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Cytotoxicity evaluation of Amoxicillin and Potassium Clavulanate in *Perna perna* mussels

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Abstract

Pharmaceutical compounds are identified in environmental matrices in the order of magnitude of ng L^{-1} to µg L^{-1}. Among the drugs, antibiotics have been receiving special attention due to the problems caused to aquatic biota. The aim of this study was to evaluate the cytotoxicity of Amoxicillin and Potassium Clavulanate, in isolated and associated forms, to marine mussels *Perna perna* through neutral red retention time assay (NRRT), which assesses the stability of lisossomal membrane of test-organisms hemocytes. Amoxicillin caused cytotoxicity to the mussels in concentrations of OEC= 1 ng L^{-1}, $IC_{25\text{-}24h}$= 0.44 ng L^{-1}, $IC_{25\text{-}48h}$= 1.19 ng L^{-1} and $IC_{25\text{-}72h}$= 0.85 ng L^{-1}, Potassium Clavulanate was cytotoxic at concentrations of OEC= 10 ngL^{-1} in 24h; 50 ng L^{-1} and 100 ng L^{-1} at 48h and 72h. The inhibitory concentration values (IC_{25}) were: 3.11 ng L^{-1}, 3.45 ng L^{-1} and 3.43 ng L^{-1} at 24h, 48h and 72h respectively. In the test conducted with the combination of drugs, all concentrations were cytotoxic to mussels in 48h. In 72h only 40 ng L^{-1} Amoxicillin + 10 ng L^{-1} Potassium Clavulanate and 200 ng L^{-1} Amoxicillin + 50 ng L^{-1} Potassium Clavulanate were cytotoxic. The IC_{25} for Amoxicillin was 1.67 ng L^{-1} in 48h and 1.36 ng L^{-1} in 72h. For Potassium Clavulanate was 0.42 ng L^{-1} in 48h and in 72h was 0.34 ng L^{-1}.

Key-words: Cytotoxicity; *Perna perna*; Neutral Red; Amoxicillin; Potassium Clavulanate; Pharmaceutical compounds; Micropollutant.

INTRODUCTION

Pharmaceutical compounds are produced to accomplish a specific therapeutical purpose, however, after the consumption are excreted almost unaltered (Cooper *et al*., 2008). They are part of a micro pollutants class that is found in low concentrations in the aquatic environment, in the order of nanograms to micrograms per liter (Bila & Dezotti, 2003).

The current model applied in sewage treatment plants is not sufficient to remove all drug residues. The effluents obtained after the treatment are discharged in environment water such as rivers and seas, which provide the persistence of these substances. The presence of these compounds originated from illegal sewage systems, the leaching of contaminated soil, and incorrect discharge of drugs have aroused the attention of the scientific community, once that it is necessary to understand the damages caused to biota and water quality. This way preventive and mitigatory action can be taken to control the conscious use of medications and their correct way of disposal and removal (Cooper *et al*., 2008; Regitano & Leal, 2010).

The antibiotics have been receiving more attention once the low concentrations allow the formation of more resistant bacteria lines (Zou *et al*., 2011). The antibiotics are also used in veterinary, agriculture and aquaculture (Kümmerer, 2009). In this group, the beta-lactam class penicillin is the best known. This class is considered a milestone since their discovery by Alexander Fleming, for it allowed advances in infection treatments, and is still used nowadays (Ozcengiz *et al*., 2013). Amoxicillin is the most representative drug due to its wide prescription and worldwide use. It acts by

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inhibiting the cell wall synthesis of Gram-positive and Gram-negative bacteria.

Amoxicillin has already been detected in some regions at concentrations as:< 10 ng L^{-1} in surface water in Northwest Germany (Christian *et al*., 2003), > 120 ng L^{-1} in sewage treatment plants in Italy (Andreozzi *et al*., 2004), 190 ng L^{-1} in Australia and 12.64 µg L^{-1} for affluent wastewater (Watkinson *et al*., 2007; Lee *et al*., 2008).

Nonetheless, with the resistance of some microorganism to Amoxicillin, it has become necessary to include another component to improve the effectiveness of the drug. Consequently, the compound found was the Clavulanic Acid isolated from *Streptomyces clavuligerus* bacteria (Saudagar *et al*., 2008).

The Clavulanic Acid is used in the form of Potassium Clavulanate. By itself, this compound has weak antibiotic action, however, associated to Amoxicillin, it inhibits the beta-lactamase enzyme, which interrupts the peptioglycan synthesis that is part of the structure of the cell wall.

The environmental information is scarce for this drug, although, based on this drug's consumption data in France, Besse & Garric (2008) estimated the presence of Potassium Clavulanate on surface water at a concentration of 520 ng L^{-1}.

In marine environmental studies, mussels are frequently used due to their characteristics as bioindicator organisms and their potential to accumulate xenobiotics on their tissue (Pereira *et al*., 2014). In Brazil, the *Perna perna* species is the most used due to its abundance.

The biomarkers help on the early warning adverse effects of a compound. Among the biomarkers, through the neutral red retention time (NRRT) assay which assesses the stability of hemocytes lysossomal membrane has been used in environmental studies. The lysosome is the responsible organelle for encapsulation of xenobiotics, however, in the presence of a toxic compound, the lysosomal membrane permeability becomes brittle and all the absorbed content is extravasated to the cytosol of the cells (Lowe *et al*., 1994).

The aim of this study was to evaluate the cytotoxicity of Amoxicilin and Potassium Clavulanate, in isolated and associated forms, through the NRRT assay, which assesses the stability of lisossomal membrane in mussels *Perna perna* hemocytes.

MATERIAL AND METHODS

Collection of water and organisms

For the assays, natural marine water and mussels *Perna perna* from a farm in Toque Toque Grande, in São Sebastião, coastal city in São Paulo, were used.

Marine water was filtered in 120 µm plankton net for removal of particles and then physicochemical parameters were adjusted: salinity 35 ± 2; dissolved oxygen > 5; pH ≥ 8.00. The organisms were kept in a tank with flowing marine water.

Solutions preparation

Amoxicillin, Potassium Clavulanate and all reagents used were purchased from Sigma Aldrich Brazil.

The physiological saline solution was prepared in distillated water in a volumetric flask of 1 L. The used reagents are described in Table 1. The pH was adjusted to 7.36 with addition of Sodium Hydroxide (NaOH) or Hydrochloric Acid (HCl).

Table 1 – Reagents used in the preparation of physiological saline solution.

Reagents	**Mass (g)**
Calcium chloride	1.47
Sodium chloride	25.48
Potassium chloride	0.75
HEPES	4.77
Magnesium sulfate	13.06
Neutral Red	0.028
Dimethylsulfoxide	1 mL

Stock solutions for each drug were prepared in the concentration of 1 mg L^{-1} in sea water.

Cytotoxicity assay

The assays were carried out in glass flasks with 9 organisms in 3 L of solution, in triplicate. During the assay the temperature was maintained at 22º ± 2º C, photoperiod of 12h light/dark, and organisms were not fed. The analysis of samples occurred in 24, 48 and 72h with changes of solutions every 24h. The drugs concentrations are presented in Table 2 and the assay flowchart are described in figure 1.

Table 2 – Amoxicillin and Potassium Clavulanate concentrations in isolated and associated forms.

Compound	Test concentration
Amoxicillin	1 ng L^{-1}, 5 ng L^{-1} and 10 ng L^{-1}
Potassium Clavulanate	10 ng L^{-1}, 50 ng L^{-1} and 100 ng L^{-1}
Amoxicillin (Amox) and Potassium Clavulanate (Clav)	4 ng L^{-1} Amox + 1 ng L^{-1} Clav (I) 40 ng L^{-1} Amox + 10 ng L^{-1} Clav (II) 200 ng L^{-1} Amox + 150 ng L^{-1} Clav (III)

Every 24h was removed 0.5 mL of haemolymph from each organism utilizing hypodermic syringe containing 0.5 mL of physiological saline solution, and kept in 2 mL Eppendorf tubes. Subsequently, an amount of 40 µL of each sample was transferred to slides, pre-treated with poly-L-lysine. The slides were placed in a humid, light-proof chamber for 15 minutes so the cells could adhere to it. After this time, the excess liquid was withdrawn and 40 µL of NR solution (10 µL stock solution of neutral red dye in 5 mL of saline) were added. The slides remained in the chamber and, every 15 minutes,

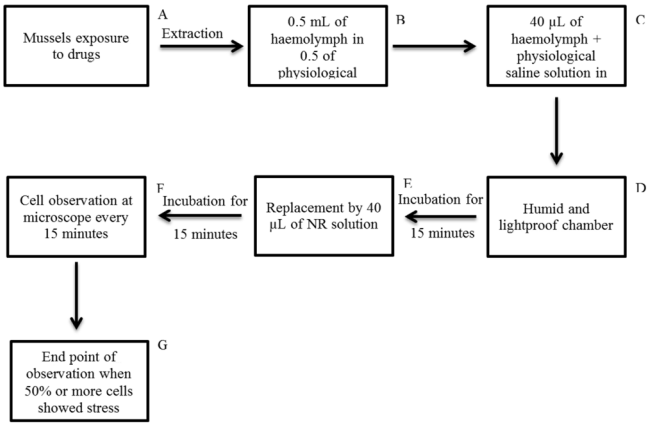

Figure 1 – Flowchart of NRRT assay

during 180 minutes, were analyzed in light microscope at 400x magnification, taking care to not expose excessively to light, to prevent photodegradation of the dye.

The results were placed in Tables using the evaluation criteria: "+" for healthy cells, "±" for those that showed signs of stress and "-" for 50% or more stressed cells and showing leakage of lysosomal content. The assay was concluded when all the slides presented "-" or after a period of 180 minutes. Images of healthy and stressed cells obtained in the assays are shown in figure 2.

Cells accepted as healthy were those wide, irregular cells with many small lysosomes of light red color. The stressed cells had become rounded and smaller, few lysosomes in extended sizes, deep red color and dye extravasation.

Statistical analysis

The average values of retention times were calculated and evaluated for normality by Chi-square method and homogeneity of variance by Bartlett's test. These data were placed on the statistical program TOXSTAT 3.5 to obtain the observed effect concentration (OEC) which is the lowest test concentration capable to cause adverse effects, and the non-observed effect concentration (NOEC), which is the highest concentration-test that does not cause adverse effects. The damage caused by drugs was also presented by 25 % Inhibitory Concentration (IC_{25}), the drug concentration which inhibit 25 % of lysosomal membrane stability measured by the NRRT assay. The IC_{25} were calculated and evaluated by linear interpolation method in ICPIN statistical program.

RESULTS AND DISCUSSION

Amoxicillin

In the assay period of 24h, 48h and 72h Amoxicillin caused cytotoxicity to mussels in all concentrations to which the organisms were exposed. Thus, in the experimental used conditions, it was not possible to calculate the non-observed effect concentration (NOEC) for this compound. Table 3 shows IC_{25} data with confidence intervals and coefficient of variation during the exposure times of the mussels to Amoxicillin and Figure 3 present the results graphically. In the figure 3 it was observed that with the increasing concentration of Amoxicillin there was decreasing of the retention time.

Amoxicillin is frequently described in the literature as a compound that does not interfere significantly with non-targets organisms, once this drug toxicity has a concentration level of mg L^{-1}, that are not likely to occur in the environment. Among the tests carried out with Amoxicillin are:

i) Algal growth inhibition assays with *Microcystis aeruginosa*, EC_{50}= 0.037 mg L^{-1}, *Selenastrum capricornutum*, NOEC > 250 mg L^{-1}, *Rhodomonas salina,* EC_{50}= 3.108 mg L^{-1} and *Synechococcus leopoliensis*, EC_{50}= 2.22 µg L^{-1}, CEO= 1.56 µg L^{-1} and NOEC= 0.78 µg L^{-1} (Lutzholft *et al*., 1999; Andreozzi *et al*., 2004).

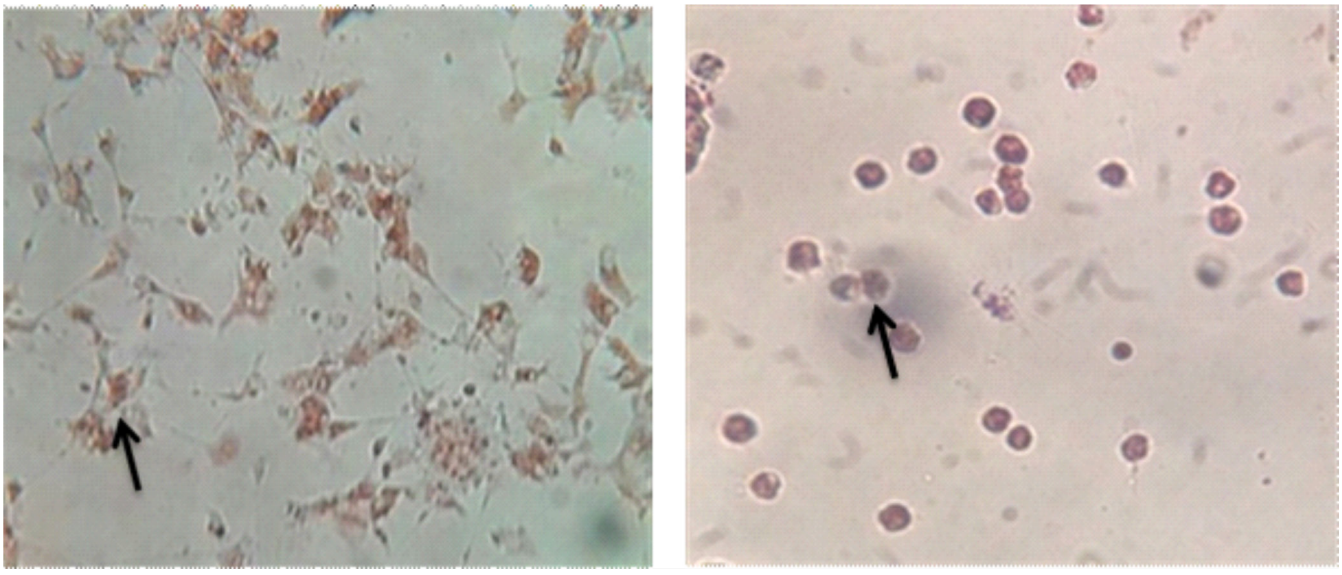

Figure 2 – Healthy and stressed cells (arrow), observed in the optic microscope (400x).

Table 3 – Amoxicillin Inhibitory Concentrations (IC_{25}) data.

Period	IC_{25} average value ± SD (ng L^{-1})	Confidence limit	VC (%)
24 h	0.44 ± 0.04	0.370 - 0.522	10
48 h	1.19 ± 0.91	0.536 - 4.125	77
72 h	0.85 ± 0.09	-	10

SD = Standard desviation; VC = Variation coefficient

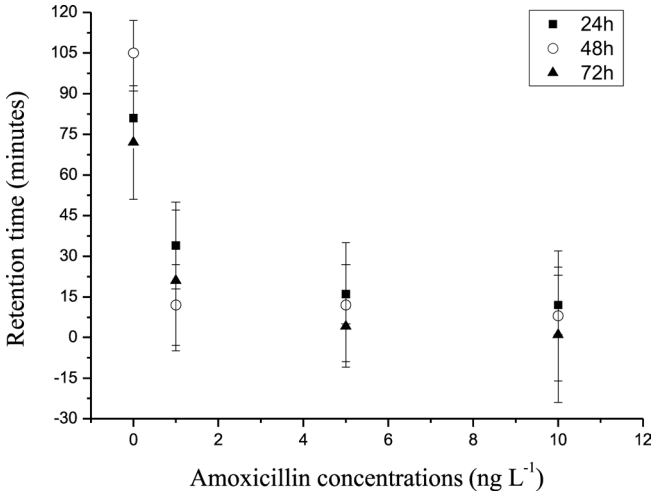

Figure 3 – Graphical representation of retention time in relation to Amoxicillin concentration.

ii) Evaluation of photosynthesis steps of *Synechocystis* sp. with damage from 50 mg L^{-1} (Pan *et al*., 2008).

iii) Studies with zebrafish, *Danio rerio*: embryos premature hatching ($EC_{50(48h)}$ = 132.4 mg L^{-1}), edema and deformities in the tail at concentrations of 221 mg L^{-1}, 380 mg L^{-1}, 654 mg L^{-1} and 1.125 mg L^{-1}, inhibition of catalase enzyme in tissues of the head and gills, as well an increase of glutathione S-transferase in tissues of adult muscle and gills exposed organisms to 1 mg L^{-1} (Oliveira *et al*., 2013).

iv) Effects on embriolarval of *Perna perna* mussels development, OEC= 1000 mg L^{-1} and IC_{50} = 835 mg L^{-1} (Bento, 2015).

However, the present study showed that concentrations in the order of ng L^{-1} of Amoxicillin caused cytotoxicity to mussel's hemocytes by the NRRT assay. These results are very similar to the occurrence in the environment such as the surface water in Northwest Germany < 10 ng L^{-1} (Christian *et al*., 2003), sewage treatment plant in Italy > 120 ng L^{-1} (Andreozzi *et al*., 2004) and affluent wastewater in Australia 190 ng L^{-1} (Watkinson *et al*., 2007), which demonstrates the risk of this antibiotic on the aquatic system.

Potassium Clavulanate

Potassium Clavulanate caused cytotoxicity to mussels at concentration of 10 ng L^{-1} in 24h and 48h periods. Under the experimental conditions used it was not possible to calculate the NOEC. At 72h the cytotoxic concentrations were 50 ng L^{-1} and 100 ng L^{-1} and NOEC was 10 ng L^{-1}. Table 4 presented IC_{25} data with confidence intervals and coefficient of variation during the exposure times of the mussels to Potassium Clavulanate and Figure 4 presented the results graphically.

The environmental data for Potassium Clavulanate are scarce once the major part of studies are focused on the medical area. The result obtained in this paper was toxic; so the level of concentrations was about 50 times more toxic than in the paper of Besse & Garric (2008) that presented the result of environmental prediction (520 ng L^{-1} in superficial water in France). These results demonstrated that it is necessary further studies about this compound and the possible damages to organisms.

Amoxicillin and Potassium Clavulanate

The assay carried out with the mixture of Amoxicillin and Potassium Clavulanate presented cytotoxicity to organisms in all test-concentrations in 48h and, in these experimental conditions used, the NOEC were not possible to be calculated. In 72h only the lowest mixture concentration of Amoxicillin 1 ng L^{-1} + Potassium Clavulanate 4 ng L^{-1} was not cytotoxic to mussels. Table 5 and 6 presents IC_{25} data with confidence intervals and coefficient of variation during the exposure times of the mussels to association of Amoxicillin and Potassium Clavulanate and Figure 5 shows the graphical results.

In the figure 5 it was observed that retention time decreased with the increasing concentrations and exposition period of Amoxicillin and Potassium Clavulanate. The drugs in

Table 4 – Potassium Clavulanate Inhibitory Concentrations (IC_{25}) data.

Period	IC_{25} average value ± SD (ng L^{-1})	Confidence limit	VC (%)
24 h	3.11 ± 0.29	2.763 - 3.654	9
48 h	3.45 ± 0.35	2.920 - 4.156	10
72 h	3.43 ± 0.24	3.139 - 4.113	7

SD = Standard desviation; VC = Variation coefficient

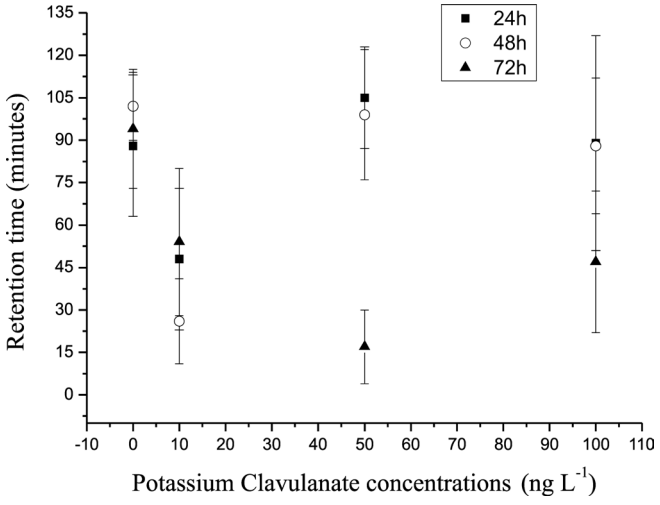

Figure 4 – Graphical representation of retention time in relation to Potassium Clavulanate concentrations.

association presented more toxicity than when tested isolated in the cytotoxicity test by NRRT method.

The toxic potential of these drugs and cytotoxic results are possibly related to the degradation of them. Amoxicillin and Potassium Clavulanate have a low half-life, approximately 1 hour, and are more stable under acidic conditions. Thus, the alkalinity of the sea water used in the assays, and high water solubility of these drugs favored the decomposition and byproducts formation. Amoxicillin generates penicilloic acid and phenol hidroxipirazin in the early stages of decomposition. From the penicilloic acid formed the peniloic acid and 2',5'- diketopiperazine. Potassium Clavulanate forms pyrazine (Haginaka *et al*., 1985; Gozlan *et al*., 2013). The lack of data on the effects of these byproducts makes it difficult to conduct a larger discussion.

The application of NRRT assay to drug toxicity for mussels was successful, with efficient responses about effects of Omeprazole, Ibuprofen, Diclofenac and Paracetamol (Mathias *et al*., 2012; Gaspar, 2015; Mazur, 2015; Fontes *et al*., 2015).

The lysosome is the first cell organelle affected by the presence of xenobiotics, since its mechanism of action is to encapsulate these compounds as a cell defense, but, when there is continuous exposure to a disturbing homeostasis agent, damage becomes irreparable and the entire function is compromised, which may cause cell death (Lowe *et. al*, 1995).

The socioeconomic factor actions of pharmaceuticals compounds in the environment should be considered, especially to antibiotics, which are related to bacterial resistance. Zhang *et al*. (2009) collected samples of wastewater effluents and in the receiver river of this material in the United States; strains isolated of *Acinetobacter* spp from collection sites, and verified that almost all strains were resistant to antibiotics Trimethroprim, Rifampicin, Chloramphenicol and Amoxicillin with Potassium Clavulanate. In Rio de Janeiro, Brazil, the Oswaldo Cruz Foundation (Fiocruz) (2014), detected a bacteria that produce KPC enzyme (Carbapenemase) in different points of the Carioca River, which flows into the Flamengo Beach. These bacteria were also found in hospitals and presented high resistance to administered treatments, being responsible for the death of many patients.

Prevention and mitigation measures are needed to decrease improper discharge of compounds and the conscious use of drugs. It is necessary to remember that the focus of this study was only Amoxicillin and Potassium Clavulanate interactions, but the degradation compounds, formation of byproducts and association which can occur in the environment are issues that need more investigation to be further elucidated.

Table 5 – Inhibitory Concentrations (IC_{25}) data of Amoxicillin in the assay with Amoxicillin and Potassium Clavulanate association.

Period	IC_{25} average value ± SD (ng L^{-1})	Confidence limit	VC (%)
48 h	1.67 ± 0.15	1.46 - 2.04	9
72 h	1.36 ± 0.09	1.22 - 1.56	7

SD = Standard desviation; VC = Variation coefficient

Table 6 – IC_{25} data of Potassium Clavulanate to assay with Amoxicillin and Potassium Clavulanate association.

Period	IC_{25} average value ± SD (ng L^{-1})	Confidence limit	VC (%)
48 h	0.42 ± 0.05	0.35 - 0.52	12
72 h	0.34 ± 0.02	0.30 - 0.39	6

SD = Standard desviation; VC = Variation coefficient

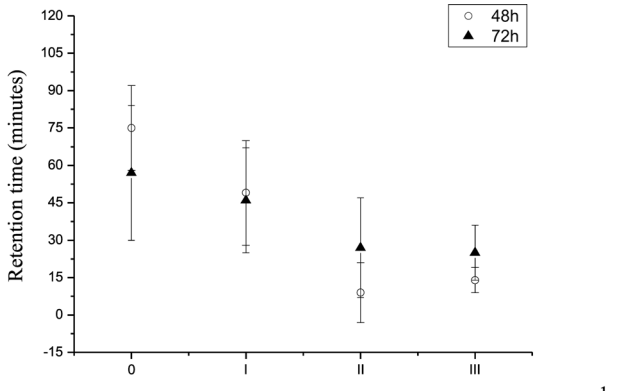

Figure 5 – Graphical representation of retention time in relation to the concentrations of Amoxicillin and Potassium Clavulanate association.

CONCLUSION

The antibiotics Amoxicillin and Potassium Clavulanate showed cytotoxic effect to *Perna perna* mussels in the cytotoxicity assay by NRRT method. The drugs presented about four times more toxicity in association than when tested isolated.

The NRRT biomarker is a good indicator of adverse effects, with accurate results that allows verify the compounds toxic potential to organisms and aquatic environment.

The mussels are good models of environment indicators due to their sensibility, easy handle, obtainment and representativity to biota.

This study allowed to determinate the drugs toxicity, an important result to prevent environment damages. These data could be complemented with analysis of compounds environmental risks, and could contribute to future aquatic environment ecotoxicity decision.

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