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In vivo genotoxicity testing of the amnesic shellfish poison (domoic acid) in piscine erythrocytes using the micronucleus test and the comet assay

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ABSTRACT

Domoic acid (DA) is a neurotoxic amino acid naturally produced in the marine environment by some diatom species belonging to the genus *Pseudo-nitzschia*. Although the neurotoxic properties of DA have been demonstrated, very little is known about *in vivo* genotoxicity of DA on aquatic organisms. In the present paper, an *in vivo* study on the genotoxic effects of domoic acid was carried out on a fish, *Oreochromis niloticus*, using the micronucleus test and the comet assay. The fish were exposed to three doses of domoic acid (1, 5 and 10 μg/g body weight) by intracoelomic injections. Ethyl methane sulphonate at a single dose of 5 mg/l was used as positive control. Analysis of micronuclei, nuclear abnormalities and DNA damage were carried out on peripheral erythrocytes sampled 24, 48 and 72 h post-treatment. Our results revealed significant increases in the frequencies of micronuclei, nuclear abnormalities as well as DNA strand breaks and thus demonstrated the genotoxic potential of DA on fish.

1. Introduction

In marine ecosystems, microalgae play an important role in the biogeochemical cycling of elements as the main primary producers (Morel and Price, 2003). On the other hand, it is also known that about 40 species among marine phytoplankton have the capacity to produce potent toxins (Sournia et al., 1991). These species make their presence known in many ways, ranging from massive "red tides" or blooms of cells that discolor the water, to inconspicuous concentrations of cells noticed only because of the harm caused by their highly potent toxins. Impacts of these events include mass mortalities of wild and farmed fish and shellfish, human illness and death, alterations of marine trophic structure, and death of marine mammals, seabirds, and other animals (Gulland et al., 2002; Shumway et al., 2003; Schnetzer et al., 2007).

Domoic acid is a naturally occurring toxin produced by microscopic algae, specifically the diatom species *Pseudo-nitzschia* (Schnetzer et al., 2007). DA was recognized in 1987 in Canada as the toxin responsible for the illness amnesic shellfish poisoning (ASP). This toxic episode caused the death of at least three elderly people and more than 100 became ill, suffering neurological problems after consuming blue mussels (*Mytilus edulis*) contaminated with DA (Quilliam and Wright, 1989; Todd, 1993). Harmful algal blooms involving toxic *Pseudo-nitzschia* occur in the many parts of the world's oceans, often resulting in the accumulation of DA in filter-feeding marine organisms and subsequent transfer of the toxin to higher trophic level predators (Scholin et al., 2000; Lefebvre et al., 2002a; Kaniou-Grigoriadou et al., 2005; Bargu et al., 2008).

Toxicity studies with DA in human and in other higher organisms such as monkeys, sea lions, as well as in laboratory mammals demonstrated the neurotoxic properties of DA (Silvagni et al., 2005; Cendes et al., 1995; Truelove and Iverson, 1994). However, the toxic effects of DA on other aquatic organisms, especially on fish, are scarcely investigated (Dizer et al., 2001; Tiedeken et al., 2005; Lefebvre et al., 2007). Additionally, little is known regarding the carcinogenity and genotoxicity of DA (Jeffery et al., 2004). Previous reports on the genotoxicity evaluation of DA are extremely limited and the obtained data is rather controversial. For example, negative results were reported on Chinese hamster lung cells (Rogers and Boyes, 1989) whereas positive results were reported on a marine mussel *in vivo* (Dizer et al., 2001) and on Caco-2 cell lines *in vitro* (Carvalho et al., 2006; Carvalho Pinto-Silva et al., in press).

The genotoxic effects of environmental toxins can be monitored using a broad range of both *in vitro* and *in vivo* biomarker assays but the micronucleus test and the comet assay have recently gained popularity over other assays in aquatic toxicity research due to their sensitivity for detecting cytogenetic and DNA damage and the short time needed to complete a study (Al-Sabti and Metcalfe, 1995; Tice et al., 2000; Cavas and Konen, 2007; Bopp et al., 2008). Micronuclei are formed by chromosome fragments or whole chromosomes that lag at cell division due to the lack of centromere, or damage or a defect in cytokinesis (Heddle et al., 1991). In the recent years,

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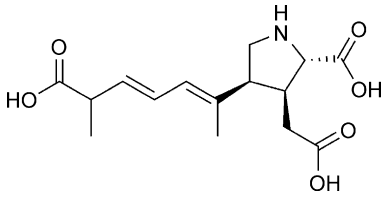

Fig. 1. Chemical structure of domoic acid.

the formation of morphological nuclear alterations in piscine erythrocytes has been used by several authors as possible indicators of genotoxicity (Cavas and Ergene-Gozukara, 2005a,b; Da Silva Souza and Fontanetti, 2006; Ergene et al., 2007a). Comet assay, also known as single cell gel electrophoresis (SCG), is a microgel electrophoresis technique which detects DNA damage in individual cells (Tice et al., 2000).

In the present study, we aimed to evaluate the cytogenetic and DNA damage in peripheral erythrocytes of a model fish species *Oreochromis niloticus* exposed to different concentrations of the marine toxin DA. The micronucleus test, the nuclear abnormalities analyses and the comet assay were used as genotoxicity endpoints. To our knowledge this is the first study devoted to the *in vivo* genotoxicity of domoic acid in fish.

2. Materials and methods

2.1. Fish and chemicals

Nile tilapia, *O. niloticus* (Family: Cichlidae), was chosen for this study because it is commonly available in most fish farms in Turkey. Specimens of *O. niloticus* with an average weight of 10 ± 1 g and length of 5 ± 1 cm were supplied by the Cukurova University fish farm (Adana, Turkey). Its relatively low chromosome number ($2n = 46$), as well as its proven sensitivity to toxic chemicals, make this species a suitable organism for genotoxicity tests (Da Silva Souza and Fontanetti, 2006; Cavas and Ergene-Gozukara, 2003; Ergene et al., 2007b). Before the experiments fish were acclimated under laboratory conditions for 1 month at a population density of 15 specimens in 50-L aquaria, and 25 °C 12/12 h dark/light modes. Fish were not fed during the experiments. Domoic acid (Fig. 1) and all the other chemicals used to perform the micronucleus test and the comet assays were obtained from the Sigma–Aldrich Chemical Company.

2.2. Experimental design

Fish were placed in aquaria containing dechlorinated tap water. Domoic acid was dissolved in water. The fish were intracoelomically (IC) injected with three different doses (1, 5 and 10 μg/g body weight) of domoic acid or ethyl methane sulphonate (EMS) which was used as positive control at a concentration of 5 mg/g body weight. Fish from the negative control group were injected with water only. For the micronucleus test, blood samples were obtained on the first, second and third days following the exposure to DA. Five fish were used for each dose/duration group. A total of 75 fish were used for the experiments.

2.3. Analysis of micronuclei and other nuclear abnormalities

Peripheral blood samples were obtained from the caudal vein of the specimens and smeared onto pre-cleaned slides. After fixation in pure ethanol for 20 min, the slides were allowed to air-dry and then the smears were stained with 10% Giemsa solution for 25 min. All slides were coded and scored blind. Three slides were prepared for each fish, and 2000 cells were scored from each slide under 100× magnification. Small, nonrefractive, circular or ovoid chromatin bodies, displaying the same staining and focusing pattern as the main nucleus, were scored as micronuclei (Al-Sabti and Metcalfe, 1995).

Nuclear abnormalities (NAs) other than micronuclei in erythrocytes were classified into four groups. Briefly, cells with two nuclei were considered as binucleated. Blebbed nuclei had a relatively small evagination of the nuclear membrane and contained euchromatin. Nuclei with evaginations larger than those in the blebbed nuclei, including those with several lobes, were classified as lobed nuclei. Nuclei with vacuoles or voids with appreciable depth into the nucleus were recorded as notched nuclei (Carrasco et al., 1990). Nuclear abnormality data was expressed as total nuclear abnormality calculated by the sum of all nuclear abnormalities.

2.4. The comet assay

In fish about 98% of total blood cells are erythrocytes. Therefore, no cell separation was performed and hereafter cells in whole blood are referred to as erythrocytes (Theodorakis et al., 1994; De Miranda Cabral Gontijo et al., 2003). The alkaline comet assay was performed according to the method of Tice et al. (2000) with some modifications. Blood samples collected from caudal veins of fish were diluted with 1 ml of PBS. 60 μl of the diluted sample were mixed with 200 μl of 0.65% low-melting-point (LMP) agarose. 75 μl of the mixture were than layered on the slides precoated with on 0.5% normal melting point (NMP) agarose and immediately covered with a coverslip and then kept for 10 min in a refrigerator to solidify. After gently removing the coverslips, the slides were covered with a third layer of 90 μl low-melting-point agarose and covered with coverslips again. After solidification of the gel, coverslips were removed and the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM Na_2-EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton X-100 added fresh) and refrigerated at 4 °C for 2 h. After lysis, the slides were placed on a horizontal electrophoresis box side by side. The tank was filled with fresh electrophoresis solution (1 mM Na EDTA, 300 mM NaOH, and pH 13.5) to a level approximately 0.25 cm above the slides. The slides were left in the solution for 20 min to allow the unwinding. Electrophoresis was performed using the same solution at 25 V, 300 mA for 25 min. The slides were than neutralized gently with 0.4 M Tris buffer at pH 7.5 and stained with 75 μl ethidium bromide (20 μg/ml).

Slides were examined using an Olympus BX40 fluorescence microscope equipped with a wide band excitation filter of 330–385 nm and a barrier filter of 420 nm. Two hundred cells (100 per replicate) were scored at 400× magnification. The DNA damage was quantified by visual classification of cells into five categories "comets" corresponding to the tail length (Anderson et al., 1994): undamaged Type 0, low-level damage: Type I, medium-level damage: Type II, high-level damage: Type III and complete damage: Type IV. The extent of DNA damage was expressed as the mean percentage of cells with medium, high and complete damaged DNA, which was calculated as the sum of cells with damage Types II, III and IV (Palus et al., 1999). From the arbitrary values assigned to the different categories (from Type 0 = 0 to Type IV = 4) a genetic damage index (GDI) was calculated for each subject (Pitarque et al., 1999).

2.5. Statistical analysis

After assessing the normality of distribution of the data, both parametric and nonparametric tests were used in order to detect differences at the 0.05 level of significance. Differences between mean values were compared using the Student's *t*-test and least

Table 1
Micronucleus frequencies (‰) in peripheral blood erythrocytes of *O. niloticus* intracoelomically injected with domoic acid (mean ± S.E.)

	Treatment groups				
	Negative control	Positive control	Domoic acid		
			1 μg/g	5 μg/g	10 μg/g
24 h (*n* = 25)	2.02 ± 0.24	9.4 ± 1.55[a]	2.73 ± 0.45	2.92 ± 0.51	5.5 ± 0.85[b]
48 h (*n* = 25)	1.87 ± 0.18	13.2 ± 2.40[a]	6.4 ± 0.80[b]	7.3 ± 1.50[c]	9.2 ± 1.90[a]
72 h (*n* = 25)	1.95 ± 0.28	17.2 ± 2.78[a]	7.1 ± 0.69[c]	8.8 ± 1.70[c]	11.3 ± 2.10[a]

n = total number of animals (*n* = 5 for each group).
[a] $P < 0.001$.
[b] $P < 0.05$.
[c] $P < 0.01$.

significant difference test for the micronuclei data. Comet assay data distributions are generally non-Gaussian, even after logarithmic transformation, which precludes the use of parametric tests (Duez et al., 2003). Thus we applied non-parametric Mann–Whitney *U*-test which is used for evaluation of visual comet data (Anderson et al., 1997; Lehmann et al., 1998; Cavas and Konen, 2007).

3. Results

Micronucleus frequencies in erythrocytes of *O. niloticus* exposed to DA as well as parallel negative and positive controls are summarized in Table 1. As can be seen from the table, the micronucleus frequencies in erythrocytes significantly increased following DA treatment ($P < 0.01$) with the exceptions of the two lowest doses of DA (1 and 5 μg/g) at the first day ($P > 0.05$). Treatment with positive control EMS significantly induced the formation of micronuclei in peripheral erythrocytes ($P < 0.001$). Analysis of total nuclear abnormalities showed significant dose and duration dependent increases following treatment with DA ($P < 0.05$). Results of total NA analyses are shown in Fig. 2.

Table 2 shows the proportion of damaged nuclei and the genetic damage index as measured in the comet assay. A clear and statistically significant increase in DNA migration was found in the DA exposed group compared to the control fish ($P < 0.05$). The differences in genetic damage index between exposed and control groups reached statistical significance, mainly by the increased percentage of type II, III, and IV cells in the exposed group, compared with the control group. These increases were most evident at the two highest concentrations (5 and 10 μg/g) of DA ($P < 0.001$). Similar responses were also observed in positive control groups ($P < 0.001$).

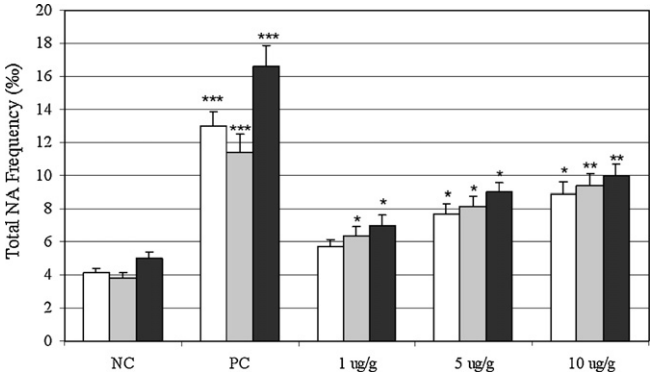

Fig. 2. Frequencies of total nuclear abnormality (NA) frequencies in peripheral erythrocytes of *O. niloticus* intracoelomically injected with domoic acid (NC: negative control, PC: positive control). *n* = 5 for each dose/duration group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 2
Analysis of DNA damage as measured by comet assay in peripheral erythrocytes of *O. niloticus* intracoelomically injected with domoic acid (*n* = 5 for each treatment/duration group)

Treatment groups	Duration (h)	Proportion of damaged nuclei (%)[a]					% of Damaged cells (II + III + IV)[b]	Genetic damage index (GDI)[c]
		Type 0	Type I	Type II	Type III	Type IV		
Negative control	24	80.0	14.9	4.1	1.0	0.0	5.10	0.26
	48	73.0	21.5	4.5	0.7	0.3	5.53	0.34
	72	77.5	18.5	2.3	0.7	1.0	3.98	0.29
Positive control	24	39.4	18.7	18.3	14.4	9.2	41.90	1.35***
	48	32.7	18.3	21.4	16.5	11.1	49.00	1.55***
	72	30.0	19.2	17.9	19.3	13.6	50.80	1.67***
1 μg/g DA	24	70.1	20.2	4.0	3.7	2.0	9.70	0.47*
	48	65.6	22.0	5.1	5.0	2.3	12.40	0.56*
	72	66.4	18.8	7.8	4.8	2.2	14.80	0.58*
5 μg/g DA	24	59.0	22.4	11.5	4.0	3.1	18.60	0.70**
	48	52.0	20.0	15.5	8.5	4.0	28.00	0.93**
	72	48.5	20.5	12.4	11.0	7.6	31.00	1.09***
10 μg/g DA	24	43.2	25.0	15.6	7.2	9.0	31.80	1.14***
	48	41.8	14.3	21.3	14.0	8.6	43.93	1.33***
	72	33.0	11.0	34.6	12.0	9.4	56.00	1.54***

$^{*}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.00$.
[a] 0–IV indicate grades of DNA damage (after Anderson et al., 1994).
[b] Percentage of damaged cells = Type II + III + IV (after Palus et al., 1999).
[c] GDI, Genetic damage index = (Type I + 2.Type II + 3.Type III + 4.Type IV)/(Type 0 + I + II + III + IV) (after Pitarque et al., 1999).

Table 3
Domoic acid levels detected in viscera of various fish species collected from different locations

Common name	Species name	DA (μg/g body weight)	Location	Reference
Chub Mackerel	*Scomber japonicus*	7.3	San Diego USA	Busse et al. (2006)
Jack Mackerel	*Trachurus symmetricus*	5.5		
Pacific Sanddab	*Citharichthys sordidus*	50.1		
Longspine Combfish	*Zaniolepis latipinnis*	9.7		
Sanddab	*Citharichthy sardinus*	2–7.2	California, USA	Lefebvre et al. (2002b)
Chub Mackerel	*Scomber japonicus*	1.4		
Albacore	*Thunnas alalunga*	4.6		
Jack Smelt	*Atherinopsis californiensis*	275		
Northern Anchovy	*Engraulis mordax*	75–444		
South American Pilchard	*Sardinops sagax*	244		
Sardine	*Sardina pilchardus*	0.1–74.2	Portugal	Vale and Sampayo (2001)
European Anchovy	*Engraulis encrasicolus*	0.9		
Northern Anchovy	*Engraulis mordax*	0.5–275	California, USA	Lefebvre et al. (2001)

4. Discussion

Domoic acid (DA) is a neurotoxin mostly produced by phytoplankton belonging to the *Pseudo-nitzschia* group. It is known that elevated concentrations of DA can be produced during phytoplankton blooms which are stimulated by environmental conditions (Trainer et al., 2007). It was also demonstrated that fish can accumulate considerable amounts of DA by dietary consumption of DA produced by toxigenic *Pseudo-nitzschia* species during or after harmful algal blooms. In some cases DA concentrations were observed to be well above the FDA (Food and Drug Administration) regulatory limit value of 20 μg/g (Table 3). Although toxicity data indicated that fish are neurologically susceptible to DA (Lefebvre et al., 2001), there are no published data on the genotoxic effects of DA in fish. The goal of the present study was to determine if treatment with ecologically relevant doses of DA cause genotoxic damage in fish. This report provides the first results of observable DA genotoxicity in fish.

Previous reports on the genotoxicity evaluation of DA are extremely limited and the obtained data is rather controversial. The first study on the genotoxic effects of DA was performed by Rogers and Boyes (1989) who tested DA for mutagenicity at two loci (6-thioguanine resistance at HGPRT locus and ouabain resistance at the Na^+–K^+-ATPase locus) and for two cytogenetic parameters (sister-chromatid exchange and micronucleus test) in a hepatocyte mediated assay with V9 Chinese hamster lung cells. The authors reported that in this *in vitro* study none of the determined genetic endpoints were significantly affected by exposure to domoic acid at dose levels of 27.2 and 54.4 μg/ml with or without activation by freshly isolated rat liver hepatocytes. Dizer et al. (2001) carried out an *in vivo* study on a marine mussel *M. edulis* to evaluate the genotoxic effects of DA exposure using the DNA unwinding assay. They observed significant increases in DNA damage after 48 h in digestive glands of mussels injected with different concentrations (1, 10, 100, 500 ng/g body weight) of DA. Furthermore, Carvalho et al. (2006) reported that *in vitro* treatment with 30, 60 and 100 ng/ml concentrations of DA significantly induced micronucleus formation in of Caco-2 cell lines in 24 h. In the same study, comparative evaluation of micronuclei percentages with and without centromeric signals using FISH assay with pan-centromeric probes revealed that DA is able to induce centromere-negative micronuclei. These results showed the clastogenic potential of DA. In our study, we found that treatment with 1, 5 and10 μg/g body weight doses of DA-induced significant increases in the frequencies of micronuclei and nuclear abnormalities as well as DNA damage in peripheral erythrocytes of *O. niloticus*. Our results are in agreement with those of Dizer et al. (2001) and Carvalho et al. (2006) who reported DNA damaging and clastogenic actions of DA.

Recently, Carvalho Pinto-Silva et al. (in press) reported that DA-induced genotoxic effects in Caco-2 cells after exposure as measured by DNA fragmentation for concentrations of 15, 30, 60 ng/ml. Additionally they reported that Caco-2 cells incubated with high (100 ng/ml) concentration of DA for 24 h showed DNA fragmentation pattern characteristic of apoptotic cells. They concluded that DA causes DNA damages in Caco-2 cells at low concentrations, which accumulated probably because of overloaded of repair systems that lead to apoptosis at higher concentrations. In our study we observed significant increases in the frequencies of types II, III and IV (hedgehog) cells in comet assay. When almost all the DNA is in the tail of a comet, the head is reduced in size, and the image has been refereed to as a "hedgehog" comet. For some reason, the idea has grown that hedgehog comets represent apoptotic cells. However, it has been suggested that they cannot be described as apoptotic for two reasons. Apoptosis is irreversible, but cells with damage revealed as hedgehog comets can repair their damage so that hedgehogs are no longer seen (Collins, 2004). Additionally, apoptosis is characterized by fragmentation of DNA to the size of nucleosome oligomers. Due to the very low molecular weight of DNA in apoptotic and necrotic cells, it was suggested that the DNA of many of these cells will be lost from the gels under the typical electrophoretic conditions (Vasquez and Tice, 1997; Collins, 2004). Singh (2000) described a further visualization method for apoptic cells in which cells are embedded in agarose and lysed as for the normal comet assay, and then – instead of electrophoresis – the DNA is precipitated with ethanol, so called Halo assay. On the other hand, the micronucleus test detects the damage of chromosome or mitotic apparatus caused by chemicals. In our study we found that the frequencies of micronuclei and nuclear abnormalities significantly increased following exposure to DA. Some studies emphasized the sensitivity of Comet assay viz-a-viz micronucleus test (Zhong et al., 2001; Vuyyuri et al., 2006). Thus, the DNA damage can be correlated with the clastogenecity of the DA because the increase in both micronuclei and DNA migration due to environmental mutagens has been reported in fish (Minisi et al., 1996; De Andrade et al., 2004).

The exact mechanism of DA-induced genotoxicity is currently unknown. However results of some previous and recent studies may provide some clues on this issue. For example, the structure of DA includes a butadiene moiety, a well known carcinogen/mutagen (Pye and Glister, 2003; Zhao et al., 2000; Madhusree et al., 2002). It was suggested that the butadiene moiety present in the chemical structure of DA raises the possibility of the formation of DNA-reactive epoxides *in vivo* (Jeffery et al., 2004; Koivisto et al., 1999). Furthermore, in a study performed by Li et al. (2004), it was observed that DA can directly bind to specific double stranded DNA fragments. Similarly, Zhou et al. (2005) reported a strong interaction between 18-mer dsDNA and DA. Additionally, DA has been

shown to induce formation of reactive oxygen species in mouse cerebellar granule cells (Giordano et al., 2007). Induction of genes involved in DNA damage such as B-cell translocation gene 2 (Btg2) has also been demonstrated in rat brain following exposure to DA (Ryan et al., 2005). It is possible to consider that all or some of the mechanisms mentioned above may contribute the genotoxicity of DA.

At the beginning of experiments, we observed some behavioral symptoms such as circular swimming, spiral swimming and upside-down swimming approximately 25 min following IC DA injection. However, these symptoms disappeared in approximately 3 h. The same symptoms were previously reported in anchovies (Lefebvre et al., 2001) and in Coho salmon (Lefebvre et al., 2007) shortly after exposure to DA. It is known that toxic responses can vary substantially depending on the exposure route. For example, it was reported that Coho salmon were neurologically susceptible to DA when they were treated via IC injection whereas neurotoxic effects were not observed in fish exposed to DA by oral gavage administration (Lefebvre et al., 2007). In our study, we used IC injection method for administration of DA and our results showed significant induction of genotoxic damage in DA exposed fish. Although intracoelomic injection does not fully represent an ecologically relevant exposure pathway, it has been used as an alternative administration method in the piscine micronucleus test (Castano et al., 1998; Ayllon and Garcia-Vazquez, 2000; Masuda et al., 2004). On the other hand, although oral exposure is the ecologically relevant route of exposure, it has been shown that fish are able to sequester DA via urinary and biliary pathways and that they may be able to do it better than mammals and birds, therefore at least partially explaining the apparent tolerance of fish to DA during blooms compared to mammals (Lefebvre et al., 2007).

In conclusion our results demonstrated that DA is genotoxic to fish since it induces the formation of micronuclei and DNA strand breaks in peripheral erythrocytes. The study highlights the need for further studies to evaluate the genotoxic effects of DA in fish using ecologically relevant exposure routes (i.e. oral exposure). These findings may provide biomarkers for DA-induced genotoxicity that could be useful for investigating the impacts of acute and long term effects of toxic blooms on wild fish populations.

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