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Toxicity evaluation of parboiled rice effluent using sperm quality of zebrafish as bioindicator

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ABSTRACT

This study aimed to assess the acute toxicity of raw and treated wastewater generated by the rice parboiling industry using zebrafish (*Danio rerio*) sperm quality as a bioindicator. Toxicity bioassays were conducted comparing physicochemical parameters of sperm quality for zebrafish at sublethal conditions ($n = 150$ fish, 50 per treatment). Acute toxicity was detected in all sperm quality parameters assessed for both raw and treated wastewater, when contrasted to the control ($p < 0.05$). For zebrafish exposed to raw effluent, negative correlations with parameters of sperm quality were observed for the concentration of iron, phosphorus and total suspended solids ($p < 0.05$). Salinity, the biochemical oxygen demand and the concentration of total suspended solids were negatively correlated with parameters of sperm quality for zebrafish exposed to treated effluent ($p < 0.05$). In comparison with the levels observed for the raw effluent, most physicochemical parameters of the treated effluent were reduced to levels within the limits required by the environmental legislation. Despite the physical and chemical parameters measured in the treated wastewater meeting environmental legislation thresholds, acute toxicity persisted. These results show that the sperm quality can be used as a bioindicator for wastewater toxicity and release of wastewater to surface water could affect the fertility of fishes.

1. Introduction

Although Asia concentrates the largest rice production in the world (Haefele et al., 2014), Brazil is the world's largest rice producer outside Asia, harvesting more than 12 million tons of rice grains in 2014 (Childs, 2014). Rice is the most consumed cereal in Brazil, with the consumption of polished parboiled rice corresponding to nearly 25% of the total rice consumption (Paraginsky et al., 2014).

Nonetheless, the rice industry has high potential for environmental pollution due to the substantial production of effluents containing organic materials and nutrients (Gil de Los Santos et al., 2012). Environmentally relevant pollutants contained in effluents cannot be detected solely by physicochemical evaluations. The parameters commonly evaluated for effluents do not distinguish substances that truly affect biological systems from those that are inert on the environment (Hernando et al., 2005; Smital et al., 2011; Bohórquez-Echeverry et al., 2012).

The conduction of toxicity trials prior to the discharge of industrial effluents is mandatory in some countries, but in others, only the physicochemical characterization is required (Kim et al., 2008; Sousa et al., 2013). That was the case of Brazil until 2005, when its environmental legislation was upgraded to include toxicity trials (Magalhães and Ferrão Filho, 2008; Pimentel et al., 2011). Although the physicochemical characteristics of rice parboiling effluents have been studied (Behera et al., 2010; Zepka et al., 2010; Ramprakash and Muthukumar, 2014), research about their potential toxicity is still scarce.

Toxicity tests do not replace traditional chemical analysis, but complement each other, because while the physicochemical analysis only quantify contaminants, toxicity tests evaluate the synergistic effects, additives or antagonists of these contaminants in the environment (Libralato et al., 2010). In this context,

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ecotoxicological tools, biomarkers and bioassays contribute to the integration of chemical and biological indicators, providing an overall insight into the quality of surface water and effluents (Cazenave et al., 2014; Martinez-Haro et al., 2015).

Evaluations of wastewater toxicity are relatively recent in Brazil and studies on that field are still incipient (Krul and Barros, 2012). Although the mortality of test-organisms is the most frequently parameter used to determine acute or chronic toxicity, new evaluation criteria need to be developed in order to upgrade the current environmental regulations (Arenzon et al., 2013).

Parameters of fish sperm quality are frequently used in toxicity trials, since fish gametes and embryos are highly sensitive to toxic effects of water contaminants (Fabrocini et al., 2010), compromising their fertility (Hatef et al., 2013). For instance, under natural conditions, the motility of fish sperm is induced immediately after sperm release in aqueous medium. That motility is influenced by some characteristics of the medium, such as ionic and osmotic pressure (Alavi and Cosson, 2006; Dzyuba and Cosson, 2014). Evaluations of sperm quality have been used in toxicity assays for guppy (*Poecilia vivipara*) (Harayashiki et al., 2013) and zebrafish (*Danio rerio*) (Lopes et al., 2014).

This way, the aim of this work was to evaluate the acute toxicity of the rice parboiling industry wastewater by using zebrafish (*Danio rerio*) sperm quality as a bioindicator.

2. Materials and methods

2.1. Sampling

The study was conducted in a rice parboiling industry located in southern Brazil (−31.7701 °C and −52.3423 °C). That industry has a system for effluents treatment and is currently licensed according to the Brazilian environmental regulations. The annual production is 144,000 tons of parboiled rice and generate 600 $m^3\ d^{-1}$ effluents. The effluents treatment system is composed of a pumping tank; a hydrodynamic sieve; an equalization/neutralization tank; an upflow anaerobic sludge blanket (UASB) reactor; and lagoons with emergent plants.

Five sampling were conducted in a period of six months. All samples were packaged, conditioned at 4 ± 1 °C and transported to the laboratory, according to APHA (2012). At the sampling moment, pH, temperature, electric conductivity (EC), and salinity were measured using a HI 9828 portable meter. The raw effluent was collected from the pumping tank, before pH adjustment; whereas treated effluent was collected after the effluent passed through the lagoons with emergent plants.

For all samples, the physicochemical parameters were evaluated in duplicates: biochemical oxygen demand – BOD (5120B); chemical oxygen demand – COD (5220D); total suspended solids – TSS (2540D); total Kjeldhal nitrogen – TKN (4500NORG B,C); ammoniacal nitrogen – N-NH_3 (4500 NH_3 B,C); total Phosphorus (4500P,E); chlorides (4500C); surfactants (5540D); sulfides (4500S,D); hardness (2340D); Al (3500B); Mn (3500B); Fe (3500B); Zn (3111B); and phenols (5530C), as recommendation by the APHA (2012).

2.2. Animal model

All procedures used in the fish toxicity assay followed recommendation of the EPA (1996) and were approved by the Ethics on Animal Experimentation Committee of the Federal University of Pelotas (Protocol 1525; May 2013). Adult zebrafish males were acclimated in reconstituted water for 15 days, prior to exposure to water and effluent samples.

Previously to the bioassay, a sublethal toxicity level was experimentally defined as 6.25% (effluent/reconstituted water), with a dilution factor of 16, considering the maximum concentration of effluent to keep sublethal conditions of the bioassays.

The toxicity bioassay was conducted using 10 fish per sample, reaching 50 fish per treatment, totalling 150 fish. Three glass aquariums (10 L volume) were used, composing three treatments: control (reconstituted water); diluted raw effluent; and diluted treated effluent. The bioassay lasted 96 h with no renewal of the tested solution.

2.3. Sperm quality evaluations

All fish were euthanized and dissected after 96 h. Their testis were extracted and conditioned in Eppendorf tubes containing 100 μL of Beltsville Thawing Solution – BTS (Pursel and Johnson, 1975). Chemicals were obtained from Sigma Chemical Company (Saint Louis, MI, USA), unless stated otherwise. All evaluations of sperm quality were conducted by the same trained technician.

To determine the motility period, sperm was activated with distilled water (2 μL sperm in 20 μL distilled water). The motility rate and sperm motility period was determined as the interval from activation to the moment sperm stopped moving (Viveiros and Godinho, 2009).

Sperm membrane viability was determined by counting 200 spermatozoa in a slide containing a staining solution including 5 g eosin Y and 10 g nigrosin, homogenized in BTS, using a bright field microscope with an oil immersion objective at 100×. Intact spermatozoa remained unstained and spermatozoa having ruptured membrane were stained either in pink or red (Maria et al., 2010).

The integrity of sperm membrane and DNA and the mitochondrial functionality were evaluated by counting 200 spermatozoa in a slide, using an epifluorescent microscope (Olympus BX 51) at 400× (Varela Junior et al., 2012).

Sperm membrane integrity was evaluated using two fluorescent markers: carboxyfluorescein diacetate and propidium iodide. Spermatozoa with integer membrane showed green fluorescence, whereas those having damaged membrane presented heads with either red or red and green fluorescence (Harrison and Vickers, 1990).

Sperm DNA integrity was evaluated using acridine orange marker. Spermatozoa stained with green fluorescence had intact DNA and those stained with red or orange fluorescence had denatured DNA (Bencharif et al., 2010).

Mitochondrial functionality was analyzed using the Rhodamina 123 marker. Green fluorescence indicated spermatozoa having functional mitochondria, whereas spermatozoa having nonfunctional mitochondria presented no fluorescence (He and Woods, 2004).

2.4. Statistical analyses

The normality of the responses of interest was evaluated using the Shapiro–Wilk test. Those that presented normality were compared through analysis of variance, with comparisons of means conducted with the Tukey Test. Due to lack of normality, salinity and hardness were compared through Kruskal–Wallis analysis of variance for non parametric data. Correlations among the evaluated parameters were determined by the Spearman's coefficient. Results were considered significant when $p < 0.05$. All analyses were evaluated using Statistix® (2008).

3. Results and discussion

3.1. Physicochemical characterization

The results of physicochemical characterization of the raw and treated parboiled rice effluent and Brazilian environment limits are shown in Table 1.

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