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# Altered brain activity in brevetoxin-exposed bluegill, *Lepomis macrochirus*, visualized using in vivo $^{14}$C 2-deoxyglucose labeling ☆

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**Abstract**

This study investigated the neurological effects of sublethal brevetoxin (PbTx-2) exposure in bluegill (*Lepomis macrochirus*) by measuring alterations in 2-deoxyglucose (2-DG) uptake in the brains of exposed fish. Changes in regional brain activity were quantified using digitized autoradiographs from exposed and control fish. Brains of brevetoxin-exposed fish had significantly higher labeling of 2-DG than brains of control fish. Regional increases in labeling were observed in the optic lobes, telencephalon, and cerebrum of PbTx-2 exposed fish. From these observations, we conclude that sublethal brevetoxin exposure in vivo in bluegill increases neurological stimulation, measured through quantification of [$^{14}$C]2-DG uptake in the brain. Increases in the uptake of [$^{14}$C]2-DG from this study may be indicative of differences in neural activity in the PbTx-exposed fish and are likely associated with the action of PbTx-2 on voltage-gated sodium channels (VGSC), as well as neurological alterations in calcium and neurotransmitter release downstream resulting from VGSC activation. These techniques quantify physiological alterations in fish brain activity resulting from exposure to brevetoxin and possibly other harmful algal bloom toxins.


*Keywords:* 2-Deoxyglucose; Brevetoxin; Brain; *Karenia brevis*; Fish toxicology

## 1. Introduction

Red tides were first recorded in the late 1800s in the Gulf of Mexico on the coast of Western Florida (Fleming and Baden, 2000). The causative agent of these harmful red tide events is the dinoflagellate *Karenia brevis*, which releases neurotoxins following ingestion by predators and through increased wave action. Ten structural isomers of brevetoxin exist, with PbTx-1, PbTx-2, and PbTx-3 the most commonly found and most toxic to organisms and humans. Brevetoxins have the capability to biomagnify once released into the environment, affecting marine mammals such as dolphins, whales, and manatees (Trainer and Baden, 1999). In addition, *K. brevis* is responsible for large-scale fish kills, bioaccumulation of neurotoxins in shellfish, and neurotoxic shellfish poisoning in humans, a syndrome that includes respiratory, neurological, and gastrointestinal symptoms (Baden and Mende, 1982).

Brevetoxins, PbTx-*n*, are a family of lipophilic polyether neurotoxins with a specific binding affinity for voltage-gated sodium channels (VGSC) in excitable membranes (Deshpande et al., 1993). PbTx-*n* are lipophilic, allowing rapid passage through membranes and acting at the lipid border of the VGSC, particularly in mylenated nerves and central nervous system (CNS) neurons (Deshpande et al., 1993; Purkerson-Parker et al., 2000). Specifically, PbTx-*n* binds to receptor site 5 near the S5-S6 extra cellular loop of domain IV of the

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α-subunit (Jeglitsch et al., 1998). The α-submit of the VGSC consists of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6), and one re-entrant segment (SS1/SS2), connected by external loops. The S4 segments are positively charged and serve as voltage sensors for initiating activation. VGSC inactivation is mediated through the connected domains of III and IV (Cestéle and Catterall, 2000). The binding of PbTx-*n* to site 5 inhibits channel inactivation and increases the VGSC open time two-fold, shifting activation to more negative membrane potentials (Jeglitsch et al., 1998). This leads to an influx of sodium ions, promoting depolarization, and may reduce the number of channels available for impulse conduction depending on the tissue (Deshpande et al., 1993; Franz and Leclaire, 1989).

It is believed that the A ring and overall length of the PbTx molecule may interact with the inactivation gate, leading to inactivation of the channel (Gawley et al., 1992; Jeglitsch et al., 1998). PbTx-*n* flexes around binding site 5, held by a combination of hydrophobic interactions and hydrogen bonds. This binding induces conformational change, activates channel opening, and inhibits channel inactivation through indirect allosteric interactions (Cestéle and Catterall, 2000; Gawley et al., 1992). However, a contrasting view is that it is not a conformational change in the channel, but rather an interaction of PbTx-*n* with the S5–S6 extra-cellular loop inhibiting inactivation (Purkerson-Parker et al., 2000). Modifications to the side chain of PbTx-3 can alter the activity of the toxin, with large additions blocking, activating, or eliciting no effect on the channels (Purkerson-Parker et al., 2000). Ultimately, VGSC activation can then lead to alterations in ion flux, leading to neural excitation.

The resultant VGSC activation and membrane depolarization initiates a cascade of events leading to excitotoxicity of neural tissue. PbTx mediated VGSC activation induces excitatory amino acid (EAA) release resulting in calcium influx and NMDA channel activation (Berman and Murray, 1999; Meunier et al., 1997). In addition, PbTx-*n*-induced release of endogenous EAA subsequently activates glutamate and NMDA receptors (Berman and Murray, 1999; Lepage et al., 2003). NMDA receptor ion channel, L-type voltage-sensitive calcium channel, and plasma membrane Na/Ca exchange are associated with the increase in calcium into the cell (Lepage et al., 2003). PbTx-1–3 produce a rapid concentration-dependent increase in calcium influx in rat cerebellar granule neurons downstream of VGSC activation (Lepage et al., 2003). A concentration-dependant release of EAA (LDH, L-glutamate, and L-aspartate) with an increase of PbTx was correlated with neuronal injury in rats. However, toxicity was mediated by NMDA receptors resulting from EAA release and not from VGSC activation alone (Berman and Murray, 1999). In addition, the action of PbTx increases AcH release, halts synaptic vesicle recycling, induces terminal swelling, and is dependant upon extra cellular sodium ions (Meunier et al., 1997). The effects of PbTx-*n* on nonmammalian vertebrates, such as fish, remain poorly understood.

In fish, access of PbTx-*n* to binding sites is direct and not hampered by the biological barriers encountered by other exposure routes, such as ingestion, since uptake occurs through the gills (Trainer and Baden, 1999). Currently, there is one study investigating the effects of brevetoxin on the sensory systems of fish. Lu and Tomchik (2002) describe a significant loss in auditory sensitivity in fish resulting from brevetoxin exposure and state that PbTx-3 can affect the CNS of fishes as well as the peripheral auditory system. This current study investigated CNS alterations resulting from brevetoxin exposure in a teleost fish.

In order to measure alterations in neural activity due to brevetoxin exposure, we applied a newly developed [$^{14}$C]2-deoxyglucose method to discern gross metabolic changes in the brains of exposed fish (Choich et al., 2002). This method is based on the previous work of Louis Sokoloff (1977), who demonstrated a direct relationship between glucose metabolism and brain activity at the regional level. When an area of the brain is activated by a stimulus, neural activity in that area increases and is accompanied by a relative increase in cellular glucose uptake and metabolism. This increased glucose demand can be measured using radiolabeled 2-deoxyglucose (2-DG) as a tracer. When the 2-DG is taken up by cells, its metabolic product is deoxyglucose-6-phosphate. Deoxyglucose-6-phosphate lacks the necessary oxygen for further enzyme recognition by glucose-6-phosphatase and, as a result, does not undergo further metabolism through the glycolytic pathway. Instead, the radiolabeled deoxyglucose-6-phosphate remains trapped in the cells and can be visualized using autoradiography.

We utilized this marker of cellular metabolic activity to measure changes in neural activity due to brevetoxin exposure. The goal of our study was to investigate changes in the CNS of a piscine research model, bluegill (*Lepomis macrochirus*), associated with waterborne exposure to a sublethal and environmentally relevant concentration of brevetoxin.

## 2. Materials and methods

### 2.1. Animal model

Juvenile bluegill (110–125 mm) were obtained from a local pond source and were laboratory acclimated for at least 1 month prior to the experiments. Laboratory acclimation conditions consisted of flow-through,

activated carbon-treated, chlorine-free freshwater (pH 7.6–7.8, hardness 80 mg/L as $CaCO_3$ equivelents, 25°C) with a photoperiod of 14:10 light:dark. Fish were fed a diet of trout chow (38% protein; Zeigler Bros, Gardner, PA, USA) and were inspected for health conditions throughout acclimation.

### 2.2. Treatment protocol

Bluegill were exposed for 30 min in 4 L glass vessels containing 2 L of exposure media at 25°C. The following three treatments were carried out in separate exposure vessels: (1) 40 ppb PbTx-2 (obtained from Dan Baden, University of North Carolina, Wilmington, NC, USA) with 0.001% emulfer vehicle (Alkamuls EL-627, Rhodia, Cranbury, NJ, USA); (2) 0.001% emulfer only (vehicle control); and (3) dilution water only (control). This 40 ppb PbTx-2 concentration approximates the 24 h LC50 response to a 1 h brevetoxin exposure (van der Schalie et al., 2001). The emulfer vehicle was used to prevent the toxin from sorbing to the walls of the glass vessels. Water samples taken at the beginning and end of the exposures verified the intended exposure concentration. The experiment was run twice, with three fish per treatment group in each replication.

Animal exposure and radiography techniques followed the methods of Choich et al. (2002). Fish were allowed to swim freely in their respective exposure vessels for 30 min. After 30 min, fish were injected intramuscularly in the hypaxial musculature with 2 μCi of [$^{14}$C]2-DG (Amersham Chemicals, Piscataway, NJ, USA), subsequently placed into separate 4-L recovery vessels containing 2 L of clean dilution water only (25°C), and allowed to swim freely for 30 min. Subsequently, fish were sacrificed by severing the spinal cord, and whole brains were removed (Kane, 1996). Brains were immediately quick-frozen on aluminum foil dipped in 2-methyl butane chilled over dry ice. Brains were stored at –80°C until processing.

### 2.3. Autoradiography

Whole fish brains were cryosectioned on the horizontal (coronal) plane, dorsal to ventral (10 μM thickness), and thaw-mounted directly onto frost-free microscope slides. Slides were coated with liquid emulsion using a Type E-107 automated emulsion dipping machine (VAvarlaid, Toronto, Canada) in a darkroom and placed flat in light-tight desiccator black boxes for 4 weeks at room temperature to develop.

After 4 weeks, slides were removed from the black boxes in a darkroom and were immersed into photographic D-19 developer (Eastman Kodak Co., Rochester, NY, USA) for 4 min. Slides were rinsed in distilled water for 30 s and then fixed with photographic fixative (Eastman Kodak) for 2 min. Slides were then washed in distilled water for 1 min.

### 2.4. Analysis of 2-DG autoradiograms

Developed slides were evaluated under dark and bright field optics and photomicrographed using a Nikon Labophot-2 microscope (Tokyo, Japan) with an attached video-based digitizing system (Alpha Innotech Corp., San Leandro, CA, USA). Measurements of $^{14}$C labeling intensity were sub-sampled from the telencephalon, optic lobes, and cerebellum of each fish using AlphaImager software (version 5.5).

Digitized autoradiographs were analyzed using the aforementioned techniques, and measurements of silver grain quantification were recorded from the brains of each individual fish separately. For measurement, whole brain sections were viewed at 2× magnification with a dark field optic lens. Developed silver grains appeared white due to the dark field optics, and the attached digital computer system automatically quantified silver grain density. When counting specific brain regions, i.e., telechephalon, optic lobes, and cerebellum (refer to Fig. 2), the specific regions were manually selected and the digital system individually counted each selected region.

### 2.5. Statistical analysis

To analyze the quantitative autoradiography data, a randomized complete block design (three treatments, 2 days, 18 fish), with replicated experimental units within blocks, was used with experimental day as a random block term. $P<0.05$ was used to detect differences between treatments. This design removed variation due to experimental day, reducing experimental error and increasing the detection of treatment effects. Data were log-transformed to meet the assumptions of normality and homoscedasticity (SAS, proc mixed, Version 8.1, Cary, NC, USA).

## 3. Results

Brain activity, measured as 2-DG uptake, was evaluated in specific brain regions in the fish. There were marked increases in autoradiographic labeling in all PbTx-exposed fish compared to the control fish (Fig. 1). There were significantly higher ($P<0.001$) densities of accumulated radioactivity observed in brains from PbTx-2 exposed fish compared to either vehicle-exposed or control fish (Table 1). Further, telencephalon, optic lobes, and cerebrum counts from PbTx-exposed fish were significantly different from vehicle control and control brains ($P<0.001$). Areas of silver grain development in PbTx-exposed fish were greatest in the optic lobes followed by the telencephalon

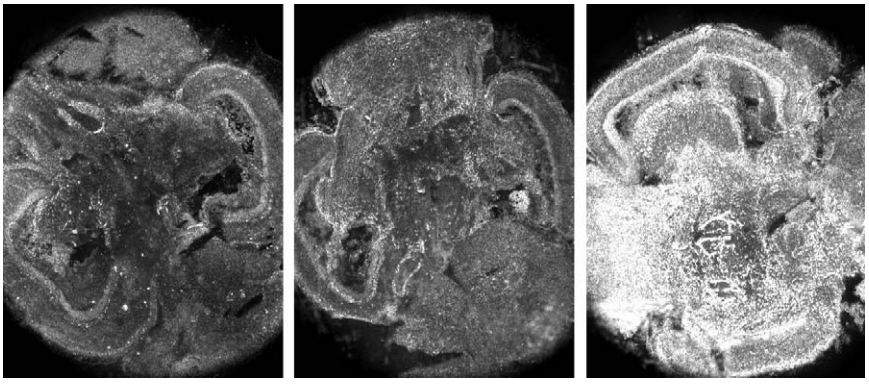

Fig. 1. Representative photomicrographs of bluegill midbrains (primarily optic lobes) showing differences in [$^{14}$C]2-deoxyglucose labeling between treatments. Fish were exposed to dilution water (left), emulfer vehicle water (middle), or brevetoxin (right). Images shown are typical of 3–5 slices per fish with 6 fish per exposure.

Table 1
Individual grain count measurements of $^{14}$C labeling intensity from different brain regions of individual fish brains exposed to brevetoxin, emulfer vehicle water, and dilution water

| Treatment/regions of development | Day 1 | | | Day 2 | | | Treatment means (±SE) |
|---|---|---|---|---|---|---|---|
| | Fish 1 | Fish 2 | Fish 3 | Fish 4 | Fish 5 | Fish 6 | |
| *Brevetoxin* | | | | | | | |
| Optic lobes | 92,273 | 65,701 | 91,783 | 62,767 | 97,701 | 62,285 | 78,752* (6852) |
| Cerebellum | 30,127 | 38,544 | 31,214 | 31,998 | 31,241 | 34,045 | 32,862* (1255) |
| Telencephalon | 73,940 | 64,176 | 70,997 | 60,229 | 57,532 | 64,214 | 65,181* (2553) |
| Whole brain | 267,751 | 263,213 | 265,174 | 214,525 | 235,656 | 220,117 | 244,407* (9814) |
| *Vehicle control* | | | | | | | |
| Optic lobes | 13,242 | 13,230 | 20,120 | 18,797 | 15,486 | 15,869 | 16,124 (1159) |
| Cerebellum | 15,550 | 14,860 | 19,576 | 21,999 | 19,835 | 17,908 | 18,288 (1114) |
| Telencephalon | 28,091 | 15,850 | 21,478 | 21,946 | 18,833 | 15,666 | 20,311 (1899) |
| Whole brain | 96,565 | 76,075 | 93,572 | 94,908 | 86,563 | 85,431 | 88,853 (3155) |
| *Dilution control* | | | | | | | |
| Optic lobes | 18,510 | 16,796 | 19,811 | 17,547 | 18,270 | 18,090 | 18,171 (412) |
| Cerebellum | 16,508 | 15,636 | 12,215 | 20,376 | 24,907 | 18,620 | 18,044 (1780) |
| Telencephalon | 25,144 | 21,768 | 21,886 | 14,396 | 17,163 | 21,022 | 20,230 (1563) |
| Whole brain | 91,086 | 91,345 | 86,837 | 83,344 | 88,648 | 87,290 | 88,093 (1220) |

Individual fish were subsampled from the optic lobes, cerebellum, and telencephalon using AlphaImager software.
*Significant difference compared with both vehicle control and dilution water control data ($P<0.001$).

and cerebellum, with the least in vehicle and dilution-water control groups (Table 1). There were no differences observed between vehicle and dilution-water control groups ($P>0.05$).

## 4. Discussion

This study demonstrated that neurochemical responses to a sublethal exposure of PbTx-2, a naturally occurring neurotoxin, can be detected in fish, which has not previously been documented. Autoradiography of bluegill brains demonstrated visible differences in [$^{14}$C]2-DG between all brevetoxin-treated fish compared to either vehicle controls or dilution-water controls. All regions of the brain measured (telencephalon, optic lobes, cerebellum) had increased [$^{14}$C]2-DG uptake compared with both vehicle control and control fish.

Previous studies have illustrated that a variety of neurotoxins that depolarize neurons through different effectors may be capable of releasing excitatory neurotransmitters, resulting in cell death in the CNS (Berman and Murray, 1999). This release of endogenous neurotransmitters results in subsequent activation of glutamate and NMDA receptors, inhibiting synaptic vesicle recycling and inducing terminal swelling (Berman and Murray, 1999; Meunier et al., 1997). Increases in uptake of [$^{14}$C]2-DG from this study may be indicative of differences in neural activity in the PbTx-exposed fish and are likely associated with the action of PbTx-2 on

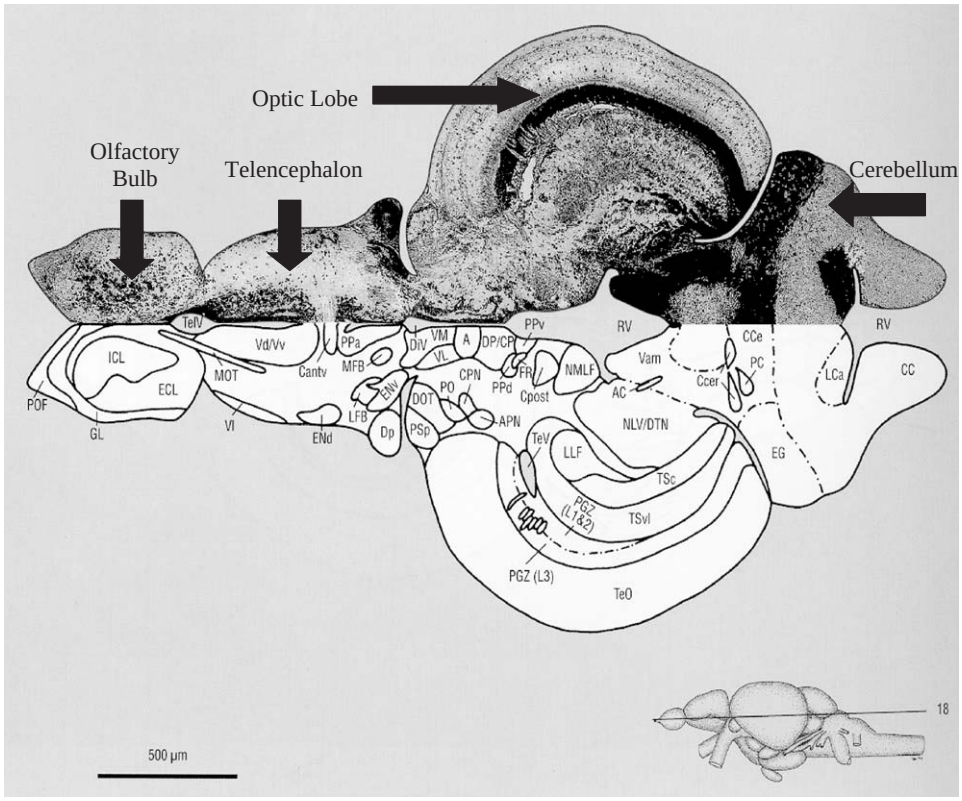


Fig. 2. Horizontal diagram of a fish brain depicting major neuroanatomic structures. Taken from: Wullimann et al. (1996).

VGSC as well as neurological alterations downstream resulting from VGSC activation.

Depolarization of nerve terminals has been postulated to cause major neurotransmitter release, causing a wide variety of responses in effector organs (Rodriguez et al., 1994). In rats, PbTx uptake results in Ca influx, activated by the NMDA receptor ion channel, L-type voltage-sensitive calcium channel, and the plasma membrane Na/Ca exchanger, downstream of VGSC activation (Lepage et al., 2003). Metabolic acidosis and increased lactic acid production leading to altered metabolism can result from PbTx exposure (Franz and LeClaire, 1989). This combination of multiple channel activations and ion fluctuations causes alterations in neurological stimulation and, more importantly, increased energy requirements. The increase in glucose uptake, represented by a proportional increase in [$^{14}$C]2-DG labeling, was therefore expected, although we were not certain that it could be detected after in vivo exposures to a sublethal concentration of PbTx.

Previous studies with fish have shown that sublethal PbTx exposure may lead to decreased auditory sensitivity, loss of balance, lethargy, uncoordinated swimming, twisting and corkscrewing, and changes in ventilatory patterns (Lu and Tomchik, 2002; Kane, unpublished data; Steidinger et al., 1998; van der Schalie et al., 2001). High concentration or chronic exposure has been associated with violent convulsions and death (Steidinger et al., 1998). Alterations of auditory capabilities by neurotoxins suggest that PbTx can effect the CNS of fishes in addition to the peripheral auditory system (Lu and Tomchik, 2002). All areas of the brain (telencephalon, optic lobes, and cerebellum) from PbTx-exposed fish in this study showed significantly altered 2-DG uptake associated with PbTx-2 exposure. Each of these brain regions have important biological functions pertinent to perceiving the surrounding environment and ultimately the survival of the fish. The telencephalic region in teleost fish is an area of sensory concentration, in particular the mechanoreception system (lateral line system). Changes in telencephalic functioning are associated with altered schooling behavior and navigation. Additional areas of sensory concentration are located in the cerebellum and optic tectum, which dominate functions related to motor learning, visual functioning, and perception (Wullimann et al., 1996).

## 5. Conclusions

Application of the 2-DG system to fish presents a unique, in vivo method for detection of physiological alterations resulting from neuroactive agents. This application provides a functional technique to characterize effects of neurotoxins on the CNS. Further investigation into specific areas of the brain effected by

neurotoxin exposure may link behavioral and physiological change with brain alterations resulting from harmful algal bloom exposure.

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## References

Baden, D.G., Mende, T.J., 1982. Toxicity of tow toxins from the Florida red tide marine dinoflagellate, *Gymnodinium breve*. Toxicon 20, 457–460.

Berman, F.W., Murray, T.F., 1999. Brevetoxins cause acute excitotoxicity in primary cultures of rat cerebellar granule neurons. J. Pharmacol. Exp. Ther. 290, 439–444.

Cestéle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883–892.

Choich, J.A., Sass, J.B., Silbergeld, E.K., 2002. A novel system applying the 2-deoxyglucose method to fish for characterization of environmental neurotoxins. Toxicol. Mech. Methods 12, 35–43.

Deshpande, S.S., Adler, M., Sheridan, R.E., 1993. Differential actions of brevetoxin on phrenic nerve and diaphragm muscle in the rat. Toxicon 31, 459–470.

Franz, D.R., LeClaire, R.D., 1989. Respiratory effects of brevetoxin and saxitoxin in awake guinea pigs. Toxicon 27, 647–654.

Fleming, L.E., Baden, D.G., 2000. Florida Red Tide and Human Health. URL: http://www.rsmas.miami.edu/groups/niehs/page11.html

Gawley, R.E., Rein, K.S., Kinoshita, M., Baden, D., 1992. Binding of brevetoxins and ciquatoxin to the voltage-sensitive sodium channel and conformational analysis of Brevetoxin B. Toxicon 30, 780–785.

Jeglitsch, G., Rein, K., Baden, D.G., Adams, D.J., 1998. Brevetoxn (PbTx-3) and its derivatives modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. J. Pharm. Exp. Ther. 284, 516–525.

Kane, A.S., 1996. FishGuts: A Multimedia Guide to the Art and Science of Fish Anatomy, Health and Necropsy. URL: http://aquaticpath.umd.edu/fg. APC Press, University of Maryland, Baltimore, MD, USA.

LePage, K.T., Baden, D.G., Murray, T.F., 2003. Brevetoxin derivatives act as potential agonists at neurotoxin site 5 on the voltage-gated $Na^+$ channel. Brain Res. 959, 120–127.

Lu, Z., Tomchik, S.M., 2002. Effects of red tide toxin on fish hearing. J. Comp. Physiol. A 188, 807–813.

Meunier, F.A., Colasante, C., Molgo, J., 1997. Sodium-dependant increase in quantal secretion induced by brevetoxin-3 in Ca-free medium is associated with depletion of synaptic vesicles and swelling of motor nerve terminals in situ. Neuroscience 78 (3), 883–893.

Purkerson-Parker, S.L., Fieber, L.A., Rein, K.S., Podona, T., Baden, D.G., 2000. Brevetxoin derivatives that inhibit toxin activity. Chem. Biol. 7, 385–393.

Rodriguez, F.A., Escobales, N., Maldonado, C., 1994. Brevetoxin-3 (PbTx3) inhibits oxygen consumption and increases $Na^+$ content in mouse liver slices through a tetrodotoxin-sensitive pathway. Toxicon 32, 1385–1395.

Sokoloff, L., 1977. The $^{14}C$ deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized rat. J. Neurochem. 28, 897–916.

Steidinger, K., Karen, K., Gabriel, G., Tester, P., Tomas, C., 1998. Bloom Dynamics and Physiology of Gymnodinium breve with Emphasis on the Gulf of Mexico. In: Anderson, D.M., Lembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, New York, pp. 133–153.

Trainer, V.L., Baden, D.G., 1999. High affinity binding of red tide neurotoxins to marine mammal brain. Aquat. Toxicol. 46 (2), 139–148.

van der Schalie, W.H., Shedd, T., Widder, M., Kane, A.S., Silbergeld, E., Reimschuessel, R., Poli, M., Sarabun, C., Burkholder, J., Glasgow, H., 2001. Real-time monitoring for toxicity caused by harmful algal blooms and other water quality perturbations. EPA/600/R-01/103, US Environmental Protection Agency, Washington, DC.

Wullimann, M.F., Rupp, B., Reichert, H., 1996. Neuroanatomy of the Zebrafish Brain: A Topological Atlas. Birkhauser Verlag, Basel, Switzerland.