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Evaluation of sensitivity to zinc and copper of *Diophrys appendiculata* (Protozoa, Ciliophora) and their associated bacteria, both isolated from a tropical polluted bay

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Abstract. Ciliates are an essential component of microbial food webs, connecting biomass production to higher trophic levels and providing substrates for bacterial growth. Bacteria are widespread in sea sediment and the first to metabolize organic matter. Heavy metals are toxic and bind to particulate organic matter. This work aims to study the influence of heavy metals (Zn and Cu) on *D. appendiculata* and their naturally associated bacteria, both from Guanabara Bay during 96 h bioassay with 0, 0.001, 0.009, 0.05, 0.1 mg L^{-1} and maximum concentration stipulated by CONAMA 357 (Zn 5.0; Cu 1.0 mg L^{-1}). It was analyzed for tolerance, resistance and biomass production. In Zn control, ciliate produced $1.24x10^{2}$-$2.47x10^{3}$ µg C cm^{-3} (0-96 h), with 0.009 mg Zn L^{-1} reaches $2.49x10^{2}$-$1.93x10^{3}$ µg C cm^{-3} (0-96 h). Their naturally associated bacteria, in Zn control, produced $5.0x10^{-2}$-$5.67x10^{-2}$ µg C cm^{-3} (0-96 h), with 0.1 mg Zn L^{-1} $4.87x10^{-2}$-$8.38x10^{-2}$ µg C cm^{-3} (0-96 h). In Cu control, *D. appendiculata* produced $1.04x10^{2}$-$3.12x10^{2}$ µg C cm^{-3} (0-96 h), with 0.009 mg Cu L^{-1} biomass was $8.31x10^{1}$-$7.27x10^{-2}$ µg C cm^{-3} (0-96 h) and with CONAMA concentration was below detection level. Their naturally associated bacteria, in Cu control, produced $1.20x10^{-2}$-$5.14x10^{-2}$ µg C cm^{-3} (0-96 h) and with 0.1 mg Cu L^{-1} $7.40x10^{-4}$-$3.81x10^{-2}$ µg C cm^{-3} (0-96 h), with CONAMA concentration $5.14x10^{-2}$ µg C cm^{-3} (96 h). *D. appendiculata* are tolerant to 0.09 mg L^{-1} and resistant to 0.1 mg L^{-1} after 24 h to Zn-Cu and LD50 stipulated was 1.17 (Zn) 0.90 (Cu) mg L^{-1}. Microbial loop was functional in low concentration of Zn and Cu, but their diversity in nature was affected.

Keywords: Bacteria, Free Living Protist, Guanabara Bay, Heavy Metals, Microbial loop, .

Introduction

Ciliates are an essential component of microbial food webs. They show great growth potential due to the high rate of metabolism that facilitates carbon and energy flux through components of the ecosystems (Fenchel, 1987; Sherr & Sherr, 1994). Plus, their phagotrophic activities, egestion metabolites, both as dissolved and particulate organic matter, which include dissolved nutrients, particularly ammonium and phosphate (Caron, 1991; Dolan, 1997), act as important influences in some bacterial processes

(Nagata & Kirchman, 1992a, 1992b). Thus, protist grazing provides substrates for the further growth of prey, which includes bacteria (Jumars *et al.*, 1989; Christakiu *et al.*, 1999).

Bacteria are widespread in sea sediment in large numbers (approximately 10^{10} cells.g^{-1}). They possess a high surface to volume ratio, which is indicative of high metabolic rates. Dissolved inorganic and organic substrates are metabolized with high substrate affinity and specificity. Particulate organic matter is decomposed in close contact with the substrate by using hydrolytic bacterial enzymes (Sobrinho da Silva *et al.*, 2010; Guerra *et al.*, 2011).

Particulate organic matter has a high affinity to binding heavy metals (Adriano, 2001). This is a group of toxic metals that have densities equal to or greater than 5 g cm^{-3} (Adriano, 2001). In highly industrialized areas, this pollutant is the main contributor of environmental degradation, and it reaches the seas through rivers, sludge, garbage or air (Nordberg *et al.*, 2007). Such contamination can affect the health of man in various ways, and the main source of heavy metals may be trophic chain through the biomagnification process. This is the natural uptake of metals by organisms in the environment, including background concentrations (Cornelis & Nordberg, 2007). The microbial loop is included in this context, however there are few studies on this subject (Fisher & Reinfelder, 1995; Fisher *et al.*, 2000; Wang & Rainbow, 2005), and this activity depends on the resistance or tolerance of microorganisms that are near sources of heavy metals.

This work aims to study the influence of heavy metals (Zn and Cu) on *Diophrys appendiculata* (Ehrenberg, 1838) Schewiakoff, 1893 and their naturally associated bacteria isolated from sediment of Guanabara Bay during 96 h bioassay, to assess resistance, tolerance of them, and to obtain a predictive base of microbial loop activity with Zn and Cu stress, by biomass production of both microorganisms, with Zn and Cu input.

Material and Methods

Site study and sampling methods

Guanabara Bay is a complex tropical estuarine environment in Rio de Janeiro State, Southeast Brazil between 22°40`-23°00`S, 043°00`-043°18`W. It is one of the largest bays along the Brazilian coastline, with an area of approximately 384 km^2. In the past, each of the 55 rivers might have created a single estuary that was distinct from the others (Amador, 2012). These estuaries originated the estuarine system of Guanabara Bay (Ribeiro & Kjerfve, 2002). However, due to human and industrial occupation, the bay lost its natural characteristics and diversity, being reduced by four large and well-demarcated areas, with different degrees of environmental pollution, as suggested by data collected (JICA, 1994; Kjerfve *et al.*, 1997; Carreira *et al.*, 2002; Ribeiro & Kjerfve, 2002; Amador, 2012, 2013; Baptista Neto *et al.*, 2013) and proposed by (Baptista Neto *et al.*, 2006). Moreover, the bay has a complex bathymetry with a relatively flat central channel that is 400 m wide, stretches more than 5 km into the bay, and is defined by the 30 m isobaths. The deepest point of the bay (58 m) is located within this channel (Kjerfve *et al.*, 1997).

The drainage basin of Guanabara Bay has an area of 4,080 km^2. It is comprised of 32 separate sub-watersheds (Kjerfve *et al.*, 1997) and 55 rivers that carry 4,000,000 tons $year^{-1}$ of solid material (JICA, 1994; Amador, 2012); however, only six

rivers are responsible for 85 % of the 100 $m^3 s^{-1}$ total mean annual freshwater input (JICA, 1994). Currently, 11 million inhabitants live in the Rio de Janeiro metropolitan area, which discharges tons of untreated sewage directly into the bay (CARREIRA *et al.*, 2002; RIBEIRO & KJERFVE, 2002). There are more than 12,000 industries located in the drainage basin, accounting for 25 % of the organic pollution released into the bay. The bay also hosts two oil refineries along its shore that process 7 % of the oil used nationally and is the homeport of two naval bases, a shipyard, and a large number of ferries, fishing boats, and yachts (FEEMA, 1990).

In this study, sediments were sampled at the closest region of Paquetá Island (22º46.9 S 43º06.7 W); this site has fine sand, coarse silt, and an input of approximated 458 g C m^{-2} $year^{-1}$ of organic matter (CARREIRA *et al.*, 2002). Their Zn and Cu concentration is approximately 256.67 and 45 mg kg^{-1}, respectively. The organic matter and organic carbon content are approximately 19.77 % and 2.46 mmol C g^{-1} of sediment. These parameters display little variability between the dry and wet seasons (CARREIRA *et al.*, 2002; RIBEIRO & KJERFVE, 2002; BAPTISTA NETO *et al.*, 2006; FONSECA *et al.*, 2013).

The sediment samples were obtained using an Eckman sampler and were stored for 2 h in sealed polythene bags, conditioned in a dark container with low temperature (4 ºC) to reduce microbial metabolism (CETESB, 2012), and were transported to the laboratory. The sediment samples were prepared in Petri dishes according to DRAGESCO & DRAGESCO KERNÉIS (1986) and incubated at 25 ºC in the dark for two weeks. *D. appendiculata* was picked from Petri dishes using glass micropipettes and was maintained in the laboratory on a plate with liquid medium containing coarse powdered rice (DRAGESCO & DRAGESCO KERNÉIS, 1986); to reduce contamination of the cultures by other microorganisms, the grains of rice and seawater were sterilized (MADONI *et al.*, 1992; MADONI *et al.*, 1994; MADONI, 2000; MADONI & ROMEO, 2006). The identification of the protists was performed using a classical methodology based on structural features (TUFFRAU, 1960; CURDS, 1975; CURDS & WU, 1983; LYNN, 2008), such as cell morphologic characteristics of the oral and somatic infraciliatures and dorsal argyromes. These features were visualized by the carbonate silver impregnation Protargol method (SILVA-NETO, 2000) and a modified Chatton-Lwoff method (FRANKEL & HECKMANN, 1968).

Bioassays and analysis

The assay was carried out with separated Zn and Cu, in triplicate, along 0, 24, 48, 72, and 96 h with and without metal exposure (control group). The assay was carried out in sterile 24-well polystyrene plates without supplementary nutrients. Plates were incubated at 25°C in the dark. Final zinc and copper concentrations used, in triplicate, were 0, 0.001, 0.009, 0.05, 0.1 mg L^{-1}, and higher concentrations were 5.0 (Zn) and 1.0 (Cu) mg L^{-1}, such CONAMA reference (BRASIL, 2005). For the Zn and Cu solution assay, analytical-grade pure zinc sulfate ($ZnSO_4.7H_2O$) and copper sulfate ($CuSo_4.5H_20$) from Sigma-Aldrich Corp. were used as the source of metal ions. A solution of metal salt in the assay was diluted with sterile seawater (MADONI *et al.*, 1992; MADONI, 2000; MADONI & ROMEO, 2006; DA SILVA *et al.*, 2014) and the pH of the tested solutions was 8.0 and did not require any adjustment.

For each metal concentration test, 30 ciliates were used. Single ciliates were picked from 72 h culture with a glass micropipette, washed

repeatedly in drops of sterile seawater, and individually inoculated into each well containing 2.0 ml of filtered (cellulose ester Millipore membrane, 0.44 µm pore diameter). The same procedure without the metal solution was performed as the control group.

D. appediculata and their naturally associated bacterial biomass were individually quantified as the organic carbon content (µg C cm^{-3}). Each well content from polystyrene plates was single fixed with paraformoldeyde (4% final concentration) and filtered through sterile Isopore polycarbonate Millipore membrane (0.22 µm pore diameter) and stained with fluorochrome acridine orange at a vacuum not exceeding 3 kPa. Intact and healthy cells were enumerated at epifluorescent microscopy at x1000 magnification (Axioskop 1, Zeiss, triple filter Texas Red – DAPI – fluorescein isothiocyanate). With this staining method with UV wavelength light, the bacterial physiology healthy cell will emit green light, and protists healthy will emit green or orange (Mirrett, 1982; Carlucci *et al.*, 1986). The data obtained was converted to bacterial and ciliate volume following methodology proposed by Kepner & Pratt (1994) and Gomes *et al.* (2007). This content was converted to bacterial organic carbon by $1.2x10^{-14}$ g C cm^{-3} (Carlucci *et al.*, 1986) and converted to ciliate organic carbon by $0.22x10^{-3}$ g C cm^{-3} (Mauclaire *et al.*, 2003).

The lethality was estimated by lethal concentration for the 50% of the *D. appediculata* (LD50 - probit) under 96h of bioassays in accordance with Bliss (1934a, 1934b); Costa *et al.* (2008); da Silva *et al.* (2014). These analyses and LD50 were performed using the SPSS 21 program. To contrast the biomass content from the microorganism along different metallic and concentration exposure, we used ANOVA parametric test with the bootstrap (x1000) and Tukey post hoc test. The biomass data were transformed using the ranging method.

Results

During Zn bioassay control, biomass of *D. appediculata* content began with $1.24x10^{2}$ µg C cm^{-3} and was enhanced during assay, showing $1.33x10^{3}$ (48 h) and $2.47x10^{3}$ µg C cm^{-3} (96 h). In contact with 0.001 mg Zn L^{-1} the biomass of *D. appediculata* enhanced from $1.90x10^{2}$ µg C cm^{-3} (0 h) to higher biomass content at 48 h ($2.00x10^{3}$ µg C cm^{-3}), and was reduced to $1.40x10^{3}$ µg C cm^{-3} at 96 h. With 0.009 mg Zn L^{-1}, their highest biomass ($2.18x10^{3}$ µg C cm^{-3}) was pointed at 24-48 h of bioassay and decreased to $1.93x10^{3}$ µg C cm^{-3} at 96 h. In contact with 0.1 mg Zn L^{-1}, *D. appediculata* biomass content was $2.49x10^{2}$ µg C cm^{-3} (0 h) and enhanced up to $2.00x10^{3}$ µg C cm^{-3} at 96 h (Figure 1a).

The naturally associated bacteria of *D. appediculata* in Zn bioassay and under ciliate's predation process had higher concentration of biomass content at 24-48 h. In control, higher bacterial biomass was reached at 24 h of assay ($1.13x10^{-1}$ µg C cm^{-3}) and decreasing content to $5.62x10^{-2}$ µg C cm^{-3} (96 h). In contact with 0.001 and 0.009 mg Zn L^{-1} highest biomass content was pointed at 48 h (1.62 and $1.45x10^{-1}$ µg C cm^{-3}) and decreased along 96 h of assay to 8.38 (0.001 mg Zn L^{-1}) and $5.60x10^{-2}$ µg C cm^{-3} (0.009 mg Zn L^{-1}). In bioassay with 0.05, 0.1 and 1.0 mg L^{-1}, the highest ciliate biomass contents were, respectively, 1.34, 1.05 and 1.50 $x10^{-1}$ µg C cm^{-3} were pointed at 24 h, decreasing to 6.20, 8.39 and $5.15x10^{-2}$ (0.05, 0.1, 1.0 mg Zn L^{-1}) at 96 h. With 5.0 mg Zn L^{-1}, the naturally associated bacteria maintained their biomass content along 0, 24, 48 h of assay, with $4.64x10^{-2}$ µg C cm^{-3} and decreased to $3.41x10^{-2}$ µg C

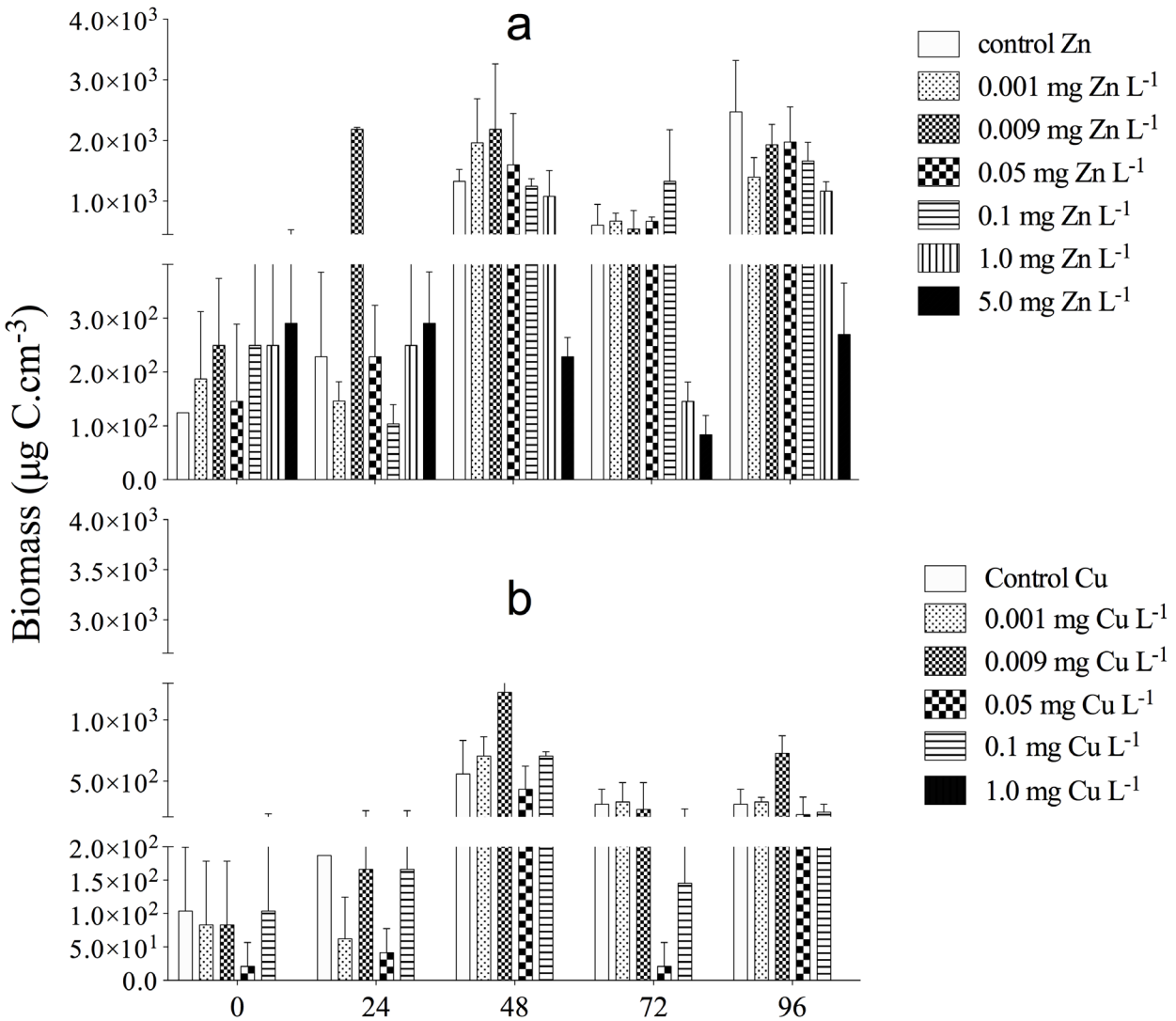

Figure 1. Biomass production of *D*. *appendiculata* during 96 h bioassay with different Zn and Cu concentration: a) with zinc and b) with copper.

cm^{-3} at 96h (Figure 2a).

In Cu bioassay, biomass of *D*. *appediculata* content began with 1.04x10^{2} µg C cm^{-3} at 0 h and enhanced to 3.12x10^{2} µg C cm^{-3} at 72-96h. Exposition to more of Cu concentration stimulated *D*. *appediculata* biomass enhancement along the first 48 h of assay, 8.31x10^{1}-7.06x10^{2} µg C cm^{-3} (0.001 Cu L^{-1}) 8.31x10^{1}-12.26x10^{2} µg C cm^{-3} (0.009 mg Cu L^{-1}), 2.01x10^{1}-4.36x10^{2} µg C cm^{-3} (0.05 mg Cu L^{-1}) and 1.04x10^{2}-7.06x10^{2} µg C cm^{-3} (0.1 mg Cu L^{-1}). After this period, the ciliate biomass content reduced. At 96 h of bioassay, under 0.001, 0.009, 0.05 and 0.1 mg Cu L^{-1} exposure, biomass contents were 3.33, 7.27, 2.30 and 2.50x10^{2} µg C cm^{-3}. In counterpart, contact with 1.0 mg Cu L^{-1}, the biomass content was lower than technical detection during bioassay (Figure 1b).

The biomass content of naturally associated bacteria reached a higher content in the same bioassay with Cu. In control, in 0 h was 1.20x10^{-2} µg C cm^{-3} and enhanced to 1.14x10^{-1} µg C cm^{-3} at 72 h. With 0.001 and 0.009 mg Cu L^{-1} at 0 h started

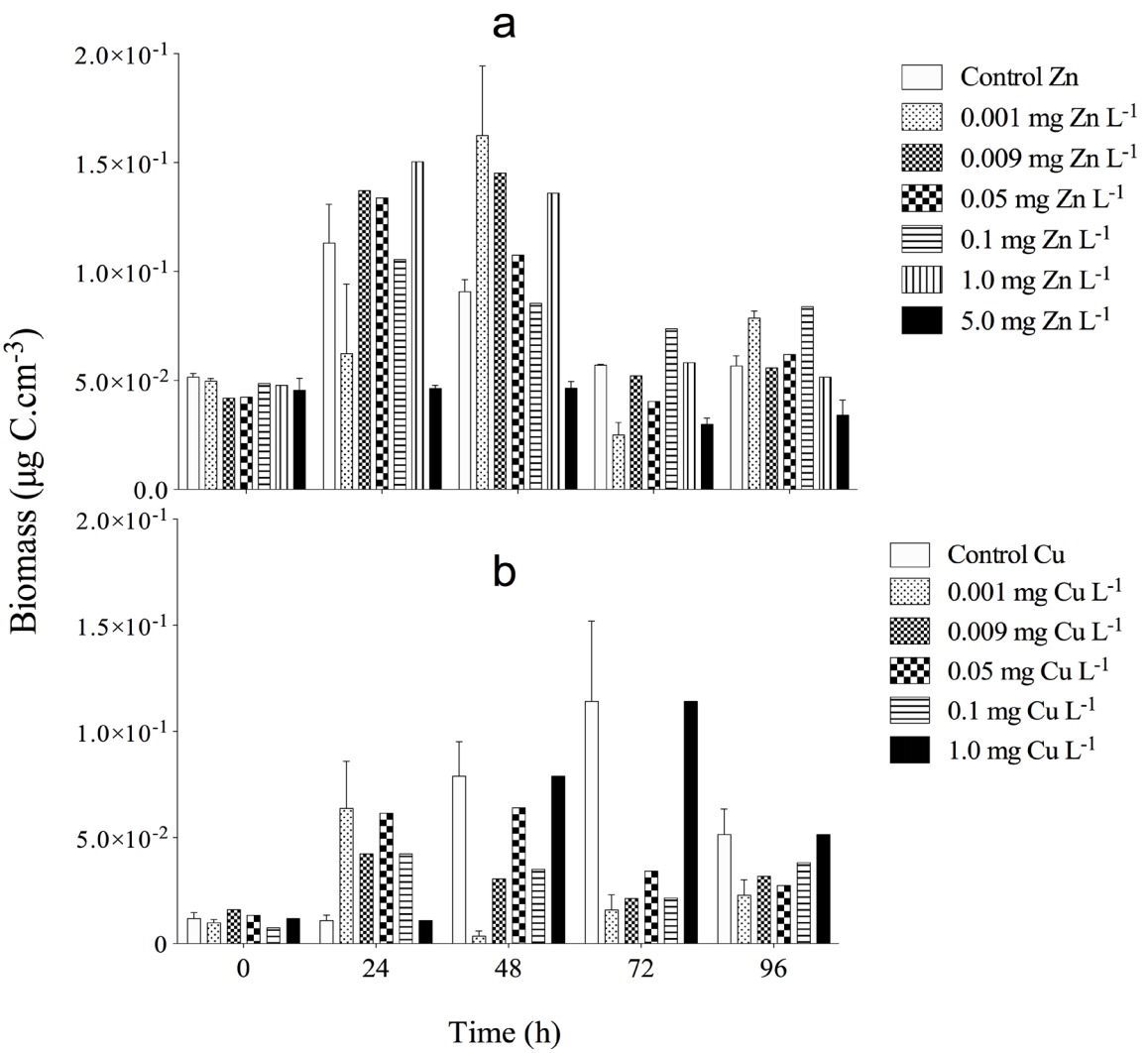

Figura 2. Biomass production of naturally associated bacteria from *D. appendiculata* during 96 h bioassay with differences Zn and Cu concentration and predation stress: a) with zinc and b) with copper.

with 1.00 and 1.60x10^{-2} µg C cm^{-3} and enhanced to the highest content at 24 h, with 6.4 and 4.23x10^{-2} µg C cm^{-3}, reducing biomasses contents to 2.27 and 3.17x10^{-2} µg C cm^{-3}. Under 0.05 and 0.1 mg Cu L^{-1} exposure, the biomass content from 24-96h was 6.15-3.18x10^{-2} µg C cm^{-3} (0.05) and 4.23-3.81x10^{-2} µg C cm^{-3} (0.1 mg Cu L^{-1}). With 1.0 mg Cu L^{-1} the highest biomass content was pointed and equal control content at 48-72 h, with 7.90x10^{-2} and 1.14x10^{-1} µg C cm^{-3}, respectively (Figure 2b).

ANOVA statistical analysis showed significant differences in the variation of biomass produced by the organisms and between bioassays with Cu and Zn (n=210; p≤0.007). Tukey post hoc test pointed out significant differences in concentrations of 0.1 mg L^{-1} (Zn and Cu) and predetermined content by CONAMA 357 for Zn and Cu (5.0 and 1.0 mg L^{-1}). The LD50 stipulated was 1.17 mg Zn L^{-1} (Z= 324.7; Standard Error= 0.004; p<0.05; 95% Confidence Interval, 1.16 and 1.17 mg Zn L^{-1}) and 0.90 mg Cu L^{-1} (Z= 1132.25; Standard Error= 0.001; p< 0.05; 95% Confidence Interval= 0.891 and 0.894 mg Cu L^{-1}).

DISCUSSION

Ciliates connect bacterial biomass production to high trophic level (AZAM *et al.*, 1983; POMEROY *et al.*, 2007) and may biomagnify Zn and Cu (FERNANDEZ-LEBORANS & NOVILLO, 1996; MADONI & ROMEO, 2006; MARTÍN-GONZÁLEZ *et al.*, 2006). However, there is a lack of information available on pollution resistance of ciliated protozoa, mainly with Zn and Cu (VIEIRA & VOLESKY, 2000). These metals are trace elements, but in low concentrations contents are toxic and harmful to microzooplankton and microzoobenthos (FERNANDEZ-LEBORANS & NOVILLO, 1996; MADONI & ROMEO, 2006).

The pollution of Guanabara Bay leads the selection of some organisms, like *D*. *appendiculata*, and their naturally associated bacteria (NEVO *et al.*, 1983; NEVO *et al.*, 1986). This selection prioritized the tolerance of organisms, as proposed by HARRISON *et al.* (2007). However, the data showed by the works of SOBRINHO DA SILVA *et al.* (2008) and SABADINI-SANTOS *et al.* (2014) indicated that the bacterial physiologic status of sampled area is near the limit of tolerance and loss of bacterial physiologic diversity. It may be possible that some microorganisms of this bay joined a co-resistance to survive on sediments with high metals contents (256.67 mg Zn kg^{-1} and 45.00 mg Cu kg^{-1}) due to connection of their physiological apparatus.

Microorganisms maintained the microbial loop influences without supplementary nutrients along 96 h of the bioassay. The Zn and Cu were free in the medium or closely associated with organisms, attached in bacterial biofilm (FLEMMING & WINGENDER, 2010; GONZÁLEZ *et al.*, 2010) and, probably in the ciliate mucus (GÖRTZ, 2006). The metals significantly positively influenced *D. appendiculata* biomass in a concentration-dependent manner. In this bioassay, they may Zn and Cu tolerate up to 0.09 mg L^{-1}, demonstrated by their regular biomass production after 24 h, and it resisted up to 0.1 mg L^{-1} for metal exposition. Metals in higher concentrations are lethal, but microorganisms have evolved various mechanisms to resist the heavy metal stress due to the selective pressure of the metal (Harrison *et al.*, 2007), such induction of metallothionein (KIM *et al.*, 2011). Some reports demonstrated the effect of Zn and Cu on cell growth or viability for 24 h or 48 h. PARKER (1979), MADONI *et al.* (1992) and MADONI & ROMEO (2006) suggested the survival rate of ciliate species was reduced remarkably after heavy metal exposure (Cu, Cr, Ni, Pb, Zn and Cd) for 24 h. KIM *et al.* (2011) showed toxic effects of heavy metals on population growth of *E*. *crassus* with inhibition of 50% growth for 48 h. However, we observed in bioassay a resistance that enables *D. appendiculata* to survive and to produce biomass above the detection limit of the technique and in higher concentrations than in the control.

Bacteria may reduce their metal damage through active uptake or biosorption of several toxic metals by the exopolysaccharide (EPS) (GADD & GRIFFITHS, 1977; KIRCHMAN, 2000; MATZ & KJELLEBERG, 2005; MORILLO PÉREZ *et al.*, 2008) and can have higher resistance than ciliates. Bacteria represent the basal element in the food chain, and they are very essential for the survival of *D. appendiculata* (AZAM *et al.*, 1983; POMEROY *et al.*, 2007). These processes can facilitate the bioaccumulation of metals in other organisms of the trophic web (HASSEN *et al.*, 1998; KENNISH, 2002).

MADONI postulated in some publications (MADONI *et al.*, 1992; MADONI, 2000; MADONI & ROMEO, 2006) that simple washing could remove

the naturally associated bacteria from ciliate protists. However, they remained associated with *D*. *appendicuata*, and throughout the bioassay occurred to the enhancement of bacterial biomass content. This process demonstrates the close relationship between protists and bacteria in the microbial loop (Matz & Kjelleberg, 2005).

The ciliates have been identified as significant sources of regenerated nitrogen (Bode *et al.*, 2005), and ciliates and bacteria fulfilled their function in the microbial loop (Pomeroy *et al.*, 2007; Fenchel, 2008). In the presence of Zn and Cu, the naturally associated bacteria may be able to grow vigorously using nitrogen compounds released by ciliates as an energy source (Kirchman, 2000; Hahn & Höfle, 2001). Their biomass content increased, and they are resistant to metal tested according to Harrison *et al.* (2007).

Conclusion

D. *appendiculata* are tolerant to 0.09 mg L^{-1} and resistant to 0.1 mg L^{-1} after 24 h to Zn-Cu probably due to previous selection on Guanabara Bay. Their associated bacteria are resistant too and important in ciliate biomass production. This work indicated that some microorganism in the base of microbial loop works during Zn and Cu exposition, including at the Zn limit concentrations established by CONAMA, but ciliate biomass is strongly reduced by these higher concentrations. However, the Guanabara Bay is seriously polluted, even losing its microbial diversity. This work serves to indicate the direction of further work with the focus of operation at the base of the microbial loop during the stress of pollutants, which can generate new tools for bioremediation.

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