Article

Experimental determination of the bioconcentration factors for anatoxin-a in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Anatoxin-a is a cyanobacterial neurotoxin with a worldwide occurrence in freshwater ecosystems. As a result of global climate changes, it is expected to occur an increase of eutrophication processes and of frequency and intensity of cyanobacterial blooms in several regions of the world. In these conditions, the water concentrations of cyanotoxins, including anatoxin-a, may increase reaching toxic levels, with an additional risk for organisms able to bioconcentrate it in their body. Considering the importance that these processes may have in freshwater ecosystems and the lack of knowledge that still exists on the topic, this study tested the hypothesis that rainbow trout is able to bioconcentrate anatoxin-a. A 96 h bioassay was carried out by exposing juvenile fish to three concentrations of anatoxin-a (132 $\mu g.L^{-1}$, 264 $\mu g.L^{-1}$ and 524 $\mu g.L^{-1}$) through the test media. At the end of the assay, the actual concentrations of the toxin were determined in the test media and in the whole fish body by HPLC-FLD and the bioconcentration factors (BCF) were determined. At all the tested concentrations of anatoxin-a, the fish body concentrations of the toxin were higher than the corresponding water concentrations, with BCF ranging from 30 to 47 based on fresh weight. These findings indicate that the rainbow trout is able to bioconcentrate anatoxin-a, even during short-term exposures and this process may considerably increase the risk of being intoxicated in real scenarios. Since other species of fish may have also this capability and considering that they are key species in a considerable number of freshwater ecosystems, more research should be done on this most important topic in the actual context of global climate changes.

Keywords bioconcentration factor; anatoxin-a; *Oncorhynchus mykiss*.

1 Introduction

Cyanobacteria are versatile and ancient microorganisms, highly successful, inhabiting different terrestrial and aquatic habitats (Dow and Swoboda, 2000; Falconer, 2005). They can proliferate at high densities mainly in brackish and freshwater, forming cyanobacterial blooms, which can be harmful to aquatic organisms living in contaminated waters and to terrestrial species depending on contaminated waters for drinking and/or getting their food. Although to a lesser extent, toxic cyanobacteria and their toxins can also be present in salt waters

becoming nowadays a more important subject (Osborne et al., 2001; Méjean et al., 2010; Frazão et al., 2010; Miller et al., 2010). Multiple conditions favour these blooms, eutrophication being one of the most important one, and others such as low turbulence that causes water stratification, high irradiance and high temperatures being also determinant (Chorus and Bartram, 1999; Hudnell, 2008).

Cyanobacterial blooms can cause oxygen depletion and high turbidity that are detrimental to aquatic organisms especially for fish that can be very sensitive to low concentrations of oxygen. In addition to this ecological consequences, several species of cyanobacterial produce highly toxic secondary metabolites (cyanotoxins), that have caused several fatalities, including humans, worldwide (Zimba et al., 2001; Azevedo et al., 2002). The first cyanotoxic incident was reported in 1878 (Francis, 1878) and since then, especially in the late decades, more incidents have been reported worldwide (Chorus and Bartram, 1999; Chorus, 2001; Alonso-Andicoberry et al., 2002; Handeland and Østensvik, 2010), indicating that these phenomena may be increasing as suggested by Paerl and Huisman (Paerl and Huisman, 2009). They considered the climate changes and the increasing of water temperature as the main factors for the increasing of toxic cyanobacterial blooms. As a result of global climate changes and human population increment, eutrophication is expected to increase in several regions of the world likely leading to more frequent and perhaps also more intense toxic cyanobacterial blooms.

Cyanotoxins can be produced by different cyanobacterial genera in different quantities, depending of the genetic characteristics of the strains and/or environmental conditions, making impossible to predict their toxic potential. Cyanotoxins are diverse in terms of chemistry and toxicity, being divided in (1) peptides, such as the hepatotoxic microcystins and nodularins, (2) alkaloids, the neurotoxic and/or cytotoxic anatoxins, aplysiotoxin, lyngbiatoxin, saxitoxins and cylindrospermopsin (3) organophosphate as the neurotoxin anatoxin-a(s) and (4) lipopolissacharides with dermotoxic effects (Botana, 2008; Sivonen, 2008).

Anatoxin-a, is produced by several cyanobacterial genera: *Aphanizomenon*, *Anabaena*, *Microcystis*, *Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium* and *Oscillatoria* (Park et al., 1993; Park, 1993; Bumke-Vogt et al., 1999; Namikoshi et al., 2003; Viaggiu et al., 2004; Araóz et al., 2005; Ballot et al., 2005; Gugger et al., 2005). Anatoxin-a has a 50% median lethal dose (LD_{50}) of 250 $\mu g.g^{-1}$ for the mouse after i.p. administration (Rogers et al., 2005). It is a very potent cholinergic agonist that competes with the acetylcholine for nicotinic and muscarinic receptors (Aronstam and Witkop 1981; Campos et al., 2006). Intoxication in terrestrial vertebrates, leads to symptoms such as loss of muscle coordination, difficulty in breathing, staggering, muscle fasciculation, gasping and convulsions (Smith, 2008); When at lethal doses, death occurs due to respiratory failure. Symptoms in fish have not yet been fully described but the mechanism of action is expected to be very similar due to the similarities in the nervous system among vertebrates, nevertheless this should be investigated.

Despite being a natural aquatic toxin, the toxic effects of anatoxin-a have been studied mainly in terrestrial mammals (Aracava et al., 1987; Thomas et al., 1993). Thus a scarcity of information regarding its toxicity to aquatic organisms, capability of aquatic species to bioconcentrate and bioacumulate it, and its potential impacts at population, community and ecosystems levels still exists. This knowledge is fundamental to support the ecological risk assessment of toxic cyanobacterial blooms to aquatic freshwater ecosystems being also useful to human risk assessment (Barron, 1990). Under this context of lack of knowledge about the fate and effects of anatoxin-a in aquatic ecosystems, this study aimed to test the hypothesis that rainbow trout is able to bioconcentrate anatoxin-a from the water.

The only available data about environmental fate of anatoxin-a is its partition coefficient octanol:water (K_{ow}) and a BCF estimated by non experimental approaches (SRC, 2009).

To the best of our knowledge, this is the first experimental based assessment of the capability of the rainbow

trout to bioconcentrate anatoxin-a. This species was selected as model organism because it has an important ecological function in many freshwater ecosystems, it is consumed by humans, it has been widely used as test organism in toxicity assays (Bury et al., 1997; Best et al., 2003; Finne et al., 2007; Rymuszka et al., 2007) and it is recommended by OECD guidelines for testing of chemicals (OECD, 1992).

By adding new information about toxic effects in fish, characterization of the risk of anatoxin-a can be ameliorated and consequently an ecological risk assessment of anatoxin-a can be achieved.

2 Material and Methods

The experiments were carried out in accordance with the current guidelines for the care of laboratory animals and ethical guidelines for investigation in conscious animals set by the General Directorate of Veterinary of Portugal.

2.1 Organisms

One month old rainbow trout fry (*Oncorhynchus mykiss*) were obtained from a local aquaculture in the north of Portugal at Coura River (Castro & Gabero Lda., Portugal) and held in a stock tank during two months before the experiments. The tank was maintained in the bioterium with aeration and filtration using bio-balls, at room temperature and natural light:dark regime. During this period, fry were fed *ad libitum* with commercial food for trout (A. Coelho & Castro, Portugal).

2.2 Instrumentation and reagents

Fish samples and reagents were weighted in a laboratorial analytical balance Kern 770. Fish tissue was freeze-dried in a Lyophilizer FTS Systems (Stone Ridge) and the obtained dried mass was macerated with a mechanical blender Silentcrusher M. (Heidolph) and sonicated with a sonicator Vibra Cell (Sonic & Materials). To remove tissue debris before HPLC analysis, a Thermo Electron Corporation Sorval Legend RT Centrifuge (SupplyLAB) was used. A N_2 sample concentrator Techne Dri-Block® DB.3 was used to concentrate the extracts prior to derivatization.

Degradation of anatoxin-a in the water at the experimental conditions was studied previously to the toxic assay and validation of the method for analyzing anatoxin-a by SPE-HPLC-FLD (Solid Phase Extraction-High Performance Liquid Chromatography-Fluorescence Detection) in the water and in the fish was achieved by fluorescence detection (HPLC-FLD) as described previously (Azevedo et al., 2011).

Therefore, in the present study, anatoxin-a was quantified in a Merck Lachrom HITACHI high-pressure liquid chromatographer (HPLC) that included an interface D-7000, a fluorescence detector L-7480, an auto sampler L-7200 and a pump L-7100. The oven for the HPLC column was a ThermaSphere™ TS-130 from Phenomenex® used as an external device.

Solid Phase Extractions (SPE) were carried out in a vacuum manifold Sep-Pak® using Supelclean™ LC-WCX SPE tubes, 500 mg, 3 mL (lot nº SP8621D).

HPLC analyses were carried out using high-purity chromatography grade solvents (LiChrosolv, Merck). Aqueous solutions were prepared with ultrapure water supplied from a Milli-Q (Millipore™) water purification system (0.0054 μS cm^{-1}). Reagents like sodium borated and hydrochloric acid were of analytical grade and trifluoroacetic acid and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) of of spectrophotometric grade 99%. Anatoxin-a fumarate was used as the reference standard (lot nº 7A/90485, 98% purity, Tocris Bioscience).

2.3 Experimental design

The experimental design was based on the OECD guideline for testing of chemicals (OECD 1992). Thirty-six fry of 3 months old (mean standard length = 3.19±0.1 cm and mean weight = 460±100 mg) were randomly selected from the stock tank and moved to the experimental conditions into 2 L glass aquaria in groups of three per aquarium. Aquaria had been previously filled with dechlorinated tap water which was continuously aerated

via glass tubing and conditioned in the experimental room at the experimental conditions: air temperature 18±1°C and light regime of 14 h L:10 h D at 0.9 x 10-6 $E.m^{-2}.s^{-1}$. Fish were acclimated in these conditions during two weeks prior beginning of the assay. During this period, water was totally replaced every 24 h and feeding was done in the same manner as before in the stock tank.

Before starting exposure, water was contaminated in the respective aquaria using anatoxin-a fumarate. For the control aquaria, no toxin was added. Nominal anatoxin-a concentrations were sub-lethal and selected to be environmentally relevant: 132 $\mu g.L^{-1}$, 264 $\mu g.L^{-1}$ and 524 $\mu g.L^{-1}$. Each treatment was run in triplicate. Fish were then transferred to the respective treatment aquaria (three fry per aquaria) and observations of behaviour were registered through the whole experiment but were not quantified. Exposure occurred in static conditions (water was not replaced), during 4 days and the fry were not fed. This experimental design was chosen based on a previous chemical assay which demonstrated that dissolved anatoxin-a did not degrade at the exactly same laboratorial conditions, during 4 days (data not shown).

2.4 Sampling of water and fish tissue

In order to determine the bioconcentration factor (BCF) total anatoxin-a was quantified in water and in the whole fish tissue. Water samples (1 mL) were taken before transferring the fish (T0) to aquaria and from thereafter they were taken every 24 hours (T1, T2, T3, T4) at the same hour to eliminate circadian variations. Fish were sampled only at the end of the exposure period, 96 h. They were euthanized by decapitation with a bisturi, measured, weighted and frozen prior to lyophilisation.

The following methods of extraction of anatoxin-a by SPE and its HPLC-FLD quantification from water and fish tissue, have been previously validated in our laboratory (Azevedo et al., 2011).

2.5 Extraction of anatoxin-a

2.5.1 Fish samples

Three lyophilized fry from each aquarium were pooled together to guarantee that levels of anatoxin-a were within the range of detection. Anatoxin-a was extracted by immersion of the freeze-dried fish in acidified 50% aqueous methanol (TFA 0.1%) and finely blended with the mechanical homogenizer for 1 min at 10,000 r.p.m. The obtained crude extracts were treated by ultrasounds (60Hz for 1 min) and then centrifuged (3,400 g, 10 min, 5ºC). This extraction procedure was repeated twice and the three supernatants were pooled following filtration trough syringe CA- filters (0.45 µm, Orange Scientific), then they were evaporated to dryness under N_2 flow. The obtained residue was dissolved in 5 mL of ultrapure water for SPE. SPE was carried out in the vacuum manifold by loading each water extracts with pH settled at 7.0 ± 0.2, in LC-WCX cartridges, preconditioned with 6 mL of methanol and 6 mL ultrapure water. The cartridges were then rinsed with 3 mL of methanol and left to evaporate in the dark for 30 minutes before the anatoxin-a elution with 10 mL of acidified methanol (0.2% TFA). These final residues were evaporated under nitrogen flow at 30ºC and immediately processed for HPLC analysis.

2.5.2 Water samples

The 1 mL water samples were immediately evaporated under N_2 flow at 30°C, to dryness. No SPE was carried out. Dried residues were immediately processed for HPLC analysis.

2.6 HPLC analysis

Dried residues from water and fish samples, were immediately derivatized with NBD-F in acetonitrile at 1 $mg.mL^{-1}$ after alkalization with sodium borate, 0.1 M. Derivatization reaction was stopped with hydrochloric acid (1 M) after 10 min at room temperature in the dark.

Derivatized products were immediately injected in the HPLC system: Anatoxin-a analysis was performed using a column Luna® C-18, 4 × 4 mm, 5 µm (Phenomenex) after the guard column Luna®, both were kept at 35 ± 1ºC. The injected volume was 20 µL and the isocratic elution was a mixture of MeOH:H2O + 0.05 %

TFA (60:40 v/v) with a flow rate of 0.6 mL min^{-1}. All HPLC solvents were filtered (Pall GH Polypro 47 mm, 0.2 μm) and degassed by ultrasound bath. The excitation and emission were set at 546 nm and 480 nm, respectively. The system was calibrated by using a set of ten water dilutions of the anatoxin-a fumarate (23-0.1 μg mL^{-1}).

2.7 Determination of BCF

Bioconcentration defined as the proportionally constant relating anatoxin-a concentration in water to its concentration in the fish (Barron 1990), was determined according to the formula:

BCF=[anatoxina in the whole trout body (μg.g^{-1})]/[anatoxin-a in the water (μg.mL^{-1})].

Concentration of anatoxin-a in the water corresponds to the average value during the 4 days and concentration in fish corresponds to levels at day 4 at the end of the experiment. BCF was calculated for each nominal concentration.

3 Results and Discussion

Test medium parameters recorded during the assay fulfilled all the requirements for acute toxicity tests with fish (16.2 ± 0,1°C, pH = 8.1 ± 0.1, oxygen concentration = 8.8 ± 0.3 mg.L^{-1} and oxygen saturation > 89.4%) and no mortality was recorded during the assay. No relevant differences in anatoxin-a concentrations in test media among different times were found at any of the tested concentrations because the percentage of decay recorded at 96 h, was less than 20% of the initial concentration (T0) in all the anatoxin-a treatments (Fig. 1). These results confirm that anatoxin-a is stable in water at least for 96 hours at the experimental conditions used here. This is also important since the stability of the concentrations of the test-substance during the test is a pre-requisite for the use of static conditions in assays for BCF determination (OECD, 1992; Kleinow et al., 2008). These findings also indicate that the steady state of anatoxin-a concentrations in the fish body was already attained at 24h of exposure.

The calculation of BCF is very important for the ecological risk assessment of any chemical (Barron, 1990; Toropov et al., 2009). They give the indication of the concentrations that should be tested in bioassays to assess the toxicity of the chemical and provide information on the potential exposure for predators of the considered organism. The BCF factors determined for the rainbow trout exposed to water concentrations of 0.129, 0.272 and 0.499 μg.mL^{-1} were 176, 153 and 297, respectively, based on the trout dry weight (Table 1, Fig. 2). These results indicate that BCF are concentration dependent in the range of concentrations tested and that at that the BCF calculated for the highest concentration tested is about 1.7x higher than the one calculated for the lowest exposure concentration. The BCF previously calculated for anatoxin-a was 2.6, based on the estimated log Kow of 1.12 (Syracuse Research Corporation (SRC, 2009), as referred in Hazardous Substances Data Bank), indicating a low bioconcentration potential by aquatic organisms (SRC, 2009). This approach has several limitations once it is a simple kinetic model and doesn't consider relevant parameters as body size, fish species, biotransformation or environmental factors (Barron, 1990; Cowan-Ellsberry et al., 2008). According to the United Nations Economic Commission for Europe, although the estimated log Kow is the most readily available indication of the potential for a chemical to bioacumulate, an experimentally derived BCF is preferred. The BCF calculated from our experimental results are considerable higher (153-297) than BCF calculated with basis on the log Kow, thus, one must conclude that the real potential of some fish species, such as the rainbow trout, to bioconcentrate anatoxin-a is considerably higher than previously considered; although they are still bellow the limit of 500 that has been considered as the minimum required for considering that a substance has the potential to be bioconcentrated by aquatic biota (UNECE, 2009). It should be work noted that the water concentrations at which the rainbow trout was exposed in the present study were environmentally significant and that the BCF calculated for the highest concentration was higher than the

corresponding value calculated for the lowest exposure concentration. Therefore, the possibility of higher bioconcentration in real scenarios where intense toxic blooms occur cannot be excluded. Hence, it is our opinion that it should be considered that anatoxin-a as the potential to be bioconcentrated by fish.

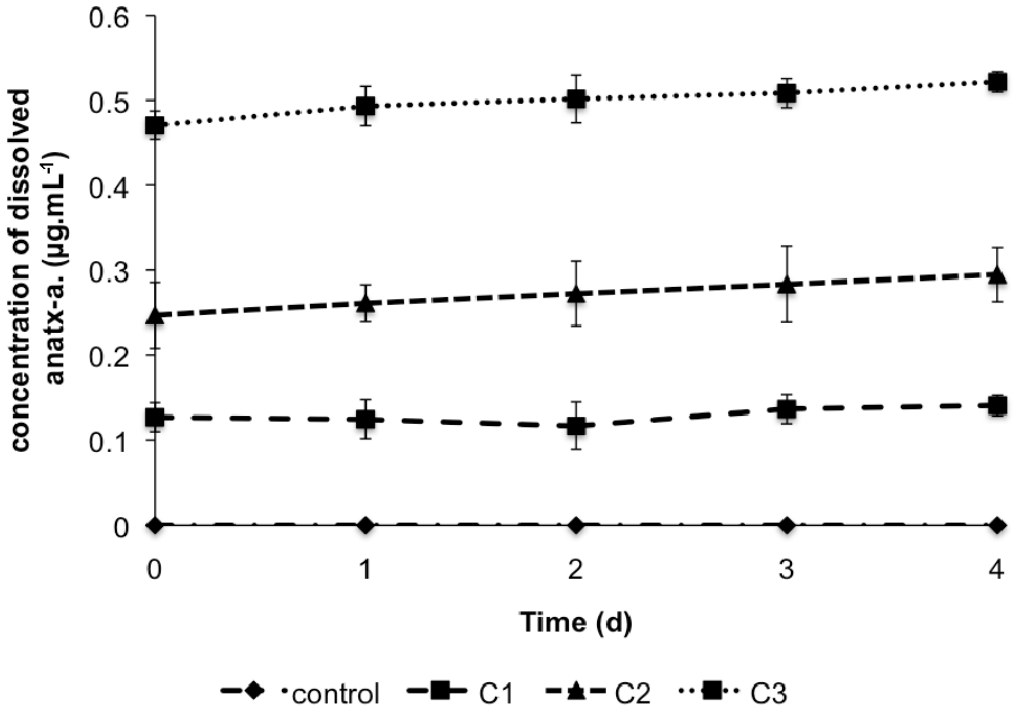

Fig.1 Actual dissolved anatoxin-a in water during the assay (4 d), at different nominal concentrations. The values are the mean of three replicates with corresponding standard errors.

Table 1 Bioconcentration factors (BCF) of corresponding nominal and actual concentrations of anatoxin-a in test media and in the fish body (whole body), after 96 h of exposure to the toxin through the water. Values are the mean with corresponding S.E.M. (n=3) f.w. - fresh weight. d.w. - dry weight.

Nominal concentrations of anatoxin-a (µg.mL^{-1})	**Actual concentrations of anatoxin-a in water (µg.mL^{-1})**	**Actual concentrations of anatoxin-a in the whole fish (µg.g^{-1}f.w.)**	**BCF (f.w.)**	**BCF (d.w.)**
(C1) 0.132	0.129 ±0.004	3.9±1.5	30.08	176.4
(C2) 0.264	0.272 ±0.008	7.1±1.9	25.95	153.8
(C3) 0.524	0.499±0.009	23.6±8.4	47.37	297.7

In relation to behaviour observations, in all treatments except in control, 5 min after the immersion, fish showed several alterations, namely, irregular swimming, jaw's spasms, gulping of air at surface, difficulty in maintaining equilibrium and erratic swimming. These symptoms were progressively aggravated with the increase of the exposure time; after 2:00 h the fish exposed to the two higher concentrations showed higher activity than in the other treatments; at 3 h after exposure, no alterations in behaviour could be observed in relation to the controls, indicating a total recovery of normal behaviour.

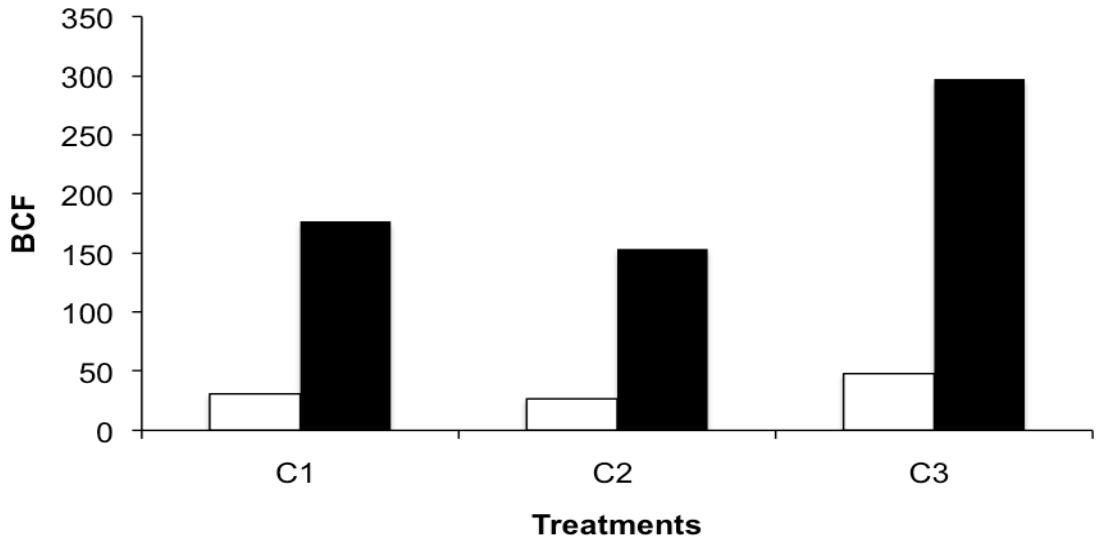

Fig. 2 Bioconcentration factors (BCF) of anatoxin-a in juvenile rainbow trout exposed through water at different actual concentrations of the toxin, during 96 h. C1 - 0.129 µg.mL^{-1}, C2 - 0.272 µg.mL^{-1} and C3 - 0.499 µg.mL^{-1}. Black bars - dry weight, white bars - fresh weight.

The behaviour alterations observed in this experiment may happen in natural environments with anatoxin-a at sublethal concentrations similar to those tested in the present study and, therefore, actions such as prey capture, predator avoidance, courtship, and mating may be affected as suggested also for the common carp (*Cyprinus carpio*) after exposure to cells containing anatoxin-a (Osswald et al., 2007; Osswald et al., 2009). Since population dynamics are dependent on prey capture and predator avoidance ability, mating and reproduction success, the alterations of behaviour observed in this experiment, may imbalance natural populations after and during anatoxin-a occurrences.

The symptoms observed in the fish are compatible to the mechanism of toxic action of anatoxin-a in the nervous system that has been described for other vertebrates and thus it is likely that they are due to the binding of the toxin to the acetylcholine receptors of the post-synaptic membrane of cholinergic synapses. Whether all the observed effects are due to the parental compound or to any possible metabolites resulting from its biotransformation by this species, it is an important question that deserves further investigation. In fact, it is well known that metabolites resulting from biotransformation may be more toxic than parental compounds (Tukey and Strassburg, 2000) and some may also have different mechanisms of toxic action. In the environment, anatoxin-a is degraded to dihydroanatoxin-a and epoxyanatoxin-a (Stevens and Krieger, 1991; James, 1998), however the metabolites resulting from its biotransformation by the rainbow trout may be different from its environmental degradation products. Therefore, the toxicity of both metabolites and degradation products should be investigated and taken into consideration in ecological risk assessment studies. Another aspect that deserves further research is the distribution of anatoxin-a inside the rainbow trout body and the bioaccumulation potential of the different tissues to understand how the fish metabolizes anatoxin-a. Having in mind that rainbow trout is consumed by humans, this study would be most important in relation human risk assessment.

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