests that the toxic components of *Pfiesteria* may degrade over time.

In Study 3, when a fresh sample of *Pfiesteria* stored only overnight was used, there was a significant *Pfiesteria*-induced deficit in choice accuracy. This deficit emerged with continued training, with the controls showing the typical learning curve on this task and the *Pfiesteria*-treated rats showing no evidence of learning during the

standard 18 sessions of training. Learning was not totally ablated by *Pfiesteria* exposure in the rats of this study. With extended training, the *Pfiesteria*-exposed rats did show significant learning during sessions 19–24.

In Study 4, the rats were pretrained on the win-shift radial-arm maze task and then exposed to *Pfiesteria* to determine the effects on memory performance after the acquisition of performance. There were no significant performance deficits in either dose group during the 18 sessions of testing over the 6 weeks after dosing. This continuation of proficiency on the radial-arm maze was an important finding regarding the specificity of the *Pfiesteria* effect because it demonstrated that *Pfiesteria* exposure at this dose range did not significantly impair neurobehavioral processes needed to perform the radial-arm maze after pretraining. Processes such as sensory function, motor function, motivation, and memory were not significantly affected by *Pfiesteria* exposure. The adverse effect of *Pfiesteria* seemed to be specific to the acquisition process as demonstrated by the significant impairment subsequently shown by the higher dose *Pfiesteria*-treated rats on repeated acquisition. Exposure to *Pfiesteria* samples that contained 106,800 cells/kg bw caused a significant impairment in accuracy in learning this new task. The lower dose group, one-third of the effective dose, did not cause a significant deficit in repeated acquisition. The deficit seen in the higher dose *Pfiesteria* group was clearly evident as attenuated improvement with

training. Perseveration of the previous win-shift strategy did not seem to be greater in the higher dose *Pfiesteria* group inasmuch as they did not differ from controls during the first block of sessions after the shift in task requirements.

Pfiesteria effects on response latency in Study 4 did not seem to be closely related to the effects on maze choice accuracy. The high-dose group showed faster performance during the win-shift and both phases of the repeated acquisition testing, but only had worse choice accuracy during the second phase of repeated acquisition testing. The low-dose group had faster performance during the second phase of repeated acquisition testing but did not show any deficit in choice accuracy. *Pfiesteria*-induced changes in response latency were not seen in Studies 1–3.

Most of the measures on the FOB did not detect any difference between the *Pfiesteria*-treated rats and control groups. The lack of effect with regard to spontaneous unconditioned behavior provides additional evidence for the specificity of the *Pfiesteria*-induced impairment to learning. The two significant effects in the FOB that were seen in the higher *Pfiesteria* dose group were related to habituation, a simple form of learning. This higher *Pfiesteria* dose caused a significant attenuation of the decline with repeated testing of arousal and rearing behavior. Many forms of activity, exploration, and awareness change during the course of a study; this type of habituation is expected with repeated testing. In this study, habituation was evident in the control and low-dose groups, even though testing was separated by days to weeks. In contrast, the high-dose group did not show lower arousal or rearing behavior over time, as evidenced by a significantly attenuated linear trend compared with controls. This lack of habituation of the FOB arousal and rearing scores could be a representation of the cognitive deficits also seen in these rats, i.e., a learning impairment, because habituation is a simple form of learning.

The *Pfiesteria*-induced cognitive impairment was quite consistent in the current set of studies. An overall analysis of Studies 1–3 documented a highly significant *Pfiesteria*-induced deficit during the first 18 sessions of training on the radial-arm maze ($p<0.005$) with 26 *Pfiesteria*-treated rats and 26 controls. This analysis includes the rats from Study 2 given the *Pfiesteria* that had been stored for a prolonged period. The specificity of the *Pfiesteria* effect to learning was demonstrated in Study 4. The rats given the higher *Pfiesteria* dose in this study (the same dose as was effective in Study 3) showed a significant deficit on another

learning task in the radial-arm maze, repeated acquisition. They also showed significant deficits in a simpler form of learning, habituation, as assessed in two measures in the FOB. These deficits in learning were relatively specific. There were no signs of obvious toxicity or compromised health in these rats. The blood cell analysis and histopathological analysis also did not detect any Pfiesteria-induced effects. There were some alterations in response latency that appeared to be not closely related to the choice accuracy deficits. The lack of effect when Pfiesteria was administered after learning had taken place in Study 4 demonstrated that the treated rats were fully able to perform all sensorimotor, motivational, and memory components of the task when new learning was not required. The lack of Pfiesteria effects on the FOB except for measures of habituation further supports the specificity of the learning deficit.

The learning deficits seen in the current studies may provide a partial model for the cognitive deficits seen in laboratory personnel who have been accidentally exposed to Pfiesteria (6). The humans accidentally exposed to P. piscicida in the laboratory showed a wide variety of cognitive disturbances including acute spatial disorientation, difficulty with memory and concentration, and reading impairments. The impairments in these individuals showed attenuation over a period of weeks to a few months after cessation of exposure. The relationship of the learning deficits seen in laboratory rats in these studies to possible risks involving human exposure in the field is as of yet unclear. The current studies, which identified a neurobehavioral effect caused by experimental exposure to P. piscicida,

should help with future work to identify critical toxins in Pfiesteria-like dinoflagellates that are responsible for learning deficits. Better measures of exposure to the toxins in the field can be made and better laboratory models of human field exposure can be developed, resulting in a more accurate definition of the possible human health risk from exposure to the toxic Pfiesteria complex in the environment (3).

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