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Original Article

The Effects of Raw and Phytoremediated Ethanol Vinasse on the Survival and Swimming Behaviour of the Amphipod *Gammarus pulex*

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Abstract

Considering the promising use of ethanol as an alternative energy source, it is important to understand how by-products such as vinasse can affect the environment, especially when they are applied directly to the environment as a fertilizer. Although treatments have been proposed for this wastewater, the focus has mainly been on the physical and chemical characteristics of vinasse. Therefore, ecotoxicology studies are necessary to confirm the efficiency of these treatments. Several studies have highlighted the acute toxicity potential of vinasse however very little is known about chronic exposure and ecologically relevant endpoints such as behaviour. In order to evaluate the toxicity of raw and phytoremediated vinasse, we exposed individuals of *Gammarus pulex* to raw, 7-day, and 15-day phytoremediated vinasses for 96 hours, recording mortality and behavioural endpoints. The animals exposed to the different vinasses showed alterations in the swimming pattern. Also, the mortality was higher in animals exposed to raw vinasse. Mortality and effects on swimming speed were related to differences in Biological and Chemical Oxygen Demand (BOD and COD), as well as low pH and zinc concentration. Our results suggest that, even after treatment, the toxic potential of vinasse remains, affecting animals' fitness. In this way, despite the ecological benefits of ethanol energy, its by-products' discharge must be evaluated to minimize effects on the biota.

Keywords: Aquatic ecotoxicology; Freshwater crustacean; Macroinvertebrate mortality; Sublethal effects; Sugarcane by-product.

INTRODUCTION

Ethanol can be an alternative source of energy to reduce fossil fuel consumption. However, one-litre ethanol production results in up to fifteen litres of by-product known as vinasse, resulting, for example, in over 328 billion liters of vinasse in the 2022/23 harvest (Azevedo-Santos *et al*., 2024; Fuess & Garcia, 2014; Marcato *et al*., 2019). This massive amount creates challenges for storage and disposal. As a management strategy, and due to its nutrient-rich composition, vinasse has been applied as fertilizer in crops since the late 1980s (Azevedo-Santos *et al*., 2024; Christofoletti *et al*., 2013), when the discharge of vinasse, direct or indirect into water bodies was officially prohibited in Brazil.

However, vinasse can still pose environmental risks. By infiltrating into the groundwater or being transported with surface runoff, it can ultimately reach lakes and rivers, thereby impacting aquatic ecosystems. Vinasse is acidic (pH from 3.7 to 5.0) and contains high concentrations of organic matter, nitrogen, phosphorus, potassium, and occasionally toxic metals, which can

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significantly alter water composition. Also, its high biochemical and chemical oxygen demand often leads to severe oxygen depletion (Azevedo-Santos *et al*., 2024).

Therefore, the long-term disposal of vinasse can cause increased soil infiltration capacity, leaching, nutrients imbalance due to its low pH, contamination by metals, disruptions to aquatic food webs, loss of ecosystem services, and eutrophication of water bodies (Azevedo-Santos *et al*., 2024; Christofoletti *et al*., 2013; Fuess *et al*., 2017; Silva *et al*., 2007; Soto *et al*., 2017). Studies have demonstrated the toxicity of this ethanol by-product across multiple taxa including fish, crustaceans, amphibians, and plants (e.g.: Correia *et al*., 2017; Fraga *et al*., 2024; Freitas *et al*., 2022; Ogura *et al*., 2022; Pinto *et al*., 2021; Silva *et al*., 2021; Velásquez-Riaño *et al*., 2019), including effects on macroinvertebrate assemblage structure (Cotta *et al*., 2023).

The remediation of vinasse impacts has been studied; however, the conventional treatments require a high investment and are unable to treat the total organic load of wastewater (Botelho *et al*., 2012; Hoarau *et al*., 2018). Marcato *et al*. (2019) proposed the hybridization of three different treatments using stabilization, filtration, and phytoremediation. The authors allied reduced cost with a highly effective treatment, turning the phytoremediated vinasse into a better option to be used as a soil fertilizer. However, the toxicity of this remediated vinasse to aquatic organisms needs to be investigated, including population and ecological effects upon exposure to both raw and treated vinasse.

Behavioural endpoints have been considered as useful as biomarkers to assess chemical exposure and/or effect, and even more sensitive in some cases. Furthermore, its alterations seem to be the most relevant adaptation mechanisms to environmental changes, including contaminant exposure (Gerhardt *et al*., 2007; Lebrun *et al*., 2017; Nørum *et al*., 2010; Pinto *et al*., 2021; Vannuci-Silva *et al*., 2019; Vellinger *et al*., 2012). Pollutants that alter locomotory behaviour can change population maintenance, reducing organisms' fitness, interfering with foraging, mating success, and predator avoidance (Vannuci-Silva *et al*., 2019; Vellinger *et al*., 2012).

Gammarids amphipods are recognized as excellent models for behavioural analyses in ecotoxicology studies because of their sensitivity to a wide range of anthropogenic contaminants (Kohler *et al*., 2018; Vannuci-Silva *et al*., 2019; Vellinger *et al*., 2012). In freshwater ecosystems, the species *Gammarus pulex* is a reliable and sensitive model for understanding the impacts of pollutants, while also playing a key ecological role in nutrient cycling, and as a food source for secondary consumers (Chaumot *et al*., 2015). These characteristics, along with the ease of laboratory maintenance, have made this species a frequent model in aquatic ecotoxicology studies, including its use in monitoring programs in Europe (AFNOR, 2019).

Accordingly, the aim of this study was to compare the potential adverse effects of raw and phytoremediated vinasses on amphipods, and to investigate whether phytoremediated vinasse remains toxic to freshwater amphipods. To address these questions, the effects of raw and phytoremediated (7 and 15 days phytoremediated) vinasses on the mortality and swimming behaviour of the amphipod *Gammarus pulex* were evaluated.

MATERIALS AND METHODS

Gammarus pulex sampling

Amphipods of the species *G. pulex* were used as the test organism. Adult individuals were caught in a 1 mm mesh net using the kick sampling method outlined by the freshwater biological association (FBA, 2005) from the second-order stream River Ems, in Westbourne (N 50°51’34.8”, W 0°55’45.8”), UK, and transported to the Institute of Marine Science (IMS) of University of Portsmouth, UK. In the laboratory, the animals were counted and separated by size and gender. Adult males were selected and acclimated in river freshwater from the samples site in incubators at 6 °C under a 12 hrs light/12 hrs dark photoperiod for a minimum of 7 days. Constant aeration was provided via an air pump and air stone, and water was renewed on alternate days. Organisms were fed twice per week with leaves from the sample site (Kohler et al., 2018).

Vinasse

The sugarcane vinasse (from sugarcane juice and molasses) used in the treatments was collected directly from the effluent outlet, during the harvest period, in 500-liter containers at an adjacent sugarcane distillery located in the municipality of Bocaina, São Paulo State, Brazil. The effluent was treated immediately after collection. The vinasse underwent a hybrid treatment system composed of three steps: (1) Stabilization: the vinasse was stored in polyethylene tanks (dimension: 1.35 × 1.00 × 0.73 m; volume: 750 L) for 147 days to allow for natural biodegradation; (2) Filtration: after stabilization, the vinasse was filtered through a 20 µm microporous filter; and (3) Phytoremediation: water hyacinth (*Eichhornia crassipes*) was used for phytoremediation. The plants were placed in tanks containing the filtered vinasse, covering approximately 80% of the surface area.

The tanks were placed in a covered and ventilated area (Marcato *et al.*, 2019). The phytoremediation step was conducted for two different durations: 7 days (P7d) and 5 days (P15d), following the protocol described by Marcato *et al.* (2019). Untreated vinasse, referred to in this study as raw vinasse (R), was used as a positive control. The vinasses were stored in a cold room at 4 °C degrees until they were used in the experiment.

Physical and chemical analyses

Physical and chemical analyses were performed to identify the differences among treatments (Table 1). The Environmental Technology Field Station, University of Portsmouth, UK, was responsible for running these analyses following the USEPA methodology - Environmental Protection Authority (USEPA, 2005). The following parameters were analysed: BOD (EPA Method 405.1), COD (EPA Method 410.1), pH (EPA Method 150.1), total suspended solids (TSS) (EPA Method 160.2), electrical conductivity (EC) (EPA Method 120.1), nitrite (NO_2) (EPA Method 354.1), nitrate (NO_3) (EPA Method 353.2), turbidity (NTU) (EPA Method 180.1) and metals: Ag, Al, As, Cd, Co, Cu, Cr, Fe, Hg, Na, Ni, Pb and Zn (EPA Method 200.7 and 200.8).

Exposure assays

To verify the vinasse effects on amphipods behaviour, after 7 days-acclimation, males were exposed to two different dilutions (1 and 5%) of each type of vinasse and natural water from the sampling site, as a negative control, totalizing 7 treatments (Figure 1). The vinasse dilutions were based on previous studies (Correia *et al.*, 2017; Fraga *et al.*, 2024; Marinho *et al.*, 2014) and prepared using the same natural water in the negative control.

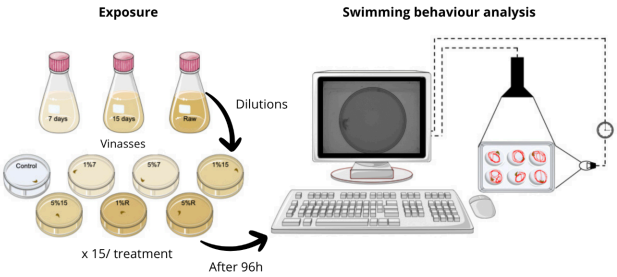

Figure 1. Exposure experimental setup and swimming behaviour analysis of *Gammarus pulex* exposed to different types of vinasses.

The animals were kept individually in plastic containers filled with 200 mL of natural river water or its dilutions, under a 12 hrs light/12 hrs dark photoperiod and at 6 °C for 96 hours, without aeration or 6 °C for 96 hours, without aeration and feeding. Plastic is commonly used in these assays because it is the least likely to interact with the test solution (e.g.: Fraga *et al.*, 2024). Fifteen individual replicates were used per treatment. The experiment was checked daily, and the animals were gently stimulated with a plastic spoon to verify mortality. Animals were considered dead when they did not react to stimulation. The behavioural analysis was conducted with the surviving animals (Kohler *et al.*, 2018).

Behavioural analysis

Behavioural assays were performed using DanionVision™ (Noldus Information Technology, Wageningen, The Netherlands) and its software EthoVision® XT. After the exposure, the surviving animals were randomly put on six-well plates filled with river water. After 1 minute, the amphipods were recorded for 12 minutes with 3 rounds of 2 minutes of lights off and 2 minutes of lights on (2000 lux), and their speed (cm.s-1) was measured (Kohler *et al.*, 2018). Statistical analyses were conducted on the speed of each amphipod.

Statistical analyses

Statistical analyses were performed (IBM SPSS® Statistics 24) to determine whether significant differences occurred over time and among exposures to dilutions of phytoremediated and raw vinasse. Highly deviant values resulting from tracking loss were identified as outliers (values > median ± 3 × interquartile range), excluded from the analysis (Kohler *et al.*, 2018, Vannuci-Silva *et al.*, 2019) and accounted for less than 3% of the total data. Mortality occurred only during the exposure period during the behavioural tracking phase, and dead animals were excluded from the analysis.

Linear Mixed-Effects (LME) statistical analysis was conducted for behaviour data using speed as the dependent variable, and time and dilutions as factors. The data were log-transformed prior to analysis to meet the required assumptions. Tukey's pairwise comparisons were used for post hoc analysis. The data were processed into 30-second time bins to analyse speed and swimming patterns precisely (Kohler *et al.*, 2018). The significance level applied in the hypothesis tests was $p < 0.05$.

RESULTS

Physical and chemical analyses

The addition of the three types of vinasses (raw, 7-day, and 15-day phytoremediated) to river water altered its parameters. The BOD and COD increased in all treatments, with the highest significant values in the raw vinasse dilutions (R), followed by the 7 days phytoremediated vinasse (P7d) and the 15 days phytoremediated vinasse (P15d). The pH decreased in both dilutions of all types of vinasses, being acid in the raw vinasse dilutions and slightly alkaline in both phytoremediated vinasse dilutions. All types of vinasse dilutions increased the Cr, Fe and Na and decreased Hg concentrations. The Cu increased in the 5% raw vinasse dilution. The Zn increased in 5% raw vinasse (5R) and, in 5% phytoremediated vinasse for 7 days (5P7d) dilution, where reached the higher value, and decreased in 5% phytoremediated vinasse for 15 days (5P15d), in 1% raw vinasse (1R), 1% phytoremediated vinasse for 7 days (1P7d) and, 1% phytoremediated vinasse for 15 days (1P15d), where they reached the lowest value. The Al increase was observed in all 5% dilutions. The detailed analyses are presented in Table 1.

Table 1. Physical and chemical parameters of water and vinasses dilutions.

	Control	1% phytoremediated for 7 days	5% phytoremediated for 7 days	1% phytoremediated for 15 days	5% phytoremediated for 15 days	1% raw	5% raw
BOD^1	2.2	9.4	31.2	4.8	13.5	**141.3**	**523.8**
COD^1	11	19	102	13	66	210	1103
EC^2	286	333	541	330	548	459	1089
pH	8.15	7.54	7.56	7.76	7.75	6.5	**4.55**
TSS^1	8.2	4.2	13.6	1.4	4.4	21.5	39.5
NTU	0.45	1.57	6.76	2.1	6.34	13.54	31.7
NO_3^3	**18**	**17**	**20**	**18**	**19**	**12**	**20**
NO_2^3	0.003	0.006	0.6	0.003	0.012	0.003	0.003
Cr^1	< 0.0004	< 0.0005	< 0.0006	< 0.0007	< 0.0008	< 0.0009	< 0.0004
Cd^1	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Cu^1	< 0.0020	< 0.0020	< 0.0020	< 0.0020	< 0.0020	< 0.0020	0.0031
Zn^1	0.0395	0.0173	0.1034	< 0.0004	0.0102	0.0141	0.0474
Pb^1	< 0.0011	< 0.0011	< 0.0011	< 0.0011	< 0.0011	< 0.0011	< 0.0011
Fe^1	< 0.0004	0.0167	0.0981	0.0187	0.1370	0.0630	**0.3641**
Al^1	0.0070	0.0045	0.0307	0.0053	0.0302	< 0.0035	0.0330
Ni^1	< 0.0008	< 0.0008	< 0.0008	< 0.0008	< 0.0008	< 0.0008	0.0012
Ag^1	< 0.0027	< 0.0027	< 0.0027	< 0.0027	< 0.0027	< 0.0027	< 0.0027
As^1	< 0.0010	< 0.0010	< 0.0010	< 0.0010	< 0.0010	< 0.0010	< 0.0010
Co^1	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002	< 0.0002
Hg^1	**0.0017**	**0.0016**	**0.0010**	**0.0012**	< 0.0004	**0.0009**	< 0.0004
Na^1	12.3	13	16.2	12.5	16.8	12.5	15.7

Note: Values in bold exceed the maximum limits established for the protection of aquatic communities by the Brazilian Ministry of the Environment (CONAMA Resolution No. 357/2005). BOD: Biochemical Oxygen Demand; COD: Chemical Oxygen Demand; EC: Electrical Conductivity; TSS: Total Suspended Solids; NTU: Turbidity. ^{1} mg.L^{-1}; ^{2} uS.cm^{-1}; ^{3} mg.L^{-1}-N.

Gammarids survival and behavioural responses

Mortality was observed in two types of vinasses. Animals exposed to 5P7d showed a mortality rate of 13%, while animals exposed to raw vinasse presented 53% mortality in 1R and 100% in 5R. The mortality rate, BOD, and COD increased with rising vinasse concentrations and from the phytoremediated to the raw treatment. Conversely, pH values decreased under these conditions (Figure 2).

For behaviour analysis, the control, 1P7d, 1P15d, and 5P15d treatments had 15 animals recorded each, while the 5P7d had 13 surviving animals for behaviour recording, and the 1R had 7 animals recorded. All the animals exposed to 5R5 died and therefore no data were generated. The LME analysis results are reported in Table 2. The animals swam significantly faster during the light phases compared to the dark phases ($p < 0.0001$) (Figure 3). Differences amongst the treatments were also observed ($p < 0.0001$). However, no significant interaction between treatments and time was found (p = 0.996). The pairwise comparisons revealed that the animals exposed to 5P7d (Figure 3B) increased (p = 0.004) their average swimming speed by ~16% when compared to the negative control group, the animals exposed to 1P15d (Figure 3C) decreased (p = 0.044) their average speed by ~13%, primarily during the light phases (Table 3). Animals exposed to vinasse dilutions with lower rates of BOD and COD decreased the swimming speed, while animals exposed to higher BOD and COD rates increased the speed. Whilst, when BOD and COD reached toxic and lethal levels, animals died.

DISCUSSION

It was observed that the raw vinasse was more lethal to exposed animals in both dilutions (Figure 1). The increase in mortality rate was followed by the increase in the BOD and COD rates and the decrease in the pH values, corroborating previous studies (Azevedo-Santos *et al.*, 2024; Botelho *et al.*, 2012; Fraga *et al.*, 2024; Freitas *et al.*, 2024). Indeed, vinasse toxicity is mainly attributed to its organic fraction (Christofoletti et al., 2013), resulting in a decrease in dissolved oxygen in the water (Von Sperling & de Lemos Chernicharo, 2015). Low dissolved oxygen may have contributed to the observed mortality, as reported in studies with other aquatic organisms (Azevedo-Santos *et al.*, 2024; Pinto *et al.*, 2021; Fraga *et al.*, 2024).

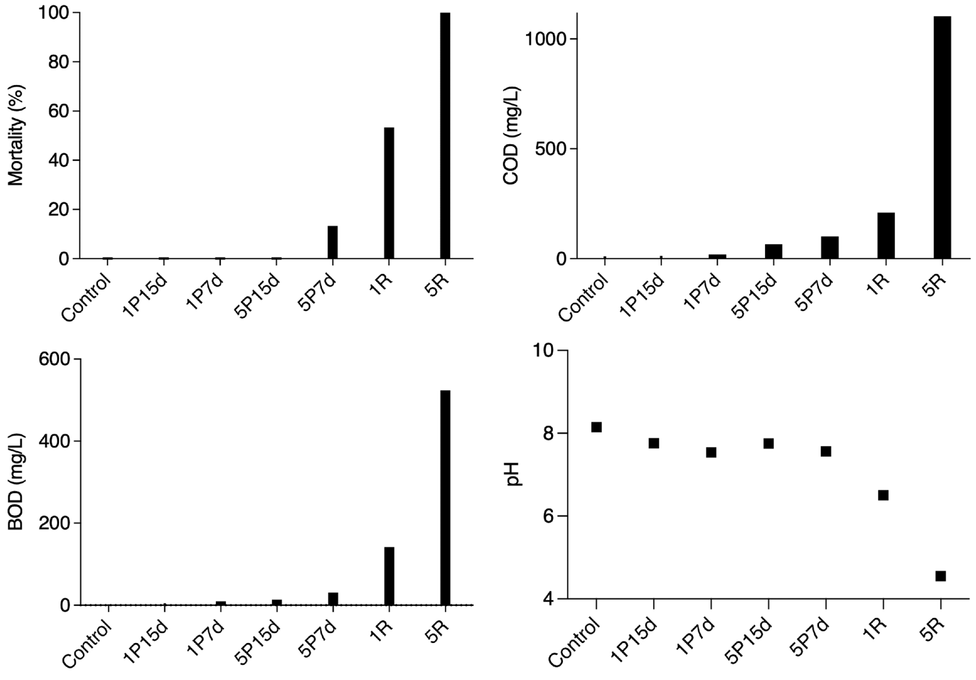

Figure 2. *Gammarus pulex* mortality rate, and exposure medium BOD (biochemical oxygen demand), COD (chemical oxygen demand) rates, and pH values.

Table 2. LME statistical analysis comparing the mean speed of *G. pulex* exposed to different types of vinasses between 30-second time bins.

	30-second time bins			
Source	N-df	D-df	F	Sig.
Intercept	1	14	696	<0.0001
Treatment	5	1767	13	<0.0001
Time	23	1762	25	<0.0001
Treatment * Time	115	1762	0.7	0.996

Note: Treatment: dilution of different types of vinasses on water. Time: represents 2-minute light/dark phases. Significance level p < 0.05.

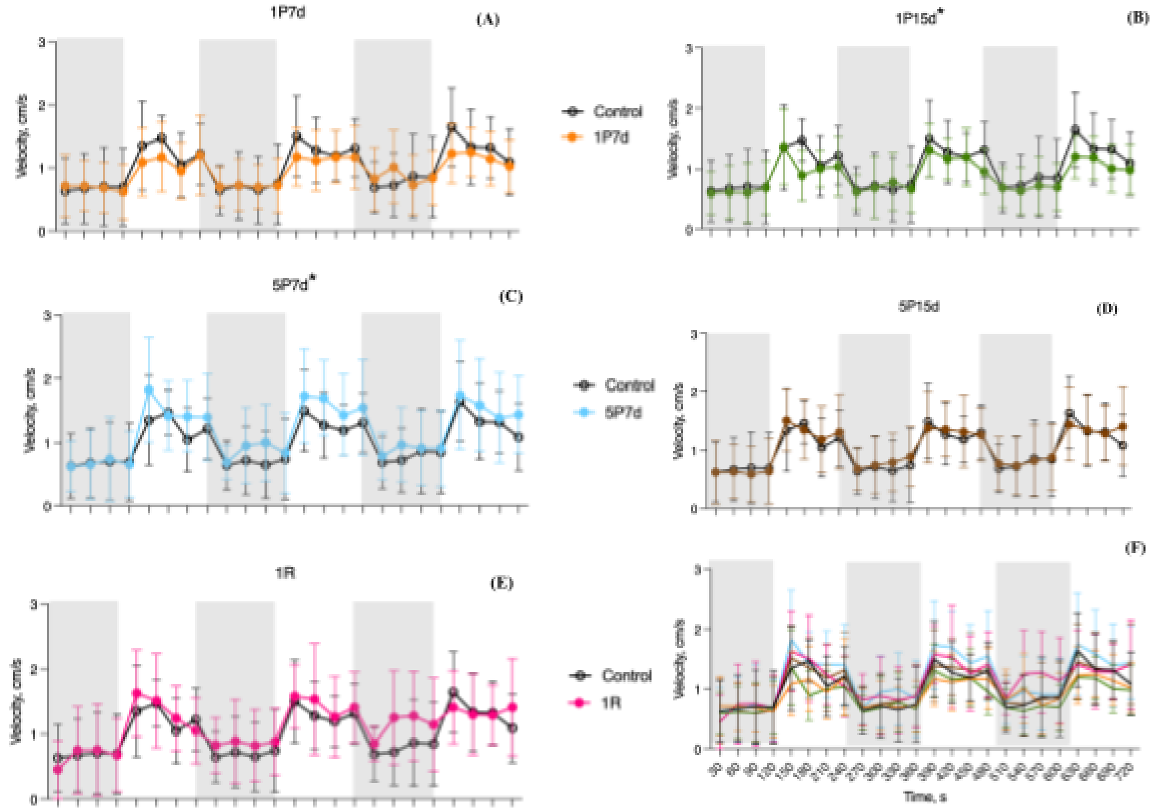

Figure 3. *Gammarus pulex* 30 s bin mean speed after exposure to different types of vinasses for 96 h: (A) 1P7d ; (B) 5P7d ; (C) 1P15d; (D) 5P15d; (E) 1R and (F) all treatments and control. Error bars represent SD. The grey background represents the dark phase, and the white background represents the light phase. (*) Significant difference from the control ($p < 0.05$).

Table 3. Results of Tukey's post hoc pairwise comparisons of the mean swimming speed of *Gammarus pulex* between the negative control and exposures to different types of vinasses.

		p
Control *vs.*	1% dilution of raw vinasse	0.276
	1% dilution of vinasse, 7 days-phytoremediated	0.595
	5% dilution of vinasse, 7 days-phytoremediated	0.004
	1% dilution of vinasse, 15 days-phytoremediated	0.044
	5% dilution of vinasse, 15 days-phytoremediated	0.982

Note: P values of <0.05 were considered significant.

In this study, nitrate and Hg concentrations exceeded the limits of 10 mg.L^{-1} and 0.0002 mg.L^{-1}, respectively, established by the Brazilian Ministry of the Environment (CONAMA Resolution No. 357/2005) to protect aquatic communities, in all treatments (Table 1). The high nitrate levels may be associated with the increase in BOD, since nitrogen released during the breakdown of organic matter can be converted into nitrates by aerobic bacteria (Marcato *et al*., 2019). Indeed, for the raw vinasse dilution treatments, BOD values were above the threshold of 3 mg.L^{-1}, as were the pH, which should be between 6 and 9, and Fe concentrations, a threshold of 0.3 mg.L-1 in the 5R (Table 1). Whereas, Hg was detected similarly in both the control and the samples, suggesting that its concentration originates from the river water used in the assays and is not exerting toxicity at these levels, since no effects were observed in the control. Additionally, the total phosphorus concentrations reported by Marcato *et al.* (2019) for similar samples also exceeded the CONAMA 357/2005 limits (0.02 mg.L^{-1}), with values of 32.7, 17.7, and 13.9 mg.L^{-1} for R and P7d and P15d, respectively.

Despite the scarcity of tests evaluating chronic effects and phytoremediated ethanol by-products, the acute toxicity of vinasse is widely reported for various taxa (e.g, Correia *et al*., 2017; Fraga *et al*., 2024; Freitas *et al*., 2022; Ogura *et al*., 2022; Silva *et al*., 2021; Velásquez-Riaño *et al.*, 2019). Pinto *et al.* (2021) found 100% of mortality in the Brazilian native epibenthic amphipod *Hyalella meinerti* exposed to raw vinasse, 1,3%

(v/v) *in situ* for 96h. Whereas Fraga *et al.* (2024) evaluated acute toxicity (96 h) of raw vinasse at concentrations ranging from 0.6 to 1.4% and toat concentrations ranging from 0.6 to 1.4% in the same species, and reported an Effective Concentration 50 (EC50) of 0.78% (confidence interval from 0.64 to 0.93%). Our results on raw vinasse, where all animals died when exposed to 5% dilution, and 7 of 15 survived to a 1% dilution of raw vinasse, suggest that both amphipod species (*G. pulex* and *H. meinerti*) exhibit comparable sensitivity to raw vinasse.

Significant differences in swimming speed were also found among treatments (Figure 3; Tables 2 and 3). Locomotion is the primary behaviour underlying many complex behaviours due to its relationship with physiological, metabolic, and neurological processes and their anatomical conditions. In this way, locomotion is important for both inter- and intraspecific interactions among the animals (Amiard-Triquet, 2009). Swimming speed is one of the most used behavioural parameters to assess the physiological state of aquatic organisms and has been widely used to assess the effects of pollutants (e.g., Kohler *et al.*, 2018; Schuijt *et al.*, 2023; Vannuci-Silva *et al.*, 2019).

In a similar study on *H. meinerti*, vinasse exposure significantly impaired the swimming behaviour, reducing both speed and distance traveled. These impairments were likely caused by physiological and neurotoxic stress from metals (e.g., Cu, Fe, Ni, and Zn) and organic pollutants present in the vinasse (Pinto *et al.*, 2021). *G. pulex* exposed to a mixture of trace metals or several organic xenobiotics, decreased 20% in locomotory activity (Gerhardt, 2007). Although metal concentrations were low in our samples, the presence of different vinasses in both dilutions altered some parameters, increasing Fe and Na in all dilutions, decreasing Hg in all dilutions, and increasing or decreasing Cr, Cu, Zn, Al, and Ni in some dilutions. Therefore, the presence of metals, even at low concentrations, could be one of the reasons for the changes observed in swimming behaviour, once they are toxic to gammarids (Lebrun & Gismondi, 2020). Indeed, the presence of metals, such as Cr, Cu, Pb, and Cd, was among the hypotheses proposed as potential causes of the acute toxicity observed in H. meinerti (Fraga et al., 2024; Pinto et al., 2021).

The acid pH increases the metal solubility in the aqueous medium by the dissociation of these metals, and the alkaline pH decreases the solubility, which increases metal aggregation and precipitation. Therefore, toxicity is also influenced by pH due to its capacity to alter metal availability (Botelho *et al.*, 2012; de Paiva Magalhães *et al.*, 2015). In fact, the acidity of raw vinasse was pointed out as one of the main factors contributing to the observed toxicity to *H. meinerti* as well as observed for other crustaceans (Botelho *et al.*, 2012; Freitas *et al.*, 2024; Pinto *et al.*, 2021; Silva *et al.*, 2021). The presence of metals that, under acidic conditions, become more bioavailable, are also reported as a cause of the vinasse toxicity (Fraga *et al.*, 2024; Pinto *et al.*, 2021). In our study, the pH decreased with the vinasse additions when compared to the control. The pH at all phytoremediated vinasses dilutions were slightly alkaline, between 7.54 and 7.76, and at both raw vinasse dilutions the pH was acidic, 6.5 in 1% dilution and 4.55 in 5% dilution. Despite the acid pH promoting an increase in the availability of some metals, it was observed that Zn and Ni are more available in slightly alkaline pH (7.3 - 8.3). Lebrun *et al.* (2017) observed that gammarids exposed to Zn showed stimulated movement behaviour, suggesting either an influence of Zn toxicity on ionic regulation in the gill structure or a survival strategy to avoid contaminated habitats. Consistent with it, the group exposed to 5P7d had the major value of Zn observed, also in this group, it was observed an increase in swim speed. These findings suggest that exposure to a 5P7d increased the speed by 16% stimulated by the zinc. The opposite was observed in the group exposed to 1P15d, which had the lowest zinc value and decreased 13% the swimming speed.

The increased speed is a well-known animal escape response (Bossus *et al.*, 2014; Kohler *et al.*, 2018). Alterations in swimming speed may compromise essential ecological functions including foraging, mate searching, migration, and predator avoidance, ultimately affecting reproduction and survival (Morillo-Velarde *et al.*, 2011; Nørum *et al.*, 2010; Pinto *et al.*, 2021). Thus, both the increase and the decrease in the swimming speed of animals exposed to different dilutions of vinasse are harmful, as they can interfere in the adequacy of the population and, consequently, in the level of the community and the ecosystem, reinforcing the ecological relevance of these behavioural endpoints.

CONCLUSION

The mortality rate in *G. pulex* was associated to the high levels of BOD, COD and Zn in the raw vinasse, which were lower in the treated ones. Nonetheless, phytoremediated vinasse still exhibited sublethal toxicity, altering amphipod swimming behaviour and potentially compromising their fitness, with consequences at population and community levels.

This study highlights the importance of incorporating behavioural endpoints into ecotoxicological assessments, as they provide sensitive and early indicators of sublethal effects in aquatic organisms. In this context, vinasse disposal as fertilizer must be carefully evaluated, since it has not been shown to be entirely safe for aquatic life, even after phytoremediation. Our findings reinforce the need to integrate bioassays with physicochemical evaluations to more comprehensively assess the environmental safety of industrial effluents.

Future research should prioritize chronic and multi-species assessments to better understand long-term ecological risks and potential impacts on trophic interactions, as well as explore complementary endpoints and behavioural metrics. Developing summary or integrative behavioural indicators, capable of revealing multivariate associations with chemical variables, will be essential to overcome current limitations and strengthen the ecological relevance of future studies.

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AUTHOR CONTRIBUTIONS

AM: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing; **MV:** Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing; **SK:** Formal analysis, Methodology, Validation, Writing – review & editing; **CF:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing; **AF:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

AM and **MV** contributed equally to this work and share first authorship.

REFERENCES

AFNOR. (2019). Qualité de l'eau - Encagement in situ de gammares pour la mesure de la bioaccumulation de substances chimiques. XP T90-721. https://www.boutique.afnor.org/fr-fr/norme/xp-t90721/qualite-de-leau-encagement-in-situ-de-gammares-p our-la-mesure-de-la-bioaccu/fa193552/82532

Amiard-Triquet, C. (2009). Behavioral Disturbances: The Missing Link between Sub-Organismal and Supra-Organismal Responses to Stress? Prospects Based on Aquatic Research. Human and Ecological Risk Assessment: An International Journal, 15(1), 87–110. https://doi.org/10.1080/10807030802615543

Azevedo-Santos, V. M., Fernandes, J. A., de Souza Andrade, G., de Moraes, P. M., Magurran, A. E., Pelicice, F. M., & Giarrizzo, T. (2024). An overview of vinasse pollution in aquatic ecosystems in Brazil. Environmental Management, 74(6), 1037–1044. https://doi.org/10.1007/s00267-024-01999-x

Bossus, M. C., Guler, Y. Z., Short, S. J., Morrison, E. R., & Ford, A. T. (2014). Behavioural and transcriptional changes in the amphipod Echinogammarus marinus exposed to two antidepressants, fluoxetine and sertraline. Aquatic Toxicology, 151(April 2016), 46–56. https://doi.org/10.1016/j.aquatox.2013.11.025

Botelho, R. G., Tornisielo, V. L., de Olinda, R. A., Maranho, L. A., & Machado-Neto, L. (2012). Acute toxicity of sugarcane vinasse to aquatic organisms before and after pH adjustment. Toxicological & Environmental Chemistry, 94(10), 2035–2045. https://doi.org/10.1080/02772248.2012.738516

Chaumot, A., Geffard, O., Armengaud, J., & Maltby, L. (2015). Gammarids as Reference Species for Freshwater Monitoring. In Aquatic Ecotoxicology (pp. 253–280). Elsevier. https://doi.org/10.1016/B978-0-12-800949-9.00011-5

Christofoletti, C. A., Escher, J. P., Correia, J. E., Marinho,

J. F. U., & Fontanetti, C. S. (2013). Sugarcane vinasse: Environmental implications of its use. Waste Management, 33(12), 2752–2761. https://doi.org/10.1016/j.wasman.2013.09.005

CONAMA, Conselho Nacional do Meio Ambiente, Brasil. (2005). Resolução nº 357, de 17 de março de 2005. Dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes, e dá outras providências.

Correia, J. E., Christofoletti, C. A., Marcato, A. C. C., Marinho, J. F. U., & Fontanetti, C. S. (2017). Histopathological analysis of tilapia gills (Oreochromis niloticus Linnaeus, 1758) exposed to sugarcane vinasse. Ecotoxicology and Environmental Safety, 135, 319–326. https://doi.org/10.1016/j.ecoenv.2016.10.004

Cotta, C. P., Pinto, T. J. S., Yoshii, M. P. C., Silva, L. C. M., Ogura, A. P., Gabriel, G. V. M., Schiesari, L. C., Carmo, J. B., Montagner, C. C., Espíndola, E. L. G., & Moreira, R. A. (2023). Exposure to fipronil, 2,4-D and vinasse influences macroinvertebrate assemblage structure: An experimental mesocosm approach. Science of the Total Environment, 888 (November 2022). https://doi.org/10.1016/j.scitotenv.2023.16425.

de Paiva Magalhães, D., da Costa Marques, M. R., Baptista, D. F., & Buss, D. F. (2015). Metal bioavailability and toxicity in freshwaters. Environmental Chemistry Letters, 13(1), 69–87. https://doi.org/10.1007/s10311-015-0491-9

FBA, F. B. A. (2005). Collecting freshwater macroinvertebrate samples. https://www.fba.org.uk/sites/default/files/CourseInvertSamplingProtocol.pdf.

Fraga, P. D., de Mello Gabriel, G. V., do Carmo, J. B., Espindola, E. L. G., & Pinto, T. J. da S. (2024). Sugarcane vinasse provokes acute and chronic responses and bioaccumulation of metals in benthic macroinvertebrates. Environmental Science and Pollution Research International, 31(3), 4067–4079. https://doi.org/10.1007/s11356-023-31446-z

Freitas, I. B. F., Duarte-Neto, P. J., Sorigotto, L. R., Cardoso Yoshii, M. P., de Palma Lopes, L. F., de Almeida Pereira, M. M., Girotto, L., Badolato Athayde, D., Veloso Goulart, B., Montagner, C. C., Schiesari, L. C., Martinelli, L. A., & Gaeta Espíndola, E. L. (2024). Effects of pasture intensification and sugarcane cultivation on non-target species: A realistic evaluation in pesticide-contaminated mesocosms. Science of the Total Environment, 922(March). https://doi.org/10.1016/j.scitotenv.2024.171425

Freitas, J. S., da Silva Pinto, T. J., Cardoso Yoshii, M. P., Conceição Menezes da Silva, L., de Palma Lopes, L. F., Pretti Ogura, A., Girotto, L., Montagner, C. C., de Oliveira Gonçalves Alho, L., Castelhano Gebara, R., Schiesari, L., & Gaeta Espíndola, E. L. (2022). Realistic exposure to fipronil, 2,4-D, vinasse and their mixtures impair larval amphibian physiology. Environmental Pollution, 299(September 2021). https://doi.org/10.1016/j.envpol.2022.118894

Fuess, L. T., Rodrigues, I. J., & Garcia, M. L. (2017). Fertirrigation with sugarcane vinasse: Foreseeing potential impacts on soil and water resources through vinasse characterization. Journal of Environmental Science and Health, Part A, 52(11), 1063–1072. https://doi.org/10.1080/10934529.2017.1338892

Fuess, L. T., & Garcia, M. L. (2014). Implications of stillage land disposal: A critical review on the impacts of fertigation. Journal of Environmental Management, 145, 210–229. https://doi.org/10.1016/j.jenvman.2014.07.003

Gerhardt, A. (2007). Aquatic Behavioral Ecotoxicology—Prospects and Limitations. Human and Ecological Risk Assessment: An International Journal, 13(3), 481–491. https://doi.org/10.1080/10807030701340839

Hoarau, J., Caro, Y., Grondin, I., & Petit, T. (2018). Sugarcane vinasse processing: Toward a status shift from waste to valuable resource. A review. Journal of Water Process Engineering, 24, 11–25. https://doi.org/10.1016/j.jwpe.2018.05.003

Kohler, S. A., Parker, M. O., & Ford, A. T. (2018). Species-specific behaviours in amphipods highlight the need for understanding baseline behaviours in ecotoxicology. Aquatic Toxicology, 202, 173–180. https://doi.org/10.1016/j.aquatox.2018.07.013

Lebrun, J.D., & Gismondi, E. (2020). Behavioural and biochemical alterations in gammarids as induced by chronic metallic exposures (Cd, Cu and Pb): Implications for freshwater biomonitoring. Chemosphere, 257, 127253. https://doi.org/10.1016/j.chemosphere.2020.127253

Lebrun, J. D., Uher, E., & Fechner, L. C. (2017). Behavioural and biochemical responses to metals tested alone or in mixture (Cd-Cu-Ni-Pb-Zn) in Gammarus fossarum: From a multi-biomarker approach to modelling metal mixture toxicity. Aquatic Toxicology, 193(November), 160–167. https://doi.org/10.1016/j.aquatox.2017.10.018

Marcato, A. C. de C., Souza, C. P. de, Paiva, A. B. de, Eismann, C. E., Navarro, F. F., Camargo, A. F. M., Menegário, A. A., & Fontanetti, C. S. (2019). Hybrid treatment system for remediation of sugarcane vinasse. Science of The Total Environment, 659, 115–121.https://doi.org/10.1016/j.scitotenv.2018.12.252

Marinho, J. F. U., Correia, J. E., Marcato, A. C. de C., Pedro-Escher, J., & Fontanetti, C. S. (2014). Sugar cane vinasse in water bodies: Impact assessed by liver histopathology in tilapia. Ecotoxicology and Environmental Safety, 110, 239–245. https://doi.org/10.1016/j.ecoenv.2014.09.010

Morillo-Velarde, P. S., Lloret, J., Marín, A., & Sánchez-Vázquez, F. J. (2011). Effects of Cadmium on Locomotor Activity Rhythms of the Amphipod Gammarus aequicauda. Archives of Environmental Contamination and Toxicology, 60(3), 444–451. https://doi.org/10.1007/s00244-010-9563-9

Nørum, U., Friberg, N., Jensen, M. R., Pedersen, J. M., & Bjerregaard, P. (2010). Behavioural changes in three species of freshwater macroinvertebrates exposed to the pyrethroid lambda-cyhalothrin: Laboratory and stream microcosm studies. Aquatic Toxicology, 98(4), 328–335. https://doi.org/10.1016/j.aquatox.2010.03.004

Ogura, A. P., Moreira, R. A., da Silva, L. C. M., Negro, G. S., Freitas, J. S., da Silva Pinto, T. J., Lopes, L. F. de P., Yoshii, M. P. C., Goulart, B. V., Montagner, C. C., & Espíndola, E. L. G. (2022). Irrigation with Water Contaminated by Sugarcane Pesticides and Vinasse Can Inhibit Seed Germination and Crops Initial Growth. Archives of Environmental Contamination and Toxicology, 82(3), 330–340. https://doi.org/10.1007/s00244-022-00914-x

Pinto, T. J. da S., Freitas, J. S., Moreira, R. A., Silva, L. C. M. da, Yoshii, M. P. C., Lopes, L. F. de P., Goulart, B. V., Vanderlei, M. R., Athayde, D. B., Fraga, P. D., Ogura, A. P., Schiesari, L., Montagner, C. C., Daam, M. A., & Espindola, E. L. G. (2021). Functional responses of *Hyalella meinerti* after exposure to environmentally realistic concentrations of 2,4-D, fipronil, and vinasse (individually and in mixture). Aquatic Toxicology, 231 (August 2020). https://doi.org/10.1016/j.aquatox.2020.105712

Schuijt, L. M., Olusoiji, O., Dubey, A., Rodríguez-Sánchez, P., Osman, R., Van den Brink, P. J., & van den Berg, S. J. P. (2023). Effects of the antidepressant fluoxetine on the swimming behaviour of the amphipod Gammarus pulex: Comparison of short-term and long-term toxicity in the laboratory and the semi-field. Science of the Total Environment, 872(January). https://doi.org/10.1016/j.scitotenv.2023.162173

Silva, L. C. M., Moreira, R. A., Pinto, T. J. S., Vanderlei, M. R., Athayde, D. B., Lopes, L. F. P., Ogura, A. P., Yoshii, M. P. C., Freitas, J. S., Montagner, C. C., Goulart, B. V., Schiesari, L., Daam, M. A., & Espíndola, E. L. G. (2021). Lethal and sublethal toxicity of pesticides and vinasse used in sugarcane cultivation to Ceriodaphnia silvestrii (Crustacea: Cladocera). Aquatic Toxicology, 241(June), 106017. https://doi.org/10.1016/j.aquatox.2021.106017

Silva, M. A. S. da, Griebeler, N. P., & Borges, L. C. (2007). Uso de vinhaça e impactos nas propriedades do solo e lençol freático. Revista Brasileira de Engenharia Agrícola e Ambiental, 11(1), 108–114. https://doi.org/10.1590/S1415-43662007000100014

Soto, M., Basso, J., & Kiang, C. (2017). Impacto da fertirrigação da cana-de-açúcar por vinhaça nas propriedades físicas, químicas e hidráulicas do solo. In Cana- de-açúcar e seus impactos: uma visão acadêmica (pp. 103–124). Canal 6.

USEPA, United States Environmental Protection Agency. (2005). National Recommended Water Quality Criteria. http://www.epa.gov/waterscience/criteria/wqctable/

Vannuci-Silva, M., Kohler, S., Umbuzeiro, G. D. A., & Ford, A. T. (2019). Behavioural effects on marine amphipods exposed to silver ions and silver nanoparticles. Environmental Pollution, 252. https://doi.org/10.1016/j.envpol.2019.06.005

Velásquez-Riaño, M., Meneses-Sánchez, J. S., & Arias, C. E. C. (2019). Evaluation of acute toxicity of vinasse by means of Daphnia magna and Aliivibrio fischeri : a comparative study. Interdisciplinary Toxicology, 12(3), 143–148. https://doi.org/10.2478/intox-2019-0017

Vellinger, C., Felten, V., Sornom, P., Rousselle, P., Beisel, J. N., & Usseglio-Polatera, P. (2012). Behavioural and physiological responses of Gammarus pulex exposed to cadmium and arsenate at three temperatures: Individual and combined effects. PLoS ONE, 7(6). https://doi.org/10.1371/journal.pone.0039153

Von Sperling, M., & de Lemos Chernicharo, C. (2015). Biological Wastewater Treatment in Warm Climate Regions Volume I. Water Intelligence Online, 4, 9781780402734–9781780402734. https://doi.org/10.2166/9781780402734