Ecotoxicology and Environmental Safety 135 (2017) 319–326

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Histopathological analysis of tilapia gills (*Oreochromis niloticus* Linnaeus, 1758) exposed to sugarcane vinasse

J.E. Correia[a], C.A. Christofoletti[a,b], A.C.C. Marcato[a], J.F.U. Marinho[a], C.S. Fontanetti[a,*]

[a] UNESP (Universidade Estadual Paulista), Av. 24-A, 1515. Bela Vista, Rio Claro, SP, Brasil
[b] Centro Universitário Hermínio Ometto (FHO-UNIARARAS), Av. Dr. João Maximiliano Baruto, 500. Jd. Universitário. Araras, SP, Brasil

ARTICLE INFO

Keywords:
Stillage
Aquatic toxicity
Histology
Ultra-morphology
Histochemical

ABSTRACT

Sugarcane vinasse is one of the main residues generated by the transformation of cane into ethanol. Because of the high organic content (COD), high biochemical oxygen demand (BOD), low pH, the large amount that this residue is generated (15 l for every liter of ethanol produced) and their use as fertilizer on the sugarcane crop, this residue is potentially polluting to the soil ecossystem and by percolation to water ecossystem too. Thus, this study aimed to assess the toxicity of vinasse by analyzing *Oreochromis niloticus* gills exposed to different dilutions (1%, 2.5%, 5% and 10%) in two bioassays. The gills were collected, fixed and analyzed using ultra morphological, histological, and histochemical techniques. After exposure to the vinasse, a statistically significant reduction of the ridges present on the surface of pavimentous cells was observed in one of the bioassays; such structures are responsible for mucus retention, which helps to protect the tissue. In addition, an intumescence of the cells was observed in the treatments with vinasse as well as an increase in the amount of chloridric cells. Some striking tissue changes detected in the treatments were epithelial detachment and loss of integrity of secondary lamellae, causing their rupture and consequent hemorrhage. In the first bioassay, the amount of these changes was statistically significant at the 5% dilution, and the focus of hemorrhage was significant at all dilution ratios. In the second bioassay, the epithelial disorganization was statistically significant only at the 2.5% dilution of vinasse. Moreover, for both bioassays performed, a significant increase in mucous cells was observed when compared with the control. Our results demonstrate the toxic action of sugarcane vinasse, which caused histopathological changes in the exposed animals at all four dilution tested. This highlights the need for caution in the disposal of sugarcane vinasse on the soil, especially due to its capacity for being leached or percolated into water resources, which could seriously damage aquatic fauna.

1. Introduction

Brazil, in the world scenario, ranks as one of the largest exporters of agricultural resources: among the major resources are soybeans, corn, coffee, and sugarcane. These monocultures require large areas of land and make use of large amounts of pesticides. They also generate a substantial amount of residues, which may contain potentially polluting substances (Ribeiro et al., 2007; UNICA, 2007). As a consequence, agribusiness has become one of the economic sectors with the greatest environmental impact.

Currently, one of the most relevant discussion themes in the scientific communities worldwide is the environmental impact of anthropogenic activities influencing the quality of water, soil, and air. The disposal of toxic products and efluents in the environment causes serious damage to organisms as well as terrestrial and, especially, aquatic ecosystems, since freshwater ecosystems, along with estuaries, are the least expensive and most convenient systems for the disposal of waste (Odum, 1988). These effluents, especially those derived from industrial activities, may contain pesticides and toxic metals that have complex compositions, which can present pesticides and toxic metals, causing serious damage to organisms (Vega et al., 1996). As a consequence, diagnosis and monitoring of aquatic environmental pollution is a priority and an extremely important concern.

Sugarcane vinasse is characterized as one of the main residues from the transformation of sugarcane into ethanol. It is generally acidic (pH 3.5–5), dark brown, malodorous to humans, and also contains a high organic content (COD 50–150 g L^{-1}) (Waliszewski et al., 1997; España-Gamboa et al., 2011). The production of ethanol from sugarcane biomass results in a considerably high amount of vinasse, with a high polluting potential (Wilkie et al., 2000). On average, for each liter of ethanol, 10–15 l of vinasse are generated as a residue (Yesilada, 1999; Kumar and Gopal, 2001; Christofoletti et al., 2013a).

* Corresponding author.
E-mail address: fontanet@rc.unesp.br (C.S. Fontanetti).

http://dx.doi.org/10.1016/j.ecoenv.2016.10.004
Received 25 September 2015; Received in revised form 19 September 2016; Accepted 5 October 2016
Available online 19 October 2016

Due to the rich organic matter and micronutrients found in vinasse, it is widely reused for the fertirrigation of sugarcane cultures. Vinasse may be release by irrigation canons, the most common, airplanes or by grooves along the crop (Christofoletti et al., 2013a.). However, the large volume of vinasse applied as fertilizer can saturate the soil and contaminate water resources that are close to crops (Silva et al., 2007). Thus, vinasse may cause several types of damage to aquatic life.

Several studies have evaluated vinasse toxicity in different organisms (Yesilada, 1999; Souza et al., 2013; Christofoletti et al., 2013b; Pedro-Escher et al., 2014). Pedro-Escher et al. (2016) evaluated the toxic and genotoxic potential of sugarcane vinasse using the *Allium cepa* test; the author observed a toxic effect evidenced by a lack of seed germination on raw vinasse and a genotoxic effect with a significant increase in chromosomal alterations and micronuclei formation in meristematic cells of groups with vinasse dilutions (50%, 25%, and 12.5%).

Fish have characteristics that make them excellent experimental models for studies on aquatic toxicology, thereby indicating the potential damaging effects of new chemical substances or the possibility of environmental pollution. They are considered good test organisms to monitor water quality, especially small aquarium species, which can be kept in the laboratory and easily exposed to toxic substances potentially harmful to human health (Harshbarger and Clark, 1990; Al-Sabti and Metclafe, 1995). Furthermore, fish gills are the main targets of several aquatic pollutants (Kikuchi et al., 1978) and their epithelium is an excellent model to examine the effects of substances dissolved in the tissues (Evans, 1987; Biagini et al., 2009). Gills are the organs most affected by constant contact with water (Machado, 1999); for this reason, they are generally considered good indicators of water quality (Fontanetti et al., 2012).

Kumar and Gopal (2001) evaluated vinasse toxicity in the fish species *Channa punctatus* at different concentrations and observed extensive mucus production, reduction in protein levels and high lactic acid concentrations in various organs such as brain, liver, kidney, and muscle.

Histopathological studies have proven to be a sensitive tool to detect direct toxic damage caused by chemical compounds within target organs of fish (Abdel-Moneim et al., 2012). Saleh and Mohamed-Assem (2016), in a pollution biomonitoring study of the Red Sea, used several biomarkers including histopathological lesions of fish *Arius thalassinus*. Semi-quantitative analysis of the observed histopathological lesions revealed that gills were the most affected organs with signs of severe alterations.

Marinho et al. (2014) evaluated the effects of sugarcane vinasse dilutions in tilapia (*Oreochromis niloticus*) livers. The authors observed significant histopathological changes, such as loss of cytoplasmic integrity and cell limit, tissue disorganization, and reduction in the accumulation of polysaccharides, with increasing vinasse concentration.

In view of the toxic effects caused by vinasse in the studies mentioned above and considering the possible environmental contamination of water resources caused by percolation/leaching of this waste on soil, this paper aimed to assess the toxic potential of sugarcane vinasse in the aquatic environment. To this end, we evaluated the histopathology of the gills of Nile tilapia after acute exposure to different dilutions of this effluent in laboratory bioassays.

2. Materials and methods

2.1. Biological material

Juveniles of the species *O. niloticus* (Perciformes, Cichlidae), popularly known as Nile tilapia, were used as test organisms with an average size of 10 cm. The specimens were obtained from fish farming and were acclimatized in plastic tanks with a capacity of 250 L with running and filtered water for 15 days. Fish were fed every day until the beginning of bioassays.

Prior to the beginning of the experiments, the present study was analyzed and approved by the Comitê de Ética no Uso de Animais do Instituto de Biociências (Ethics Committee of Animal Use of the Biosciences Institute), UNESP (Universidade Estadual Paulista), Rio Claro, São Paulo, Brazil, protocol n° 1047 from 02/16/2011.

2.2. Vinasse as a toxic substance

The sugarcane vinasse samples were collected from a sugar and ethanol plant located in Araras, São Paulo, Brazil, from two different crops (crops 2011 and 2012). The waste products were kept in a cold chamber (4 °C) in the Department of Biochemistry and Microbiology of the Bioscience Institute, UNESP, Rio Claro, until the beginning of the experiments to avoid the degradation processes and to maintain the physico-chemical properties of the vinasse.

2.3. Physico-chemical analysis of the vinasse

Physico-chemical analyses were carried out on one of the effluent samples by a specialized laboratory (TASQA Analytical Services Ltd., Paulínia, São Paulo, Brazil), to accurately determine its composition. The following parameters were measured: pH, electrical conductivity, hardness, total non-filterable residue, nitrogen, nitrate and nitrite, Kjeldahl nitrogen, ammonia, calcium, total sulphur, total phosphate, magnesium, potassium, sodium, sulphate, BOD (Biochemical Oxygen Demand), COD (Chemical Oxygen Demand), and metals (arsenic, barium, cadmium, lead, copper, chrome, mercury, molybdenum, nickel, selenium, and zinc).

2.4. Bioassays with O. niloticus

Two bioassays were assembled in aquaria with a capacity of 40 l. Both bioassays has same treatments but was performed in different days. In each aquarium, five fish already acclimatized (males and females randomly distributed) were arranged, in which they remained for 96 h, at room temperature of approximately 24 °C, and with filtration and aeration systems. There was no water exchange and the animals were not fed; hence, the ammonia levels did not rise very rapidly throughout the experiment. In both bioassays, there were five treatments: a control received water from an artesian well, whereas the others received increasing dilutions of vinasse (1%, 2.5%, 5%, and 10%) based on previous studies that used similar dilutions of vinasse to analyze its effect on different organisms (Algur and Kadioglu, 1992; Kumar and Gopal, 2001). Aquaria were checked twice a day to verify mortality and behavioral changes.

2.5. Animal dissection

After 96 h of exposure time, the specimens of *O. niloticus* were removed from the aquarium, anaesthetized using benzocaine (0.1 g of benzocaine in 1 ml ethanol per 100 ml deionized water), euthanized by excision of the backbone using surgical scissors, and then dissected in physiological saline solution. After the removal of the second branchial arch, the arch was placed in formaldehyde and in Bouin solution, according to the staining technique to be applied, remaining in the fixer for at least 2 h.

2.6. Ultra-morphology - scanning electron microscopy (SEM)

The second branchial arch of the exposed specimens was removed and fixed on a Karnovsky fixer (Karnovsky, 1965), kept in the refrigerator. This material was then dehydrated in a series of increasing concentrations of acetone, taken to the critical point and fixed on a metallic support. SEM was used to observe and quantify the alterations on paviment cells surface, as reduction on circular ridges. Chloridric

cells was also quantified. The analyses were carried out using a low-vacuum scanning electron microscope HITACHI TM3000.

2.7. Histology and histochemistry

The gills were dehydrated in a graded series of 70%, 80%, 90%, and 95% ethanol solutions. Then, they were immersed in an embedded resin (Leica Historesin-Embedding Kit) for 24 h in the refrigerator. The material was transferred to plastic moulds containing inclusion resin and thereafter, cut with the aid of a microtome (Leica RM2265). The sections were randomly placed on slides. Two slides per animal were stained with hematoxylin and eosin (Junqueira and Junqueira, 1983), according to histological methods, and two slides per animal were subjected to Periodic Acid-Schiff (PAS) staining, for the detection of neutral polysaccharides for evidence of mucous cells (Junqueira and Junqueira, 1983).

2.8. Results interpretation

Two slides were analyzed with eight cuts of 6 μm for each fish, with n=5 for each bioassay. Thus, a total of 40 slides and 160 histological sections were analyzed for each technique performed by bioassay. The results found in the control and in the treatments of the two bioassays were compared with each other.

The description and evaluation of histopathological alterations were based on the standardized protocol of Bernet et al. (1999) and adapted by Marinho et al. (2014). In the present protocol, a factor of importance (w) was defined for each injury, according to its pathological importance, which means how it affects the function of the organ and the survivability of fish. The alterations were previously classified into three factors of importance: (1) Minimal pathological importance: the injury is easily reversible when exposed to the irritant ends; (2) Moderate pathological importance: the injury is, in most cases, reversible when the stressor is neutralized; and (3) Pathological importance: the injury is usually irreversible, leading to partial or complete loss of function of the organ. The importance factor of the main alterations observed in this study with *O. niloticus* gills are shown in Table 1. For a better understanding of the results, the described histopathological abnormalities were also classified into scores (a) which ranged from 0 to 6, depending on the degree and extent of the alteration, being: (0) no occurrence; (2) little or rare occurrence; (4) frequent occurrence; and (6) very frequent occurrence. Furthermore, intermediate values were also considered. By multiplying the factor of importance by the score, we found the alteration index. For the calculation of the total index of each individual, i.e., the index corresponding to the evaluation of histopathological changes in the gill, the following calculation was used: $Index_{ind} = \sum$ (factor of importance×score). From the individual indexes, the values of average and of standard deviation for each group – control and treatments – were obtained.

Similarly, quantitative data were analyzed by basic statistics and subjected to the Shapiro-Wilk test to verify their normality. Having demonstrated the normal distribution of the data, statistical analysis was performed by ANOVA and Tukey's pos hoc test.

Table 1
Main alterations found on *O. niloticus* gills exposed to vinasse and their respective importance factors.

Characteristics analyzed	Importance factor (w)
Epithelium detachment	1
Lamellar disorganization	1
Hemorrhage	1
Ridge loss	1
Paviment cells swelling	1

Table 2
Physical-chemical analysis of vinasse samples used.

Parameter	Collection 2011	CETESB (mg/L)	Method
Ammonium (mg/L)	< LQ	–	USEPA 440/5–85–001
Calcium (mg/L)	671	–	SM21 3120 B
Electric conductivity (μs/cm)	15110	–	SM21 2510 B
DBO (mg/L)	7941	–	SM21 5210 B
DQO (mg/L)	25225	–	SM21 5220 D
Hardness(mg $CaCO_3$/L)	276	–	SM21 2340 B
Total phophate (mg/L)	–	–	SM21 4500-P C
Magnesium (mg/L)	264	–	SM21 3120 B
Nitrate (mg/L)	1,49	–	SM21 4500-NO_3^- F
Nitrite (mg/L)	0,033	–	SM21 4500-NO_2^- B
pH	4,37	–	SM21 4500-H^+ B
Potassium (mg/L)	3401	–	SM21 3120 B
Non-filtered residue (mg/L)	1800	–	SM21 2540 D
Sodium (mg/L)	114	–	SM21 3120 B
Sulphate (mg/L)	2993	–	SM21 4500-SO_4^{-2} E
Arsenic	< LQ	0,01	SM21 3120B
Barium	< LQ	0,7	SM21 3120B
Cadmium	< LQ	–	SM21 3120B
Total calcium	671	–	SM21 3120B
Organic carbon	–	–	SSSA Cap40
Lead	< LQ	0,01	SM21 3120B
Copper	0,76	0,2	SM21 3120B
Electric conductivity (μs/cm)	15110	–	SM21 3120B
Chrome	3,56	0,05	SM21 3120B
Total brimstone	1681	–	SM21 3120B
Total phosphorus	207	–	SM21 3120B
Total magnesium	264	–	SM21 3120B
Mercury	< LQ	0,001	EPA 7470[a]
Molybdenum	< LQ	0,07	SM21 3120B
Nickel	< LQ	0,02	SM21 3120B
Nitrate	1,49	–	SM21 4500-NO_3^- E
Nitrite	0,03	–	SM21 4500-NO_2^- B
Ammoniacal nitrogen	–	–	SM21 4500-NH_3 E
Kjeldahl nitrogen	171	–	SM21 4500-Norg B
Total potassium	3401	–	SM21 3120B
Selenium	< LQ	–	SM21 3120B
Total sodium	114	–	SM21 3120B
Total solid	–	–	SM21 2540B
Total volatile solid	–	–	SM21 2540B
Solid contente	–	–	SM21 2540B
Humidity	–	–	SM21 2540B
Zinc	< LQ	5	SM21 3120B

LQ: Quantification Limit. **CETESB:** Environmental Agency of São Paulo State/Brazil.

3. Results

3.1. Physico-chemical analysis of vinasse

According to the chemical analysis performed, sugarcane vinasse presented low pH and high values of BOD, COD, and potassium (3401 mg/L). The values obtained for hardness and sulphate were also high (Table 2). Regarding metal presence, copper was found at a concentration of 0.76 mg/L, which is below the maximum of 2.0 mg/L allowed by the CETESB (Environmental Agency of São Paulo State/Brazil). However, the chrome concentration of 3.56 mg/L we found was above the maximum permitted by the CETESB (0.05 mg/L)

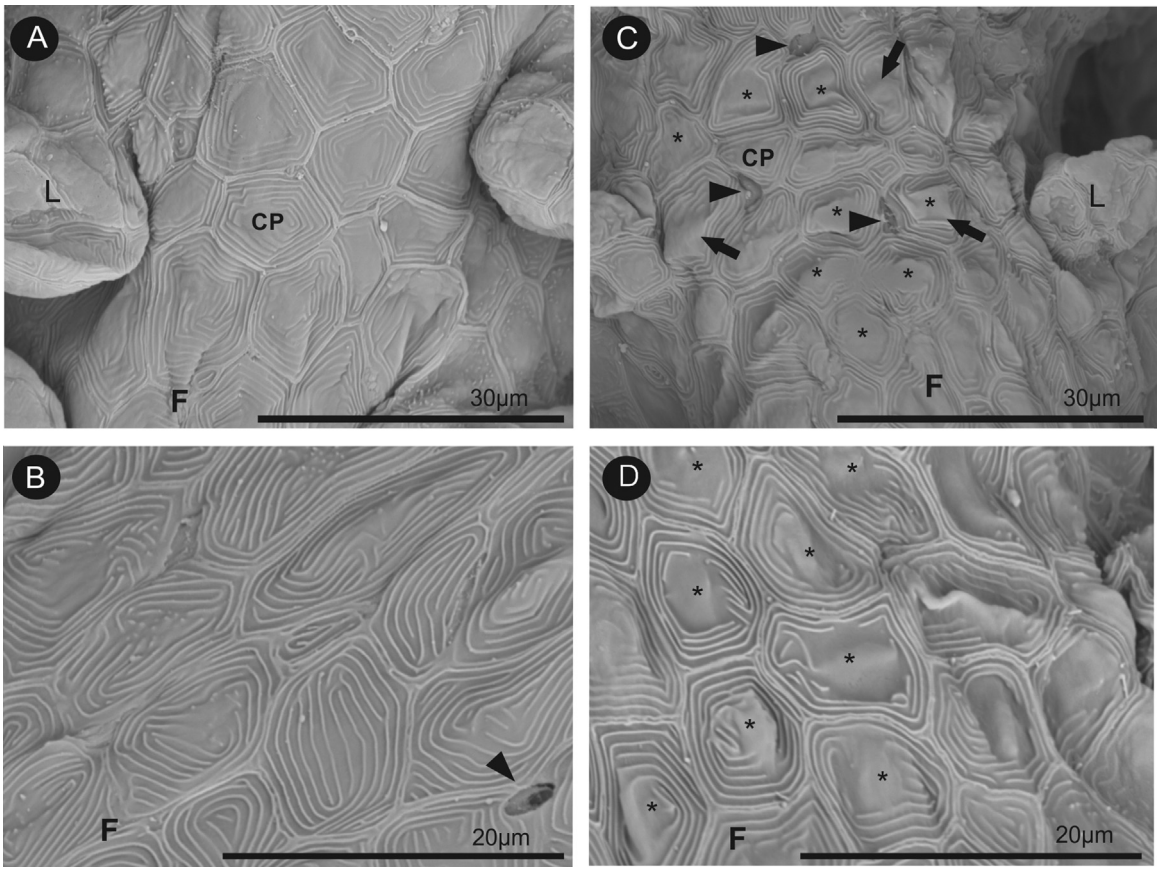

Fig. 1. Electron scanning micrographs for gill of the O. niloticus. A and B: Control group; C and D: Groups exposed to vinasse; F: primary filament; L: secondary lamellar; CP: paviment cells; *: paviment cells with ridge loss: chloridic cells; arrow: paviment cells' swelling.

(Table 2).

3.2. Bioassay observations

During the execution of the first bioassay, all individuals exposed to the 10% dilution of vinasse died; in the second bioassay, fish mortality was higher, with the death of 60% of individuals exposed to the 5% dilution of vinasse and 100% of fish exposed to the 10% dilution.

3.3. Ultramorphology

Paviment cells constitute a large part of the gill surface (Fig. 1A), with its surface having an appearance similar to fingerprints, due to the circular arrangement of its ridges (Fig. 1B). Considering the first bioassay, the results obtained through SEM did not indicate differences between the gills of the control group and those of the treatments; in both cases, the paviment cells presented few ridges (Table 3)..

In the second bioassay, there was a reduction in the number of ridges of paviment cells (Fig. 1C, D) for all of the treatments in comparison with the control group. However, there were no differences between the groups exposed to dilutions of vinasse (Table 3). A swelling of paviment cells was also observed in the treatments, as well as an increase in the quantity of chloridric cells at the 2.5% dilution (Table 4).

3.4. Histological analysis

The control group presented the gill pattern described by Fanta et al. (2002) in teleost fish (Fig. 2A). The animals presented branchial arches formed by primary filaments (primary lamellae), containing in each primary filament respiratory lamellae (secondary lamellae), which were aligned along both sides. Primary filaments were covered by stratified paviment epithelium and had blood spaces limited by pillar cells, separated from the epithelium by a thick basal membrane.

Histological alterations were observed in animals exposed to all tested dilutions. In the first bioassay, the most observed alterations

Table 3
Occurrence frequency of significant alterations found on *O. niloticus* gills exposed to vinasse.

	1° Bioassay				2° Bioassay			
Alterations	CN	V1%	V2,5%	5%	CN	V1%	V2,5%	V5%
Epithelium detachment	0,80 ± 0,97	3,20 ± 2,03	3,60 ± 1,49	4,00 ± 1,26*	0,80 ± 1,60	0	0,20 ± 0,40	0
Lamellar disorganization	1,20 ± 0,97	0,80 ± 0,97	2,40 ± 0,8	5,20 ± 0,97**	1,00 ± 1,26	2,40 ± 0,48	3,60 ± 0,80*	4,00
Hemorrhage	0	3,20 ± 0,97**	2,00**	2,00**	0,40 ± 0,48	0,20 ± 0,40	2,00 ± 2,09	0
Crest loss	5,00 ± 0,70	5,00 ± 0,70	4,80 ± 0,44	4,40 ± 1,14	1,40 ± 1,14	4,20 ± 0,44**	3,40 ± 1,34*	4,00
Hardening	1,40 ± 0,54	1,80 ± 0,83	1,20 ± 0,83	4,60 ± 0,54**	1,40 ± 1,14	2,40 ± 1,51	3,80 ± 0,44*	3,50 ± 0,70

* Significant amount for $p < 0,05$ by ANOVA site in relation to negative control.
** Significant amount for $p < 0,01$ by ANOVA site in relation to negative control.

Table 4
Mean (M) and standard deviation (DP) of mucous cells and chloridic cells quantified between secondary lamellars of *O. niloticus* from the control group and groups exposed to different dillutions of vinasse.

Treatment	1° Bioassay				2° Bioassay			
	Control	V 1%	V 2,5%	V 5%	Control	V 1%	V 2,5%	V 5%
Mucous cells	392,4 ± 64,04	524,6 ± 34,83*	505,4 ± 30,12*	494,2 ± 30,10*	305,6 ± 64,04	477 ± 74,61*	470,8 ± 55,64*	187 ± 256,74
Chloridic cells	12,00 ± 2,91	13,20 ± 3,96	13,20 ± 1,92	15,80 ± 3,42	9,00 ± 1,58	13,40 ± 2,07**	19,20 ± 1,48**	14,50 ± 0,70

Data presented with mean and standard deviations. **CN:** Negative Control; **V1%:** Vinasse 1%; **V2,5%:** Vinasse 2,5%; **V5%:** Vinasse 5%
* Significant value for p < 0,05 by ANOVA test in relation to control.
** Significant amount for p < 0,01 by ANOVA site in relation to negative control.

were epithelium detachment (Fig. 2B) and loss of epithelium integrity (epithelial disorganization), leading to its rupture and consequent hemorrhage (Fig. 2C, D). The importance factor of these alterations are presented in Table 1. These alterations were more frequent with the increase in the amount of vianasse diluted in the aquatic environment.

The histological analysis revealed significant values for the epithelium detachment for all vinasse dilutions tested here, with $p < 0.05$. For epithelium disorganization, the values were significant only for the 5% dilution, with $p < 0.01$. For the hemorrhagic foci, the values were significant at all dilutions, as well as for epithelial detachment, but with

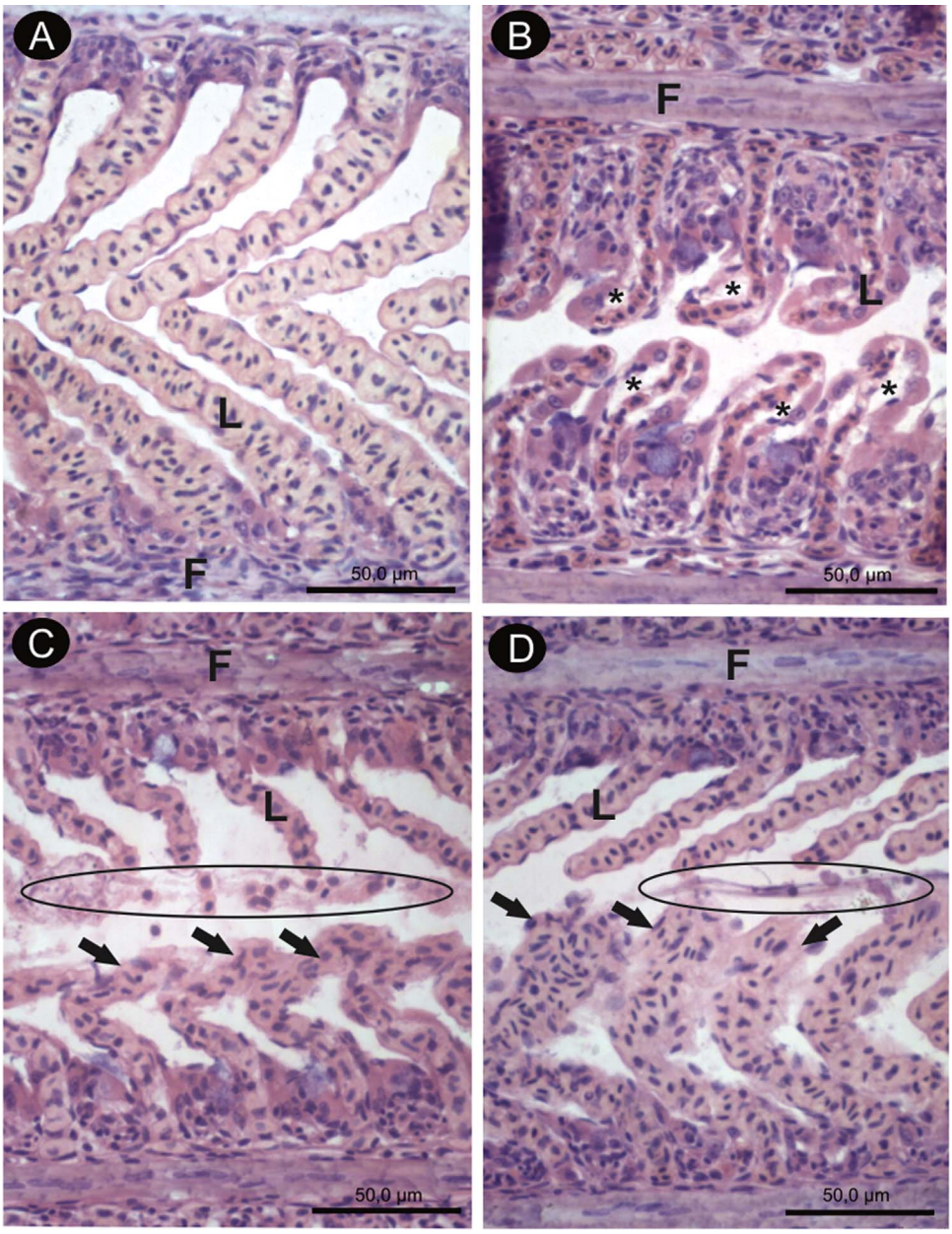

Fig. 2. Histological alterations observed more frenquently in O. niloticus. A. Control; B, C, D: Exposed groups. F: Primary filament; L: Secondary lamellar; (*) Epithelium detachment; (arrows) lamellar disorganization and lamellar integrity loss; (circle) hemorrhage.

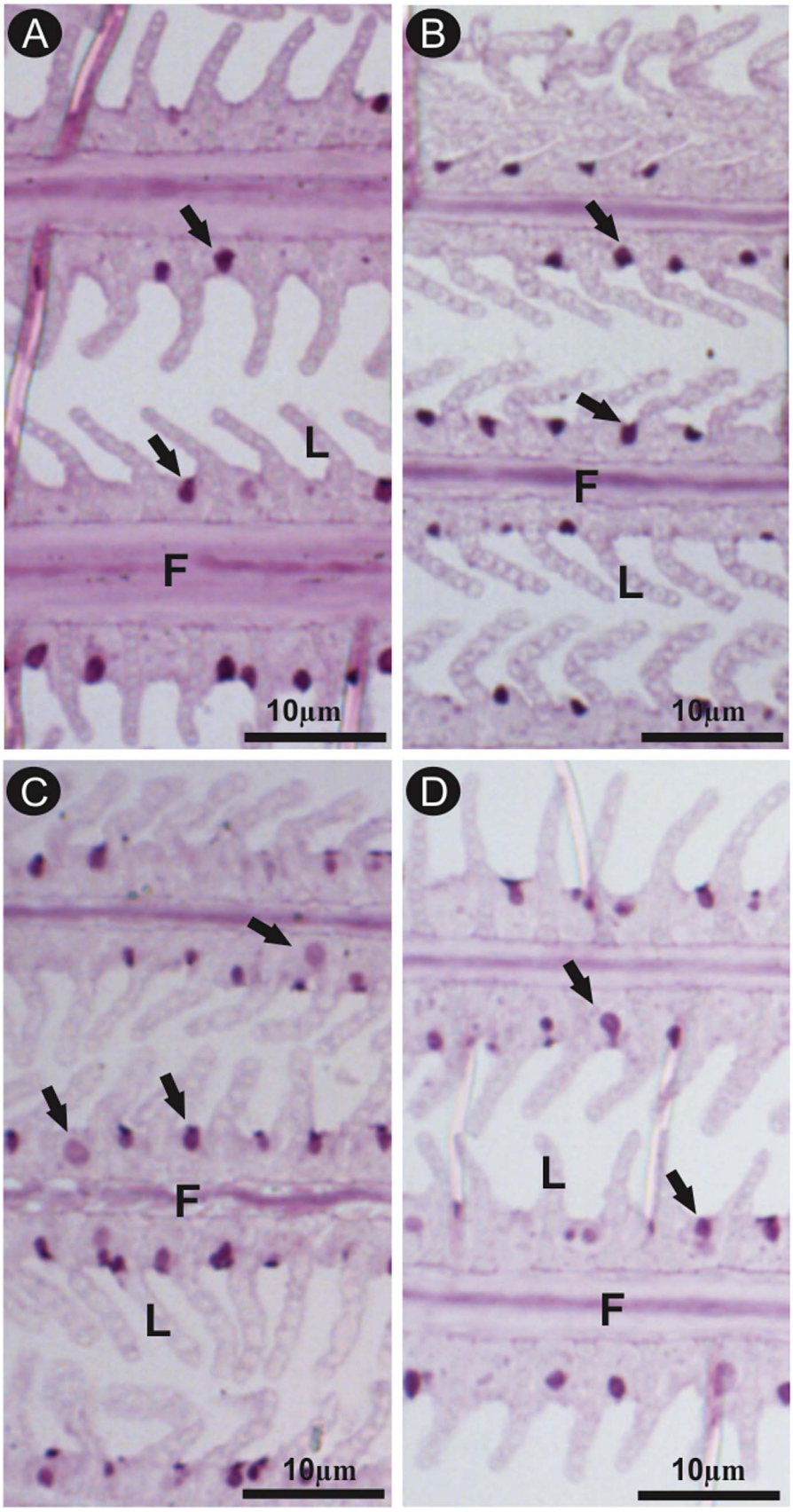

Fig. 3. Histological sections of O. niloticus gills submited to PAS technique. A: Control group; B: Vinasse dilution of 1%; C: Vinasse dilutions of 2,5%; D: Vinasse dilution of 5%. F: primary filament; L: secondary lamellar; Arrows: mucous cells.

$p < 0.01$.

In the second bioassay, the epithelial detachment was not as evident as observed in the first one, although the epithelial disorganization was greater, and increased in a dose-dependent manner, being significant at the vinasse dilution of 2.5%. Hemorrhagic foci were observed in all treatments. A results comparison of both bioassays is summarized in Table 3.

3.5. Histochemical analysis

With the analysis of the mucous cells evidenced by PAS staining (Fig. 3) it can be verified that the number of these cells have increased in the exposed groups (Fig. 3B, C, D, and E). According to ANOVA, the values were significantly different for all dilutions when compared with the control in both bioassays, ($p < 0.05$). The dilution of 1% vinasse presented the largest number of mucous cells, followed by the 2.5% and 5% concentrations in both bioassays. On the other hand, Tukey's post hoc test showed no significant difference between treatments, but differences were still found between the treatment and the control (Table 4)..

4. Discussion

This study aimed to analyze the possible histopathological alterations in the gills of tilapia after exposure to different dilutions of sugarcane vinasse. Given the large volume produced, the use of vinasse as fertirrigation is an alternative to the disposal of this residue. However, the large amount disposed of in the field may leach or percolate into soil and end up contaminating water resources.

In both bioassays, the 10% dilution of vinasse was lethal to the fish; in one experiment 60% of the individuals exposed to the 5% dilution of vinasse also died. The high mortality rates are possibly due to the high organic load of the waste and its elevated DBO and DQO, leading to the depletion of oxygen in the water. Parameters such as the high amount of potassium and low pH of the samples might also have contributed to the toxic effects of the sugarcane vinasse, as the influence of pH on an ecosystem can be both direct, with effects over the physiology of the species, and indirect, contributing, under certain conditions, to the precipitation of toxic chemicals such as trace metals (CETESB, 2013). It is worth noting that vinasse can present trace metal values far below those detectable by the analytical method used (Camilotti et al., 2007). Thus, it is not possible to measure the damage likely caused by all elements, although they can inflict numerous injuries on fish if they are present (Alvarado et al., 2006; Randi et al., 1996; Sreedevi et al.; 1992).

According to Robertiello (1982), the vinasse composition varies depending on the plant of each distillery for ethanol production and the distillation process itself. The raw vinasse sample used in the first bioassay presented a chromium concentration substantially higher than permitted by the CETESB in water resources; actually, the values were so high that even dilutions of 2.5%, 5%, and 10% showed chromium concentrations that were higher than permitted.

Fish gill tissue presents two types of cellular response: defense and compensatory (Mallat, 1985; Takashima and Hibiya, 1995). Both responses help to block the entrance of toxic substances, stopping them from reaching the bloodstream, and in smaller proportions, assist in the prevention of injuries caused by direct exposure. (Cerqueira and Fernandes, 2002).

The epithelial detachment observed in fish gills submitted to the vinasse, especially from the first bioassay, is an initial response of the branchial apparatus characterized by an increase in cellular and tissue functions, causing physiological alterations of these activities (Smart, 1976). It is a typical defense mechanism of gills that promote the increase of the water-blood barrier; this respiratory surface loss can result in death by anoxia (Rand and Petrocelli, 1985); it is a regressive alteration, the end of which is to reduce the functionality of the damaged organ, and in this way, reduce animal contamination by the toxic substance (Bernet et al., 1999).

The hemorrhage observed in all vinasse concentrations is classified as circulatory disturbance (Bernet et al., 1999). Apparently, the paviment cells lose adherence with one another, resulting in tissue rupture and consequent hemorrhage; this also makes the affected organ lose its functionality, thereby reducing contamination by the toxic agent.

Tan and Lim (1984) reported that an increase in mucous cells is the first noticeable symptom of toxicity in *Perna viridis* exposed to lead. According to Takashina and Hibiya (1995), mucous hyper-secretion might be the result of a chronic response to verminous or bacterial infections, or to irritable chemical products. Mucus is usually found on the strands, but it was also found on the respiratory epithelium of fish exposed to stressful conditions, which can suggest that the layer formed by the production of mucus protects the lamellar surfaces against toxic and/or infectious agents (Mallat, 1985). Due to the tissue protection function presented by mucus (Bernet et al., 1999), the increase in the number of mucous cells might be an indication of the presence of toxic substances in the water (David and Fontanetti, 2009; Biagini et al., 2009). As shown in the results section, the number of mucous cells was significantly higher in all treatments in comparison with the control (ANOVA test, $p < 0.05$), confirming the vinasse toxicity in all three dilutions used in this study.

The gills' paviment cells constitute the largest part of the branchial surface. They are characterized by ridges on their surfaces, which are important in mucus retention in the branchial epithelium as a protection method against environmental changes (Mallat, 1985). Wong and Wong (2000) observed a a reduction in the number of ridges on the gills of *O. mossambicus* subjected to different concentrations of cadmium. Biagini et al. (2009) also observed a reduction in the number of ridges on paviment cells on tilapia raised in polluted waters treated with flotation. Hence, a reduction of these ridges, as was observed in our second bioassay, indicates the response of the affected organ in relation to the contaminant, that being a regressive response (Bernet et al., 1999).

Overall, sugarcane vinasse has a polluting power 100 times greater than that of domestic sewage (Silva et al., 2007), being widely reused in sugarcane plantations for fertirrigation. However, through processes of leaching and percolation, this effluent can reach water resources and contaminate them with its components; therefore, it is of the utmost importance to take into consideration the toxic potential of sugarcane vinasse.

5. Conclusion

On the basis of the results obtained through histopathological analysis of the gills of O. *niloticus* fish species exposed to four different dilutions of sugarcane vinasse, it can be concluded that this residue presents toxic and cytotoxic potential in aquatic environments. This was demonstrated by the mortality rates observed at the highest dilutions, the morphological changes in gill tissue, and also by the significant increase in mucous cells for all vinasse dilutions. Thus, the reuse of this waste for fertirrigation practice must be assessed with caution, as once it reaches the aquatic environment, its effect on the fauna can be extremely harmful. Therefore, the disposal of this residue in the field should be undertaken with increased caution, being able to receive previous treatment before being released in cultures as fertilizer.

Acknowledgments

The authors thank FAPESP, Brazil (Fundação de Amparo à Pesquisa do Estado de São Paulo – Support Foundation for Research of São Paulo State, processes 2011/0679-8 and 2012/50197-2) for financial support; Gerson Mello Souza, Antonio Teruyoshi Yabuki, and Pablo Henrique Nunes for technical support; and Nilton Righetto Neto

for help during the experiments.

References

Abdel-Moneim, A.M., Al-Kahtani, M.A., Elmenshawy, O.M., 2012. Histopathological biomarkers in gills and liver of *Oreochromis niloticus* from polluted wetland environments, Saudi Arabia. Chemosphere 88, 1028–1035.

Algur, O.F., Kadioglu, A., 1992. The effects of vinasse on the growth, biomass and primary productivity in pea (*Pisum sativum*) and sunflower (*Helianthus annuus*). Agric., Ecosyst. Environ. 39, 139–144.

Alvarado, N.E., Quesada, I., Hylland, K., Marigómez, I., Soto, M., 2006. Quantitative changes in metallothionein expression in target cell-types in the gills of turbot (*Scophthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment. Aquat. Toxicol. 77, 64–77.

Al-sabti, K., Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. Mutat. Res. 323, 121–135.

Bernet, D., Schmidt, H., Meier, W., Burkhardt-Holm, P., Wahli, T., 1999. Histopathology in fish: proposal for a protocol to assess aquatic pollution. J. Fish Dis. 22, 25–34.

Biagini, F.R., David, J.A.O., Fontanetti, C.S., 2009. The use of histological, histochemical and ultramorphological techniques to detect gill alterations in *Oreochromis niloticus* reared in treated polluted waters. Micron 40, 839–844.

Camilotti, F., Marques, M.O., Andrioli, I., Silva, A.R., Junior, L.C.T., Nobile, F.O., 2007. Acúmulo de metais pesados em cana-de-açúcar mediante a aplicação de lodo de esgoto e vinhaça. Eng. Agric. 27, 284–293.

Cerqueira, C.C.C., Fernandes, M.N., 2002. Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish *Prochilodus scrofa*. Ecotoxicol. Environ. Saf. 52, 83–91.

CETESB, 2013. – Companhia Ambiental do Estado de São Paulo. Variáveis de qualidade das águas. Disponível em ⟨http://www.cetesb.sp.gov.br/Agua/rios/variaveis.asp#cobre⟩. Acessado em: 20/01/.

Christofoletti, C.A., Pedro-Escher, J., Correia, J.E., Marinho, J.F.U., Fontanetti, C.S., 2013a. Sugarcane vinasse: environmental implications of its use. Waste Manag. 33, 2752–2761.

Christofoletti, C.A., Pedro-Escher, J., Fontanetti, C.S., 2013b. Assessment of the genotoxicity of two agricultural residues after processing by diplopods using the Allium cepa Assay. Water Air Soil Pollut. (Dordr. Online) 224, 1523.

David, J.A.O., Fontanetti, C.S., 2009. The role of mucus in *Mytella falcata* (Orbigny, 1842) gills from polluted environments. Water Air Soil Pollut. 203, 261–266.

España-Gamboa, E., Mijangos-Cortes, J., Barahona-Perez, L., Dominguez-Maldonado, J., Hernández-Zarate, G., Alzategaviria, L., 2011. Vinasses: characterization and treatments. Waste Manag. Res. 29, 1235–1250.

Evans, D.H., 1987. The fish gill: site of action and model for toxic effects of environmental pollutants. Environ. Health Perspect. 71, 47–58.

Fanta, E., Rios, F.S., Romão, S., Vianna, A.C.C., Freiberger, S., 2002. Histopathology of the fish *Corydoras paleatus* contaminated with sublethal levels of organophosphorus in water and food. Ecotoxicol. Environ. Saf. 54, 119–130.

Fontanetti, C.S., Souza, T.S., Christofoletti, C.A., 2012. . The role of biomonitoring in the quality assessment of water resources. In: Bilibio, C., Hensel, O., Selbach, J. (Org). Sustainable Water Management in the Tropics and Subtropics - and Cases Study in Brazil. 1ªed. Brasil/Alemanha: UNIPAMPA/UNIKASSEL 3, 975-1005.

Harshbarger, J.C., Clark, J.B., 1990. Epizootiology of neoplasms in bony fish of North-America. Sci. Total Environ. 94, 1–32.

Junqueira, L.C.U., Junqueira, M.M.S., 1983. Técnicas básicas de citologia e histologia. São Paulo: Livraria Editora Santos, 123.

Karnovsky, M.J., 1965. A formaldehyde-glutaraldehyde fixative at high osmolarity for use in electron microscopy. J. Cell Biol. 11 (1965), 137–140.

Kikuchi, M., Wakabayashi, M., Kojima, H., Yoshida, T., 1978. Uptake, distribution and elimination of sodium linear alkylbenzene sulfonate and sodium alkyl sulfate in carp. Ecotoxicol. Environ. Saf. 2, 115–127.

Kumar, S., Gopal, K., 2001. Impact of distellery effluent on physiological consequences in the freshwater teleost *Channa punctatus*. Bull. Environ. Contam. Toxicol. 66, 617–622.

Machado, M.R., 1999. Uso de brânquias de peixes como indicadores de qualidade das águas. UNOPAR Cient. Cienc. Biol. Saude 1, 63–76.

Mallat, J., 1985. Fish gill structural changes induced by toxicants and other irritants: a statistical review. Can. J. Fish. Aquat. Sci. 42, 630–648.

Marinho, J.F.U., Correia, J.E., Marcato, A.C.C., Pedro-Escher, J., Fontanetti, C.S., 2014. Sugarcane vinasse in water bodies: impact assessed by liver histopathology in tilapia. Ecotoxicol. Environ. Saf. 110, 239–245.

Odum, E.P., 1988. Breve descrição dos principais tipos de ecossistema natural na biosfera. In: Ecologia. Ed. Guanabara Koogan, Rio de Janeiro, 367–369.

Pedro-Escher, J., Maziviero, G.T., Fontanetti, C.S., 2014. Mutagenic action of sugarcane vinasse in the *Tradescantia pallida*. Test. Syst. J. Ecosyst. Ecogr. 4, 145–149.

Pedro-Escher, J., Christofolleti, C.A., Ansoar-Rodríguez, Y., Fontanetti, C.S., 2016. Sugarcane vinasse, a residue of ethanol industry: toxic, cytotoxic and genotoxic potential using the *Allium cepa* Test. J. Environ. Prot. 7, 602–612.

Rand, G.M., Petrocelli, S.R., 1985. Fundamentals of Aquatic Toxicology. In: New York (Ed.), . Mcgraw Hill, Washington, 666.

Randi, A.S., Monserrat, J.M., Rodriguez, E.M., Romano, L.A., 1996. Histopathological effects of cadmium on the gills of the freshwater fish, *Macropsobrycon uruguayanae* Eigenmann (Pisces, Atherinidae). J. Fish Dis. 19, 311–322.

Robertiello, A., 1982. Upgrading of agricultural and agroindustrial wastes: the treatment of distillery effluents (vinasses) in Italy. Agric. Wastes 4, 387–395.

Ribeiro, B.T., Lima, J.M., Julião, L.G.F., 2007. Influência da vinhaça na adsorção e dessorção de Cd e Pb em um cambissolo. XXXI Congr. Bras. De. Ciência do Solo.

Saleh, Y.S., Mohamed-Assem, S.M., 2016. Use of Arius thalassinus fish in a pollution biomonitoring study, applying combined oxidative stress, hematology, biochemical and histopathological biomarkers: a baseline field study. Mar. Pollut. Bull. 106, 308–322.

Silva, M.A.S., Griebeler, N.P., Borges, L.C., 2007. Uso de vinhaça e impactos nas propriedades do solo e lençol freático. Rev. Bras. Eng. Agric. Ambient 11, 108–114.

Smart, G., 1976. The effect of ammonia exposure on gill structure of the rainbow trout (*Salmo gairdneri*). J. Fish Biol. 8, 471–475.

Souza, T.S., Hencklein, F.A., De Angelis, D.F., Fontanetti, C.S., 2013. Clastogenicity of landfarming soil treated with sugar cane vinasse. Environ. Monit. Assess. 185, 1627–1636.

Sreedevi, P., Siravamakrishna, B., Suresh, A., Radhakrishnaiah, F., 1992. Effects of nickel on some aspects of protein metabolism in the gill and kidney of the freshwater fish *Cyprinus carpio*. Environ. Pollut. 77, 59–63.

Takashima, F., Hibiya, T., 1995. An Atlas of Fish Histology: Normal and Pathological Features. Fischer Verlag, Tokyo: Kodanska /Stuttgart, 195, 2nd ed..

Tan, W.H., Lim, L.H., 1984. The tolerance to and uptake of lead in the green mussel, *Perna viridis*. Aquaculture 42, 317–332.

UNICA, 2007. – União da Indústria da cana-de-açúcar do estado de São Paulo. Produção de álcool do Brasil. UNICA. 2007Disponível em: http://www.portalunica.com.br/portalunica/index.php?Secao=referência & SubSecao=estatísticas & SubSacao=produção20Brasil & id=%20and%id=9. Acesso em: 10/06/12.

Vega, M.M., Fernandez, T.B., Tarazona, J.V., Castaño, A., 1996. Biological and chemical tools in the toxicological risk assessment of Jarama river, Madrid, Spain. Environ. Pollut. 92, 135–139.

Waliszewski, K.N., Romero, A., Pardio, V.T., 1997. Use of cane condensed molasses solubles in feeding broilers. Anim. Feed Sci. Technol. 67, 253–258.

Wilkie, A., Riedesel, K., Owens, J., 2000. Stillage characterization and anaerobic treatment of ethanol stillage from conventional and cellulosic feedstocks. Biomass and Bioenergy 19, 63–102.

Wong, C.K., Wong, M., 2000. Morphological and biochemical changes in the gills of Tilapia (*Oreochromis mossambicus*) to ambient cadmium exposure. Aquat. Toxicol. 48, 517–527.

Yesilada, E., 1999. Genotoxic activity of vinasse and its effect on fecundity and longevity of *Drosophila melanogaster*. Bull. Environ. Contam. Toxicol. 63, 560–566.