Interdiscip Toxicol. 2019; **Vol. 12**(3): 143–148.
doi: 10.2478/intox-2019-0017

sciendo

interdisciplinary toxicology

ORIGINAL ARTICLE

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

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ITX120319A05 • Received: 03 September 2018 • Accepted: 24 August 2019

ABSTRACT

In the bioethanol industry, per liter of the produced alcohol 9 to 14 liters of vinasse are obtained as a byproduct. If the vinasse is directly shed into bodies of water without an adequate treatment, it may have negative effects on the existing biota and human health due to its high turbidity and color, low pH and high content of organic material. 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 continuesly 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; Formagini *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

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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 (LC_{50} 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 (LC_{50} 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 (H_2O_2/Fe^{2+}) 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%), BOD_5 (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 LC_{50} 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 (EC_{50}) and the one obtained from a standard assay with *D. magna*.

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
BOD_5 (mg/l)	33,236
Total phosphates (as PO_4) (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 (P_2O_5) (g/l)	0.5
K (K_2O) (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

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 inmobilization test of *Daphnia magna*

The strain of *D. magna* used in this study was supplied by professor Maria Teresa Reguero 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 $K_2Cr_2O_7$, 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

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.

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. $KF=IC_{30}/IC_0$
2. $INH\%=100-IT_{30}/(KF\times IT_0)\times 100$

Where:

KF = Correction factor.
IC_{30} = Intensity of luminescence of the control after the time of contact (30 min) in the CPS.
IC_0 = Maximum CPS value of the control during the 5-second kinetic measurement.
IT_{30} = Intensity of luminescence of test sample after time of contact (30 min) in the CPS.
IT_0 = Maximum CPS value of the sample during the 5-second kinetic measurement.

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

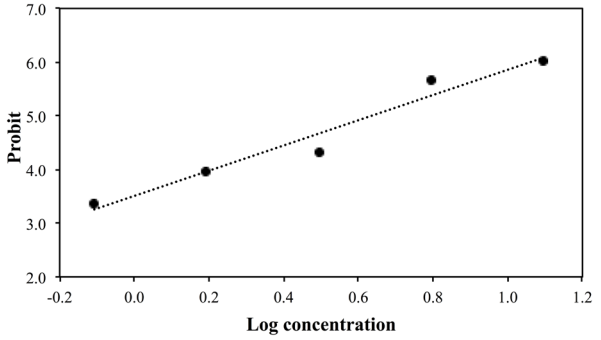

Figure. 1 Probits of lethality percentage values of *D. magna* plotted against Log10 concentrations of vinasse.

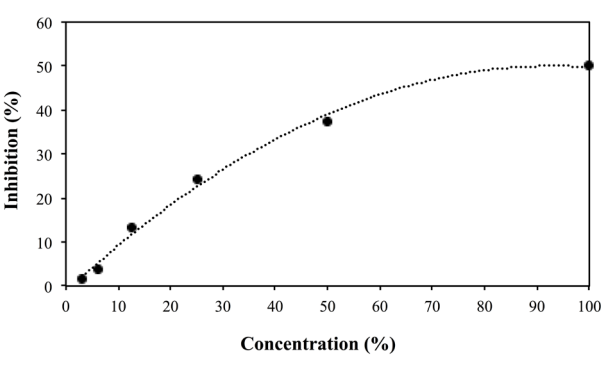

Figure. 2 Changes in *A. fischeri* light emission (INH%) as a function of toxicant concentration (%) for a contact time of 30 minutes.

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).

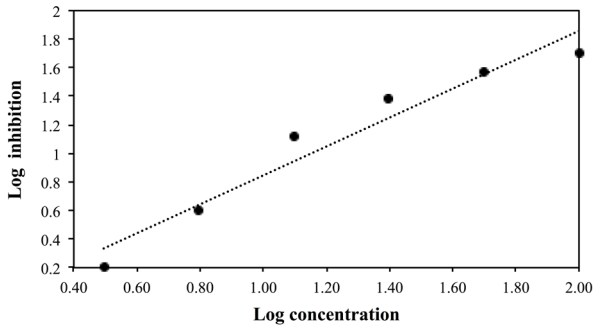

Figure. 3 Standard linear regression analysis of the linear comparison between the logarithm of the toxicant concentration and the logarithm of the intensity of the lost /remaining light in *A. fischeri*.

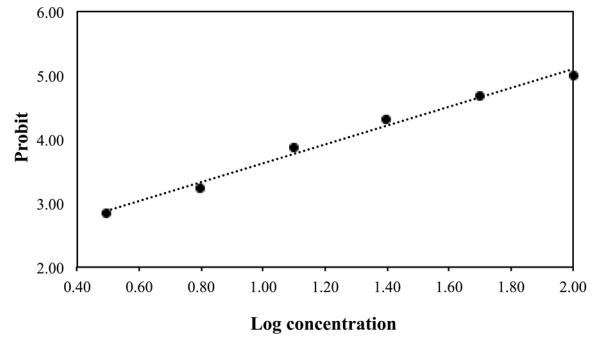

Figure. 4 Probits of inhibition percentage values of *A. fischeri* plotted against Log10 concentrations of vinasse..

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	Grossi-Botelho *et al.* (2012
C. dubia	0.7	
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

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 (*Channa 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

D. pulex, with a value of 6.9% (v/v) (Barba-Ho & García, 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 EC_{50} 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.

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