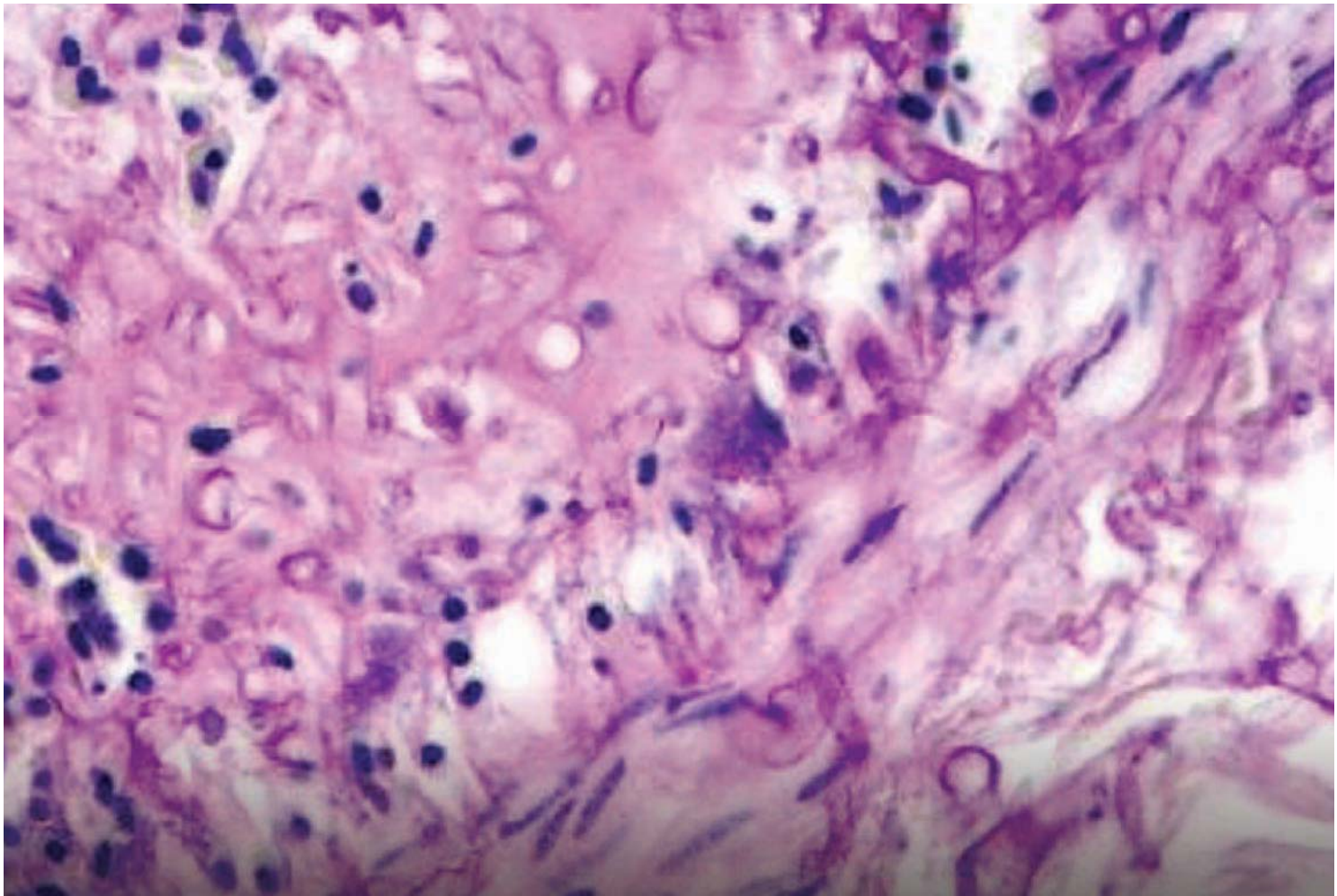

A. Mendez-Vilas
editor

Microorganisms in Industry and Environment

From Scientific and Industrial Research to Consumer Products

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From Scientific and Industrial Research to Consumer Products

Proceedings of the III International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2009)

Lisbon, Portugal 2 – 4 December 2009

editor

A. Mendez-Vilas

Formatex Research Center, Spain & University of Extremadura, Spain

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Introduction

This book contains a selection of works that were presented during the third International Conference on Environmental, Industrial and Applied Microbiology (BioMicroWorld2009), which took place at the University of Lisbon, in Lisbon (Portugal), from 2–4 December 2009. The conference was attended by more than 600 participants from 54 countries, thus consolidating the BioMicroWorld conference series, initiated in Badajoz (Spain) in 2005. The conference presentations and discussions were structured around different general and specific themes:

• Environmental Microbiology. Geomicrobiology • Agriculture, Soil, Forest Microbiology • Food Microbiology • Industrial Microbiology — Future Bioindustries • Methods — Analytical & Imaging Techniques • Medical & Pharmaceutical Microbiology. Antimicrobial Agents • Microbial Physiology, Metabolism and Gene Expression • Biofilms & Antimicrobial Surfaces • Bioremediation • Biotechnologically Relevant Enzymes and Proteins • Microfactories — Microbial Production of Chemicals and Pharmaceuticals.

The Conference could not be as successful as it was without the work of a group of professionals and researchers forming the conference Scientific Advisory Committee in charge of selecting the most relevant works submitted to be considered for presentation during the conference. In this edition, the members were:

Jose Luis Martinez, National Center for Biotechnology, Spain; **Kaarina Sivonen**, University of Helsinki, Finland; **Nuno F. Azevedo**, University of Porto, Portugal; **Yan Zhang**, Peking University, China; **Pei-Yuan QIAN**, The Hong Kong University of Science and Technology, Hong Kong; **Sergey V. Kalyuzhnyi**, Moscow State University, Russia; **Hong Kai WU**, The Hong Kong University of Science and Technology, Hong Kong; **Yves Blache**, Université du Sud Toulon-Var, France; **Megharaj Mallavarapu**, University of South Australia, Australia; **Kostas Koutsoumanis**, Aristotle University of Thessaloniki, Greece; **Gerardo R. Vasta**, University of Maryland, USA; **Elke Nevoigt**, Catholic University of Leuven, Belgium; **Peter Gerner-Smidt**, Centers for Disease Control and Prevention, USA; **Rosario Muñoz**, Institute of Industrial Fermentation, Spain; **Jan Michiels**, Catholic University of Leuven, Belgium; **Alban Ramette**, Max Planck Institute for Marine Microbiology, Germany; **Sigrid De Keersmaecker**, Catholic University of Leuven, Belgium; **Nigel Robinson**, Newcastle University, United Kingdom; **Ramesh C Kuhad**, Universiy of Delhi South Campus, India; **Raeid M. M. Abed**, Sultan Qaboos University, Sultanate of Oman; **Petr Baldrian**, Institute of Microbiology ASCR, Czech Republic; **Joseph Kreit**, Mohammed V University, Morocco; **Pilar García**, Asturias Dairy Products Institute, Spain; **Wim Crielaard**, Academic Center for Dentistry Amsterdam, Netherlands; **R. Kumar Malik**, National Dairy Research Institute, India; **Juan José Valdez Alarcón**, Michoacana University of Saint Nicolas Hidalgo, Mexico; **Rakesh K. Jain**, Institute of Microbial Technology, India; **Badal C. Saha**, National Center for Agricultural Utilization Research, USA; **Bo Mattiasson**, Lund University, Sweden; **Essaid Ait Barka**, University of Reims, France; **Ibrahim Banat**, University of Ulster, United Kingdom; **Ece Karatan**, Appalachian State University, USA; **Hermann J. Heipieper**, Helmholtz Centre for Environmental Research - UFZ, Germany; **Carme Plumed-Ferrer**, University of Kuopio, Finland; **Filip Boyen**, Gent University, Belgium; **Chao-Ying Chen**, National Taiwan University, Taiwan; **Rodney M. Donlan**, Centers for Disease Control and Prevention, USA; **Bruce A. Maguire**, Pfizer Global Research and Development, USA; **Douglas B. Weibel**, University of Wisconsin-Madison, USA; **Veronica Arthurson**, Swedish University of Agricultural Sciences, SLU, Sweden; **Götz Haferburg**, Institute of Microbiology at the Friedrich Schiller University of Jena, Germany; **Agneta Richter-Dahlfors**, Swedish Medical Nanoscience Center, Sweden; **Anil Kumar Puniya**, National Dairy Research Institute, India; **Renu Agrawal**, Central Food Technological Research Institute, India.

There were three Plenary Lectures during the conference, one per day, which attracted a large percentage of the registered participants. They were excellent examples of currently relevant interdisciplinary research in applied microbiology:

Hermann Heipieper from the Helmholtz Centre for Environmental Research – UFZ, Germany, talked about "Microbial Adaptation to Toxic Organic Solvents — Mechanisms and Biotechnological Applications".

Stefan Dübel from the Institute for Biochemistry and Biotechnology, Technical University of Braunschweig, Germany, talked about "Antibodies from Bacteria".

Rosário de Oliveira from the Department of Biological Engineering, University of Minho, Portugal, talked about "Insights into the Biofilm World".

Last but not least, we acknowledge the support of the sponsors of this edition, namely, Bertin Technologies (http://www.bertin.fr) and the organizer of the 13th International Symposium on Microbial Ecology (ISME13, http://www.isme-microbes.org/isme13). The support of publishers Springer, Elsevier, and Science Publications, through their journals *Biodegradation*, *Journal of Industrial Microbiology & Biotechnology*, *Journal of Biotechnology*, and *American Journal of Agricultural and Biological Science*, is also acknowledged.

We hope the current book accurately reflects some of the major current topics covered in the conference and we look forward to receive your presentations at the next BioMicroWorld edition.

Antonio Méndez-Vilas
BioMicroWorld2009 General Coordinator, on behalf of the Organizing Committee
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Quantification and toxicity testing of pharmaceuticals in tropical marine sediments, All Saints Bay, Bahia, Brazil

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The presence and effects of pharmaceuticals in the environment have gained increased attention lately because of their widespread use and their potential to bioaccumulate and induce negative effects in aquatic and terrestrial ecosystems. This work presents the quantification of pharmaceutical compounds in sediments of All Saints Bay, Bahia, Brazil and toxicity testing of the compounds Atenolol, Erythromycin and Caffeine on sediment microorganisms and microcrustaceans (*Artemia salina*). Pharmaceuticals were shown to be widely distributed in sediments of All Saints Bay, in areas near the capital Salvador as well as in more remote areas of the bay. Toxicity tests revealed a susceptibility of microorganisms and microcrustaceans to both Erythromycin and Caffeine at higher concentrations, while Atenolol did not cause toxic effects. The presence of pharmaceuticals at the levels detected did not imply acute toxicity to microorganisms and microcrustaceans.

Keywords: pharmaceuticals; tropical marine sediment; microbial toxicity; brine shrimp assay.

1. Introduction

Until the 1990s pharmaceuticals did not attract significant attention as a potential group of environmental contaminants. This changed when it was discovered that some compounds have the ability to interfere with the ecosystem even in lowest concentrations [1] and with the development of more sensitive analytical techniques. The main routes by which these compounds enter aquatic ecosystems are municipal wastewaters, but they are also introduced to the environment by disposal of unused or expired medicines, wastewater from manufacturers and landfill leachates [2,3]. As pharmaceuticals are developed to have some kind of biological function and persistence in the organisms applied to, they also have a potential to bioaccumulate and induce effects in aquatic and terrestrial ecosystems [1]. Monitoring studies have shown that pharmaceuticals and their metabolites are very resistant to most water treatment techniques and are present in all kinds of aquatic systems [4,5].

Biodegradation by microorganisms is the main mechanism to eliminate organic compounds from the environment. The main groups involved in degradation processes are bacteria and fungi, the latter being predominant in soils, while bacteria are of major importance in the aquatic environment, including wastewater treatment [6]. Bioactive compounds as pharmaceuticals may have toxic effects on bacteria, especially the antibiotics that are designed to combat bacterial diseases and thereby may interfere with the degradation processes of these substances in the environment. Data on toxic effects of pharmaceuticals on sediment bacteria are necessary to evaluate the fate, persistence and thereby the risk inherent to these compounds in the environment.

In Brazil, data on the presence of pharmaceuticals in the aquatic environment are still scarce, while the consumption of these compounds suffers little control and disposal of expired drugs is indiscriminate. The Brazilian government does not make demands on security or quantity limits regarding the discharge of pharmaceuticals to water bodies. Most of the data available refer to the subtropical South of Brazil [7,8].

The objective of this study was to identify and quantify the pharmaceuticals present in the tropical marine environment of All Saints Bay as well as evaluate the toxicity of some of these compounds to marine microcrustaceans and sediment microorganisms.

2. Materials and Methods

2.1 Study area

All Saints Bay is located in the state of Bahia, in the tropical northeast of Brazil, on 12°50'S and 38°38'W. It is the second largest bay in Brazil, with an extension of 1233 km² and an average depth of 9.8 m. The bay has a shoreline of 300 km and contains fifty-six islands. The largest terrestrial oil reservoirs of Brazil are located in the adjacent continent Part of the bays shoreline adjoins the third major metropolitan area in Brazil, the city of Salvador and the largest petrochemical complex in the southern hemisphere (Camaçari Petrochemical Center) [9]. Most important harbors within the bay are the harbor of the city of Salvador and the harbor of Aratu.

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Sediment structure varies from muddy to coarse sand. Muddy bottom sediment predominates in the northern part of the bay, whereas its southern part presents an accumulation of medium to very coarse sands. Since its discovery in the sixteenth century, All Saints Bay has been heavily used, from mining of calcareous sands, to petroleum exploration, over fishing, industrial sewage and intensive tourism [10].

Seventeen sampling points throughout the bay were selected for the determination of pharmaceuticals in sediments (Fig. 1).

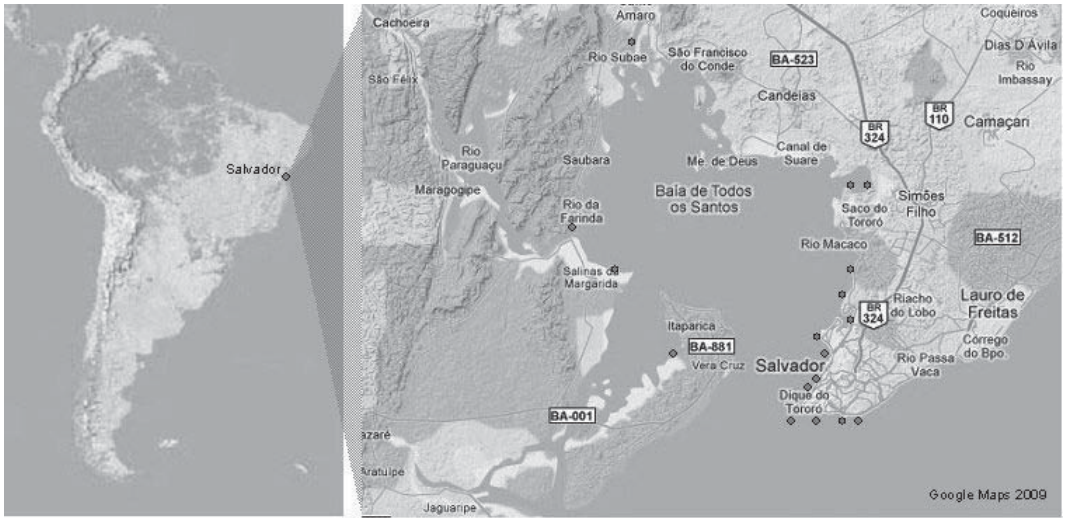

Fig. 1 Location of the 17 sampling points in All Saints Bay, State of Bahia, Brazil.

2.2 Quantification of pharmaceuticals

Sediment from 17 sampling points in the All Saints Bay were analyzed for the occurrence of pharmaceutical compounds: Samples were extracted in ultrasound, filtered, purified and quantified by Liquid Chromatography coupled to tandem Mass Spectrometry (LC-MS/MS), and Gas Chromatography and Mass Spectrometry (GC/MS). Analyses were realized at the University of La Coruña, Spain.

2.3 Toxicity tests

Test organisms (brine shrimp and sediment microorganisms) were exposed to Atenolol, Erythromycin and Caffeine in concentrations ranging between 0.002 and 2.0 mg/ml. Solutions of Atenolol and Erythromycin were made by dissolving commercially available pharmaceuticals, while caffeine was used as a pure substance. The concentration range tested was determined according to preliminary sensitivity tests. The microcrustacean *A. salina* was exposed to solutions of Atenolol and Erythromycin of 0.125 mg ml^{-1}, 0.25 mg ml^{-1} and 0.5 mg ml^{-1} and to Caffeine in concentrations of 2.0 mg ml^{-1}, 0.2 mg ml^{-1} and 0.02 mg ml^{-1}. For tests with sediment microorganisms, concentrations of 0.002 mg ml^{-1}, 0.02 mg ml^{-1} and 0.2 mg ml^{-1} were used for the three pharmaceuticals.

Cysts of *Artemia salina* were hatched in artificial seawater (Tetra Marine Salt) and 24 h old nauplii were used for toxicity testing in the Brine Shrimp Assay [11]. Five nauplii of *Artemia salina* were placed in 10 ml of test solutions and in artificial seawater as controls. After 48 hours, surviving nauplii were counted. All experiments were conducted in triplicate.

Toxicity to sediment microorganisms was tested with sediments collected from the shore area of All Saints Bay. 0.25 to 0.5 g of marine sediment was suspended in 100 ml of sterile marine water. The microorganisms in 1 ml of sediment suspension were exposed to varying concentrations of Atenolol, Erythromycin and Caffeine in solution, added to Plate Count Agar (3.5% NaCl) by the pour plate technique [12]. Controls received sediment suspension and sterile diluting water on Plate Count Agar. For each dilution, plates were prepared in duplicate and incubation was realized at 35°C. After 24 hours, colony-forming units (CFUs) on the 65-cm^2 plates were counted with a Darkfield Quebec Colony Counter. On plates with less than 10 CFUs, 13 cm^2 with a representative colony distribution were counted, while on plates with more than 10 CFUs only 4 cm^2 were counted and results were extrapolated to the whole plate area of 65 cm^2. Thus results could be expressed in CFU ml^{-1}.

3. Results and Discussion

3.1 Quantification of pharmaceuticals

Atenolol and Caffeine were present at all seventeen sampling points in All Saints Bay (BTS), while Erythromycin was only present at 29% of the sampling sites. Atenolol was also predominant with a mean concentration of 6.0 ng g^{-1} dry weight (dw). Caffeine, which has been shown to be an effective tracer of organic pollution from wastewater sources [13], was present in all samples with a mean concentration of 2.57 ng g^{-1} dry weight and reaching maximum concentrations of 23.45 ng g^{-1} dw (Fig. 2). These findings are consistent with/ contrary to concentrations measured by Stackelberg [14] in United States in 2007.

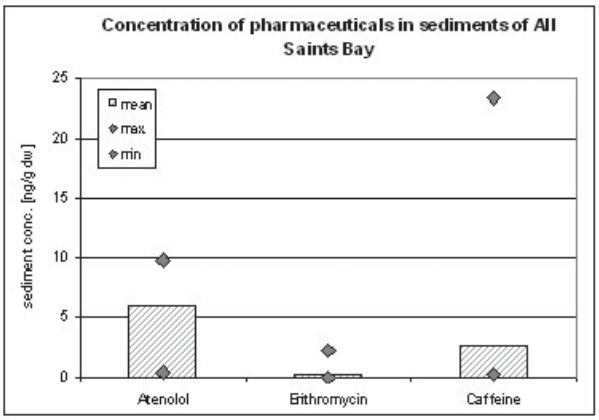

Fig. 2 Concentration of Atenolol, Erythromycin and Caffeine in sediments of 17 sampling points in All Saints Bay. Columns show the mean concentration of all sampling points; Points indicate the highest and lowest concentrations measured.

Atenolol belongs to the beta-blockers and is a commonly used pharmaceutical in cardiovascular diseases. Erythromycin is a macrolide antibiotic and inhibits protein synthesis interacting with bacterial ribosomes. Caffeine is most commonly consumed as an ingredient of coffee, tea, chocolate and soft drinks, but is also medicinally used as a cardiac, cerebral, and respiratory stimulant and also functions as a diuretic [15]. Due to its high consumption rate it may serve as a tracer of organic pollution [16].

Pharmaceuticals enter the environment primarily through wastewater discharges due to their low removal rate in wastewater treatment plants (WWTPs). The removal rate of Atenolol in WWTPs is lower than 10% [17]. Jones [18] gives a total removal of 1.85%, of which only 0.09% is due to biodegradation. Also for Erythromycin total removal rates and total biodegradation rates are low, with 6.23% and 0.13%, respectively. Caffeine on the other hand was proved to be readily biodegradable, resulting in high removal rates during sewage treatment processes [19].

3.2 Toxicity tests

Nauplii or cysts of *Artemia* have been shown to be sensitive to wide variety of contaminants, including antibiotics, and have been found suitable for testing seawater soluble drug pollutants [20,21].

In our toxicity tests with nauplii of *Artemia salina*, a mortality rate of >20% was considered toxic effect. The test organisms showed highest susceptibility to the antibiotic Erythromycin, resulting in more than 90% mortality at 0.5 mg mL^{-1}. Caffeine had a comparable effect, while Atenolol did not seem to cause toxic effects in the concentration range tested, considering the limit of 20%, even though increasing concentration resulted in higher mortality rates (Fig. 3a). Higher concentrations of Atenolol were not tested due to the high turbidity of the solution, which would have made it impossible to count surviving nauplii. LC50 values reported in literature for Atenolol is 33.4 mg L^{-1} for cladocerans and 620 mg L^{-1} for algae [22]. Fish may be more affected by beta-blockers as they contain ß2-receptors in heart and liver [23] and the related drug propanolol indicated chronic toxicity effects on the cardiovascular system [17]. Nauplii of *Artemia salina* were much more sensible to Erythromycin and Caffeine than *Daphnia magna* used in standardized toxicity assessments. Thus, LC50 of Erythromycin for daphnids was reported to be 211 mg L^{-1} [24], while in our experiments the lethal dose for 50% of *Artemia* was between 0.25 and 0.5 mg L^{-1}. For Caffeine LC50 values of 182 mg L^{-1} for cladocerans have been reported [25], while we found that about 0.2 mg L^{-1} of caffeine resulted in 50% mortality of Artemia nauplii. As nearly 84% of caffeine is demethylated to paraxanthine, the concentrations of caffeine found in sediment might be significantly lower than the load of caffeine received by the water column.

During tests with sediment microorganisms, a reduction of CFUs of 20% compared to controls was considered toxic effect. Sediment microorganisms, particularly aerobic superficial colonies were strongly inhibited by the antibiotic Erythromycin at concentrations of 0.2 mg L^{-1}. Caffeine also inhibited bacterial growth

at concentrations ten times higher than the antibiotic (2.0 mg L^{-1}), while Atenolol had no effect on the number of colony forming units (CFUs) (Fig. 3b).

Caffeine has been shown to have antimicrobial effects on a variety of microorganisms, including human pathogens. For example the enterobacteria *Salmonella enterica* was inhibited in growth by a caffeine concentration of 2.6 mg mL^{-1} [26].

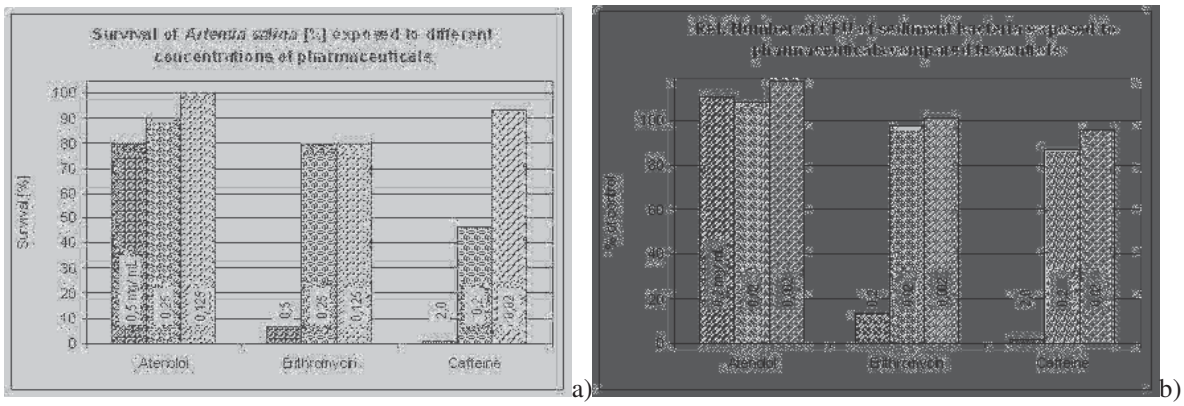

Fig. 3 Results of toxicity test with a) Artemia salina and b) colony forming sediment microorganisms.

The presence of compounds exhibiting antimicrobial activity in the environment is of concern, as microorganisms play a crucial role in degradation and therefore bioremediation processes. Even if the concentrations of these compounds measured in the environment are much lower than inhibition concentrations, the possibility of synergistic and additional effects of several compounds cannot be ruled out. Another concern regarding antibiotics in the environment, is the creation of low-level resistance in bacteria [27].

4. Conclusions

It can be assumed that the presence of pharmaceuticals at the levels detected do not imply acute toxicity to microorganisms and microcrustaceans, because the concentrations tested in toxicity assays were higher than the ones detected in the environment and the lower concentrations did not affect the test organisms.

Nevertheless it cannot be ruled out that these compounds do not have any influence on organisms, such as endocrine disruption, and synergistic or additive toxicity effects interacting in a mixture of chemical compounds. Seiler [28] emphasized the importance of more "mechanism-based" approaches to the investigation of potential environmental hazards, considering that pharmaceuticals, besides the primary effect they are designed for, may cause secondary effects considered irrelevant for the therapeutic activity in humans but may play a role in other organisms. Effects by exposure to pharmaceuticals may be sub-lethal, affecting reproduction or growth and thereby threaten the species survival. These effects may only be detectable during chronic long-term exposure.

Further studies should focus on persistence/ degradation dynamics of these compounds in the marine environment and on their chronic and combined toxicity to marine organisms.

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