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TESI DI LAUREA

Ecotoxicity assessment of fluoroquinolones and sulfonamides, in two saltwater organisms

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ABSTRACT

Fluoroquinolones (FQs) and Sulfonamides (SAs) are widely used as antimicrobials in both medical and veterinary fields due to their effectiveness and broad spectrum of action against various pathogenic microbial species. However, this has resulted in their widespread presence in the aquatic environment. Sources of contamination include pharmaceutical discharges, urban sewage, aquaculture facilities, and agricultural lands fertilized with manure/slurry from animals subjected to mass medication. The effect of these antimicrobials on the aquatic environment is well documented in the scientific literature, both concerning surface waters and river and lake sediments; however, very little is known about their impact on marine ecosystems. This thesis evaluated the ecotoxicity of eight antibiotics, four FQs, and four SAs, on two salt-water organisms: the algae *Phaeodactylum tricornutum* and the crustacean *Artemia salina*. This choice was motivated by the importance of assessing effects on the first two trophic levels, which can have bottom-up repercussions on the marine ecosystem. Algal growth inhibition tests (72h) were run in *P. tricornutum* whilst acute immobilization tests (48h) were performed on *A. salina*. The results highlighted significant toxic effects of SAs on *P. tricornutum*, while with FQs, effects were observed only at rather high concentrations. Unlike FQs, SAs, in three out of four cases, demonstrated algicidal effects, which apparently are not limited by the addition of folic acid to the culture medium. Regarding *A. salina*, adverse effects were observed only with Ciprofloxacin, suggesting its potential use in future chronic toxicity tests. By comparing the EC_{50} values measured in *P. tricornutum* with the environmental concentrations reported in the literature, a preliminary assessment of the Risk Quotient (RQ) was possible, highlighting Sulfadiazine and Sulfamethoxazole as the two antibiotics that deserve the most attention.

1. INTRODUCTION

Ecotoxicology is a term derived from the combination of "eco", from the Greek word oikos (home), meaning and including everything related to the environment, and toxicology, from the Greek word toxicon (poison) and logos (science), indicating the study of poisons (Férard, 2013). This modern discipline was defined by Renè Truhaut, as the analysis of the effects of toxic substances that reach the environment, including both natural and man-made contaminants, on living organisms (Truhaut, 1977) .
Ecotoxicological studies, which are characterized by an interdisciplinary approach that integrates ecology and toxicology, are essential for understanding the impact on different ecosystems, thus distinguishing themselves from classical toxicology, which focuses primarily on the human species. These studies extend their analysis from the level of the individual organism down to the ecosystem population and communities, including holistic approaches and taking into consideration the different trophic levels (Chapman, 2002).
The goal of these studies is environmental protection; this links ecotoxicology to regulatory processes and procedures concerning the preservation and protection of ecosystems (Belden, 2020).
The relevance of ecotoxicology in the dynamics of protecting the environment and the life forms that inhabit it has been emphasized by distinguished scientists such as the aforementioned Rene Truhaut and the American scientist Rachel Carson. The latter contributed, as early as the 1960s, to raising public awareness of the environmental impact of pesticides.
Over the years, agencies such as the US-Enviromental Protection Agency (US-EPA) have developed standardized protocols for assessing ecotoxicity. These protocols, with tests of different durations, from acute to chronic, allow comparative evaluation of results among different laboratories and contribute to the regulation of the substances assayed.
In modern ecotoxicological studies the approach has evolved, defining more diverse endpoints and refining the parameters for measuring ecotoxicity, with the addition to the already existing NOAEC (No Observed Adverse Effect Concentration) and LOAEC (Lowest Observed Adverse Effect Concentration) of the calculation of EC_{05} and EC_{10}, thus providing more precise information on the exposure concentration at which the toxic effect begins.
Through the application of evaluation criteria and assessment factors for the lowest NOEC or EC_{50} found in at least three species, each belonging to a different trophic level, the threshold dose of environmental safety, termed PNEC (Predicted No Effect Concentration), is determined, which is then compared to the maximum concentration found in the environmental compartment

of interest, to calculate the RQ (Risk Quotient) which if ≥1 indicates that the risk is not negligible (CHMP, 2006).

1.1 The use of antibiotics in human and veterinary medicine

The discovery of antibiotics and the ability to synthesize them marked a fundamental turning point in the history of medicine and humanity. Together with vaccines and the general improvement of hygienic conditions, antibiotics have made it possible to heal and prevent infectious diseases that only a century ago were often lethal or could result in serious, even permanent, damage.
The term antibiotic (from the Greek anti, against, and bios, life) originally referred only to substances produced by microorganisms (bacteria or fungi) and capable of inhibiting the growth of other microorganisms and/or causing their destruction. In current common usage, however, the term antibiotic denotes antibacterial drugs both of natural and synthetic origin.

The history of antibiotics began in 1928, when Alexander Fleming, a Scottish physician, working on some strains of bacteria, observed that a fungus that contaminated one of his cultures had inhibited their growth. Because the mold belonged to the genus *Penicillium*, Fleming named this antibacterial substance 'penicillin.' More than a decade later, thanks to the work of Ernst Chain and Howard Walter Florey who were able to isolate and purify the antibiotic, its production and use for the treatment of bacterial infections began. Meanwhile, in 1935, German physician Gerhard Domagk identified antibacterial capabilities in Prontosil red, a dye used in the textile industry. Later, French physician Daniel Bovet identified sulfanilamide as the active component of the dye substance from which Sulfonamides (SAs) would be made.
With the discovery and synthesis of penicillin and SAs, therefore, came a particular group of drugs, the antibiotics, which would lead to the treatment of many diseases of the respiratory system, the skin, the urinary tract, but also serious intestinal infections, such as typhoid, that were quickly fatal in the past.

The antibiotic works by blocking certain vital functions of the bacterium, stopping its growth, thus preventing it from multiplying (bacteriostatic), or killing it (bactericidal). Of course, with the least possible harm to the host organism. This is made possible by selective toxicity, which is the ability of antibiotics to selectively hit a target that is present in the bacterium but absent in the host cells. Indeed, selective toxicity is based on the differences between prokaryotic and eukaryotic cells.

Today, the main problem associated with the widespread use of antibiotics is that of bacterial resistance, which is the other side of the coin. Nearly 100 years after the discovery of penicillin, we have several classes of antibiotics (penicillins, sulfonamides, cephalosporins, aminoglycosides, tetracyclines, macrolides, quinolones etc.), but the spread of antibiotic-resistant superbugs (superbugs) threatens to take us back to the pre-antibiotic era, when many surgical procedures and even some dental treatments exposed patients to serious infections.

Antibiotic resistance indicates a partial or complete loss of activity by an antibiotic against a previously susceptible bacterium. The phenomenon of antibiotic resistance is complex and has a multifactorial genesis. One of the main causes is the excessive, and sometimes unnecessary, use of antibiotics. From this perspective, prudent use of antibiotics is an individual's responsibility to his or her own health and to the community. Antibiotic resistance is a phenomenon that involves the use of antibiotics not only in human but also in veterinary medicine. Indeed, intensive livestock farms are very often under indictment for antibiotic abuse.

As early as the middle of the last century, the food industry faced an increasing demand for its products, which prompted it to accentuate and expand its activities. This expansion, both in land-based farming and aquaculture, led to an overloading of facilities, resulting in hygienic problems and frequent occurrence of diseases in the farmed species. This was worstened by the increase in stress that any reduction in usable space brings to the animals. Stress, on the other hand, having a negative impact on the immune system, contributes to increased episodes of infection. This situation made it imperative in animal husbandry to apply a rapid and effective response to the onset of infection. Therefore, in cattle and poultry farming, and even in aquaculture, the use of antibacterial drugs gained ground (Cabello, 2006).

The massive use of antibiotics in animal husbandry, for not only therapeutic but also prophylactic, metaphylactic, and growth-promoting purposes, began around 1940, proving to be particularly cost-effective (Cherian *et al.*, 2023). The growth-promoting use allowed for higher yields, while prophylactic use allowed for damage limitation at critical steps in the farming cycle (weaning, arrival of new animals, transportation etc.).
Today there is a very strict legislation in Europe on the use of antibiotics in the livestock sector. Mainly because of the fight against antibiotic resistance, the European Union has recently banned the use of antibiotics in animals for preventive purposes

and authorizes it only for therapeutic or metaphylactic purposes. Another use permanently banned in the EU, since 2006, is the growth-promoting one, which, by employing small doses of pharmaceuticals over long periods, has probably made a major contribution to the spread of antibiotic resistance.

Since the years following their first use, antibiotics have gone through a period of steady development, both in quantitative terms, with the increase of antibiotic classes, and in qualitative terms, due to the gradual increase in their efficacy. SAs, which were the first synthetic antibacterials, have seen a gradual decline in interest in the human sector, giving way to more modern molecules; however, this decline has not occurred in the livestock sector, due to the cost-effectiveness of this class of antimicrobials, and their ability to also act on protozoa, which are microorganisms often responsible for disease in livestock. Instead, among the most interesting antibiotics that have emerged over the years, mention should be made of Fluoroquinolones (FQs); these compounds have found wide application both in the human field and in the context of animal production, due to their remarkable therapeutic efficacy (Miller & Harbottle, 2018).

1.2 The sources of antibiotics and their pathways to the aquatic environment

Major sources of antibiotic release into the environment include pharmaceutical manufacturers, urban discharges, land-based farms, and aquaculture.
The problem of discharges from pharmaceutical companies is particularly serious in some areas of India where there is a high concentration of this industry, which is not matched by adequate implementation of controls and verification of compliance with emission limits. For example, in Patancheru, near Hyderabad, concentrations of antibiotics in the order of mg L^{-1} and as much as 33 mg L^{-1} of Ciprofloxacin, a FQ widely used in human medicine, were found in the effluent of a wastewater treatment plant receiving wastes from 90 pharmaceutical manufacturers (Larsson *et al.*, 2007).
Urban discharges, particularly those from highly populated areas, inevitably contribute to antibiotic contamination of the aquatic environment, as the unmetabolized portion of the drug is excreted. Wastewater treatment plants, on the other hand, allow only partial elimination of this contamination, with appreciable differences depending on the type of drug considered.
Animals raised for the production of meat, eggs and milk, when treated with antibiotics, like humans, eliminate a certain amount

of them in active form. The transfer of these antibiotic residues to the environment is then accomplished through the use of manure or slurry to fertilize farmland. From the soil, antibiotics, which are generally weak acids or bases endowed with a certain degree of water-solubility, will be transported to surface waterways by rainwater, through infiltration and runoff phenomena.
Microbial agents can cause the emergence and occurrence of diseases in aquaculture farms. Due to the vast economic impact of these events, the prompt administration of antibiotics has been and still is considered indispensable. Some intrinsic properties of these molecules, such as non-biodegradability and a relative water-solubility, lead them to be easily transported and stored in the environment surrounding the farm facilities.
In aquaculture, antibiotics are generally administered via medicated feed to the entire group of animals or, in rare cases, via injection to individual, valuable fish (Miller & Harbottle, 2018). After medicated feed has been administered to fish, antibiotics may contaminate the surrounding environment, either because a portion of the dose taken up is excreted without being metabolised, or because part of the administered feed is not ingested. Another portion that is dispersed in the environment is the drug that, although taken with the feed, is not absorbed and transits through the digestive tract to be eliminated as such with the faeces. It can therefore be argued that the administration of antibiotics via medicated feed is not the most efficient solution from both an economic and an environmental point of view; however, it is an extremely practical method to treat large numbers of fish. Typically, the dispersion of drugs in the aquatic environment surrounding aquaculture favours their progressive accumulation in sediments. For example, Flumequine has been found at concentrations of up to 578.8 μg kg^{-1} d.w. in sediments surrounding Italian aquaculture farms. Concentrations of up to 1.1 μg kg^{-1} d.w. have also been found in sediments of watercourses downstream the same farms (Lalumera *et al.*, 2004).

1.3 The impact of antibiotics on the aquatic environment

One of the adverse effects due to the presence of antibiotics in the aquatic environment is the selective pressure they exert on bacterial populations, resulting in the development of resistant strains. Species that exhibit antibiotic resistance, even if not pathogenic, can horizontally transfer resistance to pathogenic organisms that, in turn, can infect other animal species or humans (Miller & Harbottle, 2018). In addition, in many cases, the same mechanism of action that these antibiotics exhibit against bacteria also proves effective on non-target organisms, especially of the

lowest trophic levels (primary producers and primary consumers). Assessment of the environmental impact of pharmaceuticals involves conducting tests on model organisms to define certain toxicity parameters such as EC_{50} and NOEC. In addition, sensitive species could be used as biological indicators of the presence of antibiotics in the aquatic environment (Thompson, 1985).

2. FLUOROQUINOLONES

The introduction of quinolones, for antibiotic therapy, dates back to 1965, through the approval of Nalidixic Acid. This compound was not, however, as successful as hoped. It, in fact, showed limited clinical application because of poor oral absorption, moderate antibacterial activity after intravenous administration, high binding to plasma proteins, and low patient tolerance (Bryskier, 2005). In the following years, other quinolones were approved for clinical use; then, in the 1970s, the progenitor of the FQs, Flumequine, was synthesized and marketed. In the early 1980s, development in the synthesis of these compounds led to the addition to the basic structure of the quinolones, not only of the fluorine atom at position 6, but also of a piperazine molecule at position 7; this led to a significant improvement in both the antimicrobial and pharmacokinetic characteristics of the FQs.

In Figure 1 changes in the basic structure of quinolones are shown.

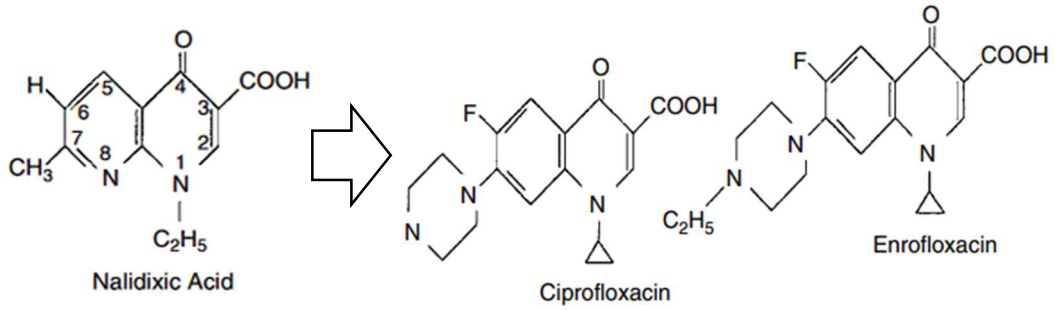


Figure 1. Chemical structure of quinolones and fluoroquinolones.

Among the first FQs to be approved and administered in human medicine were Norfloxacin and, later, Ciprofloxacin. Then, in the year 1988, Enrofloxacin was approved in the US for use in veterinary medicine. In the years to follow, seven other fluoroquinolones were approved for marketing (Giguère *et al.*, 2013).

2.1 FQs are classified according to two specific distinguishing keys: chemical structure and biological activity.

The classification based on chemical structure takes into consideration the number of rings associated with the pyrimidine-beta-carboxylic nucleus. In summary, Group I of FQs includes compounds derived from monocyclic molecules, Group II from tricyclic molecules, and Group III from quadricyclic molecules. Most FQs currently on the market belong to Group II.
The classification based on biological activity, on the other hand, takes into consideration the activity that the compounds perform, which defines their spectrum of action. In the first-generation FQs, one finds molecules that manifest specific activity against enterobacteriaceae, such as Flumequine and Cinoxacin (no longer on the market).

In the second generation of FQs we find, however, most of the approved compounds administered in humans and animals. These FQs have broad-spectrum antimicrobial activity and include, for example, Ciprofloxacin, Norfloxacin, and Enrofloxacin.

As for the third generation of FQs, it includes drugs such as Levofloxacin and Pradofloxacin, which exhibit strong antimicrobial activity against streptococci and obligate anaerobic bacteria (Bryskier, 2005).
Finally, Moxifloxacin and Gemifloxacin are part of the fourth Generation, with further expanded spectrum against both Gram+ and anaerobes (Idowu & Schweizer, 2017).
Flumequine, the first FQ, proved essential in tackling bacterial infections that began to emerge in intensive aquaculture farms, supporting their economics and maintaining their production efficiency. The first uses of Flumequine are documented in the literature from 1970 onward (Ruiz, 2019). As noted above, further and subsequent developments in the synthesis of these molecules led to the emergence of other FQs. New synthesized molecules such as Ciprofloxacin and Levofloxacin found application in human medicine, enjoying enormous success. The remarkable efficacy of this family of antibiotics in producing the bactericidal effect stems from their mechanism of action, which was initially thought to be highly selective. FQs, in fact, inhibits two specific enzymes, DNA Gyrase and Topoisomerase IV, which play a key role in DNA replication in bacterial cells but are not present in mammalian cells (Blondeau, 2004). Subsequent research, however, revealed that DNA gyrase is not exclusive to bacteria, as its presence has been demonstrated both in green algae (Thompson, 1985) and plants (Wall *et al.*, 2004).

In addition, more recent studies have shown that Ciprofloxacin can interfere with topoisomerase II (Top2α and Top2β) found in mammalian mitochondria, leading to mitochondrial damage: a phenomenon referred to as 'mitotoxicity' that is likely responsible for some side effects observed in humans, such as nephropathy and tendinopathy (Badal *et al.*, 2015).
Lastly, FQs can act at the level of RNA and protein synthesis through trapping of Topoisomerase IV complexes on DNA (Martinez *et al.*, 2006; Maxwell A. and Critchlow, 1998).

2.2 Fluoroquinolones and the aquatic environment

Based on investigations carried out on the concentration of FQs in riverine and estuarine environments, it is possible to affirm their presence and distribution, particularly in sediments. Indeed, FQs are characterized by a high affinity for organic material, despite their relatively low K_{ow}.

Concentrations in sediments can reach mg kg^{-1}, while in the water column they usually do not exceed a few μg L^{-1}. Exceptions to this rule are Asian countries where surveys, particularly in the vicinity of manufacturing activities or aquatic farms, report concentrations of FQs as high as mg L^{-1}.

To date ecotoxicity test show the following ranking of ecotoxicity in the freshwater environment: cyanobacteria > aquatic plants > unicellular algae > crustaceans > fish. In Figure 2 the lowest EC_{50s} measured in the different taxa are reported (Pauletto & De Liguoro, manuscript in preparation). Of particular note is the toxicity to aquatic plants which is surprisingly higher than that to unicellular green algae.

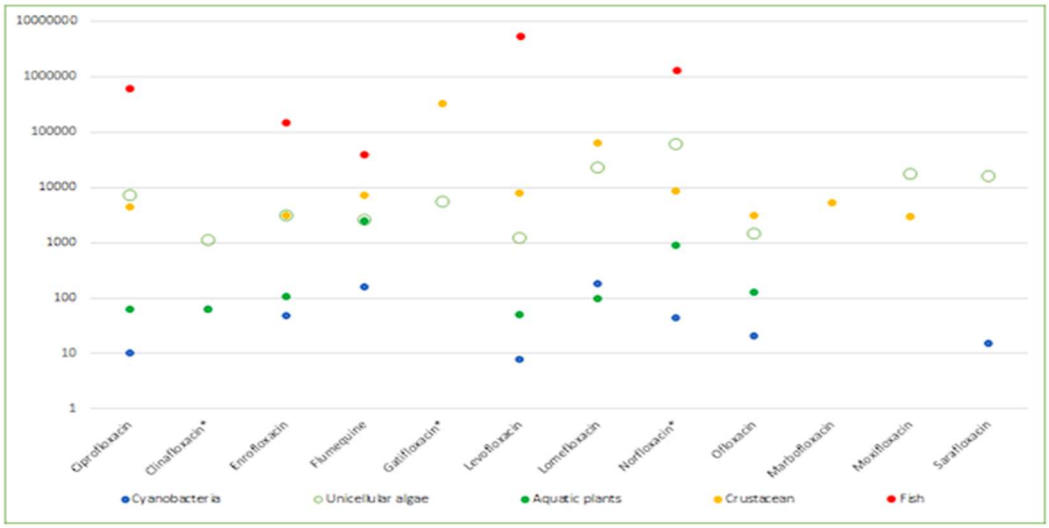


Figure 2. Lowest EC_{50} values (µg L^{-1}) of the various fluoroquinolones, for different trophic levels of freshwater ecosystems.

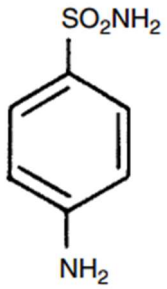
3. SULFONAMIDES

SAs were the first effective chemotherapeutic agents to be used for prophylaxis and treatment of bacterial infections in humans. The first SA introduced into therapy was an azo dye, p-sulfanylcrisoidine, which was given the name Prontosil Rubrum because of its red color. Produced in Germany in 1932, it was officially introduced to medical science by Domagk, in 1935. The first clinical study was reported, in 1933, by Foerster, who administered Prontosil to a 10-month-old infant with staphylococcal septicemia and achieved a sensational recovery. Later, through studies conducted by two British researchers, Colebrooke and Kenny, it was understood that the therapeutic effects of Prontosil in meningococcal meningitis were due to its active metabolite, Sulfanilamide.

In addition to human medicine, SAs have attracted a great deal of interest in veterinary medicine and have been part of animal husbandry practice for more than 40 years because of their broad spectrum of action and relatively low cost.

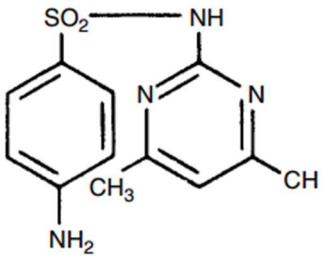
3.1 Chemical structure

Each of these compounds is derived from possible modifications of the chemical structure of Sulfanilamide, which has specific structural prerequisites that justify its antibacterial activity.

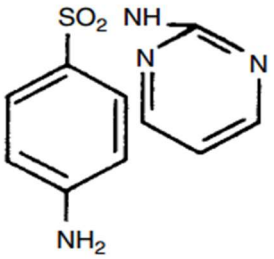


Sulfanilamide

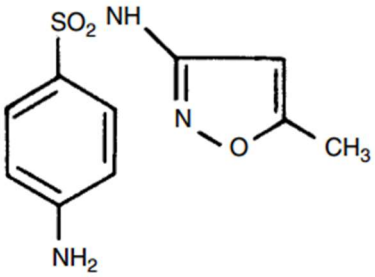
SAs result, in fact, from modification of the radical (R) that is docked to the amide group (-SO_2NHR) or, only occasionally, from substitution of the group (-NH_2). There are currently on the market at least a dozen antimicrobials belonging to this family.



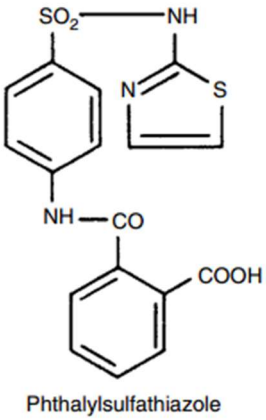
Sulphamethazine



Sulfadiazine



Sulfamethoxazole



Phthalylsulfathiazole

This class of antibiotics is distinguished by a modest solubility that, however, becomes high in alkaline environment (Giguère *et al.*, 2013).

3.2 Therapeutic characteristics of Sulfonamides

SAs have antimicrobial, broad-spectrum action, being active against both Gram+ and Gram- bacteria. Their activity is only bacteriostatic, so the host's humoral and cellular defense mechanisms are essential for complete eradication of the infection.
Many organisms are susceptible to the action of SAs, including *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Chlamydia trachomatis*.
Among the various chemotherapeutic agents in the veterinary sector, SAs are still recommended as particularly useful drugs to fight bacterial infections. SAs also have the merit of being effective against protozoan diseases such as, leukocytozoonosis, malaria (plasmodium) of chickens, toxoplasmosis of pigs, and coccidiosis

of cattle, against which other antibacterial agents are totally ineffective.
All these aspects, combined with their modest cost, make SAs excellent candidates for use in animal husbandry. In fact, they are used for a number of diseases:

- septicemias;
- listeriosis, pasteurellosis, salmonellosis, colibacillosis, tetanus, anaplasmosis, coccidiosis;
- pneumonia, diphtheria, gangrenous laryngitis;
- foreign body syndromes, enteritis;
- metritis, endometritis, placental retention, genital infections;
- acute bacterial arthritis;
- acute mastitis;
- umbilical infections, purulent processes, phlegmon, postoperative prophylaxis.

3.3 Antimicrobial activity and mechanisms of action

SAs are broad-spectrum agents having high efficacy against Gram+ and some Gram- bacteria. The bacteriostatic action of SAs is due to their ability to interfere with the tetrahydrofolic acid (THF) biosynthetic pathway within microorganisms. THF is the starting point for the biosynthesis of purines, pyrimidines and amino acids.
In mammals, THF synthesis occurs from folic acid (vitamin B9), which is taken in through the diet. In bacteria, however, THF is obtained from dihydropteroic acid: a molecule of dihydropteroate diphosphate is converted to dihydropteroate by the enzyme dihydropteroate synthetase and the addition of a molecule of p-aminobenzoic acid (PABA).
SAs are structural analogs of PABA: the similarity is both in spatial structure, in bond length and angles, and in functional group reactivity. As a result, SAs enter the biosynthetic pathway in place of PABA, exerting competitive inhibition of dihydropteroate synthetase and preventing THF formation. The enzyme's affinity for SAs is 10,000 times greater than that for PABA. Bacteriostatic action is preceded by a dormancy phase that is due to folate and PABA accumulated in the cell. The latency period continues until the folic acid reserve is depleted (Ovung & Bhattacharyya, 2021).

3.4 Environmental impact of Sulfonamides

SAs are polar molecules with amphoteric properties, classified as photo and thermally stable. By virtue of their stability, they accumulate in ecosystems and in various organisms of the trophic chains. The presence of SAs residues has been repeatedly detected in aquatic environments in different parts of the world (Giang *et al.*, 2015); (Perret *et al.*, 2006); (Xu *et al.*, 2007); (Perret *et al.*, 2006); (García-Galán *et al.*, 2009); (Santos *et al.*, 2010); (Guedes-Alonso *et al.*, 2013).

SSs adsorb little to the soil, so they are easily transferred to the aquatic environment where they are potential micropollutants, capable of having adverse effects in key organisms (fish, invertebrates, unicellular algae).
Bacteria can easily acquire resistance to SAs. This phenomenon is of particular concern because there has been a significant increase in SAs-resistant bacterial strains in recent years and because this class of antibacterials can generate cross-resistance.

In the bacterium, resistance to SAs can occur through multiple mechanisms:

- increased production of PABA at the intracellular level;
- decreased membrane permeability to the drug;
- overexpression of the gene encoding the enzyme dihydropteroate synthetase;
- synthesis of folic acid via alternative pathway.

4. MODEL SPECIES

4.1 Phaeodactylum tricornutum

P. tricornutum is a pleomorphic, brown marine diatom whose habitat are supralittoral pools. Depending on environmental conditions, it can take a fusiform, oval or triradiate shape. This microalga was initially not included among the diatoms, given the absence of silica-containing structures, but after the observation of the presence of a silicate raphe in the oval forms, it was placed in the class *Bacillariophyceae*. This diatom is the only one in the suborder of the *Phaeodactylineae* (Lewin, 1958).

4.1.1 Classification

Empire	*Eukaryota*
Kingdom	*Chromista*
Phylum	*Heterokontophyta*
Class	*Bacillariophyceae*
Order	*Bacillariophyceae*
Suborder	*Phaeodactylineae*
Family	*Phaeodactylaceae*
Genus	*Phaeodactylum*
Species	*Phaeodactylum tricornutum*

(Lewin, 1958);(AlgaeBase, 2023)

4.1.2 Morphological aspects

Phaeodactylum tricornutum is classified as a marine diatom with a pinnate raphe and has three different forms: oval, fusiform and triradiate. These forms also differ from each other in terms of their habitat in the marine environment; in fact, the oval form is typical of the benthos while the fusiform and triradiate are planktonic (Round, 1990; Martino *et al.*, 2007). It was noted in fusiform and triradiate cells that there were three component layers of the cell wall (Borowitzka *et al.*, 1977). This structure is common in diatoms, which have the outer and inner layers both organic and an intermediate layer composed of silicon. There is also evidence of an organic layer, called diatotepum, surrounding the cell wall

composed of silicon. Also, in *P. tricornutum*, the equivalent of the diatotepum of the common diatom is generated during the final stage of cell wall formation, but it is composed of polyanionic polysaccharides and fibrillar material (Coombs & Volcani, 1968; Volcani, 1981; Kröger *et al.*, 1996). It has also been noted that *P. tricornutum* shares, with the common structure of diatoms, many of the essential components of the cell wall, although there is a substantial difference in the arrangement of macromolecules in this alga. Among the major macromolecules constituting the cell wall is glucomannan sulfate, which constitutes a network of linkages suitable for maintaining the structure and geometry of this diatom (Volcani, 1981). Unlike many other diatom species, *P. tricornutum* does not require silicic structures for replicative purposes. In fusiform and triradiate cells, however, silica bands have been found to be located in the epitheca: more specifically, at the valve junction. Interestingly, these bands increase in number during growth, and in older cells they were found in the hypotheca (Reimann *et al.*, 1966; Johansen, 1991; Borowitzka & Volcani, 1978).
The oval form of *P. tricornutum* has characteristics that distinguish it from other forms; in fact, a greater thickness and an elongated or rounded shape are found in this form. During the culture of this alga, its different expression of certain morphological traits was noted, not resulting from genetic differences but dictated by variations in culture conditions (Tesson *et al.*, 2009).

In this microalga, the amount of silicon is variable, influencing the structure itself, which can vary significantly, ranging from the presence of only the raphe to the presence of raphe and attached siliceous valves. The structure of the valves is characterized by an organic inner part located outside the cellular plasmalemma and a siliceous portion containing the raphe, located at the center and attached to the organic wall of the valve, often situated in the hypotheca. Beneath the raphe, the presence of organic layers can be observed, which have a greater thickness than the peripheral portion, possibly corresponding to the inner part of the cell wall in fusiform and triradiate cells (Tesson *et al.*, 2009).

As described by Borowitzka & Volcani (1978), oval-shaped cells of *P. tricornutum* bearing two raphes are not common. Additionally, it is interesting to note that in the hypotheca of these cells, only newly formed raphes are retained, whilst older ones are eliminated through mechanisms that are still not fully understood (McConville *et al.*, 1999).

Recent studies on the three different forms of this alga have shown the role of organic structures in supporting the siliceous structures and the potential expulsion of some of their parts (Francius *et al.*, 2008). It has also been revealed that the silicon valves present in oval cells give them considerable strength, which is not present in the other two forms (Iwasa *et al.*, 1971; Iwasa & Shimizu, 1972; Hoagland *et al.*, 1993; Chiovitti *et al.*, 2006).
P. tricornutum can have the three typical morphological forms (Figure 3) already described, but it can also, albeit more rarely, appear cruciform. In theory, only the oval form can be considered a true diatom. The difficulty in taxonomic classification of this microalga arises from the differences in its structural composition which vary across its four forms and in the presence or absence of silicon (Bourrelly & Dragesco, 1955).

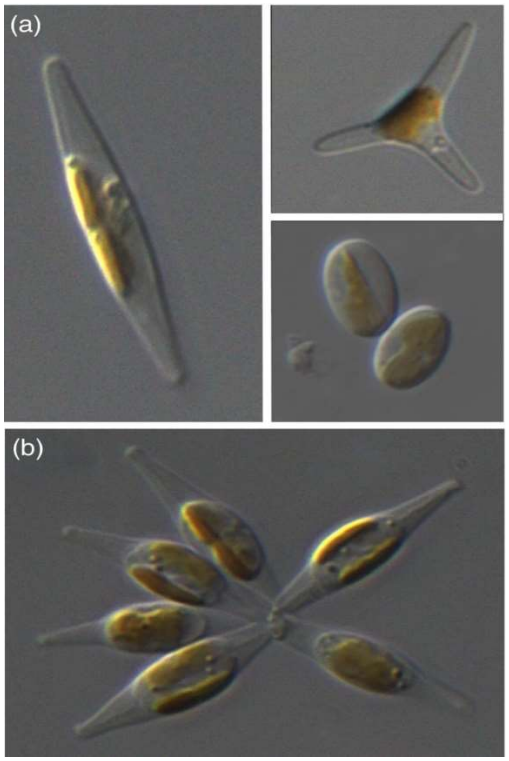


Figure 3. The three morphotypes of *P. tricornutum*: fusiform (a); top right, triradiate; bottom right, oval. A small cluster of fusiform cells of *P. tricornutum* (b) (Vardi *et al.*, 2008).

4.1.3 Ecological aspects

P. tricornutum in the supralittoral pools surpasses in number and distribution the other species of microalgae, and it is found in the triradiate and fusiform shapes that distinguish it. However, it is not exclusively associated with this habitat; its presence has also been evidenced in the benthic ecosystem where it typically appears in the oval form.
This marine alga has euryhaline characteristics: according to various studies, it would adapt to a range of salinity from 2.5 to 87.5 ‰. However, further research has indicated the ability of *P. tricornutum* to grow at minimal salinity levels, while optimal growth has been located in a salinity range between 12‰ and 36‰ (Bonin *et al.*, 1986).

4.2 Artemia salina

The genus *Artemia* is ubiquitous and found in diverse environments. *A.* salina, in particular, is a crustacean that has adapted to live in very hostile environments that ensure the absence of predators. It has been found in ecosystems where very high saline concentrations can be reached, such as salt lakes, lagoons, and salt flats (Sorgeloos *et al.*, 1987; Triantaphyllidis *et al.*, 1998). Indeed, this family of crustaceans can live in a range of salinity varying from 10 to 340 g L^{-1}, without being particularly influenced by possible variations in temperature or the ionic composition of the surrounding environment (Dhont & Sorgeloos, 2002).

The genus *Artemia* is spread all over the world, and this wide distribution is matched by a variety of species. Its presence is found along the coasts of tropical, subtropical, and temperate regions (Santhanam *et al.*, 2018).

4.2.1 Classification

Kingdom	*Animalia*
Phylum	*Arthropoda*
Subphylum	*Crustacea*
Class	*Branchiopoda*
Order	*Anostraca*
Family	*Artemia*
Species	*Artemia salina*

(Belk & Bowen, 1990)

The genus *Artemia* includes seven species divided into 50 different strains. Among these species, the most common are *A. salina*, including its variety *A*. *franciscana*, and *A. parthenogenetica*, followed by *A. tunisiana*, *A. urmiana*, *A. persimilis*, and *A. sinica*.

4.2.2 Morphological traits of Artemia salina

The male and female of *A. salina* share morphological traits including the presence of two compound eyes, two pairs of antennae, thoracopods, a long and straight intestine, and a caudal fork (Figure 4).

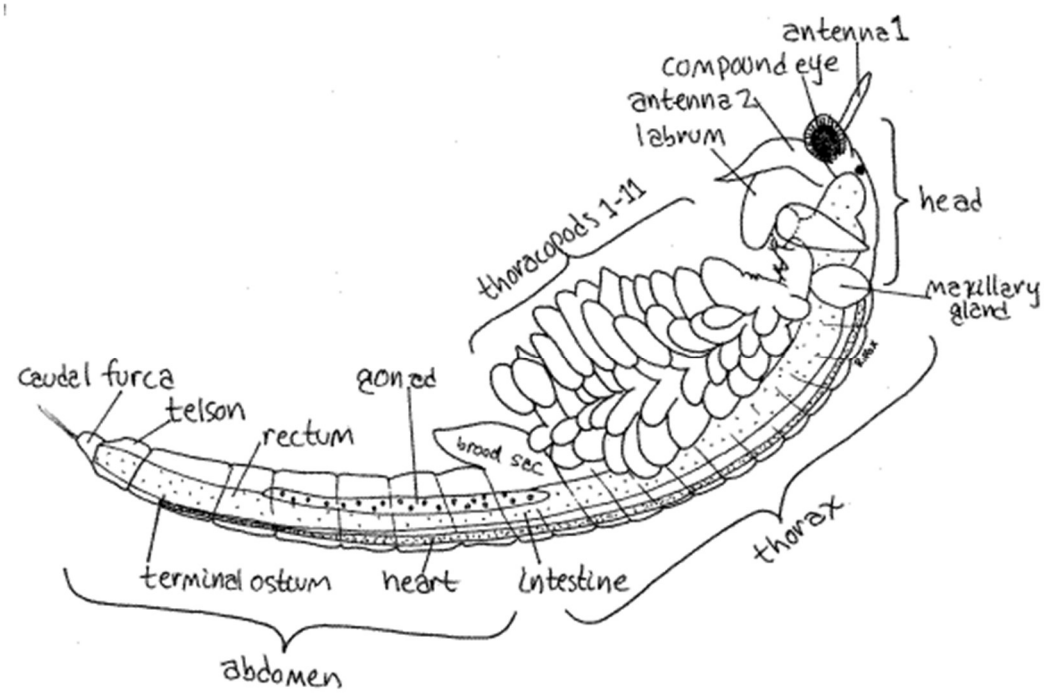


Figure 4. Morphological traits of *Artemia salina*

In the initial growth phase, *A. salina* shares, in both sexes, the presence of a simple eye that exhibits photosensitivity and persists even after the appearance of the other two compound eyes. It is interesting to note that individuals in the early growth phase are attracted to light, while older individuals are averse to it, moving away from direct light sources. The distinction between male and female is present in all species of *Artemia*, except for *A. parthenogenetica*, which exhibits a colony of only female individuals.
In all other species, there is a morphological differentiation between male and female (Figure 5). Indeed, the two sexes are

distinguished by the presence of two large reproductive organs called 'Graspers' in males, which are additional antennae located cranially that allow for attachment to the female during mating.

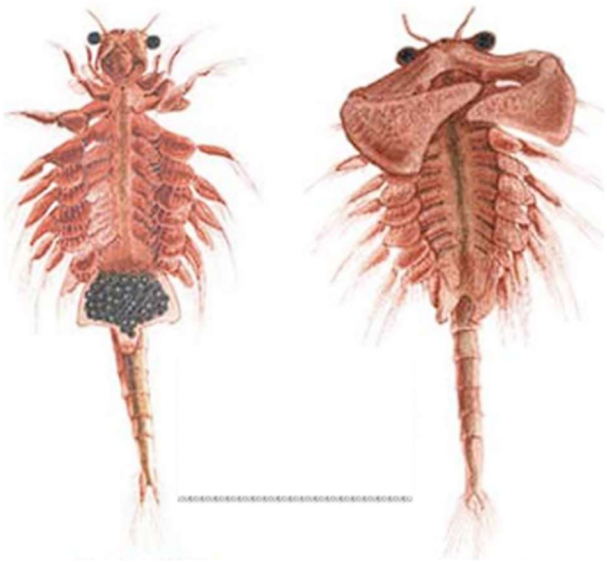


Figure 5. Anatomical differences of female and male individual of *Artemia salina*.

On the other hand, female individuals, unlike males, possess only a single small antenna and a large sac, near the gonad, capable of containing more than 200 eggs. The reproductive habits of this family of crustaceans vary from oviparous to ovoviviparous, depending on environmental conditions (Emslie, 2003).

4.2.3 Ecological characteristic of Artemia

Artemia exhibits high tolerance to salinity, as it can withstand salinity levels from 10 to 350 ‰, up to saturation levels. In addition to this marked tolerance, *Artemia* shows great resistance to oxygen-deficient environments, down to a tolerable minimum level of 0.3-0.5 mg L^{-1}.
These organisms are filter feeders and have a non-selective diet composed of microalgae, organic material, various types of detritus, and even some bacteria. Under optimal conditions, these crustaceans can live for several months and take 8 days to pass from the nauplius stage to adulthood, undergoing about 15 molts.

Depending on environmental conditions, whether favorable or adverse, *Artemia* can exhibit two different types of reproduction. Indeed, in the case of favorable conditions, *Artemia* displays an ovoviviparous reproductive model, giving birth directly to nauplii. In the case of adverse conditions, it exhibits an oviparous reproductive model, with up to 300 cystic eggs that, once laid, will hatch only under more favorable conditions. In both cases, females are able to lay or release up to 300 eggs or nauplii every four days. In the case of oviparous reproduction, fertilized eggs have characteristics that allow them to remain dormant with interruption of embryonic development in the early stages of gastrula. When environmental conditions become favourable, embryonic development resumes rapidly, and hatching occurs after 24-48 hours; within the next 12 hours, the nauplius becomes fully developed and enters the umbrella stage of development.

The growth stages that *Artemia* undergoes are numerous (Figure 6). In the nauplius phase, there are two stages. In the first, called stage 1 or instar 1, the nauplius acquires swimming ability, reaching a size of 0.4-0.5 mm and acquiring an orange to brown color. In this stage, the initial development of appendages and antennae, always paired and located cranially, the formation of mandibles, a large labrum covering the ventrally located oral apparatus, and a slightly developed and photosensitive eye are distinguishable. Following stage 1, nauplii enter the second stage of development, defined as instar 2 and are called 'metanauplius larvae.' In this stage, the nauplius reaches a length of 0.6 mm and develops a translucent color, the secondary antennae, and the ability to grasp organic material through filter-feeding and channel it into the now functional digestive tract. Then, *Artemia* pass through post-naupliar stages, which are seven, with complete development of thoracic appendages and compound eyes, definition of thoracic segments, and reduction of the labrum. This leads to the post-larval phase in which there are several stages

resulting in the formation of sexual organs together with a reduction in the length of the antennae and the ocular peduncles. Moving on to the juvenile stages, *Artemia* undergoes significant morphological-functional changes with the complete development of organs leading it to the pre-adult phase. The pre-adult phase involves several stages in which there is sexual differentiation, morphologically visible with the development of Graspers by males and, by females, the separation of thoracopods and sensory appendages into three distinct parts.
Through all the described stages, *Artemia* reaches adulthood, taking 15 molts and 3 weeks of development. At this time, the crustacean reaches a size ranging from 0.7 to 1.2 cm in length; under optimal environmental conditions, it can survive up to 4 months in this adult form (Santhanam *et al.*, 2018).

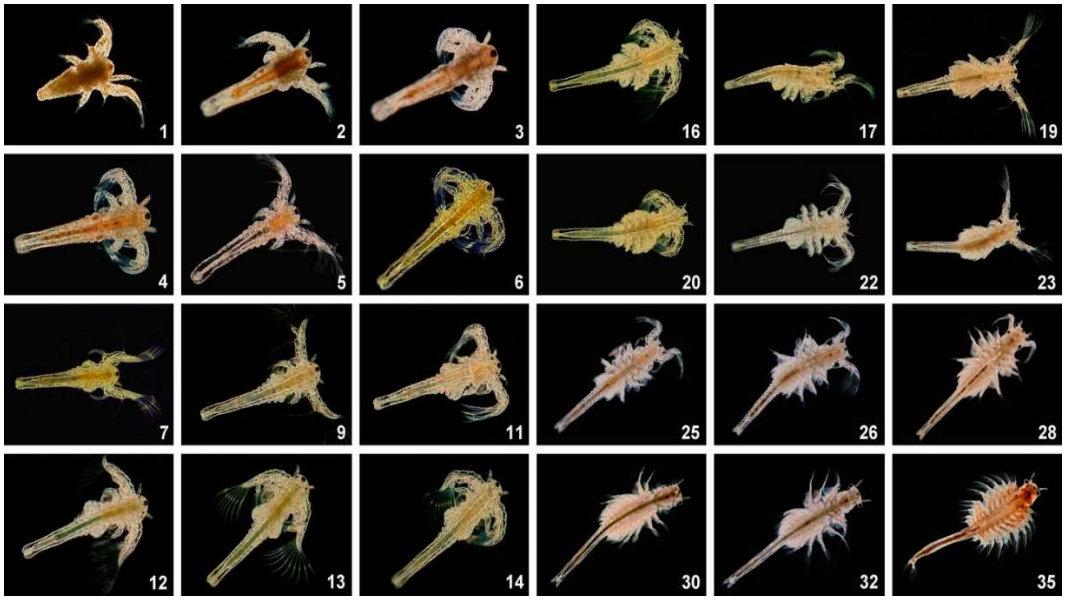


Figure 6. Morphological evolution of *Artemia salina*, each single image represents the individual's age in days (Piper, 2018).

5. AIM OF THIS THESIS

Contamination by antibiotics can disrupt the delicate balance of aquatic ecosystems. This disruption arises not only from their effects on bacterial populations but also from the impact of these pharmaceuticals on the homeostasis of non-target organisms across various trophic levels. Ecotoxicity tests offer a means to gauge specific toxicity parameters, enabling the establishment of concentration thresholds to safeguard ecosystem integrity.

Among the plethora of antibiotics commonly utilized in human and veterinary medicine, FQs and SAs warrant particular scrutiny. Their notable persistence and capacity to induce toxicity, even at minute concentrations (<mg L^{-1}) in certain species, underscore the urgency of their examination. While extensive literature exists on the ecotoxicity of these antibiotics toward model organisms of freshwater habitats, information regarding their impact on marine environments remains limited.

Consequently, the aim of this experimental thesis is to assess the acute ecotoxicity of eight antibiotics, comprising four FQs and four SAs, using two foundational organisms within marine ecosystems: the unicellular alga *P. tricornutum* and the small crustacean *A. salina*. Selection of these specific antibiotics was based on their prevalent usage in human medicine (Ciprofloxacin, Levofloxacin, and Sulfamethoxazole), veterinary medicine (Enrofloxacin and Sulfamethazine), and aquaculture (Flumequine, Sulfadiazine, and Sulfadimethoxine).

The findings of this study may furnish an initial assessment of the potential repercussions of the tested antibiotics on the marine ecosystem. While existing analytical data predominantly pertain to freshwater environments, evidence suggests antibiotic presence in coastal waters, particularly near populous urban centers, offshore aquaculture installations, and estuarine regions, reflecting widespread contamination originating from major river systems (Zheng *et al.*, 2021).

6. MATERIALS AND METHODS

6.1 Marine medium preparation

The basic marine medium for the culture of this algae is prepared following the instructions provided by ISO (BSI Standards Publication, 2020). After adding 33 g L^{-1} of synthetic marine salt 'Instant Ocean' (Aquarium Systems, Italy) to distilled water (Table 1), the medium is enriched with nutrient and vitamin solutions (Table 2) and finally supplemented with 2 mL L^{-1} of the Biobloom fertilizer (BioBizz, The Netherlands). The salts used for the production of stock solutions are of certified quality and supplied by (Sigma-Aldrich, Milan).

Salt	**Concentration**
NaCl	22 g L^{-1}
$MgCl_2 \cdot 6H_2O$	9.7 g L^{-1}
Na_2SO_4	3.7 g L^{-1}
$CaCl_2$	1 g L^{-1}
KCl	0.65 g L^{-1}
$NaHCO_3$	0.2 g L^{-1}
H_3BO_3	0.023 g L^{-1}

Table 1. Components of the basic medium.

Nutrient	Concentration in stock solution	Final concentration in test solution
Stock solution 1		
FeCl3·6H2O	48 mg l^{-1}	149 µg l^{-1} (Fe)
MnCl2·4H2O	144 mg l^{-1}	605 µg l^{-1} (Mn)
ZnSO4·7H2O	45 mg l^{-1}	150 µg l^{-1} (Zn)
CuSO4·5H2O	0,157 mg l^{-1}	0,6 µg l^{-1} (Cu)
CoCl2·6H2O	0,404 mg l^{-1}	1,5 µg l^{-1} (Co)
H3BO3	1 140 mg l^{-1}	3,0 mg l^{-1} (B)
Na2EDTA	1 000 mg l^{-1}	15,0 mg l^{-1}
Stock solution 2		
Thiamin hydrochloride	50 mg l^{-1}	25 µg l^{-1}
Biotin	0,01 mg l^{-1}	0,005 µg l^{-1}
Vitamin B12 (cyanocobalamin)	0,10 mg l^{-1}	0,05 µg l^{-1}
Stock solution 3		
K3PO4	3,0 g l^{-1}	3,0 mg l^{-1}; 0,438 mg l^{-1}P
NaNO3	50,0 g l^{-1}	50,0 mg l^{-1}; 8,24 mg l^{-1}N
Na2SiO3·5H2O	14,9 g l^{-1}	14,9 mg l^{-1}; 1,97 mg l^{-1}Si

Table 2. Composition of the stock solutions to be added to the basic medium in order to obtain the final medium.

6.2 Culture of Phaeodactylum tricornutum

The algae used for ecotoxicological assays were obtained from axenic cultures of *Phaeodactylum tricornutum* (strain: 1090-1a; SAG-Collection of Algal Cultures, University of Göttingen). The algae were shipped in agar medium; therefore, it was necessary to transfer them to liquid marine medium under a laminar flow hood. The culture of *Phaeodactylum tricornutum*, for setting up assays in the laboratory, is run in 2 L glass flasks, maintained at 24±1°C, and positioned on a transparent platform allowing the passage of light emitted by the fluorescent lamps positioned underneath, with an intensity of 4000 LUX. The culture was continuously aerated with filtered air using 0.2 µm PTFE membrane filters (Cytiva, UK).
Tests were initiated after reaching a concentration of 4 x 10^6 cells mL^{-1} in the flask, with the algae in exponential growth. Through appropriate dilutions during the experimental period, the culture was maintained in exponential growth, with an average concentration of 9 x 10^6 cells mL^{-1}.

6.3 Culture of Artemia salina

For the preparation of the *A.* salina culture, a quantity of 25 mg of cystic eggs (Hobby, Germany) was weighed and transferred to a Petri dish containing 20 mL of medium identical to that used for the culture of *P. tricornutum* but not supplemented with the Biobloom fertilizer. The Petri dish was incubated for 30 hours under continuous light at 4000 lux and at a temperature of 21°C.

6.4 Compounds Used in the Tests

The compounds Levofloxacin, Flumequine, Enrofloxacin, Ciprofloxacin, Sulfadiazine, Sulfamethoxazole, Sulfadimethoxine, and Sulfamethazine used for the tests, with certified minimum purity (Table 3), were provided by Sigma-Aldrich (Milan, Italy). Drug solutions were prepared just before each assay by dissolving the active ingredient in marine medium. If necessary, the solutions were adjusted to the original pH with 1M NaOH. The pH was measured using the BASIC 20 pH meter (CRISON, Carpi, Italy).

Compounds	**Chemical structure**	**CAS No**	**Purity**
Levofloxacin	$C_{18}H_{20}FN_3O_4$	100986-85-4	≥ 98.0 %
Flumequine	$C_{14}H_{12}FNO_3$	42835-25-6	≥ 98.0 %
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	93106-60-6	≥ 98.0 %
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	85721-33-1	≥ 98.0 %
Sulfadiazine	$C_{10}H_{10}N_4O_2S$	68-35-9	≥ 98.0 %
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	723-46-6	≥ 98.0 %
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	122-11-2	≥ 98.0 %
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	57-68-1	≥ 98.0 %

Table 3. Antibiotics Used in the Tests.

6.5 Algal Growth Inhibition Test on Phaeodactylum tricornutum

The ecotoxicity tests on *P. tricornutum* were conducted following the protocol 'Water quality - Marine algal growth inhibition test with *Skeletonema sp*. and *Phaeodactyulum tricornutum*' (BSI Standards Publication, 2020).

Based on the results of preliminary tests, it was decided to use a different concentration range for the two classes of antibiotics under examination. Therefore, the FQs were assayed at 3.5-400 mg L^{-1}, with the exception of the poorly soluble ciprofloxacin (3.5-100 mg L^{-1}), and the SAs at 0.39-100 mg/L (Table 4). Further details regarding the algal growth inhibition tests are reported in Table 5 and Table 6.

Organisms	**Compounds**			
Phaeodactylum tricornutum	**FQs**			
	Levofloxacin	Flumequine	Enrofloxacin	Ciprofloxacin
	3.125-400	3.125-400	3.125-400	3.125-100
	Sulfonamides			
	Sulfadiazine	Sulfamethoxazole	Sulfadimethoxine	Sulfamethazine
	0.391-100	0.391-100	0.391-100	0.391-100

Table 4. Range of nominal concentrations (mg L^{-1}) used in algal growth inhibition tests (dilution factor of 2).

Experimental group	CTRL	C8	C7	C6	C5	C4	C3	C2	C1
Drug concentration (mg/L)	0.00	3.125	6.25	12.5	25	50	100	200	400
Initial algal concentration (cells/ml)	10^4	10^4	10^4	10^4	10^4	10^4	10^4	10^4	10^4
Volume in each well (ml)	10	10	10	10	10	10	10	10	10
Number of replicates	6	3	3	3	3	3	3	3	3

Table 5. Assays with Fluoroquinolones on *Phaeodactylum tricornutum*.

Experimental group	CTRL	C9	C8	C7	C6	C5	C4	C3	C2	C1
Drug concentration (mg/L)	0.00	0.391	0.781	1.563	3.125	6.25	12.5	25	50	100
Initial algal concentration (cells/ml)	10^4	10^4	10^4	10^4	10^4	10^4	10^4	10^4	10^4	10^4
Volume in each well (ml)	10	10	10	10	10	10	10	10	10	10
Number of replicates	6	3	3	3	3	3	3	3	3	3

Table 6. Assays with Sulfonamides on *Phaeodactylum tricornutum*.

6.5.1 Preparation of the dilutions

For the preparation of dilutions of the test substance, an initial stock solution (C1) of the compound under examination was first prepared. Subsequently, consecutive 1:1 dilutions in the marine medium were made to obtain solutions with progressively lower concentrations (C2, C3, C4, etc.).

6.5.2 Preparation of Plates for the Test on Phaeodactylum tricornutum

For the assay, cell culture plates with 6 wells of 10 mL each were used. For the negative control, 6 wells were filled with pure medium, while for each of the drug concentrations to be tested, 3 wells were filled. The volume of the algal inoculum was adjusted to achieve an initial concentration of 10,000 cells mL^{-1} in each well. The plates were maintained at 20±2°C under continuous illumination of 10,000±1000 LUX throughout the test duration (72 hours). Cell counting, using a Burker chamber, was performed every 24 hours. At the end of each test, in the case of significant inhibition of algal growth, the algistatic or algicidal effect of the compounds was determined using one of the two methods described in the test guideline (EPA, 2012). For this purpose, a subculture was prepared by transferring a 0.5 mL aliquot from each of the three wells of the highest tested concentration to three new wells containing 10 mL of pure marine medium; the same procedure was carried out for the preparation of subcultures from three replicates of the control.
The plates were incubated for up to nine days under the same conditions as the tests and were randomly rearranged every day, with cell counting performed every two days to check for growth within the wells. As soon as obvious algal growth recovery was observed, the verification was concluded, and the effect was identified as algistatic. If no growth recovery was observed within nine days, the effect was identified as algicidal.

6.5.3 Evaluation of the ability of Folic Acid to minimize the Toxic Effect of Sulfonamides in Phaeodactylum tricornutum

Since the pharmacological mechanism of action of Sulfonamides (SAs) is based on the inhibition of folate synthesis, it was considered to verify whether the addition of folic acid to the medium could reduce or nullify the growth inhibition effects caused by these antibiotics. Therefore, an additional test was conducted, exposing *P. tricornutum* to 100 mg L^{-1} of each of the four SAs, in the presence of 100 ng L^{-1} of Folic Acid. The concentration of folic acid to be used was established based on a similar experiment previously performed on the freshwater green alga *Raphidocelis subcapitata* (Eguchi *et al.*, 2004).

6.6 Immobilization Test on Artemia salina

For the ecotoxicity tests on *A.* salina, the protocol of the "*Artemia* Toxicity Test" assay, included in the kit commercialized by MICROBIOTEST INC, Belgium (MICROBIOTESTS, 2019), was followed. It is an acute test that takes place in the absence of light and involves the exposure of *Artemia* nauplii for 24 hours. Based on preliminary tests where the negative controls were observed to survive for over 48 hours, it was decided to extend the duration of the test to 48h in order to increase its sensitivity. The FQs and SAs were tested at concentrations ranging from 6.25 to 100 mg L^{-1} (Table 7).
The decision not to test concentrations higher than 100 mg L^{-1} was based on the complete absence of effects at the concentration of 100 mg L^{-1} during preliminary tests. Indeed, it was believed that to measure the EC_{50} values, extremely high concentrations, of little ecotoxicological significance, would have to be tested. Besides, the only compound that showed some effect at 100 mg L^{-1} in preliminary tests was Ciprofloxacin, but at higher concentrations it was practically insoluble in marine medium.

6.6.1 Preparation of the dilutions

For the preparation of dilutions of the test substance, initially, a stock solution (C1) of the compound under examination was prepared. Subsequently, consecutive 1:1 dilution were made in marine medium to obtain solutions of lower concentrations (C2, C3, C4, and C5) (Table 7).

Experimental group	Control	C5	C4	C3	C2	C1
Drug Concentration (mg L^{-1})	0.00	6.25	12.5	25	50	100
Volume in each well (mL)	1	1	1	1	1	1
Number of replicates	3	3	3	3	3	3

Table 7. Fluoroquinolones and Sulfonamides Assay on *Artemia salina*.

6.6.2 Setting up the plates for the test on Artemia salina

The acute toxicity test on *Artemia* involves exposing the nauplii at the larval instar II-III stage, obtained from the hatching of cysts. For this reason, 30 hours before the start of the assay, 25 mg of *Artemia* salina cysts were transferred to a Petri dish containing 20 mL of medium and incubated under continuous illumination of 4000 LUX at a temperature of 25°C.
After the incubation period, an extremely abundant number of nauplii were available, and they were randomly transferred to the wells for the assay. Each of the 24 wells of the plate was first filled with 1 mL of solution, starting from the left column of the plate with the negative control, and continuing towards the right in the other 5 columns with solutions at increasing concentrations (C5-C1).
The wells in the bottom row were used for washing, while the other three wells of each column accommodated the three replicates of the test. According to the protocol, using a stereoscope, 50 specimens were initially placed in each well of the bottom row of the plate (washing wells), from which groups of 10 were taken and transferred to the three wells above. At this point, the plate were covered, sealed with parafilm, and placed inside a box to ensure absence of light during the incubation period (48h) at a constant temperature of 25°C.

6.6.3 Reading of the plates

After 48 hours of incubation, the endpoint was determined by counting the number of immobile specimens in each replicate of each concentration, and then calculating the EC_{50}. Individuals were considered immobile if they showed no movement for 15 seconds, even when stimulated by tapping the edge of the plate with the fingers.

6.7 Chemical analysis

To verify the stability of the compounds during each algal toxicity test, 10 mL of the lowest and highest exposure concentrations were collected both at the beginning and at the end of each test. For the samples collected at the end of the test, which were taken directly from the wells used for the tests, centrifugation and subsequent collection of the supernatant were necessary in order to remove algal cells. The analysis of these samples was conducted using HPLC-MS by the chemical analysis laboratory of the Department of Comparative Biomedicine and Food.

6.8 Data analysis

Dose-response curve for the tests on *P. tricornutum*: using the GraphPad Prism 8.4.3 software, the data were best fitted to a four-parameter (variable slope) Inhibitor vs. Response model, and the EC_{50} and EC_{10} with their 95% Confidence Interval (C.I.) were determined.

7. RESULTS

7.1 Algal growth inhibition test on Phaeodactylum tricornutum

Table 8 presents the EC_{50} and EC_{10} values obtained from the exposure tests of *P. tricornutum* to each of the eight antimicrobials analyzed. During the 72-hour incubation period, no significant variations were observed in either the temperature, which was continuously monitored, or the pH.

Compounds	**EC_{50}**	**EC_{10}**
Fluoroquinolones		
Levofloxacin	429.8 (263.9-2473)	41.65 (27.59-60.26)
Flumequine	670 (517.6-1154)	214.0 (148.2-275.4)
Enrofloxacin	120.4 (77.58-320.5)	10.06 (5.917-16.37)
Sulfonamides		
Sulfadiazine	0.3989 (0.2501-0.6112)	0.00131 (-∞ - 0.0054)
Sulfamethoxazole	1.308 (0.9840-1.866)	0.3908 (0.2329-0.5777)
Sulfadimethoxine	57.75 (51.90-64.59)	13.21 (11.11-15.65)
Sulfamethazine	1.133 (0.643-1.724)	0.0591 (0.0329-0.1007)

Table 8. EC_{50} and EC_{10} resulting from the exposure of *Phaeodactylum tricornutum* to the eight antimicrobials under investigation.

7.1.2 Fluoroquinolones

The graphs in Figure 7 show the dose-response curves related to the percentage inhibition of growth of *P. tricornutum* exposed to Levofloxacin, Flumequine, and Enrofloxacin.

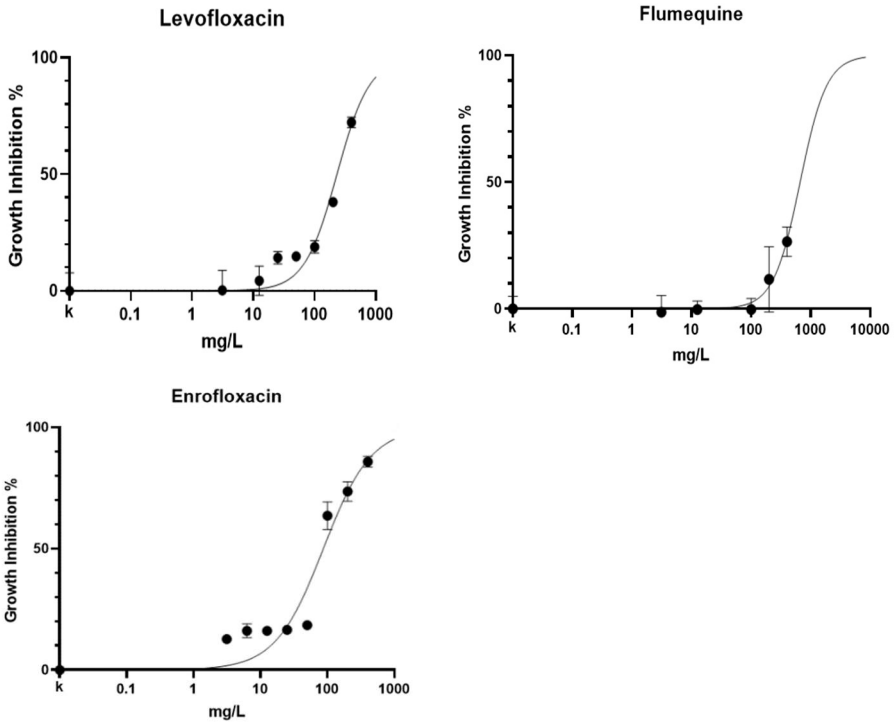


Figure 7. Inhibition of *Phaeodactylum tricornutum* growth after exposure to Fluoroquinolones.

With Ciprofloxacin, instead, effects were only observed at the highest concentration (100 mg L^{-1}), and surprisingly, they were stimulatory rather than inhibitory. Although nearly doubled in number compared to the control, the cells showed clear alterations in shape (Figure 8). It should also be noted that the results of control chemical analyses showed that Ciprofloxacin, above a certain concentration, did not adequately dissolve in the medium, and therefore the observed effects cannot be attributed to the nominal concentration (100 mg L^{-1}), but must be referred to an average of the initial and final concentrations measured by chemical analyses (27.58 and 16.86 mg L-1, respectively); this average concentration is 22.22 mg L-1. In the case of all other FQs, however, the concentrations, both at the beginning and at the end of the test, were maintained within ± 20% of the nominal values; therefore, for the calculation of the EC_{50s} nominal concentrations were used (US-EPA, 2012).

Although samples for chemical analyses of SAs solutions were also collected, these analyses have not yet been performed by the appointed laboratory. However, it is to be expected that, under the specific experimental conditions, SAs are even more resistant than FQs since, unlike the latter, they are considered photostable.

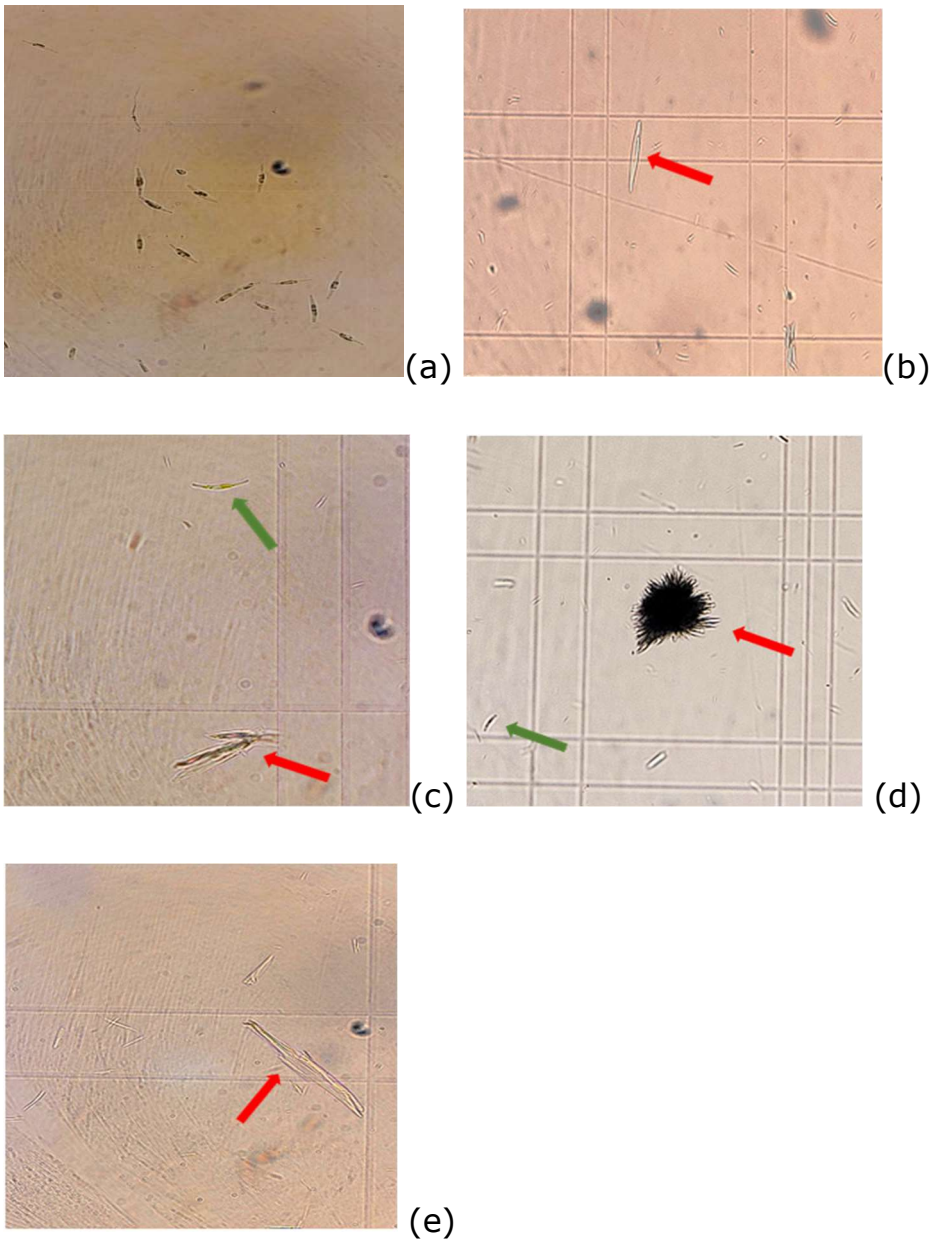


Figure 8. Morphological modifications observed in *Phaeodactylum tricornutum* following exposure to Ciprofloxacin at a nominal concentration of 100 mg L^{-1}. (a) normal algal cells; (b) particularly elongated algal cell (red arrow); (c) comparison between a normal algal cell (green arrow) and an elongated cell fused with other cells (red arrow); (d) comparison between a normal cell (green arrow) and an aggregate of cells (red arrow); (e) elongated and fragmented cell.

7.1.2 Sulfonamides

The graphs in Figure 9 show the percentage inhibition of growth of *P. tricornutum* exposed to Sulfamethazine, Sulfamethoxazole, Sulfadimethoxine, and Sulfadiazine.

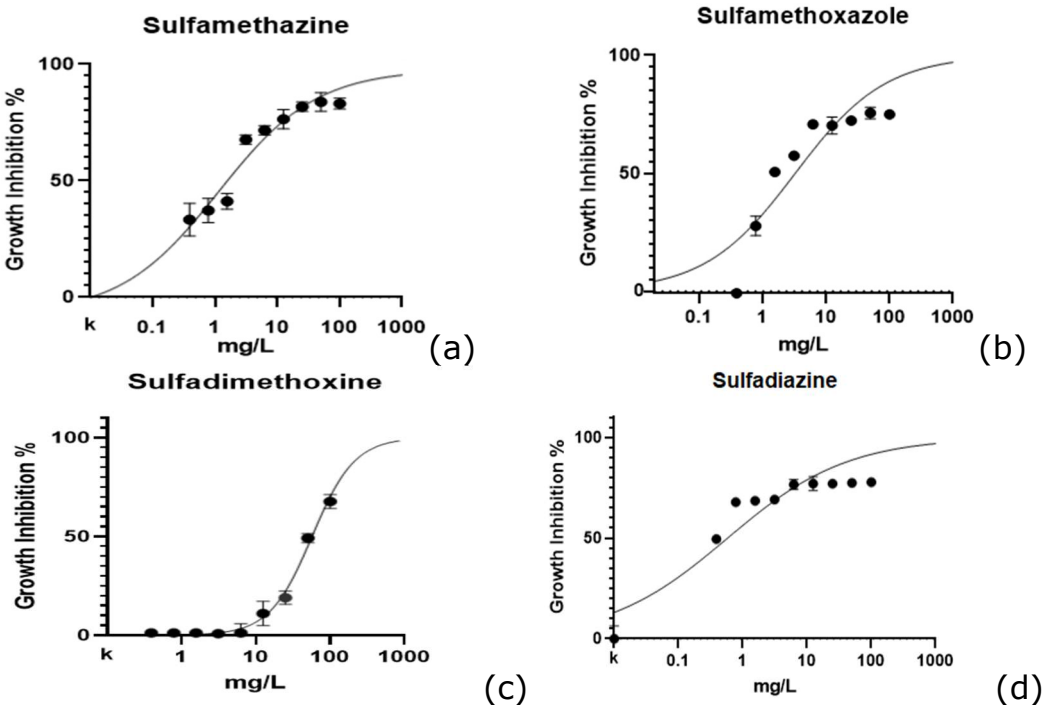


Figure 9. Growth inhibition of *Phaeodactylum tricornutum* after exposure to Sulfonamides.

7.1.3 Identification of algistatic or algicidal effect

The compounds Flumequine and Enrofloxacin were not considered for this evaluation since, even at the highest dose, their inhibitory effect was respectively rather limited or completely absent. As for the other two FQs, Levofloxacin and Enrofloxacin, a clearly algistatic effect emerged. With the Sulfonamides, on the other hand, an algistatic effect was only observed with Sulfadimethoxine, whilst an algicidal effect was observed with Sulfamethazine, Sulfamethoxazole, and Sulfadiazine. The test results and details of these effects are reported in Table 9.

Compounds	Concentration	Inhibition % (72h)	Time needed for regrowth (days)	Effect
Fluoroquinolones				
Levofloxacin	400 mg L^{-1}	74.84	2	Algistatic
Enrofloxacin	400 mg L^{-1}	88.39	2	Algistatic
Sulfonamides				
Sulfamethazine	100 mg L^{-1}	85.86	>9	Algicide
Sulfamethoxazole	100 mg L^{-1}	76.06	>9	Algicide
Sulfadimethoxine	100 mg L^{-1}	71.69	2	Algistatic
Sulfadiazine	100 mg L^{-1}	79.06	>9	Algicide

Table 9. Results of tests on the algistatic or algicidal effect of compounds inhibiting the growth of *Phaeodactylum tricornutum.*

7.2 Immobilization test on Artemia salina

In this assay, as elucidated in the Material and Methods section, the maximum concentration evaluated was 100 mg L^{-1}. Among the compounds tested, only Ciprofloxacin exhibited toxic effects, with no more than 10% immobilization observed for any other antibiotics, even at the highest concentration tested. Notably, as per the test protocol, 10% immobilization is deemed acceptable within the negative control group, rendering it entirely incidental. In Table 10, results of the assay with Ciprofloxacin are reported. Theoretically, an EC_{50} of 100 mg L^{-1} can be inferred; however, analytical investigation has shown that at this concentration the solubility of Ciprofloxacin in marine medium is scarce. For this reason, the real EC_{50} may be lower than 100 mg L^{-1}.

48h	Control	C5	C4	C3	C2	C1
Replicate (a)	0\10	0\10	1\10	3\10	3\10	6\10
Replicate (b)	2\10	1\10	0\10	3\10	5\10	4\10
Replicate (c)	1\10	2\10	0\10	2\10	4\10	5\10
Total	3\30	3\30	1\30	8\30	12\30	15\30

Table 10. Results of the *Artemia salina* immobilization test with Ciprofloxacin.

8. DISCUSSION

FQs and SAs, being relatively stable drugs, after contaminating surface water, can be transferred to the sea, particularly to estuarine areas. For some of these compounds, authorized for use in aquaculture as well (Flumequine, Enrofloxacin, Sulfadiazine, Sulfadimethoxine), a significant contribution to the contamination of marine ecosystems may also come from offshore fish farms.

The presence of traces of pharmaceuticals in the marine environment has been repeatedly confirmed, highlighting in particular that 49% of the antibiotics detected in this ecosystem belong to the FQs family, with SAs following closely in terms of frequency. For FQs, detection has occurred both in the water column and in sediments, with maximum concentrations of 460 ng L^{-1} (Norfloxacin) and 406 ng g^{-1} (sum of FQs), respectively. For this class of antibiotics, Enrofloxacin and Ciprofloxacin are most frequently present in marine waters, with concentrations ranging from 0.56 to 139 ng L^{-1} and from 14.94 to 110 ng L^{-1}, respectively (Maghsodian *et al.*, 2022). There are few studies regarding the presence of Flumequine in marine waters; the highest value reported so far is only 7 ng L^{-1} (Umweltbundesamt-UBA, 2020). Instead, apparently, there are no published data on the presence of Levofloxacin in salt waters.

Regarding SAs, Sulfamethoxazole is the most frequently detected compound in marine waters, with concentrations up to a maximum of 3082 ng L^{-1} (Umweltbundesamt-UBA, 2020); Trimethoprim, a potentiator that is always present in medicinal products containing SAs, was frequently detected as well. Along the northeast coast of Spain, concentrations of up to a maximum of 596 ng L^{-1} of Sulfamethazine have been measured (Zhou *et al.*, 2022). Furthermore, in the estuarine area of Bilbao, Spain, the presence of Sulfadiazine has been detected at a concentration of 581 ng L^{-1} (Mijangos *et al.*, 2018). However, Sulfadimethoxine has been rarely detected, and concentrations have not exceeded 1.36 ng L^{-1} (Umweltbundesamt-UBA, 2020). In coastal marine sediments, concentrations on the order of ng g^{-1} d.w. have been measured for a number SAs (Siedlewicz G. *et al.*, 2011).

8.1 Phaeodactylum tricornutm

In this experimental thesis, the acute toxicity in *P. tricornutum* of four FQs (Levofloxacin, Flumequine, Enrofloxacin, Ciprofloxacin) and four SAs (Sulfamethazine, Sulfamethoxazole, Sulfadimethoxine, and Sulfadiazine) was evaluated. As highlighted by the results, Levofloxacin, Flumequine, and Ciprofloxacin showed weak toxicity towards *P. tricornutum*, with EC_{50} values in the order of hundreds of mg L^{-1}. However, Enrofloxacin was found to be more active, with an EC_{50} lower than 100 mg L^{-1}.

In contrast to what was observed for FQs, SAs exhibited significant toxicity, with EC_{50} values in three out of four cases being close to 1 mg L^{-1}. If we observe the graphs related to the inhibition of algal growth caused by SAs (Figure 9) and relate them to the results regarding algistatic/algicidal effects (Table 9), it is possible to notice that the three compounds with algicidal effects show a flattening of the response as doses increase. This tendency is particularly pronounced in the case of Sulfadiazine, the most toxic compound. This phenomenon could be explained by the fact that, from a certain concentration, cells grow for a time until they exhaust their folate reserves, and then die. However, as they appear intact under the microscope, they are still counted. In other words, to obtain a correct dose-response curve, it would be necessary to distinguish between live and dead cells during cell counting, a procedure that is generally not required in the official guidelines for algal growth inhibition test.

Surprisingly, the addition of folic acid to the medium did not limit the toxicity of SAs towards *P. tricornutum*. This suggests that the inhibition of algal growth caused by these antibiotics may not be due to the blockage of folate synthesis but to another mechanism, different from the one responsible for their antimicrobial activity. However, it is also possible that the dose of folic acid added (100 ng L^{-1}) was not sufficient. It should be noted, in any case, that in a similar experiment conducted on the freshwater green alga *Raphidocelis subcapitata*, the same concentration of vitamin was able to completely nullify the growth inhibition caused by 2.19 mg L^{-1} of Sulfadiazine. Further experiments using higher concentrations of folic acid may clarify these aspects.

As many data are available regarding the toxicity of FQs and SAs in freshwater unicellular algae, it is possible to compare their sensitivity with that of the marine alga *P. tricornutum*. In Table 11, the EC_{50} values measured in this experiment are therefore shown alongside the minimum and maximum EC_{50} values reported in the literature for freshwater unicellular algae. It can be

observed that, while for SAs the sensitivity of freshwater unicellular green algae is comparable to that of *P*. tricornutum, with FQs there is a difference of two orders of magnitude. In this case, the first hypothesis is that the different toxicity is the result of a dissimilar affinity for the target, which in the case of FQs is presumably represented by DNA gyrases, whose presence has been demonstrated not only in bacteria but also in green algae (Robert John Thompson, 1985) and higher plants (Wall *et al.*, 2004). Another possible hypothesis is that, compared to freshwater algae, the saltwater alga has a lower capacity to absorb FQs.

Antibiotic	**EC_{50} in *P. tricornutum*[a]**	**Lowest EC_{50} in fw algae[b]**	**Highest EC_{50} in fw algae[b]**
Ciprofloxacin	n.d.	6.7*(Raphidocelis subcapitata)*	40.7 (*Chlorella vulgaris*)
Enrofloxacin	120.4	3.1 *(Raphidocelis subcapitata)*	111.0 *(Chlorella spp)*
Flumequine	670.0	2.6 *(Raphidocelis subcapitata)*	9.3 *(Raphidocelis subcapitata)*
Levofloxacin	429.8	1.2 *(Raphidocelis subcapitata)*	7.4 *(Raphidocelis subcapitata)*
Sulfadiazine	0.4	1.3 (*Chlorella vulgaris*)	2.2 (*Chlorella fusca*)
Sulfadimethoxine	57.7	9.8 (*Chlorella fusca*)	11.2 (*Chlorella vulgaris*)
Sulfamethazine	1.1	8.7*(Raphidocelis subcapitata)*	19.5 (*Chlorella fusca*)
Sulfamethoxazole	1.3	0.5*(Raphidocelis subcapitata)*	1.6 (*Chlorella vulgaris*)

Table 11. Comparison of EC50 values (mg L^{-1}) measured in *P. tricornutum*, with minimum and maximum values reported in the literature for freshwater algae.[a] Data obtained in this experiment.[b] Data provided by Ecotox Database (EPA, 2023).

8.2 Artemia salina

With the sole exception of Ciprofloxacin, *A. salina* has shown high resistance to various concentrations of all the antimicrobials tested. Although this crustacean is commonly used in various laboratories for toxicological testing, it does not seem to possess adequate sensitivity to toxic substances. In fact, there is no official ecotoxicological test that recommends its use, and standardized ecotoxicological tests on saltwater crustaceans involve the use of copepods (ISO, 1999). However, due to the ease of hatching cystic eggs in the laboratory and managing cultures, *A. salina* can offer considerable practical advantages in experimental settings. In this regard, it would be interesting to develop official protocols for

chronic tests on *Artemia*, as they may give answers that acute tests seem unable to provide.

8.3 Risk assessment

Through the results obtained from ecotoxicological studies such as those conducted in the present experimental thesis, it is possible to make a provisional estimation of the environmental risk. This assessment is based on calculating the Risk Quotient (RQ), obtained by comparing the maximum environmental concentration (MEC) with the concentration threshold at which no toxic effect of the substance is expected (PNEC). The PNEC can be calculated by applying an assessment factor of 100 to the EC_{50} obtained in short-term algal toxicity tests (EMEA, 2005).

If RQ ≥1, the risk cannot be excluded and measures should be taken to minimize it (Nikinmaa, 2014). Regardless of such calculations, however, PNEC values are a useful reference for monitoring water quality and understanding which compounds deserve attention.
In the specific case, for the FQs for which the RQ calculation was possible (Flumequine and Enrofloxacin), no risk is expected. In the case of SAs, however, a low risk can be predicted for Sulfadiazine and Sulfamethoxazole (Table 12 and Table 13). Sulfadiazine is a compound largely used in veterinary practice, particularly in the aquaculture sector. In Italy, it is the only SA authorized for this type of use; therefore, it would be important to measure its actual concentrations in waters near offshore farming facilities. Sulfamethoxazole, on the other hand, is the only SA still widely used in human medicine orally and parenterally; for this reason, it would be appropriate to continue monitoring its presence in estuarine waters, where it has frequently been found. The risk posed by Sulfadimethoxine, an antibiotic licensed for aquaculture in other countries, is of little importance; for this reason, at least in offshore farms, Sulfadimethoxine would be preferable to Sulfadiazine. Moreover, Sulfadiazine has shown a marked tendency for synergistic toxicological interactions with other SAs (De Liguoro *et al.*, 2018). Lastly, the risk posed by Sulfamethazine seems negligible; however, it may not be so in coastal areas affected by river inputs in particularly livestock-rich areas. Indeed, this drug is the most widely used SA in intensive livestock farming.

Compounds	MEC (ng/L)	EC50 (mg/L)	PNEC (ng/L)	RQ
Fluoroquinolones				
Levofloxacin	\	429.8	4290000	\
Flumequine	7	670.0	6700000	0.000001
Enrofloxacin	139	120.4	1204000	0.0001
Sulfonamides				
Sulfadiazine	581	0.4	4000	0.145
Sulfamethoxazole	3082	1.3	13000	0.237
Sulfadimethoxine	1.31	57.7	570000	0.000002
Sulfamethazine	596	1.1	11000	0.054

Table 12. Risk Quotient (RQ) calculation for Fluoroquinolones and Sulfonamides, based on their toxicity to *Phaeodactylum tricornutum*.

Environmental risk level	Risk quotient (RQ)
Insignificant	<0.1
Low	0.1–1.0
Moderate	1.0–10
High	>10

Table 13. Interpretation of the risk quotient (RQ)

9. CONCLUSION

Given the limited availability of data in the literature regarding the toxicity of antibiotics towards saltwater organisms, this thesis evaluated the effects of four FQs and four SAs on the microalga *P. tricornutum* and the small crustacean *A. salina*. From the results obtained in *P. tricornutum*, it emerged that the FQs showed toxicity at high concentrations, which are not plausible under ordinary conditions of environmental contamination. On the contrary, the SAs showed considerable toxicity, comparable to that already observed in freshwater unicellular green algae. Although the risk assessment indicated, even for the most toxic compounds, a low risk for the saltwater ecosystem, a possible negative impact of Sulfadiazine cannot be excluded near offshore aquaculture facilities that use this antibiotic. In particular, for a more accurate risk assessment, the impact on benthic organisms should be evaluated, given the tendency of SAs to accumulate in sediments, and chronic toxicity towards crustaceans should also be considered. In particular, *A. salina* showed very low sensitivity in acute toxicity tests, but it could be reconsidered in the future for chronic toxicity tests, which consider finer endpoints such as inhibition of growth, reproduction, and swimming activity. Based on the results obtained in this experimentation, the best candidate for future tests in this species is, however, Ciprofloxacin, being the only compound that caused mortality in *A. salina*, despite its poor solubility in saltwater, as evidenced by the chemical analyses conducted so far.

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