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Review

A review on the effects of antimicrobials use in cultures of planktonic organisms: a procedure for ecological experiments

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ABSTRACT. Many questions about the role of planktonic organisms remain unanswered because of the difficulty in obtaining a medium where bacteria and fungi are not present. Moreover, an excess load of these microorganisms in phytoplankton cultures and zooplankton organisms may cause nutrient competition or diseases and consequently the death of the organisms of interest. For this reason, we reviewed several methods that have been used to obtain axenic planktonic cultures through specific metabolic inhibitors, such as antibiotics and antifungal agents. From 1940 to 2016, most research was related to microalgae and crustacean's cultures, with the antimicrobials: penicillin, streptomycin, chloramphenicol, oxytetracycline, neomycin and nystatin being the most frequently used. The studies that applied antimicrobials agents to planktonic cultures were mainly focused on being able to culture them and to answer questions about the role of bacteria in aquatic communities without previous testing their effectiveness or their effects on non-target organisms. Therefore, this review sought to determine the correct use of antimicrobials in cultures of planktonic organisms to prevent bacterial and fungal growth, without causing damage to non-target organisms and may assist in the implementation of ecologically-oriented scientific experiments where bacterial and/or fungal inhibition is necessary.

Keywords: aquaculture; artificial aquatic systems; bacterial and fungal contamination; hypothesis test; production of algae; production of invertebrates

INTRODUCTION

Plankton is the term used for all living organisms floating or drifting in the water column that have little or no ability to swim against currents (Haeckel, 1893), including virus (virioplankton), bacteria (bacterioplankton), filamentous fungi and yeasts (mycoplankton), microalgae (phytoplankton) and phagotrofic organisms (zooplankton) (Johnson & Allen, 2012). These organisms, as a whole, play a vital role in the food chain (classical and microbial loop), in some cases as prey and in others as predators (Hopcroft & Roff, 1998), maintaining the balance of aquatic populations and cycling nutrients in this environment (biochemical cycles) (Calbet & Saiz, 2016).

In addition, plankton plays a key role in benthic-pelagic coupling, marine snow formation and biological pump (Schnack-Schiel & Isla, 2005; Turner, 2015;

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Agostini *et al*., 2018). It can also be used as biological indicators of water masses, ocean currents, pollution and climate changes (Reid & Edwards, 2001; Johnson & Allen, 2012). For this reason, there is great interest in understanding how the processes involving the planktonic community work, and how these organisms intereact with other organisms, being the phytoplankton and zooplankton the groups most studied, usually through the establishment of laboratory cultures.

Laboratory cultivation has been established in order to answer a series of questions about the biology and ecology of these organisms, facilitating genetic, cytology, morphology, physiology, development and taxonomy studies, as well as intra and inter-specific interaction studies (Trottet *et al*., 2011). Furthermore, planktonic organisms are commonly cultivated intensively for use in the aquaculture sector (Schipp *et al*., 1999), principally as the live food of commercial species of fish, mollusks and crustaceans (Creswell, 2010). However, when at high densities these cultures result in large loads of waste and organic matter (Olafsen, 1993), favoring the proliferation of virioplankton, mycoplankton and particularly free and/or adhered bacterioplankton, which can benefit or harm the culture, depending on the researcher aim.

Effects of bacteria and fungi in cultures

In aquaculture, for example, the biofloc tec (BFT) system comprises an association (aggregates) of bacteria, phytoplankton, fungi, virus and particulate organic matter, as well as bacterial grazers which benefit shrimp culture (Hargreaves, 2006). These aggregates or biofilms (bacteria systematically arranged in a self-produced polymeric matrix) (Vasudevan, 2014) that develop in the walls of the production tanks of crustaceans and fish reduce the cost of production by minimizing the overload of waste components, consequently reducing water exchange (Pandey *et al*., 2014).

Even though bacteria in BFT and biofilm in tanks formation can be considered to be beneficial in aquaculture, it is notable that in small-scale laboratory cultures, bacterial loads at high densities may result in the mortality of planktonic organisms (*e.g.*, phytoplankton and zooplankton) (Shishlyannikov *et al*., 2011). In the absence of dense organic matter, such as those in laboratory cultures, pathogenic microorganisms can adhere to cultured organisms (Torkildsen & Magnesen, 2004), and the intense aeration necessary to establish the BFT (Avnimelech, 2007) is unfeasible for cultures of smaller organisms, since the turbulence caused by the strong aeration could stress the organisms. Even if the laboratory culture has a high concentration of solids to which the microorganisms can adhere, this will cause a drastic consumption and reduction of dissolved oxygen because the volume of the medium is significantly lower than that of commercial cultures (Emerenciano *et al*., 2012). Also, the biofilm can eventually colonize the body and appendages of the cultivated organisms, resulting in a multitude of consequences for the basibiont, like: increased weight and friction, impeded trans-epidermal exchanges, altered color, smell and contour, resulting in multiple consequences. These changes may lead to the loss of buoyancy and motility, impair mating or even cause a substantial shift in the interactions among species (Wahl *et al*., 2012).

Both commercial and laboratory cultures can stimulate the selection and growth of opportunistic bacteria (Olafsen, 1993), resulting in diseases (Alday *et al*., 2006), lower production (Lipton *et al*., 2003), and consequently even the death of algae, invertebrates or fish (Wyban & Sweeney, 1991). The increased occurrence of diseases in aquatic species of economic interest due to the rapid multiplication of pathogens has resulted in significant losses in aquaculture, affecting the development of this sector in many countries (Pillay & Kutty, 2005). These diseases were principally caused by pathogenic microorganisms belonging to the bacterioplankton, mycoplankton, phytoplankton and protozoans (Uriarte *et al*., 2001). Among these microorganisms, the bacterioplankton is the one that can cause the most harm to aquatic species. Vibriosis and photobacteriose are the two most common types of bacterial diseases that can afflict marine, estuarine and sometimes freshwater farming systems and are principally responsible for the greatest impacts already reported in aquaculture (Defoirdt *et al*., 2007). In addition to the damage posed by the presence of bacterioplankton and mycoplankton in cultures, these microorganisms also interfere in aquatic scientific experiments, generating methodological problems that will be reflected on results (Spencer, 1952; Yetka & Wiebe, 1974), or making it impossible to test hypothesis about the role of bacteria and fungi in an ecosystem or community (DeLorenzo *et al*., 2001; Trottet *et al*., 2011).

Solutions for microbial control in cultures

Despite many years of research, bacterial and fungal loads are still a primary concern in the production and culture of planktonic organisms (Torkildsen & Magnesen, 2004) with several methods and techniques developed in an attempt to inhibit bacterial and fungal growth in cultures. According to Couch (1939) and Vieira (1977), the control of temperature, inoculum size, pH, the organic content of the medium, washing the cells (using filtration and centrifugation) and

reisolation, are the main techniques used to inhibit and/or eliminate bacteria and fungi from cultures. Furthermore, these microorganisms can be controlled through sterilization by ultraviolet light (UV) (Rentachler *et al*., 1941; Alexandre *et al*., 2008), membrane filtration (Bobbitt & Betts, 1992; Zhou *et al*., 2011), autoclaving the water (Ferris & Hirsch, 1991; Creswell, 2010), detergent (McCracken, 1989) or through chlorination (Creswell, 2010; Liu *et al*., 2012). These methods can be used singly or in combination.

However, when the research involves the production and/or culture of live organisms, it is difficult to avoid contamination of sterile water through the air because microalgae or zooplankton cultures/production involve transferring and/or handling organisms at distinct time intervals (Lourenço, 2006; Agostini, 2014; Agostini *et al*., 2016).

Currently, the use of probiotics to inhibit the growth of pathogenic bacteria has been proposed as an important nutritional factor influencing gastrointestinal physiology and function in aquaculture (Diplock *et al*., 1999), and it was defined as a live microbial feed supplement that could beneficially affect the host animal by improving its intestinal balance (Fuller, 1989). However, these measures have rarely, if ever, completely prevented the incidence of infectious diseases in farm animals. According to Alday *et al*. (2006), with the possible exception of ranching, some degree of the antimicrobial cover has been necessary for all animal production systems with probiotics. Antimicrobial agents can be defined as natural, semi-synthetic or as synthetic substances that can kill (bactericides and fungicides) or inhibit the growth of microorganisms (bacteriostatic, fungistatic), principally based on the action mechanism and concentration dose (Dixon, 2000). After the discovery of penicillin by Fleming in 1928, antibiotics have become important drugs for human and animal welfare.

Antibiotics should be safe (non-toxic) for the host, allowing their use as chemotherapeutic agents for the treatment of infectious bacterial diseases. In addition to their use in human and veterinary, antimicrobials are also used in cultured animals for human consumption, and their use can be categorized as therapeutic, prophylactic or metaphylactic (Serrano, 2005). According to Shaw *et al*. (1994) and Campa-Córdova *et al*. (2006), there are advantages to the application of antimicrobials to the control of microbial density in culture mediums relative to other methods, and the main ones include the ease of handling and implementation, as well as the maintenance of its effect on the artificial environment. Shishlyannikov *et al*. (2011) and Agostini *et al*. (2016) believe that the sole use of antimicrobials is possible to obtain bacteria and fungi-free cultures without damage to non-target organisms.

The importance of healthy and well-established cultures of planktonic organisms are a goal on a variety of scientific (biology and ecology) or economic studies focused on genetic, cytology, morphology, physiology, taxonomy, ecology and as a food source for aquaculture or human use. For this reason, we reviewed antibiotic and antifungal methods that have been used to obtain zooplanktonic and phytoplanktonic cultures free of bacterioplankton and mycoplankton. These procedures are widely dispersed in the literature. We now summarize them to establish an accessible guide to new scientists about the use of antimicrobials in the culture medium of planktonic organisms for i) assisting ecology studies to answer questions about the role of bacteria and fungi in aquatic communities, ii) enhancing or treating cultures of planktonic organisms used as food in aquaculture, and iii) facilitating biological studies (genetic, taxonomy, development, growth) preventing bacterial and fungal growth, without causing damage to non-target organisms.

Antimicrobials in planktonic cultures: historic

The use of prokaryote inhibitors (such as antibiotics) to control bacterial growth in cultures of planktonic organisms began in the 1940s, when penicillin was used to obtain bacteria-free cultures of the protozoan *Tritrichomonas foetus* (Riedmuller, 1928) (Ithaca & Mahmoud, 1944), and their use increased after the discovery of streptomycin in 1943, tetracycline in 1945 and neomycin in 1949. Between 1950 until 1969 there was an increase in the number of studies on this topic (Fig. 1). A series of papers documented the negative correlation between bacterial density and survival, and development of mollusk larvae (Loosanoff & Davis, 1963), leading to studies regarding the use of antibiotics in planktonic cultures of other organisms such as microalgae and crustaceans (Shaw *et al*., 1994; Agostini *et al*., 2016).

From the 1970s until 1999, the use of antimicrobials in cultures of planktonic organism cultures declined with the application of other chemicals to culture ponds due to the contamination of the environment, of the final product, or both (Collier & Pinn, 1998) (Fig. 1). From 2000 onwards, the problems concerning the use of antibiotics in aquaculture continued. However, a new field of application arose with studies using antibiotics and/or antifungals to acquire controlled environments free of microorganisms, enabling the test of hypotheses involving the contribution of bacterioplankton and mycoplankton in aquatic communities (DeLorenzo *et al*., 2001; Fouilland *et al*., 2007; Trottet *et al*., 2011) (Fig. 1).

A review on this topic using specialized books (Ray, 1966; Walne, 1970; Roberts, 1972; Brown, 1973; Kinne,

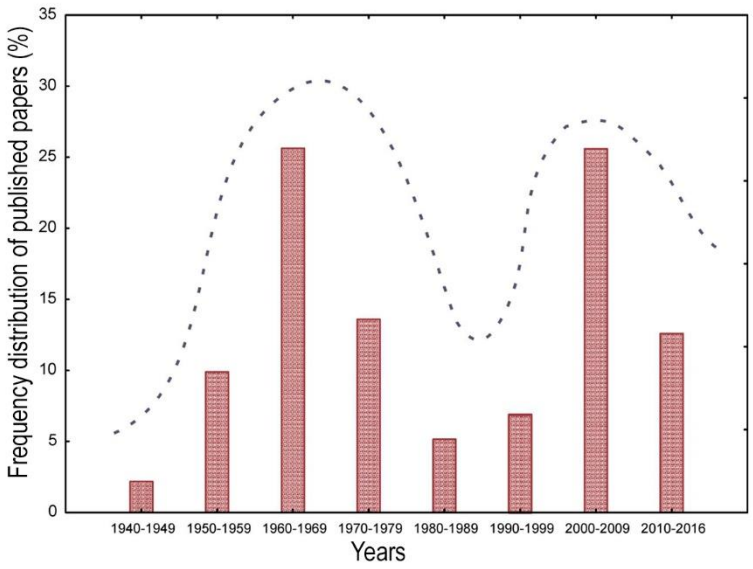

Figure 1. Frequency distribution of published papers (%) that used antimicrobial agents in cultures of planktonic organisms between the years 1940 and 2016. The review was conducted using specialized books and ASFA database (http://www.fao.org/fishery/asfa/en) for papers published after 1971 using the keywords, plankton and antibiotics, plankton and antimicrobials, and aquaculture and bacteria-free. Google scholar (https://scholar.google.com.br) and CAPES periodic portal (http://www.periodicos.capes.gov.br) were also used to complement the information.

1977; Ukeles, 1977; Hetrick *et al*., 1981; Olafsen, 1993; Dixon, 2000; FAO/WHO, 2002; Pillay *et al*., 2005; Serrano, 2005; Alday *et al*., 2006; Lourenço, 2006; Creswell, 2010) and trough ASFA database (http://www.fao.org/fishery/asfa/en) using the keywords: plankton and antibiotics and antifungal, plankton and antimicrobials, aquaculture and bacteria-free returned 204 publications between 1940 and 2016 related to the use of antimicrobials in planktonic cultures. Google scholar (https://scholar.google.com.br) and CAPES periodic portal (http://www.periodicos.capes. gov.br) were also used to complement the information. From the review, it was seen that the decades starting on the 1960s and 2000 were the ones when most of the papers on this topic were published, corresponding each with 25.5% of the retrieved papers. The lowest number of publications goes back to the 1940s (2.2%), at the beginning of this line of study, and when the associated problems in aquaculture arose in the 1990s (7%). Fifty-seven antimicrobial agents were applied to plankton cultures between 1940 and 2016 (Annex 1).

The reviewed material indicated that the application of antibiotics in cultures not only ensures the inhibition of bacterial growth but also accelerates the development of the species of interest and increases their survival, when compared to control without antibiotics, reinforcing the idea that these substances may also be used as a tool for the hypothesis test. It was also observed that penicillin and streptomycin were the most often used in cultures of planktonic organisms; however, the percentage used about published works has decreased (Fig. 2), probably due to the discovery/formulation of new antibiotics. Currently, there is increased use of chloramphenicol (Chloromycetin®), oxytetracycline and neomycin in studies. These antimicrobials, together with penicillin and streptomycin, are the five antibiotics that are most often used (Fig. 2) due to their greater efficiency in inhibiting the bacterial community (bacterioplankton) (Annex 2) and when used in combination, ensure a broad spectrum of bacterial inhibition. Thus, antimicrobials could be associated with peptides (tryptone), acids (oxolinic, nalidixic), surfactants (Tween-80) or detergents (Eggermont *et al*., 2014). Guillard (2005) recommends the addition of organic matter with low molecular weight (for example, 0.006 mg L^{-1} glucose) when using antibiotics because this will ensure the inhibition of the synthesis of the bacterial cell wall. If bacterial growth is not happening, antibiotic will only retain the inert cells; on the other hand, if the biosynthesis of cell walls were occurring, will result in fatal bacterial damage.

Some authors emphasize that when successfully inhibiting bacteria, the niche is usually occupied by filamentous fungi and/or yeasts, which, in most cases, also needs to be controlled by applying an antifungal to avoid ill effects to the organisms of interest (Agostini, 2014; Agostini *et al*., 2016). Considering that most fungal pathogens are secondary or opportunistic invaders,

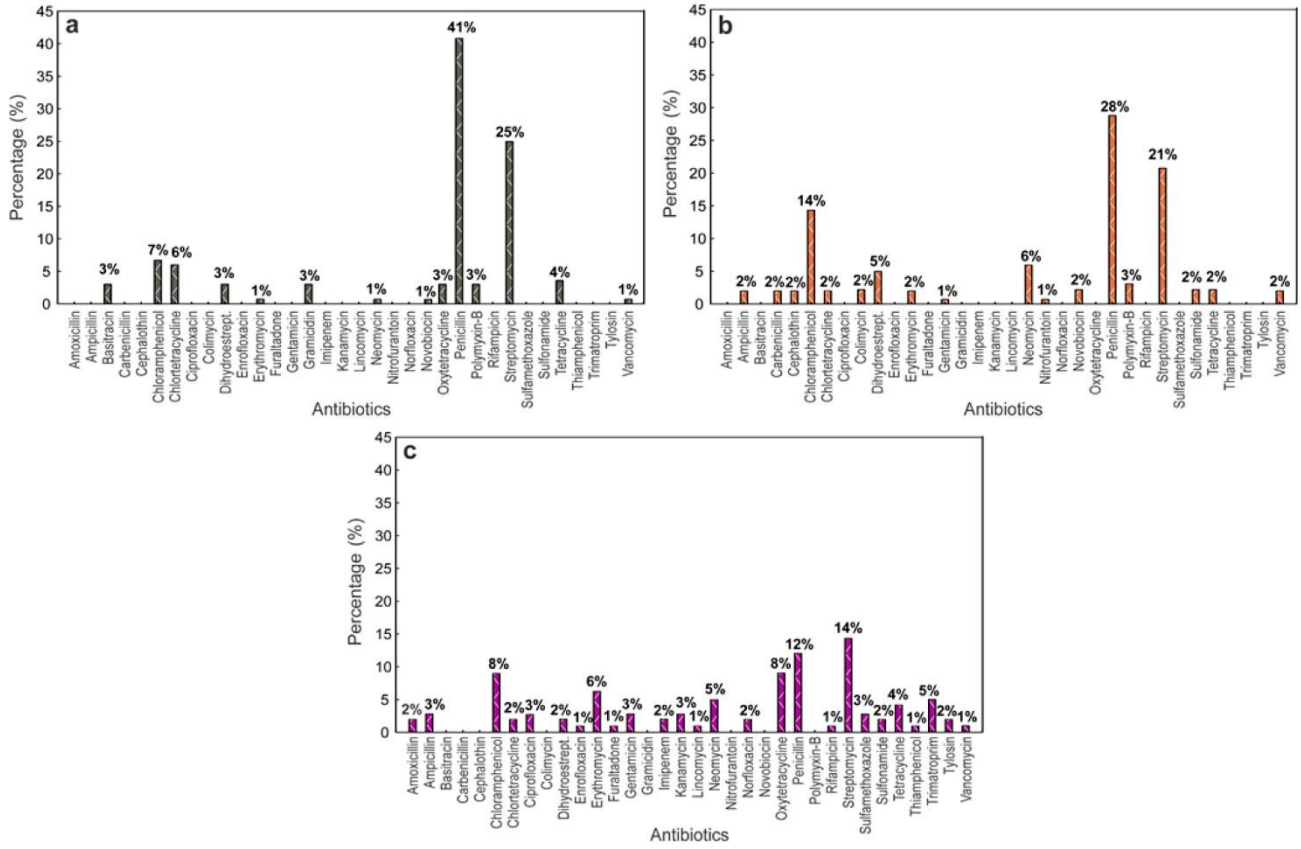

Figure 2. Percentage of published papers using a specific antibiotic formula in plankton cultures between 1940 and 2016. a) 1940-1959, b) 1960-1989, c) 1990-2016.

which already belong to the biota of the water used, they can cause problems to farmed organisms (Leaño *et al*., 2005), *e.g.*, mycoses, that have been considered one of the main factors related to economic losses in aquaculture (Zaror *et al*., 2004). Therefore, fungi can cause as much damage as bacteria; however fungi contamination is less common in marine than freshwater cultures (Lourenço, 2006).

The main antifungal agents used in cultures of planktonic organisms in the period between 1940 and 2016 included nystatin (51%), cycloheximide (30%), amphotericin-B (14%) and candicidin (5%) (Fig. 3). Many authors reported nystatin (Micostatin®) for the inhibition of fungal growth of filamentous fungi and yeasts in cultures (Lee *et al*., 1970; Sande & Mandell, 1987; Groll *et al*., 1999; Wilkens & Maas, 2012). This antifungal was first discovered in the early 1950s and is produced by the growth of the bacteria *Streptomyces noursei*. This antifungal bind to ergosterol in the plasmatic membrane of the fungal cell, forming pores that will lead to the loss of K as well as other small molecules to the medium, causing the death of the fungus (Lourenço, 2006). Cycloheximide is another often used antifungal, that will inhibit the protein synthesis in eukaryotes, by preventing the elongation of peptides through the transferal peptidase activity in ribosomes 60S (Lourenço, 2006). It is also produced by a bacteria, namely *Streptomyces griseus*, and was first used by Ray (1966) in cultures of the oyster *Crassostrea virginica*. Another antifungal, amphotericin-B (Fungizone®), was originally extracted from *Streptomyces nodosus* in 1955, and employed by Lee *et al*. (1970) in combination with antibiotics in nematode cultures to inhibit filamentous fungi. Candicidin is an antifungal compound obtained from *Streptomyces griseus* and was only used by Provasoli & Gold (1962) in cultures of the protozoan *Crypthecodinium* (=*Gyrodinium*) *cohnii* (Seligo) Javornicky, 1962 in combination with antibiotics. Both amphotericin B and candicidin have the same active principle of nystatin (Lourenço, 2006).

Combining antibiotics and antifungals could be an alternative to maintain or reduce the initial bacterial load and thus reducing the risk of handling contamination; however, using these substances to prevent bacterial and fungal infections should be done carefully to avoid harming the organisms of interest (Trottet *et al*., 2011).

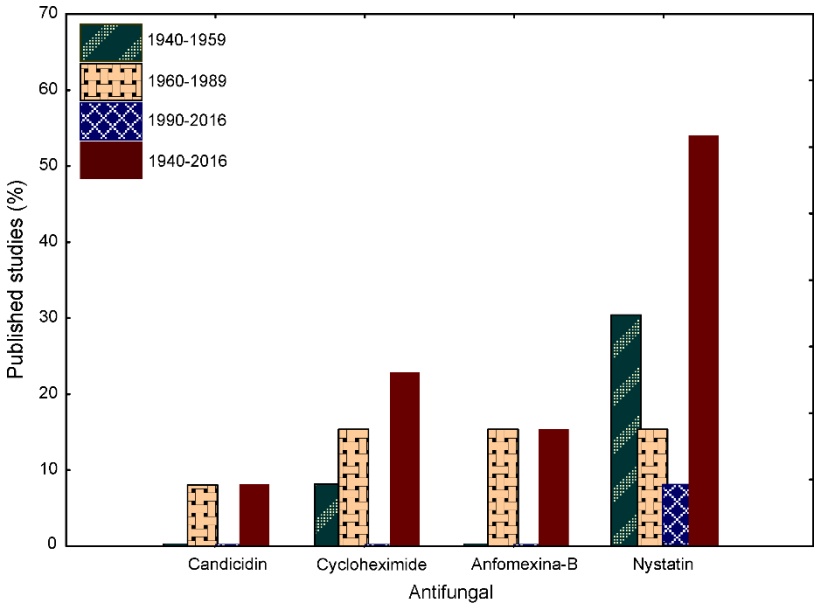

Figure 3. Percentage of antifungal used in plankton cultures between 1940 and 2016.

Antimicrobials in planktonic cultures: major contributions

Antimicrobial and other chemical agents have been used in conjunction with physical methods for many years to obtain axenic cultures of planktonic organisms. These procedures are widely dispersed in the literature since the 1940s. The present report summarizes this information for researchers that needs to minimize bacterial proliferation in planktonic cultures. The majority of the papers published between 1940 and 2016 regarding the areas of application for antimicrobial agents (Annex 3) were related to the inhibition of bacteria and fungi in cultures/production of phytoplankton (*e.g.*, Campa-Córdova *et al*., 2006), followed by crustaceans (*e.g.*, Agostini *et al*., 2016), hypothesis testing involving the planktonic community (*e.g.*, Trottet *et al*., 2011), mollusks (*e.g.*, Roberts *et al*., 2007), protozoan (*e.g.*, Provasoli & Gold, 1962), fish (*e.g.*, Struhsaker *et al*., 1973), planktonic phase of seaweed (*e.g.*, Kobayashi *et al*., 2003), periphyton (spores can be found in planktonic enviromental) (*e.g.*, Vieira, 1977), Rotifera (*e.g.*, Dougherty *et al*., 1960), Cnidaria (*e.g.*, Claybrook & Eakin, 1960), Cyanobacteria (*e.g.*, Guo & Chen, 2012), tychoplanktonic nematodes (zooplankton that oscillate periodicaly their position in water column) (*e.g.*, Lee *et al*., 1970), Porifera (*e.g.*, Borojevic, 1966), Platyhelminthes (*e.g.*, Miller & Johnson, 1959) and meroplanktonic Nemertea (zooplankton with a life cycle splited in pelagic and benthic enviromental) (*e.g.*, Tuker, 1959) (Fig. 4).

We believe that the major use of antimicrobials in cultures of planktonic microorganisms i) allow/improve the culture of planktonic organisms and ii) increase our knowledge about the role of bacteria and fungi in aquatic communities, testing hypothesis. Through the use of specific inhibitors in planktonic cultures, it is possible to i) eliminate or decrease bacteria and/or fungi load in the medium (Youn & Hur, 2007), ii) inhibit the growth of pathogenic microorganisms (Castro-Mejía *et al*., 2007), iii) increase zooplankton and phytoplankton growth/survival (Campa-Córdova *et al*., 2006), development (Uriarte *et al*., 2001) and production (Agostini, 2014), and iv) reduce competition between phytoplankton and bacterioplankton (Youn & Hur, 2007) and eliminate bacterial aggregates on microalgae wall´s (Vieira, 1977). In phytoplankton and periphyton cultures, Vieira (1977) compare different methods and antimicrobials to maintain cultures of 12 different species of microalgae bacteria-free. He noted that the use of antibiotics boosted results. However, some criteria such as i) the evaluation of the physiological state of the algae cells to be treated (algal cells in the decline phase of growth are strongly attacked by bacteria, and are more sensitive to chemicals substances), and ii) the sensitivity of microalgae to antimicrobials (which varies according to the species, antibiotic, concentration and exposure time), should be followed to ensure the success of the method. Similarly, Youn & Hur (2007) determined the extent of bacteria contamination and resistance to various antibiotics commonly used in different microalgal cultures (*Chlorella ellipsoidea* Gerneck, 1907; *Isochrysis galbana* Parke, 1949; *Heterosigma akashiwo* (Hada) Hada ex Hara & Chihara, 1987; and *Cyclotella didymus*

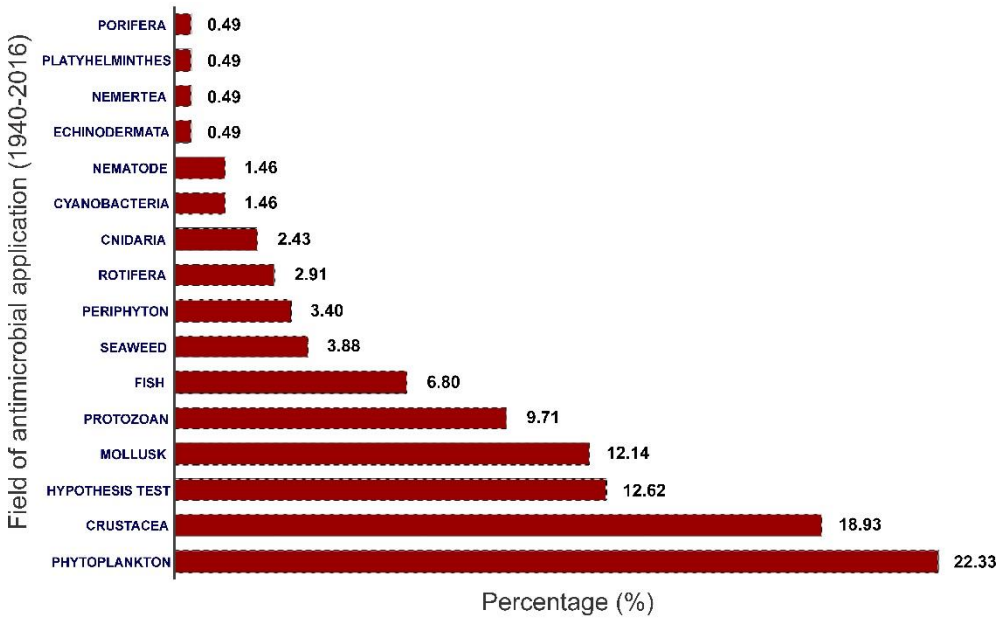

Figure 4. Field of study where antimicrobials were applied to inhibit the growth of bacterioplankton and/or mycoplankton.

and *Thalassiosira alleni* Takano, 1965). Seven different dosage levels of chloramphenicol, dihydrostreptomycin sulfate, neomycin, penicillin G, streptomycin sulfate, penicillin G + streptomycin sulfate and penicillin G + streptomycin sulfate + chloramphenicol were added to each culture of microalgae. The authors verified that axenic culture of Bacillariophyceae and Dinophyceae was more difficult to obtain than those of Chlorophyceae and Haptophyceae because of them intricated external morphology. The efficiency of different antibiotics and their concentrations to obtain axenic cultures varied depending on the microalgal species used. The same conclusion was also observed by Vieira (1977).

For zooplankton cultures, the findings of Walne (1956, 1958, 1963, 1970) on molluscan production encouraged the application of antimicrobials in other zooplankton cultures (*i.e.*, heterotrophic protozoans, rotifers, crustaceans and fish). This author used antibiotics (penicillin + streptomycin) to benefit oyster, through direct application in the culture medium and food (microalgae) of this molluscan in order to eliminate pathogenic microorganisms and increase the survival of oysters (More information in zooplankton cultures can be seen in Annex 2.2).

Regarding hypotheses tests, Müller (1969) initiated the use of antibiotics for test ecological relationship on the biofouling process, however involving just one planktonic invertebrate species (*Hydractinia echinata* (Fleming, 1828)). While Yetka & Wiebe (1974) used specific inhibitors in an aquatic laboratory system, involving all community, without success because they concluded that the antibiotics tested could not be used to delineate bacterial respiration in mixed microbial communities. However, this initial approach leaded Sherr and collaborators in 1986 to estimate the grazing rates of heterotrophic nanoplankton on bacterioplankton using inhibitors, as well as Wheeler & Kirchman (1986) to evaluate the utilization of inorganic and organic nitrogen by bacteria in marine systems applying antimicrobials in the medium, thus opening the way for other research using antimicrobials in different fields. For example, Maurin *et al.* (1997) evaluated the relationship between phytoplanktonic excretion and bacterial reassimilation in an oligo-mesotrophic lake, using antibiotics to get treatment without bacteria, making it possible to prove the hypothesis that bacterial heterotrophic reassimilated most of the products excreted by phytoplankton. Meanwhile, Bidle *et al.* (2003) experimented in paleoceanography area with treatment with antibiotics to prove that marine bacteria accelerate biogenic silica dissolution rates in the sea.

In aquatic ecology, the main contributions on the application of antimicrobials for hypothesis tests were the works of DeLorenzo *et al.* (2001) and Trottet *et al.* (2011). DeLorenzo *et al.* (2001) use of metabolic inhibitors (penicillin+streptomycin+neomycin+cycloheximide) to characterize ecological interactions of the com-munity (free-living and adherent heterotrophic bacteria, ciliate and flagellate) in an estuarine microbial food web proved their hypotheses that there are seasonal shifts in microbial food web structure and function related to the season. In their study, they showed the important role of microbial loops in driving

primary and secondary production in estuaries, using antimicrobials as a tool. Trottet *et al*. (2011) selected, tested and compared the most commonly reported antibiotics to assess their effect on bacterial growth and functional diversity of natural communities and their efficiency involving phytoplankton and bacterioplankton interactions such as competition and mutualism, or lack of interaction between the different components of the microbial communities to study their relative importance in biogeochemical fluxes. They found that penicillin and streptomycin at a final concentration of 0.1 g L^{-1} each significantly reduced bacterial growth within two hours. They also reported that there was a greater impact on bacterial functional diversity when both antibiotics were mixed, and this mixture did not have any significant effect on the growth of selected cultured phytoplankton strains. The most interesting aspects of these works is the community level study (DeLorenzo *et al*., 2001) which differs from those that evaluated the interaction of bacterioplankton with only one functional group (*i.e.*, phytoplankton) and the comparison of different antimicrobials (individually or in combination) (Trottet *et al*., 2011).

While in population level, Shaw *et al*. (1994) used antibiotics with success as tools to evaluate situations in which marine phytoplankton use chemical feeding deterrents to reduce or inhibit grazing by the copepod *Tigriopus californicus* (Baker, 1912). Whereas Tartarotti & Torres (2009) used treatments with the antibiotic gentamicin (50 g L^{-1}; applied at a concentration of 1 mL L^{-1}) in cultures of the copepod *Acartia tonsa* Dana, 1849 to successfully test the hypothesis that bacteria affect the formation of the copepod exoskeleton. Although it is noteworthy that prokaryotic and eukaryotic inhibitors have been successfully used also to evaluate: i) the role of bacteria in the production of metabolites (Ringelberg & Van Gool, 1998), ii) the role of microbes in the decomposition process (Tang *et al*., 2006), iii) phytoplankton-bacteria interactions (Hamdan & Jonas, 2007), iv) competition for dissolved nitrogen (Fouilland *et al*., 2007), v) influence of bacterioplankton activities on nitrogen uptake rates (Tungaraza *et al*., 2003), vi) interaction on biofouling process (Roberts *et al*., 2007) and vii) the potential protein synthesis by bacteria (Tartarotti & Torres, 2009).

Adverse effects of antibiotic use on cultures

Despite all these benefits, due to improper disposal, the development of bacterial resistance and ecological problems, the current trend is to reduce the use of antibiotics. Furthermore, import restrictions by the presence of residues in tissues of cultured organisms and possible damage to public health, result even in the banning and limitation of the use of these antimicrobials in many countries when used in cultures of organisms for human consumption (Defoirdt *et al*., 2007). Antimicrobial agents are usually administered to marine fish (Buschmann *et al*., 2012), and its feces containing unabsorbed antimicrobials and secreted antimicrobial metabolites in the environment of fish farming sites often retain their antimicrobial activity and can remain in the aquatic environment for variable periods (Burridge *et al*., 2010). For example, McCracken *et al*. (1976) established that the antibiotic trimethoprim could remain in the system for 77 days after the cessation of treatment. Therefore, environmental contamination can occur if the tank water is discharged into the environment within this period.

Antibiotics act as inducers of bacterial genes encoding mechanisms of drug resistance (Butaye *et al*., 2003) and the selection of antimicrobial-resistant bacteria in the marine environment could have detrimental impacts on animal and human health (Buschmann *et al*., 2012). Nevertheless, there is a hypothesis that antibiotics resistance varies between environments and regions, depending primarily on the selection pressure imposed on the ecosystem (Pereira *et al*., 2006), and the selection power is proportional to the exposure time of the bacteria to the antimicrobial (Aliabadi & Lees, 2000).

Antibacterial agents have been frequently detected in sewage effluents, surface waters and groundwater (Ying & Kookana, 2007) through the improper disposal of water with antimicrobial residues (by aquaculture, hospital, industrial and domestic sewage). According to Hektoen *et al*. (1995), a large portion of the antibiotics administered in farms (70-80%) has been reported to reach the environment, promoting the impact of the disposal on the adjacent community. Møster (1986) observed detectable levels of antibiotic in blue mussels (*Mytilus edulis*) located 80 m from a fish farm where antibiotics were used. The ecological effects in the environment are related to the lack of biodegradation of these substances. Only a few of the compounds were partially biodegraded in aquatic systems (Kümmerer *et al*., 2000), accumulating in the water and sediment and this allows the appearance of resistant bacteria to affect the food chain directly (Kümmerer, 2003). For example, according to Lourenço (2006), continuous exposure to antibiotics generates damage with various extensions in microalgae. Thus, this author suggests only short-term treatments. Furthermore, it is reported in the literature that the use of antibiotics can cause in planktonic organisms: genetic mutations (Kumar, 1964; Droop, 1967), inhibiting the nuclear DNA synthesis in algae (Vieira, 1977), loss of color in microalgae and protozoa (Provasoli *et al*., 1948),

inhibition of chloroplasts (Vieira, 1977) and mitochondrial synthesis (Lloyd, 1974) in algae, abnormalities in the morphology and behaviour of the clam larvae (Fitt *et al*., 1992), toxicity (Wollenberger *et al*., 2000) and inhibition of growth (Kviderova & Henley, 2005).

As noted in section 1.2.1, the use of probiotics (live microorganisms that when administered in adequate amounts confer a health benefit for the organism cultivated, inhibiting possible pathogens) (FAO/WHO, 2002) can be a solution for large-scale cultures destined for human consumption, replacing the use of antimicrobials. Rego *et al*. (2012) observed that the concentration of *Vibrio* spp. on the water and postlarvae, shrimps of *Litopenaeus vannamei* were significantly reduced with the use of *Bacillus* spp. probiotic. Riquelme & Avendaño-Herrera (2003), for example, used streptomycin/oxytetracycline + furaltadone to compare the efficiency of probiotics for the same species culture. Probiotics had the same results regarding mollusk survival than antibiotics; however, the antibiotic treatment decreased bacterial density more than probiotic treatment, being the most indicated for the hypothesis test, that requires a culture medium with minimum bacteria as possible. Still, short-time cultures without transfer of organisms and with low organic matter can be maintained without bacterial and fungal contamination by traditional methods (sterilization, chlorination or filtration of the culture medium), preferably maintaining the cultures covered (*i.e.*, PVC film), but with light penetration, avoiding contamination via air, in order to maximize antimicrobial efficiency.

There are other chemical procedures for the purification of marine and freshwater cultures, particularly of microalgae, involving the use of bacteriostatic agents such as potassium tellurite (K_2TeO_3) or sulfonamides, which oxidizes bacterial cells and prevents the synthesis of nucleic acids by bacteria, respectively. Besides the application of the enzyme lysozyme, iodine in alcoholic solution or ionic detergent (Triton and Tween), as well as of Dakin's solution (0.45 to 0.5% sodium hypochlorite + 4% boric acid) developed during the First World War (Lourenço, 2006). The problem with these methods is that they often need to be applied in conjunction with antimicrobial or accompanied by physical procedures (*i.e.*, washing and centrifuging) to ensure its efficiency.

Future directions: remarks and conclusion

Planktonic organisms are involved in the major ecosystem processes in the aquatic community. However, ecological laboratory studies require precise and replicable protocols that allow the understanding of the biological and ecological factors that directly and indirectly affect a population or a community, respectively, without affecting organisms of interest. The application of antimicrobials in laboratory cultures of planktonic organisms seems to be an alternative to obtain responses at the population level or the community level without the microbial influence, although care must be taken to obtain accurate results. A pilot investigation on the characteristics and effects of antimicrobials should be conducted to obtain precise results, ensuring maximum efficiency of antimicrobials in the aquatic system. According to McCracken (1989), there is no overall applicable method for all species because every culture has different communities of microorganisms and the degree of sensitivity of organisms may show different responses to the same chemical. Thus, we have established remarks for the use of antimicrobials in planktonic cultures safely either for the hypothesis test or to obtain healthy cultures:

i) Penicillin, streptomycin, oxytetracycline and neomycin are the antibiotics most often used in planktonic cultures with success, individually or in combination and they must be prioritized in the choice, either for phytoplanktonic or zooplanktonic organisms.

ii) Note the tolerance of the organisms to the antimicrobial chosen. Give preference to antibiotic combinations of a broad spectrum, because studies indicate that they are more efficient than individual antimicrobials alone (Pappas & Hoffmann, 1952; Kinne, 1977; Agostini *et al*., 2016). However, attention should be paid to the burnout effect among them. Speck *et al.* (1951) reported that chlortetracycline (Aureomycin®) and oxytetracycline (Terramycin®) antagonized the action of penicillin in cultures. Furthermore, the antagonistic effects between species that can minimize the effects of antibiotics used must also be checked. Tchan & Gould (1961) found that bacteria tested alone were sensitive to some of these antibiotics, but not when associated with the blue-green algae, suggesting a protective effect of the algae.

iii) In the development of bacteria-free cultures, previous sterilization of the equipment, such as glassware and medium, will probably be beneficial, because this will reduce the initial bacterial load (Alexandre *et al*., 2008; Creswell, 2010; Zhou *et al*., 2011). Cover the cultures (*e.g.*, PVC film) could also be an alternative to avoid contamination.

iv) The stock solution of antimicrobials (diluted in distilled water) must be prepared, if possible, immediately before use; otherwise, it must be frozen after being filtrated trough sterilized filters (0.2 µm). Two variables define the final result: the dose of the antimicrobial and the exposure time (Lourenço, 2006).

v) Evaluate the effectiveness of the selected antibiotics through bacterial density estimated by checking the onset of action and its half-life in the culture medium.

vi) Test the antimicrobials previously in the salinity and culture medium of interest, since studies have shown that the variation of the salinity medium results in an increase of the toxicity of the chemical substances in invertebrates (Kwok & Leung, 2005; Pedroso *et al*., 2007), due to alteration of the water content and concentration of inorganic ions (Ferguson & Hogstrand, 1998).

vii) The use of the appropriate antibiotics will result in the inhibition of bacteria in the culture medium. However, this will likely cause an overgrowth of fungi, making it necessary to include a small concentration of a eukaryotic inhibitor in the combination after a previous sensitivity test. Tang *et al*. (2006), Agostini (2014), and Agostini *et al*. (2016) reported the prevalence of fungi when the bacterial load was reduced, suggesting the inclusion of an antifungal. We suggest the application of nystatin (the antifungal most often used in cultures), but in low concentration, because this inhibits eukaryotes organisms.

viii) There may be different sensitivities to the same treatment of antimicrobial between development stages of the same animal species; usually, earlier stages are more sensitive. For the adult mollusk *Ostrea edulis* Linnaeus, 1758, for example, 0.25 g L^{-1} of streptomycin in combination to 50,000 IU L^{-1} of penicillin is applied in its culture medium (Walne, 1963), however for larval stages just 0.05 g L^{-1} of streptomycin + 50,000 IU L^{-1} of penicillin will be required (Walne, 1958). For egg culture of the barnacle *Fistulobalanus albicostatus*, Chen *et al*. (2007) suggest the use of 0.01 g L^{-1} of penicillin + 0.005 g L^{-1} of streptomycin, however for barnacle larvae of the same species it is advisable to use 0.02 g L^{-1} of penicillin + 0.03 g L^{-1} of streptomycin (Yoshimura *et al*., 2006). For this reason, even if the combination of antimicrobials has been utilized for the cultivation of an adult of a particular species, it is advisable to test this treatment with the development stage of interest.

ix) It is necessary to evaluate the water quality (ammonia, nitrite, nitrate, phosphate, sulfate) and estimate possible changes in the use of antimicrobials in culture medium caused by the withdrawal of bacteria. The bacterioplankton is responsible for nutrient cycling, and its removal could result in the accumulation of nutrients to toxic levels (Alongi, 1994; Silva *et al*., 2012).

x) Make sure to process and appropriately dispose of the culture and the production medium with antimicrobials avoiding environmental contamination and/or bacteria resistance.

Even with the limitations in the application of antimicrobials in farming systems intended for human consumption, these substances have the potential to be used in controlled laboratory cultures of planktonic organisms intended to be used in bioassays, and also for testing hypotheses aimed at evaluating the contribution of the bacterial and/or fungal in the aquatic community (DeLorenzo *et al*., 2001; Trottet *et al*., 2011; Agostini *et al*., 2016). Therefore, with proper care, scientists may use these substances in scientific experiments where systems without bacterioplankton and/or mycoplankton contamination are needed to provide a better understanding of the real effect of the microbial community such as competition and mutualism, or the lack of interaction between the different components of the planktonic community. Besides, it could be used to assess the relative importance of these microorganisms in biofouling, marine snow, biogeochemical fluxes, microbial loop, benthic-planktonic coupling and the biological pump.

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SUPPLEMENTARY DATA

Annex 1. Main antimicrobials (prokaryote inhibitors) used in plankton cultures between 1940 and 2016.

Antimicrobials	Used between 1940 and 2016 (%)	Years		
		1940-1959	1960-1989	1990-2016
Aerosporin	0.2		X	
Amoxicillin	0.9			X
Ampicillin (Binotal®)	1.6		X	X
Basitracin	0.7	X		
Carbenicillin (Geopen®)	0.5		X	
Cefazolin	0.2			X
Cefuroxime	0.3			X
Cephalothin (Keflin®)	0.5		X	
Chloramphenicol (Chloromycetin®)	9.5	X	X	X
Chlortetracycline (Aureomycin®)	3.2	X	X	X
Ciprofloxacin	1.4			X
Colomicin	0.5		X	
Dihydroestreptomicine	2.9	X	X	X
Doxycycline	0.2			X
Enrofloxacin	0.5			X
Erythromycin (Clarithromycin®, Iloticina®)	3.2	X	X	X
Florfenicol (Nuflor®)	0.3			X
Furaltadone	0.5			X
Furazilidone	0.2			X
Gentamicin	1.4		X	X
Gramicidin	0.7	X		
Imipenem	0.7			X
Kanamycin	1.4			X
Levofloxacin	0.2			X
Lincomycin	0.5			X
Neomycin	4.3	X	X	X
Nitrofurantoin	0.2		X	
Norfloxacin	0.9			X
Novobiocin	0.7	X	X	
Ofloxacin	0.2			X
Oxytetracycline	4.1	X		X

Continuation

Antimicrobials	Used between 1940 and 2016 (%)	Years		
		1940-1959	1960-1989	1990-2016
Penicillin	23.9	X	X	X
Polymyxin-B	1.6	X	X	
Pyrimethamine	0.2			X
Rifampicin	0.5			X
Ristocetin	0.2		X	
Roxithromycin	0.3			X
Spiramycin	0.2		X	
Streptomycin	18.5	X	X	X
Sulfamerazine	0.5		X	
Sulfamethoxazole	1.1			X
Sulfonamide, (Sulfadiazine®, Sulfadimethoxine®)	1.1		X	X
Tetracycline	3.2	X	X	X
Thiamphenicol	0.5			X
Triclocarban	0.3			X
Trimethoprim	2.0			X
Trisulfapyrimidine (Triple sulfa®)	0.2		X	
Tylosin	0.9			X
Vancomycin	0.9	X	X	X

Annex 2. Inhibiting concentrations (g L^{-1}) of the five most frequently cited antibiotics for different bacterial strains (modified from Berland & Maestrini, 1969; Lourenço, 2006). *Partial activity.

Bacterioplakton species	Antibiotic				
	Penicillin	Streptomycin	Chloramphenicol	Oxytetracycline	Neomycin
Action	Gram +	Gram ±	Gram ±	Gram ±	Gram ±*
Pseudomonas aestumarina	4	>10	1	2	0.02
Psedomonas cruciviae	0.001	0.03	0.002	0.05	0.03
Pseudomonas marinoglutinosa	2.5	4	0.15	1	1
Pseudomonas riboflavina	0.05	0.02	0.025	0.02	0.02
Pseudomonas stereotropis	0.00025	0.025	0.002	1.75	0.05
Vibrio algosus	1.75	0.003	0.01	1.5	2
Vibrio phytoplanktis	0.015	0.08	0.0015	1.7	1.75
Agarbacterium mesentericus	3	0.02	0.005	1.5	0.03
Xanthomonas	0.03	>10	0.001	1	1.5
Achromobacter parvulus	0.001	>10	0.002	1,5	1.25
Achromobacter stationis	0.00025	0.015	0.0015	0.05	0.01
Achromobacter stenohalis	0.05	0.005	0.0015	0.02	0.015
Flavobacterium aquatile	0.05	0.04	0.03	1.25	>10
Flavobacterium lutescens	1	>10	0.075	0.075	>2.1
Flavobacterium peregrinum	0.002	10	0.005	10	0.01
Micrococcus	0.015	0.025	0.002	2.5	4
Staphylococcus aureus	0.007	0.02	0.005	1.5	0.03

Antibiotic	Mechanism of action
Penicillin G	interferes with the synthesis of bacterial cell wall by inhibiting enzymes involved in transpeptidation, making them fragile and subject to rupture
Streptomycin	interrupts bacterial growth by binding to the 16S rARN subunit, preventing the synthesis of polypeptide chains
Chloramphenicol	inhibits the activity of the enzyme peptidyl transferase, preventing the coupling of amino-acyl-rARN to the site of the 50S subunit of the ribosome, preventing bacterial protein synthesis
Oxytetracycline	inhibits cell growth by preventing translation: coupled to the part of the 16S subunit of 30S ribosomes and prevents the action of amino-acyl-tARN in the site A of organelle
Neomycin	prevents the translocation site acyl peptidyl-tARN to the site A to site P resulting in mRNA reading errors which lead to the interruption of protein synthesis

Annex 3. List of important published papers on the use of antimicrobials in culture/production and in hypothesis testing to obtain a bacteria-free medium. *Unspecified.

Antimicrobials used	Application	Author(s)
penicillin	*Trichomonas foetus* (protozoan)	Ithaca & Mahmoud (1944)
*	*Trichomonas foetus* (protozoan)	Morgan (1946)
penicillin	*Trichomonas foetus* (protozoan)	Williams & Plastridge (1946)
penicillin	Protozoan	Seaman (1947)
*	Phytoplankton community	Fish (1950)
*	Nematode	Epps (1950)
penicillin, dihydrostreptomycin	*Euglena gracilis* (protozoan)	Goodwin (1950)
aureomycin + oxytetracycline hydrochloride + penicillin	*	Speck *et al*. (1951)
streptomycin $CaCl_2$ + penicillin	*Nitzschia closterium* (periphyton)	Spencer (1952)
penicillin	Phytoplankton community	Spencer (1952)
penicillin + streptomycin	*Euglena gracilis* (protozoan)	Pappas & Hoffman (1952)
nystatin	Phytoplankton community	Donovick *et al*. (1955)
penicillin + streptomycin	*Sardinops caerulea, Gadus callarias, Pleuronichthys* sp. (fish)	Oppenheimer (1955)
penicillin + streptomycin	Oyster (mollusk)	Walne (1956)
penicillin	*Amphibalanus eburneus* (barnacle)	Costlow & Bookhout (1957)
aureomycin	*	Velankar (1958)
penicillin + streptomycin	Oyster (mollusk)	Walne (1958)
streptomycin	Copepod	Marshall & Orr (1958)
terramycin, streptomycin, polymyxin-B, tetracycline, penicillin, bacitracin, chloromycetin and gramicidin	*Scenedesmus obliquus, Chlorella pyrenoidosa, Chlorella vulgaris* (phytoplankton)	Galloway & Krauss (1959)
penicillin	*Podophrya collini* (protozoan)	Palincsar (1959)
streptomycin	*Ramphogordius sanguineus, Amphiporus formidabilis* (nemertine)	Tuker (1959)
aureomycin, chloramphenicol, tetracycline, streptomycin, neomycin, erytromycin, novobiocin, vancomycin, penicillin, trypaflavin, filipin	*Dugesia dorotocephata* (platyhelminthes)	Miller & Johnson (1959)
penicillin + streptomycin	*Ectopleura crocea* (Cnidaria)	Fulton (1959)
chlortetracycline + chloramphenicol + streptomycin	*	Fell *et al*. (1960)
bicyclohexylamine	*Hydra oligactis, Hydra viridissima* (Cnidaria)	Claybrook & Eakin (1960)
penicillin + streptomycin	*Lecane inermis* (rotifer)	Dougherty *et al*. (1960)
penicillin	*Ectocarpus siliculosus, Hincksia secunda* (seaweed)	Boalch (1961)
nystatin	Phytoplankton community	Lampen & Arnow (1961)
*	Cyanobacteria	Tchan & Gould (1961)
*	*Neurospora crassa* (Fungi)	Kinsky (1961)
chlortetracycline + citric acid	Phytoplankton community	Suehiro & Tomiyase (1962)
penicillin + streptomycin/ chloramphenicol	*Mercenaria mercenaria* (mollusk)	Guillard (1959)
penicillin + dihydrostreptomycin	*Detonula confervacea, Cyclotella nana* 3H (phytoplankton)	Guillard & Ryther (1962)
penicillin + chloramphenicol + neomycin + polymyxin-B + dihydrostreptomicin + tetracycline + candicidin	*Gyrodinium cohnii* (protozoan)	Provasoli & Gold (1962)
streptomycin, penicilin	*Calanus hyperboreus* (copepod)	Conover (1962)
penicillin + streptomycin	Fed to *Ostrea edulis* (mollusk)	Walne (1963)
amphotericin-B + streptomycin	Phytoplankton community	Ghosh & Ghosh (1963)
penicillin + streptomycin	*Stylonema alsidii* (seaweed)	Fries (1963)
penicillin + chloramphenicol + neomycin + polymyxin B	*Gyrodinium resplendens* (phytoplankton)	Provasoli & McLaughlin (1963)
dihydrostreptomycin + streptomycin	*Mercenaria mercenária, Crassostrea virginica* (mollusk)	Hidu & Tubiash (1963)
polymyxin-B + neomycin	Diatom (phytoplankton)	Soli (1964)
chlortetracycline + citric acid	Phytoplankton community	Suehiro & Tomiyase (1964)
penicillin + streptomycin	*	Fuller *et al*. (1964)
penicillin + streptomycin	*Mya arenaria* (mollusk)	Stickney (1964)
penicillin + streptomycin + chloramphenicol	*Acetabularia* sp. (seaweed)	Keck (1964)
penicillin + streptomycin	*Mytilus edulis* (mollusk)	Bayne (1965)

Continuation

Antimicrobials used	*Application*	Author(s)
penicillin + streptomycin + chloramphenicol + Tween-80	*Tetraselmis maculata* (phytoplankton)	Antia & Kalmakoff (1965)
cycloheximide	*Crassostrea virginica* (mollusk)	Ray (1965)
penicillin + streptomycin	*Euterpina acutifrons, Temora turbinata, Oncaea* sp. (copepod)	Neunes & Pongolini (1965)
streptomycin, penicillin	*Calanus hyperboreus* (copepod)	Conover (1966)
penicillin + streptomycin	*Mycale contarenii* (Porifera)	Borojevic (1966)
penicillin + dihydrostreptomycin	*Penaeus aztecus* (shrimp)	Cook & Murphy (1966)
cycloheximide, oxytetracycline, chlortetracycline, tetracycline	*Crassostrea virginica* (mollusk)	Ray (1966)
penicillin + streptomycin + chloramphenicol	*Mercenaria mercenaria* (mollusk)	Millar & Scott (1967)
sulfamethazine	*Crassostrea virginica* (mollusk)	Calabrese & Davis (1967)
streptomycin, penicillin	*Calanus hyperboreus* (copepod)	Conover (1967)
tetracycline, streptomycin, penicillin	Invertebrates	Stephens (1967)
chloramphenicol + chlortetracycline + streptomycin	*	Meyers *et al.* (1967)
chloramphenicol + streptomycin / penicillin + streptomycin + neomycin + chloramphenicol	Diatoms (phytoplankton)	Droop (1967)
penicillin + neomycin + nystatin	*Acetabularia acetabulum (mediterranea)* (seaweed)	Green *et al*. (1967)
penicillin + streptomycin	*Rhincalanus nasutus* (copepod)	Mullin & Brooks (1967)
penicillin + dihydrostreptomycin + nystatin	Protozoan	Khouw *et al*. (1968)
chloramphenicol	*	Ahearn *et al*. (1968)
gentamicin	*	Ahearn *et al*. (1968)
penicillin + streptomycin	*Tintinnopsis tubulosa* (protozoan)	Gold (1968)
penicillin + streptomycin	*Tintinnopsis tubulosa* (protozoan)	Gold (1969)
neomycin + streptomycin	*Pinctada maxima* (mollusk)	Minaur (1969)
*	Hypothesis test with *Hydractinia echinata* (Cnidaria)	Müller (1969)
penicillin + streptomycin	*Deontostoma californicum* (nematode)	Viglierchio *et al*. (1969)
penicillin + streptomycin	*Ulva* (*Enteromorpha*) *linza, Cladophora rivularis* (*gracilis*) (seaweed)	Berglund (1969)
chloramphenicol + novobiocin	*Foraminifera* (protozoan)	Muller & Lee (1969)
spiramycin, ristocetin, sulfamerazine, triple sulpha, vancomycin, penicillin, streptomycin	Diatom (phytoplankton)	Berland & Maestrini (1969)
penicillin + streptomycin	*Crepidula fornicata* and *Nassarius reticulatus* (mollusk)	Pilkington & Fretter (1970)
penicillin + carbenicillin + kanamycin + vancomycin	*Acetabularia mediterranea* (phytoplankton)	Shephard (1970)
novobiocin + erythromycin, novobiocin + colimycin + nystatin, novobiocin + colimycin + fungizone	Nematode	Lee *et al.* (1970)
penicillin + streptomycin	*Elminius modestus* (barnacle)	Tighe-Ford *et al.* (1970)
penicillin	*Euterpina acutifrons* (copepod)	Nassogne (1970)
penicillin + streptomycin	Oyster (mollusk)	Walne (1970)
chloramphenicol	*	Seshadri & Sieburth (1971)
penicillin	*Callinectes sapidus* (crab)	Roberts (1972)
penicillin + streptomycin + chloromycetin	Invertebrates	McCammon (1972)
dihydrostreptomycin, binotal	*	Hoppe (1972)
several	Review (invertebrates)	D'Agostino (1972)
penicillin + gentamicin + streptomycin	Phytoplankton	Hoshaw & Rosowski (1973)
penicillin + chloramphenicol + neomycin + penicillin + tetracycline + chloramphenicol + aureomycin + ceporin + neomycin	*Oscillatoria* sp., *Pediastrum boryanum, Chlorella vulgaris* (phytoplankton)	Jones *et al*. (1973)
colimycin, geopen + carbenicillin, penicillin, penicillin + ampicillin, keflin + cephalothin, aerosporin	Fish	Struhsaker *et al*. (1973)
erythromycin, carbenicillin, penicillin, ampicillin, cephalothin, nitrofurantoin, sulfadiazine	*Caranx mate* (fish)	Struhsaker *et al*. (1973)
chloramphenicol + colimycin	Foraminifera (protozoan)	Lee (1974)
colimycin, streptomycin, penicillin, neomycin	Fish	Struhsaker *et al*. (1974)
penicillin + polymyxin-B	*Scylla serrata* (crab)	Brick (1974)
penicillin + streptomycin	*Mugil cephalus* (fish)	Nash *et al*. (1974)

Continuation

Antimicrobials used	*Application*	Author(s)
chloramphenicol	Fish	Struhsaker *et al*. (1975)
Iloticina	Phytoplankton community	Sheath (1975)
*	*Euglena gracilis, Mycobacterium phlei* (protozoan)	Ebringer *et al*. (1976)
*	*Amphibalanus eburneus* (barnacle)	Landau & D'Agostino (1977)
penicillin + streptomycin + chloramphenicol + neomycin + neomycin + cycloheximide	Phytoplankton community	Vieira (1977)
chloramphenicol + streptomycin + penicillin	*Cancer magister* (crab)	Fisher & Nelson (1978)
penicillin + streptomycin	Ulva (*Enteromorpha*) *intestinalis* (seaweed)	Millner *et al*. (1979)
penicillin + streptomycin	Hypothesis test with *Cassiopea andromeda* (Cnidaria)	Neumann (1979)
streptomycin + chloramphenicol	Phytoplankton community	Tipper & Wright (1979)
dihydrostreptomycin + neomycin + penicillin + amphotericin-B	*Gonyaulax catenella, Gonyaulax excavata* (protozoan)	Divan & Schnoes (1982)
penicillin + streptomycin + chloramphenicol	*Dictyosiphon foeniculaceus* (seaweed)	Saga & Sakai (1982)
penicillin, vancomycin, chloramphenicol/ vancomycin + penicillin	Hypothesis test	Sherr *et al*. (1986)
chloramphenicol	Hypothesis test	Wheeler & Kirchmann (1986)
*	Hypothesis test with *Cassiopea andromeda* (Cnidaria)	Fitt *et al*. (1987)
penicillin + streptomycin	*Elminius modestus* (barnacle)	Harms (1987)
chloramphenicol	*Artemia* (artemia)	Benavente & Gatesoupe (1988)
chloramphenicol	*Brachionus plicatilis* (rotifers)	Benavente & Gatesoupe (1988)
ampicillin, carbenicillin, cephaloridine, cephalothin, chloramphenicol, chlortetracycline, colistin, erythromycin, gentamicin, kanamycin, lincomicin, nalidixic acid, neomycin, novobiocin, rifampin, streptomycin, triple sulfa, tetracycline, trimethoprim, vancomicin	Seaweed	Bradley *et al*. (1988)
penicillin + streptomycin + polymyxin B + chloramphenicol	Seaweed	Tatewaki (1989)
sulfamethazine	Hypothesis test with *Arachnoides placenta* (Echinodermata)	Chen & Run (1980)
*	Review (phytoplankton)	McCracken (1989)
penicillin, neomycin, streptomycin	*Tridacna deresa* (mollusk)	Fitt *et al*. (1992)
penicillin, kanamycin, erythromycin, cephalothin, chloramphenicol, ampicillin, oxacillin, carbenicillin	*Artemia fransciscana* (artemia)	Rosowski *et al*. (1992)
penicillin, neomycin, gentamycin, kanamycin	*Micromonas pusilla* (phytoplankton)	Cottrel & Suttle (1993)
*	Hypothesis test with *Amphibalanus Amphitrite* (barnacle)	Avelin Mary *et al*. (1993)
chloramphenicol, oxolinic acid	Rotifers	Hernández-Cruz *et al*. (1994)
penicillin + dihydrostreptomycin + dextrose + chloramphenicol	*Tigriopus californicus* (copepod)	Shaw *et al*. (1994)
oxolinic acid, kanamycin, erythromycin, penicillin, streptomycin	*Brachionus plicatilis* (rotifers)	Munro *et al*. (1995)
penicillin + streptomycin + polymyxin-B + chloramphenicol	*Gymnodinium* (protozoan)	Hasui *et al*. (1995)
Penicillin + streptomycin + chloramphenicol	Phytoplankton	González *et al*. (1995)
framycetin + amoxicillin	*Prorocentrum cordatum* (protozoan)	Grzebyk *et al*. (1997)
streptomycin	Hypothesis test	Maurin *et al*. (1997)
*	*Amphibalanus amphitrite* (barnacle)	Thomason *et al*. (1998)
*	*Coscinodiscus wailesii* (phytoplankton)	Nagai *et al*. (1998)
ampicillin	Hypothesis test	Ringelberg & Van Gool (1998)
*	*Amphibalanus reticulatus* (barnacle)	Lee *et al*. (1999)
chloramphenicol	*Nodipecten nodosus* (mollusk)	Bem (1999)
kanamycin, crys-4, streptomycin, chloramphenicol	*Pinctada fucata* (mollusk)	Dharmaraj & Shahmugasundaram (1999)
penicillin + streptomycin + neomycin	Hypothesis test	Middelburg & Nieuwenhuize (2000a)
penicillin + streptomycin + neomycin	Hypothesis test	Middelburg & Nieuwenhuize, 2000b
penicillin + streptomycin + neomycin + cycloheximide	Hypothesis test	DeLorenzo *et al*. (2001)
streptomycin + furaltadone	*Argopecten purpuratus* (mollusk)	Riquelme *et al*. (2001)
chloramphenicol	*Argopecten purpuratus* (mollusk)	Uriarte *et al*. (2001)
flumequine, oxytetracycline	*Brachionus plicatilis* (rotifers)	Göksan & Gökpinar (2001)
*	Rotifers	Dhert *et al*. (2001)

Continuation

Antimicrobials used	*Application*	Author(s)
chloramphenicol, gentamicin, kanamycin, streptomycin, ampicillin, tetracycline	*Alexandrium catenella* (protozoan)	Códova *et al*. (2002)
oxytetracycline, oxolinic acid	*Penaeus monodon* (shrimp)	Tendencia & Peña (2002)
benzylpenicillin + streptomycin	Diatom (phytoplankton)	Wolfstein *et al*. (2002)
benzylpenicillin + chloramphenicol	Hypothesis test	Bidle *et al.* (2003)
penicillin + streptomycin	Phytoplankton	Tavares & Rocha (2003)
gentamicin + penicillin + streptomycin	*Pseudo-nitzschia multiseries* (periphyton)	Kobayashi *et al*. (2003)
antibiotic + fungicide*	Fish	Saborido-Rey *et al*. (2003)
streptomycin	Hypothesis test	Tungaraza *et al*. (2003)
oxytetracycline	Fish	Bruun *et al*. (2003)
chloramphenicol	*Isochrysis galbana* (phytoplankton)	Subhash *et al*. (2004)
penicillin + streptomycin + neomycin	Hypothesis test	Veuger *et al*. (2004)
oxytetracycline	*Penaeus japonicus* (shrimp)	Uno (2004)
oxytetracycline	*Litopenaeus setiferus* (shrimp)	Reed *et al*. (2004)
penicillin + streptomycin	*Alexandrium tamarense* (protozoan)	Wang *et al*. (2004)
ampicillin, gentamicin, penicillin, streptomycin, erythromycin, chlortetracycline, kanamycin	*Penaeus monodon* (shrimp)	Vaseeharan *et al*. (2004)
oxytetracycline, amoxicillin, oxolinic acid	*Hippoglossus hippoglossus* (fish)	Verner-Jaffreys *et al*. (2004)
ciprofloxacin	*Lepomis gibbosus* (fish)	Richards *et al.* (2004)
ciprofloxacin	Phytoplankton community	Richards *et al.* (2004)
ciprofloxacin	Zooplankton community	Richards *et al.* (2004)
sulfadiazone, dihydrostreptomycin, tylosin, norfloxacin, erythromycin, sulfadimethoxine, cefazolin, ampicillin, trimethoprim, pyrimethamine, sulfamethoxazole, oxytetracycline, thiamphenicol	*Raphidocelis subcapitata, Chlorella vulgaris* (phytoplankton)	Eguchi *et al*. (2004)
tetracycline + oxytetracycline + doxycycline + chlortetracycline	Phytoplankton community	Wilson *et al*. (2004)
tetracycline + oxytetracycline + doxycycline + chlortetracycline	Zooplankton community	Wilson *et al*. (2004)
penicillin + streptomycin + polymyxin-B + chloramphenicol	*Cochlodinium polykrikoides* (protozoan)	Cho & Costas (2004)
erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin, clarithromycin	*Brachionus calyciflorus calyciflorus* (rotifers)	Isidori *et al.* (2005)
erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin, clarithromycin	*Thamnocephalus platyurus* (Artemia)	Isidori *et al*. (2005)
erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin, clarithromycin	*Ceriodaphnia dubia, Daphnia magna* (cladoceran)	Isidori *et al*. (2005)
erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin, clarithromycin	*Danio rerio* (fish)	Isidori *et al*. (2005)
streptomycin + penicillin	Hypothesis test	Tang *et al*. (2006a)
streptomycin + penicillin	Hypothesis test	Tang *et al*. (2006b)
penicillin + streptomycin + neomycin	Hypothesis test	Cozzi & Cantoni (2006)
several	Phytoplankton	Lourenço (2006)
chloramphenicol, erythromycin, furaltadone	*Isochrysis galabana, Chaetoceros gracilis* (phytoplankton)	Campa-Córdova *et al*. (2006)
tetracycline, oxytetracycline, amoxicillin, trimethoprim + sulphonamide, trimethoprim	*Abalone* (mollusk)	Handlinger *et al.* (2006)
oxytetracycline	Fish	Pereira Jr. *et al*. (2006)
oxytetracycline	*Penaeus monodon* (shrimp)	Uno *et al*. (2006)
*	*Chattonella marina* (phytoplankton)	Kim *et al*. (2006)
penicillin + streptomycin	*Amphibalanus amphitrite, Fistulobalanus albicostatus, Megabalanus rosa* (barnacle)	Yoshimura *et al*. (2006)
*	*Chattonella marina, Chattonella ovata* (phytoplankton)	Kim *et al*. (2007)
penicillin + streptomycin	*Fistulobalanus albicostatus* (barnacle)	Chen *et al*. (2007)
chloramphenicol, ciprofloxacin, nitrofuratoin	*Artemia fransciscana* (Artemia)	Castro-Mejía *et al*. (2007)
streptomycin	Hypothesis test	Fouilland *et a*l. (2007)
streptomycin + kanamycin	Hypothesis test	Hamdan & Jonas (2007)

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Antimicrobials used	*Application*	Author(s)
ampicillin, cefuroxina, chloramphenicol, erythromycin, gentamicin, nitrofurantoin, norfloxacin, tetracycline, sulfonamides	*Oreochromis niliticus* (fish)	Carneiro *et al*. (2007)
dihydrostreptomycin, neomycin, chloramphenicol/ neomycin + dihydrostreptomycin	*Chlorella ellipsoidea, Isochrysis galbana, Hetrosigma akashiwo, Cyclotella didymus, Thalassiosira alleni* (phytoplankton)	Youn & Hur (2007)
penicillin + streptomycin	Hypothesis test with *Mytilus galloprovincialis* (mollusk)	Bao *et al*. (2007)
penicillin + streptomycin	Hypothesis test with *Haliotis iris* (mollusk)	Roberts *et al*. (2007)
nalidixic acid, tetracycline, chloramphenicol, imipenem, cefoxitin, gentamicin, ciprofloxacin, rofurantoin, ceftriaxone, ampicillin, sulphazotrim, trimethoprim	*Litopenaeus vannamei* (shrimp)	Costa *et al*. (2008)
rifampicin	*Boeckella antiqua* (copepod)	García *et al*. (2008)
triclosan, triclocarban, roxithromycin, clarithromycin, tylosin, tetracycline, chlortetracycline, norfloxacin, sulfamethoxazole, ciprofloxacin, sulfamethazine, trimethoprim	*Pseudokirchneriella subcapitata* (phytoplankton)	Yang *et al*. (2008)
gentamicin	Hypothesis test	Tartarotti & Torres (2009)
gentamicin	*Acartia tonsa* (copepod)	Tartarotti & Torres (2009)
penicillin + streptomycin	Hypothesis test	Pringault *et al*. (2009)
chloramphenicol, florfenicol, tianfenicol	*Chlorella pyrenoidosa, Isochrysis galbana, Tetraselmis chui* (phytoplankton)	Lai *et al*. (2009)
penicillin + streptomycin/ gentamicin	*Oxyrrhis marina* (phytoplankton)	Lowe *et al*. (2011)
penicillin + streptomycin	Hypothesis test	Trotted *et al*. (2011)
*	*Gadus morhua* (fish)	Forberg *et al*. (2011)
ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin/ ampicillin + kanamycin + nalidixic acid + streptomycin	Periphyton	Suga *et al*. (2011)
ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin/ ampicillin + kanamycin + nalidixic acid + streptomycin	Phytoplankton community	Suga *et al*. (2011)
ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin/ ampicillin + kanamycin + nalidixic acid + streptomycin	Zooplankton community	Suga *et al*. (2011)
ampicillin, chloramphenicol, kanamycin, nalidixic acid, streptomycin/ ampicillin + kanamycin + nalidixic acid + streptomycin	Macroinvertebrate community	Suga *et al*. (2011)
streptomycin, chloramphenicol, kanamycin, co-trimoxazole, erythromycin, oxytetracycline, ampicillin, nystatin, gentamicin, penicillin, furazolidone, ciprofloxacin, rifampicin, neomycin	*Portunus pelagicus* (crab)	Talpur *et al*. (2011)
ciprofloxacin	*Fragilaria radians* (phytoplankton)	Shishlyannikov *et al*. (2011)
streptomycin	*Oncomelania hupensis* (mollusk)	Aina *et al*. (2012)
imipenem	*Achnanthidium minutissimum, Cymbella affiniformis, Nitzschia dissipata* (phytoplankton and periphyton)	Windler *et al*. (2012)
oxytetracycline, ciprofloxacin	*Scenedesmus obliquus* (phytoplankton)	Zhang *et al*. (2012)
chlortetracycline	*Microcystis aeruginosa, Scenedesmus obliquus* (phytoplankton)	Guo & Chen (2012)
ciprofloxacin, lincomycin, tylosin	*Ceratoneis closterium, Navicula ramosissima* (periphyton)	Hagenbuch & Pinckney (2012)
erythromycin	*Litopenaeus vannamei* (shrimp)	Rego *et al*. (2012)
streptomycin⁄ penicillin, ampicillin, rifampicin, nalidixic acid, tetracycline, gentamicin, nystatin	*Thraustochytrids* (protozoan)	Wilkens & Maas (2012)
amoxicillin, erythromycin, levofloxacin, norfloxacin, tetracycline	*Anabaena* (cyanobacteria)	González-Pleiter *et al*. (2013)
amoxicillin, erythromycin, levofloxacin, norfloxacin, tetracycline	*Raphidocelis subcapitata* (phytoplankton)	González-Pleiter *et al*. (2013)
penicillin + streptomycin	*Acutodesmus obliquus, Chlorella kessleri* (phytoplankton)	Stemmler (2013)
tylosin	Periphyton	Pinckney *et al*. (2013)
penicillin + streptomycin	Hypothesis test with *Mytilus unguiculatus* (mollusk)	Yang *et al*. (2013)
oxytetracycline + trimethoprim	*Dolichospermum flosaquae* (cyanobacteria)	Kolar *et al*. (2014)
oxytetracycline + trimethoprim	*Raphidocelis subcapitata* (phytoplankton)	Kolar *et al*. (2014)
oxytetracycline + trimethoprim	*Daphinea magna* (cladoceran)	Kolar *et al*. (2014)
enrofloxacin	Periphyton	Rico *et al*. (2014)

Continuation

Antimicrobials used	*Application*	Author(s)
enrofloxacin	Phytoplankton community	Rico *et al*. (2014)
enrofloxacin	Zooplankton community	Rico *et al*. (2014)
enrofloxacin	Invertebrates	Rico *et al*. (2014)
rifampicin, tryptone	*Mytilus edulis* (mollusk)	Eggermont *et al*. (2014)
ciprofloxacin, sulfamethoxazole	Periphyton	Johansson *et al*. (2014)
penicillin + vancomycin/ penicillin + streptomycin/ neomycin/ penicillin + streptomycin + chloramphenicol	*Isochrysis galbana, Conticribra weissflogii* (phytoplankton)	Agostini (2014)
penicillin + streptomycin/ neomycin/ penicillin + chloramphenicol + neomycin + streptomycin + tetracycline/ penicillin + streptomycin + chloramphenicol/ penicillin + vancomycin	*Temora turbinata* (copepod)	Agostini (2014)
penicillin + streptomycin + neomycin/ penicillin + streptomycin	*Acartia tonsa* (copepod)	Agostini (2014)
streptomycin + ampicillin	*Limacina helicina* (mollusk)	Howes *et al*. (2014)
enrofloxacin, ciprofloxacin	*Pangasius* (fish)	Andrieu *et al*. (2015)
ampicillin + gentamycin + kanamycin + neomycin + streptomycin	*Nannochloropsis* sp., *Cylindrotheca* sp., *Tetraselmis* sp. *Amphikrikos* sp. (phytoplankton)	Han *et al*. (2016)
ampicillin, neomycin, kanamycin, chloramphenicol, sulphate G418, streptomycin, carbencillin	*Isochrysis galbana* (phytoplankton)	Molina-Cárdenas *et al*. (2016)
penicillin + streptomycin + neomycin	*Acartia tonsa* (copepod)	Agostini *et al*. (2016)