Comparison between *Aliivibrio fischeri* and activated sludge microorganisms in the evaluation of the toxic pollutants of leachates from Brazilian landfills

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Research Article

Keywords: Landfill leachate, Acute toxicity, Bioluminescence inhibition, Respirometry assay, Ammonia removal, Humic substances, Principal component analysis (PCA)

Posted Date: June 21st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-160117/v1>

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Version of Record: A version of this preprint was published at Environmental Science and Pollution Research on August 5th, 2021. See the published version at <https://doi.org/10.1007/s11356-021-15771-9>.

Abstract

Ecotoxicological assessment of landfill leachate has become a priority to determine its impacts on the ecosystem. Toxicity assays with microorganisms stand out due to their quick response, low cost and ease of testing. In this context, the present study evaluated the acute toxic effects of leachates from two landfills of different ages and modes of operation to bacterium *A. fischeri* and activated sludge microorganisms and the ammonia nitrogen and humic substances (HS) sensitivity to these organisms. Reductions greater than 30% in leachate toxicity were observed after ammonia removal for *A. fischeri* and activated sludge microorganisms. After 97% removal of HS, the greater reductions in toxicity (44.28 to 79.82%) were verified for microbial species studied, indicating that the organic compounds (measured as chemical oxygen demand, total organic carbon and humic substances) were the primary pollutants responsible for the toxicity of the leachates. Concerning the organisms studied, *A. fischeri* showed greater sensitivity to the leachates' pollutants compared to the activated sludge microorganisms. Nevertheless, a strong correlation was observed between *A. fischeri* and activated sludge microorganisms' toxicity responses, suggesting that respirometry assay can be used to determine leachate toxicity.

Introduction

Globally, municipal solid waste management (MSW) has become a challenge in the environmental, economic and social spheres. In Brazil, the sanitary landfill is the main form of final disposal of MSW. However, despite the advance of sustainable initiatives that occurred in the last decades, about 40% of MSW collected in the country are still incorrectly disposed of in controlled landfills and open dumps, favoring the emission of toxic pollutants and the contamination of soil and water (Alfaia et al. 2017; ABRELPE 2020).

Leachate is considered as one of the most significant threats to the environment, originating in landfills (Kjeldsen et al. 2002; Matejczyk et al. 2011). Due to its variable and heterogeneous composition with high pollutants concentrations, landfill leachate is considered a highly toxic effluent (Christensen et al. 2001; Naveen et al. 2017).

The toxicity of leachate is a consequence of its numerous contaminants, additive, synergistic and antagonistic effects and physicochemical properties (Martinen et al., 2002; Prestes et al., 2020). Hence, the need to determine each pollutant's contribution to leachate toxicity has become one of the main measures to understand the aquatic ecosystem's contamination risks.

The toxicity of landfill leachate, in general, is related to four pollutants (ammonia nitrogen, alkalinity, organic matter and salinity) (Clément and Merlin 1995; Pablos et al. 2011, Miao et al. 2019). Ammonia nitrogen has been identified as the most toxic component present in leachate, being found in high concentrations for an extended period in landfills that have already closed their operations (Kurniawan et al., 2006).

Traditionally, landfill leachate quality assessment is based on the identification of pollutants using physicochemical analyses. However, these analyses individually cannot determine the complex interaction between the contaminants and their biological effects. Therefore, the use of ecotoxicity assays has become essential to complement physicochemical analyses to directly assess the impact of the leachate on living organisms (Matejczyk et al., 2011; Ghosh et al., 2017).

As an alternative to traditional ecotoxicity assays with invertebrates and fish, bacterial assays are indicated as a screening and biomonitoring tool in an environmental microscale (Johnson 2005; Norberg-King et al. 2018). Microbial assays stand out due to reproducibility, quick response, low cost, easy operation, use of small sample volume, in addition to not needing the approval of ethics committee for the use of animals in scientific research (Thomas et al. 2009; Polo et al. 2011; Hassan et al. 2016).

Bioluminescent inhibition assays using marine bacterium *Aliivibrio fischeri* are widely used to evaluate different classes of pollutants, such as pesticides, heavy metals, nanoparticles, phenols and derivatives, industrial effluents, river water, municipal wastewater, landfill leachate, among others (Abbas et al. 2018). For example, in the evaluation of leachate toxicity, *A. fischeri* presented high sensitivity to the presence of organic compounds (Clément et al. 1997; Ward et al. 2002; Pivato and Gaspari 2006; Ghosh et al. 2017) and showed the best response when compared to other assays using bacteria (Dalzell et al. 2002; Parvez et al. 2006; Abbas et al. 2018).

Respirometry or inhibition of oxygen consumption is an assay that assesses the effects of contaminants on the microbial consortium of activated sludge (composed mainly of heterotrophic and nitrifying bacteria, but also protozoa, fungi and rotifers) (Riendel et al. 2002; Oviedo et al. 2009; Aguilar et al. 2020). Although the respirometry assay has been traditionally applied in the monitoring of biological treatment systems, this test is also pointed out in the literature, as well as *A. fischeri* an important tool in determining the toxicity of landfill effluents (Cotman and Gotvajn 2010; Kalčíková et al. 2011; Corsino et al. 2020).

The objective of this work was to evaluate physicochemical characteristics and the toxic potential of leachates from two landfills of different ages and modes of operation and correlate the sensitivity of marine bacterium *A. fischeri* and activated sludge microorganisms to ammonia nitrogen and humic substances pollutants present in these leachates.

Material And Methods

Landfills leachates

The leachates used in this study were collected at Seropédica Landfill (active landfill with nine years of operation) and Gericinó Landfill (landfill operated for 27 years and currently closed) in Rio de Janeiro state, Brazil.

The Seropédica Sanitary Landfill has been in operation since 2011 and received an average of 10,000 tonnes per day of municipal solid waste from Seropédica, Itaguaí and Rio de Janeiro cities. The leachate production in this landfill is, on average, 1,000 m³/day (Ciclus Ambiental 2018; Almeida et al. 2020).

The Gericinó Controlled Landfill is located in Rio de Janeiro city that started operation in 1987 as an open dumpsite, but after being subjected to modifications, operated as a controlled landfill until its closure in 2014. The landfill received on average 2,000 tonnes per day of municipal solid waste, generating about 500 m³/day of leachate (Lima et al., 2017b).

In each landfill, leachate samples (n= 4) were collected, in a simple sampling, at the accumulation point, in different seasons from 2017 to 2019. The samples were maintained refrigerated (4 °C) for later physicochemical characterization (performed the day after collections) and preserved (-20 °C) for ecotoxicity assays (performed within a maximum period of 60 days after sample collection).

Evaluation of physicochemical parameters

The parameters pH, alkalinity, conductivity, chemical oxygen demand (COD), total organic carbon (TOC), total solids, total dissolved solids, total suspended solids, chloride, total ammonia nitrogen (TAN), turbidity, humic substances (HS) and absorbance at 254 nm, the latter related to the presence of aromatic organic compounds, such as humic substances (Zielińska et al., 2020) were evaluated for the physicochemical characterization.

The analyses were performed according to the methodology described in Standard Methods for the Examination of Water and Wastewater (APHA 2012), except for the humic substances parameter that was determined spectrophotometric/colorimetric method described by Lima et al. (2017a). All the tests were performed in triplicates, considering the standard deviation of the replicates.

Evaluation of leachates ecotoxicity

Aliivibrio fischeri (bioluminescence inhibition assay)

The marine bacterium *Vibrio fischeri*, currently designated as *Aliivibrio fischeri* (strain NRRL B-11177), was used following the Brazilian Standard NBR 15411-3 (ABNT 2012) based on the international standard ISO 11348-3:2007, using the Microtox equipment (Modern Water and Model 500 Analyser). The bacterial suspensions used in the study were commercially obtained in lyophilized form (Umwelt Biotechnologia Ambiental, Blumenau, Brazil) and stored at a temperature between -18°C and -20 °C.

For this assay, the pH of the samples was adjusted to the range of 6.0-8.5 with HCl (1.0 mol/L) and NaOH (1.0 mol/L). Before analysis, the salinity adjustment of the samples was carried out with the osmotic solution (20% NaCl). The assay was performed at 15°C.

Reference assays with Zn²⁺ (ZnSO₄.7H₂O) and Cu²⁺ (CuSO₄.5H₂O) were used to check the sensitivity of each vial of bacteria. In addition, an additional control using 3,5-dichlorophenol and potassium

dichromate were periodically evaluated for each bacterial batch used during the study, according to the methodology proposed by ABNT (2012).

Assay responses were expressed as reducing bioluminescence in EC50 (median effect concentration) after 30 minutes of exposure of the organism to the samples. All bioluminescence inhibition tests were performed in triplicate, considering the standard deviation of the replicates.

Activated sludge microorganisms (Respirometry assay)

Respirometry assay was carried out based on the Organisation for Economic Co-operation and Development methodology, method number 209 (OECD, 2010). Initially, the preparation of concentrated synthetic sewage was realized for feed for sludge during the test, as shown in Table 1.

Table 1 Composition of concentrated synthetic sewage (OECD, 2010)

Components	Concentration (g/L)
Peptone	16.0
Meat extract	11.0
Urea	3.0
NaCl	0.7
CaCl ₂ . 2H ₂ O	0.4
MgSO ₄ . 7H ₂ O	0.2
K ₂ HPO ₄	2.8

The test was conducted under constant aeration, with the following composition: sample, biological sludge (concentration of 1.5 g/ L of suspended solids), and synthetic sewage (pH = 7.5 ± 0.5) in a beaker using a stirring plate and an air compressor connected to a silicone hose and a porous stone, for 180 min at a temperature of 20 ± 2 °C. The proportions of this mixture used in the test are shown in Table 2.

Table 2 Composition of Respirometry assays

Reagents	1	2	3	4	5	Blank	Abiotic
Sample (mL)	150	75	50	25	12.5	-	150
Synthetic sewage feed (mL)	16	16	16	16	16	16	16
Activated sludge suspension (mL)	134	134	134	134	134	134	-
Deionized Water (mL)	-	75	100	125	137.5	150	134
Total volume (mL)	300	300	300	300	300	300	300

Dissolved oxygen (DO) of the mixture (300 mL in a BOD bottle) monitoring reading started. DO monitoring (oximeter WTW OXI 7310) was performed until values lower than 2.0 mg DO/L.

The blank test (prepared with activated sludge and synthetic sewage, with no sample or reference substance), abiotic control (prepared without the addition of sludge biomass), and reference with Zn^{2+} ($ZnSO_4 \cdot 7H_2O$) and Cu^{2+} ($CuSO_4 \cdot 5H_2O$) were performed. In addition, all respiration inhibition tests were performed in triplicate and conducted at a temperature of 20 ± 2 °C, based on the required test condition (OECD, 2010).

The percentage of total inhibition (TI) of the sample was calculated using Equation 1, where: R_t = Total sample respiration rate; R_{ta} = Total abiotic respiration rate, and R_{tb} = Total blank respiration rate.

$$TI = \left[1 - \left(\frac{R_t - R_{ta}}{R_{tb}} \right) \right] \times 100 \quad (1)$$

Finally, the percentages of inhibition of oxygen consumption of the tested samples were plotted, and the EC50 values (% v/v) were obtained using the Trimmed Spearman-Kärber statistical method (Hamilton et al. 1977), using the TSK program version 1.5 (USEPA, Cincinnati, Ohio).

Removal of leachate pollutants

Ammonia nitrogen and humic substances are found in high concentrations in landfill leachates and are identified as one of the main potential pollutants of this wastewater. In this context, the treatments of the raw leachate were carried out separately to remove ammonia by air stripping and humic substances by membrane filtration.

Ammonia Stripping

After pH adjustment (4 L), the raw leachate samples were submitted to air stripping at room temperature (25-27°C) to remove ammonia nitrogen. The pH was adjusted with NaOH 1.0 mol/L for pH = 11.0, and the tests were conducted in constant aeration and agitation, with the use of an air diffuser (rate of 360 L/ h) and agitation plate in order to maintain continuous contact between the air bubbles and the liquid phase.

The removal of total ammonia nitrogen (TAN) was performed for a long period, given the high ammonia nitrogen concentration in the leachate samples. Therefore, the ammonia concentration was monitored during the test period.

In order not to generate toxic effects for the studied organisms, the air stripping occurred until reaching a concentration lower than the no observed effect (NOEC) for *A. fischeri* (0.39 mg NH₃/L) (Silva et al., 2018). In relation to the activated sludge microorganisms, ammonia concentration is not a concern; studies show that most species have a high tolerance to ammonia, except the ciliated protozoa, which exhibit a decrease in their diversity (Puigagut et al. 2005).

All samples were readjusted to pH initial after the treatment by adding 37% HCl or 1.0 mol/L NaOH and stored (4 °C) for subsequent physicochemical and ecotoxicological tests.

Membrane filtration

The filtration was conducted in a batch mode, using dead-end cell equipment (PAM Membranas Seletivas Ltda, BR) and the nitrogen gas for the pressure regulation in the filtration module.

Before membrane filtration, leachate samples were filtered in the vacuum system on a 0.45 µm glass fiber filter (Macherey-Nagel, Germany) to remove suspended solids. After this process, permeation was carried out using membranes of two different molecular weight cut-offs (10 kDa and 500 Da) to remove humic and fulvic fractions from landfill leachate samples.

In leachates from intermediate and mature landfills, humic acids (HA) constitute organic molecules with a molar mass greater than 10,000 Da, while fulvic acids (FA) constitute molecules with a molar mass between 500-1,000 Da (Chian, 1977; Trebouet et al., 2001 and Zolfaghari et al., 2018).

Ultrafiltration (UF) was performed using a 10 kDa polyethersulfone membrane (UP 010 - Microdyn-Nadir, USA) and transmembrane pressure of 5 bar. Nanofiltration (NF) was performed from the permeate of UF using a 500 Da polyethersulfone membrane (NP 030 - Microdyn-Nadir, USA) and transmembrane pressure of 20 bar. The filtration tests were performed at the sample's natural pH and room temperature (25-27°C). In this way, the UF permeate (with the humic portion removed) and UF+NF (with the humic and fulvic portion removed) were evaluated.

The use of UF and NF membranes can remove a part of ammonia nitrogen from the leachate. Therefore, to evaluate only the toxic potential of humic substances and fractions, the correction in the ammonia nitrogen concentration was performed with NH₄HCO₃ for the initial concentration of the raw leachate in UF and UF+NF permeates for further ecotoxicological evaluation. The ammonia nitrogen present in landfill leachate is associated with carbonates and bicarbonates (Campos et al., 2013).

The effluents from the ultrafiltration and nanofiltration permeate were stored (4 °C) for subsequent physicochemical and ecotoxicological tests.

Data analysis

Toxicity reduction

The toxic units (TU) of the samples were calculated using Equation 2.

$$TU = \frac{100}{EC50} \quad (2)$$

The samples' acute ecotoxicity reduction was evaluated based on the TU of the raw leachates in TU of treated leachates, as calculated by Equation 3.

$$R(\%) = \left(\frac{TU_{raw\ leachate} - TU_{treated\ leachate}}{TU_{raw\ leachate}} \right) \times 100 \quad (3)$$

The Kolmogorov-Smirnov test assessed the normality of the data. The comparison among the means of the parameter's landfills leachate was performed using Fisher's test (Least Significant Difference- LSD) to correlate the studied landfills' characteristics. The bivariate relationship was assessed using the Pearson correlation, and the association between the physicochemical and ecotoxicological parameters was performed using the analysis of variance (ANOVA), principal component analysis (PCA) and linear regression. All statistical tests were performed with STATISTICA software version 7, licensed by STAT SOFT, except for the comparison test between the means of EC50 values considering the confidence intervals, in which it was performed based on the methodology proposed in USEPA (1985).

Results And Discussion

Leachate characterization

The leachate samples' physicochemical characterization and the statistical comparison of mean values between the two landfill leachates are presented in Table 3.

From the results of Table 3, it was observed that the physicochemical composition of the leachate from Seropédica and Gericinó landfills showed high concentrations of organic matter, ammonia nitrogen and alkalinity, typical characteristics of leachate from Brazilian landfills, as verified in the studies of Campos et al. (2013); Lins et al. (2015); Albuquerque et al. (2018); Costa et al. (2018).

The mean pH value (8.0 and 8.2) was observed in the leachate from Gericinó and Seropédica landfill, respectively. This pH range may indicate that the studied landfills are in the stabilization or methanogenic phase.

Average concentrations of organic matter, reported as COD, were 5.185 mg/L in the leachate from the Seropédica landfill and 2.174 mg/L in the leachate from the Gericinó landfill. In addition to the COD concentration, a great part of it corresponds to recalcitrant compounds due to the high concentration of

humic substances (2,074 and 648 mg/L) in the leachates Seropédica and Gericinó, respectively. The average concentration of humic substance found in this study for the leachate from Gericinó landfill corroborates with Lima et al. (2017a), which verified an average concentration of 616 mg/L of HS in samples collected at the same landfill from 2013 to 2016.

Kulikowska and Klimiuk (2008) investigated the composition of leachate from Wysieka landfill in Poland and noted that over the years, the leachate presented alkaline pH, low biodegradability, and an increase in the concentration of recalcitrant organic material, highlighting the significant influence of the landfill age on leachate composition.

The ammonia nitrogen was observed with an average concentration of 1,375 mg/L (Gericinó landfill) and 1,936 mg/L (Seropédica landfill). These results corroborated the studies by Costa et al. (2018), where high concentrations of TAN (2,104–2,231 mg/L) were observed in leachate samples from the Seropédica landfill.

In general, high concentrations of ammonia nitrogen are found in leachate due to protein degradation from municipal solid waste (Jokela et al., 2002). Mature landfill leachates are rich in ammonia nitrogen and are considered the primary source of pollution in the long term because there is no reduction trend over time (Kjeldsen et al., 2002).

Since the leachates studied in this study come from landfills in the methanogenic phase, as the leachate characterization indicates, heavy metals have reduced their solubilities and toxicity (Slack et al., 2005; Kulikowska and Klimiuk 2008). In addition, *A. fischeri* have low sensitivity to heavy metals, and these pollutants can induce a stimulating effect on bacterial luminescence, called hormesis (Munkittrick, Power, 1989; Pivato and Gaspari, 2006; Teodorovic et al., 2009).

Table 3 also shows a statistically significant difference between the averages of the studied landfills' physicochemical parameters. The leachate composition from the Seropédica landfill showed greater polluting potential than the leachate from the Gericinó landfill, except for the pH and chloride parameters.

Table 3 Physicochemical characterization of the raw leachate from Seropédica Sanitary Landfill (n=4) and Gericinó Controlled Landfill (n=4) collected from 2017 to 2019 and statistical evaluation of comparison of means between the parameters of landfills leachate by Fisher Test LSD (confidence interval =95%).

Parameters	Landfill leachate [average value (range)]		Statistical results (p value)*
	Seropédica	Gericinó	
	Landfill (SL)	Landfill (GL)	
pH	8.2 (8.17-8.23)	8.0 (7.8-8.41)	0.35545
Absorbance at 254nm	34.4 (33.0-36.8)	16.04 (11.17-19.83)	0.00012
Total Ammonia Nitrogen (mg TAN/L)	1,936 (1,681-2,295)	1,375 (866-1,576)	0.04274
Chloride (mg/L)	3,119 (1,322-4,448)	2,031 (1,549-2,599)	0.22322
Conductivity (mS/cm)	27.96 (23.00-31.69)	14.66 (10.03-17.80)	0.00177
COD (mg/L)	5,185 (4,787-5,546)	2,174 (1,850-2,383)	0.00002
Humic Substances (mg/L)	2,074 (2,012-2,191)	648 (586-688)	0.00001
TOC (mg/L)	2,253 (1,922-2,128)	855 (739-942)	0.00004
Alkalinity (mg CaCO ₃ /L)	11,970 (10,017-13,166)	3,583 (1,830-4,479)	0.00010
Turbidity (NTU)	219 (129-327)	6.3 (2.8-9.2)	0.00345
Total Solids (mg/L)	16,545 (13,337-18,280)	5,420 (4,530-6,365)	0.00008
Total Dissolved Solids (mg/L)	15,254 (12,575-17,594)	5,117 (4,187-6,245)	0.00016
Total Suspended Solids (mg/L)	479 (393-650)	208 (120-268)	0.00648

* Values of **p <0.05** show a significant difference between the means of landfills, and for p> 0.05, there is no significant difference between the means of the parameters of landfills

Ammonia stripping

Fig.1 shows the removal of ammonia nitrogen from landfills leachate by air stripping, during the average period of 114-132 hours for leachate samples from Gericinó and Seropédica landfills, respectively.

In Fig. 1, it was observed that the removal of ammonia reached efficiencies greater than 99% for both the leachates. These results are following Silva et al. (2004) findings which achieved 99% of ammonia removal from raw leachate with an initial concentration of 800 mg TAN/L, after 96 h of air stripping (16.5 L air/h) and 800 mL of leachate from Gramacho Metropolitan Landfill (Rio de Janeiro State, Brazil). An efficiency of 99% of ammonia removal was also reached by Ferraz et al. (2013), that evaluates air stripping in landfill leachate from São Carlos landfill (São Paulo State, Brazil). The authors utilized 12 L of leachate (at pH 11 and 25 °C) with an initial concentration of 1493 mg TAN/ L and a flow rate of 1600 L air/h for 288h and 4800 L air/h for 24h, attaining 99% of ammonia removal. Santos et al. (2020) utilized the air stripping process and removed 98% of ammonia nitrogen on average, with an operating time of 4 to 9 days and using 9 to 21 m³ of air for every 1 g of NH₃-N removed.

The long experimental period is due to the fact of the high average initial concentrations of the pollutant (1,375- 1,936 mg TAN/L), as the sample volume used in the test (4 L) and the application of moderate airflow (360 L/h) to prevent foaming. As a result of the extended test period, it was possible to observe that organic matter, reported as COD, showed reductions of 11.4% and 20.9% for leachates from Seropédica and Gericinó landfills, respectively. However, these parameters showed no significant difference between the raw leachate's mean value and after ammonia stripping (Fisher test LSD, more than 5%).

Ultrafiltration and Nanofiltration

The results of humic substances removal from raw leachate and after membrane filtration (the UF and UF+NF permeates) are shown in Fig. 2.

In Fig. 2, it was observed that UF permeate of the leachate from the Seropédica (SL) landfill had significant removals: about 40% of organic material (COD) and 69.1% of humic substances (in this case, the fraction of humic acids with a molar weight greater than 10kDa). The average organic matter removal averages were higher for the mature leachate from the Gericinó landfill, 51.1% of COD) and 70.8% of humic substances. These results corroborate with the studies of Lima et al. (2017a), who performed the fractionation of recalcitrant organic matter with precipitation (pH< 1.0) and XAD-8 resin and found that the fraction of humic acids in mature landfills leachate corresponds to 60-70% of the total concentration of humic substances. Zielińska et al. (2020) reached removals of 90.4% of humic substances and 86.3% of COD using a fine-UF membrane (5 kDa) for treating leachate generated in a 6-year-old municipal landfill located in north-eastern Poland.

Humic acids constitute an important group of organic material of high molar mass (> 10,000 Da) found in leachate, but which decrease more rapidly with the age of the landfill, considering the fulvic acids present in the fractions of 500-1,000 Da (Kang et al., 2002).

Ammonia nitrogen removals were observed in UF, and UF+NF permeate. Permeate from UF presented about 45% of removal for both leachates, and the permeates from UF + NF, removals of 59.3% and 69.9%

for Seropédica and Gericinó landfill leachates, respectively; these losses were corrected to the initial ammonia concentration of the samples.

Fig. 2 also shows that UF+NF permeate achieved a removal average greater than 97% of humic substances for both studied landfills, which evidences in this study the efficiency of removing recalcitrant organic matter using membrane fractionation. In addition, the filtration with membranes shows high efficiencies in removing the humic substances from leachate (Gu et al., 2019).

Permeate from UF+NF showed an average remaining COD of 34.58% and 21.39% in the leachate from Seropédica and Gericinó landfills, respectively, with a molecular size of less than 500 Da, corresponding to a non-humic fraction. These results corroborated with Trebouet et al. (2001), who observed after nanofiltration that a fraction of 38% of the COD present in the leachate from the Saint-Nazaire landfill in France corresponded to molecules < 500 Da and with Campagna et al. (2013) that observed a value of 44% for this same fraction, after the sequential filtration treatment in leachate from the Odayeri landfill in Istanbul.

The organic matter with a molecular weight of less than 500 Da and ammonia indicate small organic acids or amino acids derived from biological degradation (Jarusutthirak and Amy, 2007; He et al., 2015).

Toxicity assays

In this study's initial phase, the bacterium *A. fischeri* and the activated sludge microorganisms were subjected to analytical quality control to carry out ecotoxicological assays. The tests were performed (n=8) with the reference substances Zn²⁺ and Cu²⁺, as shown in Table 4.

Table 4 Toxicity results of reference substances with organisms

Reference substances	<i>Aliivibrio fischeri</i> (EC50 _{30min} , mg/L)		Activated Sludge microorganisms (EC50 _{180min} , mg/L)	
	Average	Confidence Interval (95%)	Average	Confidence Interval (95%)
Zn ²⁺	0.56	0.48-0.62	51.62	48.62-56.70
Cu ²⁺	0.31	0.27-0.38	31.7	28.56-42.10

In Table 4, the EC50 values for Zn²⁺ and Cu²⁺ showed agreement with the values reported in the literature for *A. fischeri* (Utgikar et al. 2004; Zhou et al. 2006; Rosen et al. 2008; Kurvet et al. 2011) and activated sludge microorganisms (Fiebig and Noack 2004; Aguilar et al. 2020). The inhibition values of 3,5-dichlorophenol and potassium dichromate ranged between 36-50%, values between 20-80% inhibition being acceptable, based on the assay methodology (ABNT, 2012).

3,5-dichlorophenol is a widely recommended reference compound with *A. fischeri* and activated sludge microorganisms (Broecker and Zahn 1977, Gendig et al. 2003, Strotmann et al. 2020). In their studies, Strotmann et al. (2020) found that the chlorinated phenol compounds have high chemical stability and do not vary with incubation time, culture medium, storage method, and do not form complexes such as Cr^{6+} and Zn^{2+} , which may influence the bioavailability of the chemical compound.

In the test with activated sludge microorganisms, the reference test with 3,5 dichlorophenol was not carried out, because according to the methodology used (OECD, 2009), both 3,5 dichlorophenol and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ could be chosen as the reference substance, and in this study, we chose to use $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.

The toxicity results of the raw and treated leachates are shown in Fig. 3 and more detailed in Table S1 and Table S2 (Supplementary Material).

Fig 3 shows that raw leachates from active (Seropédica) and closed (Gericinó) landfills were toxic to *A. fischeri* and activated sludge microorganisms. In addition, the Seropédica landfill leachates showed greater toxic potential than the Gericinó landfill, confirmed by the low EC50 values for both test organisms studied.

In this study, bacterium *A. fischeri* showed greater sensitivity to leachate samples than activated sludge microorganisms in respirometry assay. These results are in accordance with the studies by Kalčíková et al. (2015), who verified a higher tolerance of the activated sludge microorganisms in the leachate samples ($\text{EC50}_{180 \text{ min}} = 46.6 \pm 5.8\% \text{ v/v}$) and greater sensitivity of the bacterium *A. fischeri* ($\text{EC50}_{30 \text{ min}} = 27.6 \pm 5.3\%$).

The activated sludge microorganisms require a longer contact time with the sample to determine the effluent's actual toxicity (Kalčíková et al., 2015). Therefore, the test period of 180 minutes is considered the most appropriate compared to the 30 minutes since low toxicity or non-toxic results are observed with the shortest exposure time.

Cotman and Gotvajn (2010) evaluated leachate samples from a landfill in Slovenia. The leachates were collected from an old cell (closed in 2006) and a new cell (started in 2006) and mixed in a proportion of 36% volume of mature leachate and 64% of young leachate. The authors observed the inhibition values for raw leachate samples and after air stripping, activated carbon, zeolite, and Fenton oxidation treatments, the *A. fischeri* assay showed higher sensitivity than respirometry assay.

Luo et al. (2019) showed in their review the use of *A. fischeri* as a preliminary ecotoxicological investigation on different leachates from traditional and sustainable landfills. The authors highlighted that the leachate toxicity was considerably lower in sustainable landfills where the ammonia had been degraded.

Gutiérrez et al. (2002) carried out a comparative study between respirometry and *A. fischeri* assays with organic and inorganic pollutants. The authors concluded that the low sensitivity verified in the

respirometry assays is due to the heterogeneous composition and the high adaptability of the activated sludge microorganisms.

Statistically significant differences were observed between the averages of the raw leachate samples and after treatments for ammonia nitrogen and humic substances removals in both landfills, according to the methodology proposed by USEPA (1985).

The leachates of both landfills reduced toxicity after ammonia stripping and membrane filtration treatments, as shown in Table 7.

Table 7 Toxicity removal efficiency (%) after landfill leachate treatments

Toxicity Removal Efficiency (%)	Seropédica Landfill		Gericinó Landfill	
	<i>Aliivibrio fischeri</i>	Activated sludge microorganisms	<i>Aliivibrio fischeri</i>	Activated sludge microorganisms
Ammonia stripping	65.47%	37.16%	38.06%	31.33%
UF	73.96%	34.15%	44.80%	30.97%
UF+NF	79.82%	49.56%	55.91%	44.28%

Table 7 shows that for the leachate from the Seropédica landfill, the toxicity reduction was greater than 70% for *A. fischeri* after ultrafiltration and nanofiltration treatments. Concerning activated sludge microorganisms, the greatest reductions, around 50%, were obtained only after nanofiltration (with the removal of HA and FA).

For the leachates from the Gericinó landfill, the toxicity reduction values were lower than the leachates from the Seropédica landfill. After nanofiltration, leachates showed the highest percentages of toxicity reduction for *A. fischeri* (55.91%) and activated sludge microorganisms (44.28%) comparing to the other treatments performed. Table 7 also shows that toxicity reduction after ammonia stripping to *A. fischeri* for the leachates studied was superior to that found by Kalčíková et al. (2015), who observed a reduction of about 26% for *A. fischeri* toxicity (leachate with a final ammonia concentration of 5.0 mg/ L). From this, it can be inferred that the removal of ammonia performed in this study (which reached a concentration <0.3 mg/ L) was fundamental for the high efficiency in reducing the toxicity of the leachates analyzed.

The correlation between the physicochemical parameters and the leachate toxicity assays was evaluated using PCA statistical analysis, as shown in Fig. 4.Z

In Fig. 4, the two main axes (PC1 and PC2) represented between 97.35% and 98.32% of the total data variance. Since the analyzed variables are on opposite sides, the increase in the physicochemical

parameter values represents a reduction in the EC50 value. In other words, it contributes to increased toxicity.

In the leachate from the Seropédica landfill, a strong correlation was found mainly with the organic parameters: COD (-0.988), TOC (-0.978), humic substances (-0.958), and a weak correlation with ammonia nitrogen (-0.343). In the leachate from Gericinó landfill, as noted in Seropédica landfill, a strong correlation was observed with the organic parameters: COD (-0.990), TOC (-0.989), humic substances (-0.934), and a weaker correlation with ammonia nitrogen (-0.509) for EC50 *A. fischeri* and activated sludge microorganisms.

The correlation observed in Fig. 4, between the test organisms and the physicochemical parameters, indicates that the organic compounds (COD, TOC and humic substances) were the main pollutants responsible for the leachate samples' toxicity from Seropédica and Gericinó landfills. Corroborating with studies by Clément et al. (1997), Ward et al. (2002), and Pivato and Gaspari (2006), from landfill leachate samples analyses, they verified a high sensitivity of *A. fischeri* to organic compounds and low sensitivity to inorganic compounds.

The leachate's organic material fractions present different toxicity values to the bacterium *Aliivibrio fischeri* (He et al., 2015). Humic acids presented a greater toxicity level ($EC_{50_{15\text{ min}}} = 9.2\text{ mg/L}$), followed by fulvic acids ($EC_{50_{15\text{ min}}} = 130.8\text{ mg/L}$) and low molecular weight molecules, mainly carbohydrates, which show low toxicity or no toxicity.

Although the present study found a strong correlation between organic compounds from the landfill leachate and the toxicity to the activated sludge microorganisms, a scarcity of studies in the literature on this correlation was observed. Furthermore, as the respirometry assays use a non-pure culture, different from the bioluminescence inhibition assay with *A. fischeri*, difficulties in reproducibility studies are observed (Elnabarawy et al., 1988) and Ricco et al., 2004). As a result, the respirometry assay has become a frequent tool for monitoring treatment processes with activated sludge, and few studies are carried out to evaluate effluent toxicity.

A regression analysis was performed to determine a correlation between the toxicity responses obtained from test organisms studied, as shown in Fig. 5.

Although *A. fischeri* was more sensitive to leachate pollutants, as noted in previous results, the linear regression (Fig. 5) revealed a high degree of correlation ($R^2 = 0.9734$, $p < 0.05$) between toxicity responses with *A. fischeri* and activated sludge microorganisms.

Conclusions

The physicochemical characterization of the leachates showed that the Seropédica landfill (active landfill) presented characteristics, such as COD, humic substances, TOC, ammonia nitrogen, alkalinity, absorbance 254nm, conductivity, turbidity, and series of solids, in concentrations statistically higher than

the Gericinó landfill (closed landfill). These characteristics reflect the age, the operating mode of the landfill, and the fact that the Seropédica landfill still receives MSW daily, unlike the Gericinó landfill. Only the pH and chloride parameters showed statistically equal values between the landfills.

Regarding toxicity, the raw leachate from Seropédica and Gericinó landfills showed high toxicity to *A. fischeri* and activated sludge microorganisms. However, the Seropédica landfill has greater toxic potential than the Gericinó landfill, which the low EC50 values can confirm for both test organisms.

Reductions in the toxicity of landfill leachate were observed after the removal of ammonia nitrogen pollutants and humic substances. The removal of ammonia nitrogen (efficiency greater than 99%) contributed to mean toxicity reductions of 38.06% and 65.47% for *A. fischeri* and 31.33% and 37.16% for activated sludge microorganisms for the Gericinó and Seropédica leachates, respectively. After UF (molar weight greater than 10 kDa), the humic acid fraction's removal was 69.1% for the leachate from the Seropédica landfill and 70.8% for the Gericinó leachate. After this treatment, the toxicity reduction was greater compared to landfills leachates after ammonia stripping.

For removals of humic and fulvic acids, efficiencies were greater than 97% after UF + NF processes were achieved for both leachates. The remaining material (molecular weight below 500 Da) corresponded to a fraction of 34.58% for the leachate from the Seropédica landfill and 21.39% for leachate from the Gericinó landfill. Leachates without humic substances obtained a greater reduction in toxicity (44.28–79.82%) for *A. fischeri* and activated sludge microorganisms, highlighting the studied organisms' sensitivity to recalcitrant organic material.

About the test organisms, it was possible to verify that the bacterium *A. fischeri* presented higher sensitivity to the leachate samples from both landfills when compared to activated sludge microorganisms, and this is due to the fact the high sensitivity of bacterium to organic compounds, which are found in high concentrations in landfill leachates. Furthermore, principal component analysis between the physicochemical and ecotoxicological parameters indicated that the organic compounds (COD, TOC and humic substances) were the main pollutants responsible for the toxicity of the leachate samples for both landfills.

This study observed a strong correlation between the toxicity responses of *A. fischeri* and activated sludge microorganisms with the regression analysis. Therefore, the respirometry assay can be used to determine landfill leachate toxicity. Furthermore, respirometry assay can be an alternative for developing countries, such as Brazil, due to its simplicity and low cost. Finally, it can be inferred that the respirometry assay is an important toxicity assessment tool. However, it is emphasized that the use of test organisms of different trophic levels is essential for assessing the impacts of this complex wastewater, which is the landfill leachate, on the aquatic ecosystem.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its Supplementary Material.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was financed in part by the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil) - Finance Code 001 and by FAPERJ (Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro) – Project codes: E-26/010.001868/2015; E-26/202.923/2018, and E-26/010.002468/2019.

Authors' contributions

AMC performed all experiments, analyzed data, and wrote the manuscript; MRSV and LFS contributed to carrying out the experiments; RA co-wrote the manuscript; SADD, BRQ, and JCC supervised experiments, contributed to data interpretation and co-wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank the Municipal Urban Cleaning Company of Rio de Janeiro City (COMLURB) for collecting and sending leachate samples

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76. **Comparison between Aliivibrio fischeri and activated sludge microorganisms in the evaluation of the toxic pollutants of leachates from Brazilian landfills**
77. Alyne Moraes Costa¹, Marllon Robert dos Santos Valentin¹, Livia Ferreira da Silva¹, Ronei de Almeida¹, Sarah Dario Alves Daflon¹, Bianca Ramalho Quintaes² and Juacyara Carbonelli Campos¹

Tables

Table 5 and 6 not available with this version

Figures

Figure 1

TAN concentration profile over to experimental time in ammonia stripping of leachates

(a)

(b)

Figure 2

Humic substances average concentration of raw and treated leachate after UF and UF +NF. (a- Seropédica landfill and b- Gericinó landfill).

Figure 3

Average toxicity results of *A. fischeri* (EC50 30 min %) and activated sludge microorganisms (EC50 180min %v/v) for raw leachates and treated for removal TAN, HA and HA+FA. (a- Seropédica landfill (n = 4) and b- Gericinó landfill (n = 4))

(a)

Figure 4

Graphical representation of the main axes (PC1 and PC2) of the principal components of the correlation matrix for the Seropédica (a) and Gericinó (b) landfills

Figure 5

Determination of a relationship between the activated sludge microorganisms results ($EC_{50_{180min}}$ %) and the *A. fischeri* ($EC_{50_{30min}}$ %) results

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