Evaluation of acute toxicity of vinasse by means of *Daphnia magna* and *Aliivibrio fischeri*: a comparative study

Móritz VELÁSQUEZ-RIAÑO, Juan Sebastián MENEGES-SÁNCHEZ, Carel Elizabeth CARVAJAL ARIAS
Facultad de Ingeniería, Universidad El Bosque, Bogotá, Colombia

ABSTRACT

In the bioethanol industry, for each liter of alcohol a residue of 9 to 14 liters of vinasse is obtained which is produced depending on whether a process of recirculating the vinasse is used or not (Jimenez et al., 2003). If the vinasse is directly shed into bodies of water without an adequate treatment, due to the above mentioned characteristics, it may have negative effects on the existing biota and human health (Jimenez et al., 2003). The purpose of this study was to assess the acute toxicity of vinasse by means of a rapid test with *Aliivibrio fischeri* and compare it with a standard immobilization assay with *Daphnia magna*. The standard assay of *D. magna* by means of its EC$_{50}$ of 4.7% showed that organism was more sensitive to the contaminant, in comparison with the 69.6% obtained with the A. fischeri which suggests that it should be continuously used as one of the organisms of first choice for the evaluation of the acute toxicity of this effluent.

KEY WORDS: wastewater distillery; bioindicators; water fleas; luminescent bacteria

Introduction

Vinasse is the industrial residue of the process of distilling alcohol produced by the fermentation of molasses (from sugar cane, beetroot, timbers) by yeasts like *Saccharomyces cerevisiae* (Parnaudeau et al., 2008; Velásquez-Riaño et al., 2013). Depending on their origin their characteristics vary but they are generally brown in color, have a high turbidity (due to suspended solids) and toxicity, a low pH (which ranges from 3.5–5.0) and a high content of dissolved and suspended organic material with values for biochemical oxygen demand (BOD) between 7,000 and 20,000 mg/l and values for chemical oxygen demand (COD) between 50,000 and 150,000 mg/l. They also contain a noticeable amount of inorganic salts composed of calcium sulfates and phosphates, potassium, sodium and magnesium (España-Gamboa et al., 2011).

In the bioethanol industry, for each liter of alcohol a residue of 9 to 14 liters of vinasse is obtained which is produced depending on whether a process of recirculating the vinasse is used or not (Jimenez et al., 2003). If the vinasse is directly shed into bodies of water without an adequate treatment, due to the above mentioned characteristics, it may have negative effects on the existing biota and human health (if that resource is used for consumption), (Figaro et al., 2006).

Over the years, several alternatives, either physico-chemical or biological, have been proposed for the use and degradation of vinasses. These strategies include the production of energy (methane) as in the case of anaerobic biodigestors (Marques et al., 2013; Choeisai et al., 2014; Formaginini et al., 2014) and the production of microbial biomass or some metabolite of interest (Marques et al., 2013; Nitayavardhana et al., 2013; Sydney et al., 2014). However, there are few studies which have included parameters for the analysis of the toxicity of this byproduct after a physico-chemical, biological or coupled treatment was made. Evaluating these toxicological parameters is fundamental for a correct analysis of the treatment effectiveness for removing contaminating substances, since the traditional parameters on their own cannot show whether new substances, maybe even more...
toxic than the original one, are or are not being produced (due to the treatment) (Rodrigues & Umbuzeiro, 2011; Romanholo-Ferreira et al., 2011).

Among the studies which have evaluated the parameters of vinasse toxicity there is one from Romanholo-Ferreira et al. (2011) that analyzed the treatment of vinasses with Pleurotus pulmonarius, employing Pseudokirchneriella subcapitata, Hydra attenuata, Daphnia magna and Daphnia similis as toxicological indicators. This study concluded that the proposed treatment serves to reduce the color and degrade the complex compounds and, in turn, diminish the toxicity of vinasses. Grossi-Bothelo et al. (2012) analyzed the toxicity of vinasse in cladocerans and fish before and after an adjustment of the pH employing an acute toxicity assay. The average lethal concentration (LC50 48h) of vinasse, before the adjustment of the pH, was 0.7% for Ceriodaphnia dubia and 0.8% for D. magna, and the average lethal concentration (LC50 96h) for Danio rerio was 2.6%. After adjusting the pH, the values increased for all of the organisms in the study showing a decrease of the toxicity. Barba-Ho & García (2012) compared a photo-Fenton system (H2O2/Fe2+) with an anaerobic biological system coupled with the photo-Fenton one with the aim to evaluate the effectiveness of such treatment in removing the organic material and toxic compounds (using Daphnia pulex as a toxicity assay). At the end of the experiment, they obtained high percentages of the removal of COD (83–97%), BOD5 (96%), TOC (88%), phenols (99%), hardness (87%) and chlorides (91%). The evaluation of toxicity with D. pulex showed an increase in the toxicity of vinasse after the photo-Fenton treatment with a value of LC50 48 h of 6.9% (v/v) for the crude vinasse at 5.5% (v/v) and a final value of 16.7% (v/v) for the one treated with the coupled system. They concluded that the coupled treatment with anaerobic microorganisms was the most suitable for removing the organic material and reducing the toxicity of this compound.

As showed above, most studies have evaluated the toxicity of vinasse before and after its treatment by using standard indicators belonging to the Daphnia genus. Although that test is relatively simple and economical, it requires the cultures to be maintained in suitable conditions including a constant cleaning of the culture vessels, the growing of their food (Chlorella sp.), their periodical feeding with Chlorella sp., and the synchronization of the cultures to maintain a sufficient amount of neonates for the implementation of the assays. Caring for the cultures thus takes a great deal of a time.

For that reason, the aim of this study is to broaden the battery of toxicity assays for vinasse, by means of a rapid assay with an analysis of the inhibition of the luminescence of A. fischeri and a comparison between its average effective concentration (EC50) and the one obtained from a standard assay with D. magna.

Materials and methods

The vinasse

The vinasse for this study was kindly supplied by the Fábrica de Licores de Antioquia (Antioquia Liquor Distillery, Medellín, Colombia), which was stored at 4°C until it was used in the different assays (it was stored for 15 days). Table 1 shows the main physical and chemical characteristics of the vinasse used in this study.

The acute immobilization test of Daphnia magna

The strain of D. magna used in this study was supplied by professor Maria Teresa Regueiro Reza of the Universidad Nacional de Colombia (National University of Colombia, Bogotá). D. magna was fed with Chlorella vulgaris at a proportion of 0.1–0.2 mg of culture/day/Daphnia and it was kept at a photoperiod of 16:8 h of light-darkness at 22±1°C. The assay followed the next TG202 protocol of the OECD (OECD, 2004) in which 20 neonates younger than 24 h of age in groups of 5 individuals/10 ml (4 replicas in plastic cells) were exposed to each of the vinasse concentrations evaluated: 100, 50, 25, 12.5, 6.3, 3.1 (% v/v); a negative control was reconstituted water and a positive control was 1.2 mg/l of K2Cr2O7, while the temperature and initial photoperiod were maintained. The number of immobilized animals was recorded after 48 h. The daphnids which were unable to move after 15 s of a gentle shaking were regarded as immobile.

Inhibition assay using Aliivibrio fischeri

Acute toxicity was measured in vitro with the BioTox™ kit (Aboatox Oy, Finland). This kit employs bacteria which emits a natural luminescence (A. fischeri NRRL B-11177). For this assay, we followed the manufacturer’s

Table 1. Main physical and chemical characteristics of the vinasse used in this study

| Parameters                        | Value |
|-----------------------------------|-------|
| Turbidity (NTU)                   | 241   |
| pH                                | 4.8   |
| Conductivity (NaCl) (ms/m)        | 0.3   |
| COD (mg/l)                        | 97,000|
| BOD5 (mg/l)                       | 33,236|
| Total phosphates (as PO4) (mg/l)  | 40.7  |
| Total Kjeldahl Nitrogen (mg/l)    | 1,059.6|
| Total solids (mg/l)               | 58,329.9|
| Total volatile solids (mg/l)      | 39,581.8|
| P (P2O5) (g/l)                    | 0.5   |
| K (K2O) (g/l)                     | 11.0  |
| C (CaO) (g/l)                     | 1.6   |
| Mg (MgO) (g/l)                    | 2.7   |
| Cu (mg/l)                         | 1.1   |
| Mn (mg/l)                         | 3.4   |
| Fe (mg/l)                         | 27.0  |
| Cr (VI) (mg/l)                    | 0.3   |
instructions; briefly, the flask which contains the lyophilisate of *A. fischeri* was reconstituted by the addition of a whole flask of the reagent at a dilution of +4 °C, balanced for a minimum of 30 min at +4 °C, and stabilized at +15 °C for a minimum of 30 min. The pH of the initial sample of vinasse was measured and adjusted to 7.0±0.2, the salinity was adjusted to 2% with a 20% of NaCl, and the sample was oxygenated to reach an initial concentration of dissolved oxygen to more than 3.0 mg/l. Afterwards, the sample of pure vinasse was diluted with a 2% of NaCl to obtain a series of dilutions of 3.1, 6.3, 12.5, 25, 50, 100 (% v/v). A sample of the 2% NaCl was used as a control. Later, 500 μl of the dilutions and the control were placed in the test tubes (in duplicate). The flask which contains the reconstituted bacteria was placed in a self-injector connected to a luminometer (Triathler, Hidex Oy) with a maximum count rate of 30,000,000 counts per second (CPS) previously calibrated. Following that, it proceeded to individually place each of the previously prepared tubes (starting with those which contain the control sample), into which the device injected 500 μl of bacteria, and after counting five seconds, the reading of the initial bioluminescence in CPS was made. Finally, each of the samples was subjected to a contact time of 30 min at +15 °C, and after that the bioluminescence was measured again.

### Results and discussion

The evaluation of the eco-toxicity of a substance is an essential stage in the analysis of its possible environmental impacts and serves as a tool for taking decisions about its final disposal. Unfortunately, an evaluation of the toxicity of these byproducts is not obligatory and even today it is still incorrectly thought that the traditional parameters for the degradation of a substance like the BOD, COD, color, turbidity, pH, etc. are sufficient to establish if those substances may or may not be harmful for the ecosystems into which they are shed (Rodrigues & Umbuzeiro 2011).

In the assay of acute immobilization with *D. magna*, the EC$_{50}$ 48 h was only calculated with the Probit analysis method, obtaining a value of 4.7% (Figure 1). Figure 2 shows a curve which represents the changes in the emission of light from several concentrations of vinasse measured by the BioTox™ kit (and expressed as INH%). One can see that the emission of light declines as the concentration of the sample increases (inversely proportional) and at the same time the percentage of inhibition increases as the concentration of vinasse increases (directly proportional). The EC$_{50}$ values given by el BioTox™ kit were calculated by means of a standard linear regression analysis of the

### Statistical analysis

Only nominal concentrations were used in this study because it is very difficult to quantify measured concentrations once the vinasse has been dilute because this effluent is a mixture of many compounds. In the tests of acute toxicity in *D. magna*, the EC$_{50}$ value and its 95% confidence limits were calculated by the Probit analysis (Finney, 1971). For the acute inhibition assay with *A. fischeri*, the inhibition percentage (INH%) of each sample dilution was calculated in accordance with the equations shown below (1 and 2) and plotted on log-log scale. The EC$_{50}$ value was determined by using a standard linear regression analysis of the linear comparison between the logarithm of the toxic concentration and the logarithm of the intensity of the lost/remaining light and with a Probit analysis (Finney, 1971).

1. \[ \text{KF}=\frac{\text{IC}_30}{\text{IC}_0} \]
2. \[ \text{INH\%}=100-\frac{\text{IT}_{30}}{(\text{KF} \times \text{IT}_0)} \times 100 \]

Where:

- \( \text{KF} \) = Correction factor.
- \( \text{IC}_{30} \) = Intensity of luminescence of the control after the time of contact (30 min) in the CPS.
- \( \text{IC}_0 \) = Maximum CPS value of the control during the 5-second kinetic measurement.
- \( \text{IT}_{30} \) = Intensity of luminescence of test sample after time of contact (30 min) in the CPS.
- \( \text{IT}_0 \) = Maximum CPS value of the sample during the 5-second kinetic measurement.
linear comparison between the logarithm of the toxicant concentration and the logarithm of the intensity of the lost /remaining light, which yielded a linear plot. The EC$_{50}$ value for the vinasse at an exposure of 30 min. was 69.6% (Figure 3). The EC$_{50}$ value was also calculated by means of the Probit analysis. However, the EC$_{50}$ value that method yielded differs from that obtained by the linear regression method, reaching a value of 85.6% (Figure 4).

As can be seen in the EC$_{50}$ calculations, the organism which is the most sensitive to the vinasse by far was D. magna, with an EC$_{50}$ 48 h value of 4.7%, which is 14.4 times less than that obtained from A. fischeri (69.6%). To undertake the evaluation of the eco-toxicity of a substance it is recommend the use of different organisms in the same study which correspond to different levels of the trophic chain (Choi & Meier 2001). Thus, the evaluation of the toxicity of vinasse has been done with different bacteria (A. fischeri) (Guerreiro et al., 2016), algae (Pseudokirchneriella subcapitata) (Romanholo-Ferreira et al., 2011), onion seeds (Allium cepa) (da Silva-Souza et al., 2010; Christofoletti et al., 2013), sugar cane (Saccharum officinarum) (Srivastava & Jain, 2010), cladocerans (Ceriodaphnia dubia, Daphnia magna, Daphnia similis, Daphnia pulex) (Romanholo-Ferreira et al., 2011; Grossi-Botelho et al., 2012; Barba-Ho & García, 2012; Paz-Pino et al., 2014), cnidarians (Hydra attenuata) (Romanholo-Ferreira et al., 2011), the eggs of nematodes (Meloidogyne javanica and Meloidogyne incognita) (Pedrosa et al., 2005) and fish (Chaunna punctatus, Danio rerio) (Kumar & Gopal, 2001; Grossi-Botelho et al., 2012) (Table 2).

The choice of the organism to be evaluated is going to depend on the environment which may be affected by the shedding of the substance: daphnids are mainly used in studies of the toxicity of water. Due to their sensitivity, these cladocerans are widely recommended as the standard organisms for evaluating acute toxicity by a variety of international organizations and agencies like EPA (2002), OECD (2004) and ISO (2012) and, as has been mentioned above, they have also been used in the evaluation of acute toxicity in vinasse. Among the assays, which use bacteria regarded as the main trophic level in many aquatic ecosystems in terms of energy flows and the cycling of nutrients, one of the most widely used on a world level and which is also standardized by the ISO (2007) is the assay of inhibition with A. fischeri. In view of the above, we decided to work with those two organisms in this study.

The characteristics of the vinasses in terms of conventional parameters like BOD, COD, pH, turbidity, color, etc., may differ depending on the process of fermentation and distillation used in each factory and the same applies to the different batches in the same factory (Kumar & Gopal 2001; Naik et al., 2008) which may have a direct influence on their toxicity. The EC$_{50}$ 48 h value obtained with D. magna in this study was 4.7% at a pH of 4.8. This value is 5.9 times greater than that reported by Grossi-Botelho et al. (2012), who, working with sugar cane vinasse with a pH of 4.0, obtained a LC$_{50}$ 48 h value of 0.8%. These authors showed that the acute toxicity of vinasse changes as a function of the adjustment of the pH and that it is more toxic at a low pH (4.0) than a neutral pH which may explain the difference in the results. However, the EC$_{50}$ 48 h value obtained in the present study was very similar to that obtained in previous ones which employed

### Table 2. Toxicity bioassays conducted with vinasse at different trophic levels.

| Bioassay     | EC$_{50}$ (%) | Source                      |
|--------------|---------------|-----------------------------|
| D. magna     | 3.6           | Romanholo-Ferreira et al. (2011) |
| D. similis   | 2.2           |                             |
| H. attenuata | 2.3           |                             |
| P. subcapitata| 1.6           |                             |
| D. pulex     | 5.5           | Barba-Ho and García (2012)  |
| D. magna     | 0.8           |                             |
| C. dubia     | 0.7           | Grossi-Botelho et al. (2012) |
| D. rerio     | 2.6           |                             |
| A. cepa      | 11.2          | Christofoletti et al. (2013) |
| D. pulex     | 3.9 (LC$_{50}$) | Paz-Pino et al. (2014)       |
| A. fischeri  | 28.5          | Guerreiro et al. (2016)      |
| D. magna     | 4.7           | This study                   |
D. pulex, with a value of 6.9% (v/v) (Barba-Ho & Garcia, 2012) and 3.9% (v/v) (Paz-Pino et al., 2014).

At the start of the present study, no reports that would deal with the use of A. fischeri to evaluate acute toxicity in vinasse were found in the literature and that is the reason why we decided to assess the potential of this rapid assay for evaluating acute toxicity in this byproduct of the ethanol industry. However, a short while ago the first study which included the use of this bacteria (Guerreiro et al., 2016) was published and while the authors show that the toxicity to A. fischeri was eliminated after the proposed treatment was done, they did not take into account its toxicity for several organisms, since, as the present study has shown, A. fischeri is not an organism which is suitable for making tests of acute toxicity with vinasse. Because it has such a low sensitivity to this byproduct, it should not be used as a criterion in making the decision of evaluating its toxicity reduction and much less making decisions of how it should be disposal.

One of the probable reasons for its low sensitivity to this byproduct is that vinasse is a rather complex solution. This byproduct is made up of various elements which are needed for the growth of this bacteria, and which are also found in seawater, (the origin of this microorganism or a common medium for its culture in the laboratory) like Na, K, Mg and Ca, among others. It is also a good source of nitrogen which favors the growth of this bacteria (Romanholo-Ferreira et al., 2011). High sensitivity to this effluent by D. magna, which was previously demonstrated, must be due, in the first place, to the complexity of its mixture since it is made up of compounds like glycerol, lactic acid, sorbitol, citric acid, quinic acid, β-fructofuranose, α-glucopyranose, trehalose, saccharose, among others (such a mixture is a challenge for the metabolism of any organism) (Morales et al., 2000). In the second place, it must be due to its low pH which, as also been demonstrated, is a factor that negatively affects this cladoceran. And finally, it must be due its large amount of organic material and high turbidity which are directly related to the rapid exhaustion of the dissolved oxygen in the medium (Christofoletti et al., 2013) and in turn makes it very difficult for this organism to survive.

Although many authors describe the acute immobilization test for D. magna as “simple” and rapid, it needs a lot of time and effort to standardize the test in the laboratory. The daphnids must grow in optimum conditions of alkalinity, pH, temperature and photoperiod, they must be fed, usually with algae (which likewise should be grown in optimum conditions), and finally, at least 120 neonates less than 24 h old are needed for the test which means that this assay is really complex. Despite the above, due to its strong sensitivity to the vinasse, D. magna should be one of the top choices for an organism used for testing the toxicity of this byproduct in water, however, scientists should also continue to evaluate others organisms and rapid tests to see if they would be more sensitive, less complicated and cheaper to use in such assays than this cladoceran.

Conclusion

The standard assay of Daphnia magna by means of its EC50 of 4.7% showed that the organism was more sensitive to the contaminant in comparison with the 69.6% obtained with the A. fischeri which suggests that it should continue to be used as one of the organisms of first choice for the evaluation of the acute toxicity of this effluent.

REFERENCES

Barba-Ho LE, Garcia LA. (2012). Evaluación de la factibilidad de acopio de un sistema fotocatalítico biológico para el tratamiento de vinazas mediante estudios de toxicidad (Evaluation of the coupling feasibility of a biological photocatalytic system for the treatment of vinasse through toxicity studies). EIDENAR 11: 63–71.

Choeiasi P, Jitkam N, Silapanoraset K, Youbolsi C, Yooyachatcha, W, Yamaguchi T, Onodera T, Suyusubo K. (2014). Sugarcane molasses-based bio-ethanol wastewater treatment by two-phase multi-staged up-flow anaerobic sludge blanket (UASB) combination with up-flow UASB and down-flow hanging sponge. Water Sci Technol 69: 1174–1180.

Choi K, Meier PG. (2001). Toxicity evaluation of metal plating wastewater employing the Microtox® Assay: A comparison with cladocerans and fish. Environ Toxicol 16: 136–141.

Christofoletti CA, Pedro-Escher J, Fontanetti CS. (2013). Assessment of the genotoxicity of two agricultural residues after processing by diplo pods using the Allium cepa assay. Water Air Soil Pollut 224: 1–14.

Da Silva-Souza TS, Henklein FA, De Angelis DF, Fontanetti CS. (2013). Clastogenicity of landfarming soil treated with sugar cane vinasse. Environ Monit Assess 185: 1627–1636.

EPA. (2002). Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fifth edition, Washington, DC.

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

Fígaro S, Louisy-Louis S, Lambert J, Ehrhardt JJ, Ouensanga A, Gaspard S. (2006). Adsorption studies of recalcitrants compounds of molasses spentwash on activated carbons. Water Res 40: 3459–3466.

Finney DJ. (1971). Probit analysis. Cambridge University Press, Cambridge.

Formagini EL, Marques FR, Serejo ML, Paulo PL, Boncz MA. (2014). The use of microalgae and their culture medium for biogas production in an integrated cycle. Water Sci Technol 69: 941–946.

Grossi-Botelho R, Tornisielo VL, Alves de Olinda R, Maranzo L A, Machado-Neto L. (2012). Acute toxicity of sugarcane vinasse to aquatic organisms before and after pH adjustment. Toxicol Environ Chem 94: 2035–2045.

Guerreiro LF, Rodrigues CS, Duda RM, de Oliveira RA, Boaventura RA, Madeira LM. (2016). Treatment of sugarcane vinasse by combination of coagulation/flocculation and Fenton’s oxidation. J Environ Manage 181: 237–248.

ISO 11348. (2007). Water quality - Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminous bacteria test). Part 3: Method using freeze-dried bacteria.

ISO 6341. (2012). Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) - Acute toxicity test.

Jimenez AM, Borja R, Martín A. (2003). Aerobic-anaerobic biodegradation of beet molasses alcoholic fermentation wastewater. J Environ Manage 69: 1275–1284.

Kumar S, Gopal K. (2001). Impact of distillery effluent on physiological con-
sequences in the freshwater teleost Channa punctatus. Indian J Microbiol 41–48.

Kumar S, Almeda PF, Chinalia, FA. (2013). Growth of Chlorella vulgaris on Sugarcane Vinasse: The Effect of Anaerobic Digestion Pretreatment. Appl Biochem Biotechnol 171: 1933–1943.

Morales A, Larrahondo J, Victoria H, Jaramillo A. (2000). Compuestos orgánicos en vinaza (Organic compounds in vinasse). Carta trimestral 3: 5–6.

Naik NM, Jagadeesh KS, Alagawadi AR. (2008). Microbial decolorization of spentwash: a review. Indian J Microbiol 48: 41–48.
Evaluation of acute toxicity of vinasse
Móritz Velásquez-Riaño, Juan Sebastián Meneses-Sánchez, Carel Elizabeth Carvajal Arias

Nitayavardhana S, Issarapayup K, Pavan, P, Khanal SK. (2013). Production of protein-rich fungal biomass in an airlift bioreactor using vinasse as substrate. *Bioresour Technol* **133**: 301–306.

OECD. (2004). Test No. 202: *Daphnia* sp. Acute Immobilization Test, OECD Publishing, Paris.

Parnaudeau V, Condom N, Oliver R, Cazevoieille P, Recous S. (2008). Vinasse organic matter quality and mineralization potential, as influenced by raw material, fermentation and concentration processes. *Bioresour Technol* **99**: 1553–1562.

Paz-Pino OL, Barba-Ho LE, Marriaga-Cabrales N. (2014). Tratamiento de vinazas acoplando electrodisolución, heterocoagulación y digestión anaerobia (vinasse treatment by coupling of electrodissolution, heterocoagulation and anaerobic digestion). *DYNA* **81**: 102–107.

Pedrosa EMR, Rolim MM, Albuquerque PHS, Cunha AC. (2005). Supresividad de nematóides em cana-de-açúcar por adição de vinhaça ao solo (Supressivity of nematodes in sugarcane by adding vinasse to soil). *Revista Brasileira de Engenharia Agrícola e Ambiental* **9**: 197–201.

Romanholo-Ferreira LF, Aguiar MM, Messias TG, Pompeu GB, Lopez AM, Silva DP, Monteiro RT. (2011). Evaluation of sugar-cane vinasse treated with *Pleurotus sajor-caju* utilizing aquatic organisms as toxicological indicators. *Ectotoxicol Environ Saf* **74**: 132–137.

Rodrigues ES, Umbuzeiro GA. (2011). Integrating toxicity testing in the wastewater management of chemical storage terminals - a proposal based on a ten-year study. *J Hazard Mater* **186**: 1909–1915.

Srivastava S, Jain R. (2010). Effect of distillery spent wash on cytomorphological behaviour of sugarcane settlings. *J Environ Biol* **31**: 809–812.

Sydney EB, Larroche C, Novak AC, Nouaille R, Sarma SJ, Brar SK, Letti LA Jr, Soccol VT, Soccol CR. (2014). Economic process to produce biohydrogen and volatile fatty acids by a mixed culture using vinasse from sugarcane ethanol industry as nutrient source. *Bioresour Technol* **159**: 380–386.

Velásquez-Riaño M, Lombana-Sánchez N, Villa-Restrepo AF, Fernández-Calle EP. (2013). Cellulose production by *Gluconacetobacter kokkisztai* GMS in two batch process using vinasse as culture media. *Water Sci Technol* **68**: 1079–1084.