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Effects of Antibiotics on Marine Benthic Algae

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Abstract

The aim of this study is determination of toxic effect of three antibiotics tylosin, lincomycin and norfloxacin and their mixtures on two marine diatoms species, which is algae class consists of eukaryotic primary producers. *Nitzschia acicularis* and *Bacillaria paradoxa Gmelin* subculture were done in 24-well microtiter plates with addition of antibiotic even in single form or mixtures with serial dilution. Algael growth rates were monitored through microscopic cell counts them. IC_{50} were utilized as component of toxic unit to assess the mixture reactions. $IC_{50's}$ for single-compound were 0.25 mg/l, 13.15 mg/l and 54.39 mg/l for tylosine, lincomycin and norfloxacin, respectively for *N. acicularis*. The $IC_{50's}$ for *B. paradoxa Gmelin* were 0.93 mg/l, 10.87 mg/l and 71.58 mg/l for tylosine, lincomycin and norfloxacin, respectively. $IC_{50's}$ for antibiotic mixture were additive for *B. paradoxa Gmelin* and synergistic against *N. acicularis*. Single antibiotic treatment and antibiotics mixtures eliminated or reduced algeal motility. Responses to single antibiotic treatment were similar in both studied species and were not important in prediction of mixture interactions. Antibiotic mixtures showed species-specific and compound-specific effects.

Key words: Antibiotics, Marine benthic algae, Toxic effect

Introduction

Antibiotics have been of interest to scientists with increasing concerns about drug-resistant microorganisms (Khan et al., 2009). There are international efforts to identify concerned antibiotics and report their toxic and environmental effects (Grenni et al., 2018). However, most studies did not mentioned marine systems and focused on the effects of these compounds on freshwater (Ge et al, 2010). Białk-Bielińska et al. (2017) studied the effect of the herbicide mix and mentioned that few researches have studied the effect of antibiotic mixtures on non-target micro-organisms.

Coastal areas, especially estuaries consider from the most productive habitats worldwide and the most affected sites by human as a result of receiving sewage and other sources of population (Liu et al., 2013). Marine water contains a complex chemicals mixture including antimicrobial compounds that are close to molecules, increasing their potential for deposition in the benthos (Hagenbuch & Pinckney, 2012).

Many estuaries feed on eukaryotic algae. In coastal organisms, the primary microbial production is fixing about sixty percent of the carbon (Nisbet, 2012).

Much of primary productivity of microbes is achieved by eukaryotic benthic microalgae in marshland systems. Benthic microalga is the main pillar for benthic consumers that contribute at least 40 percent of carbon in various species in photic waters (Oakes et al., 2010). Benthic microalga are usually controlled by numbers of diatoms in numerical terms and photosynthesis, they are memorable among those species where pollutant response should be quantified (Duong et al., 2007).

Fluoroquinolones are new synthetic antibiotics group with having strong antibacterial action. Fluoroquinolones are used in treatment of urinary tract infections, respiratory tract, the skin and gastrointestinal system in the medicine (Orzoł & Piotrowicz-Cieślak, 2017).). In intensive breeding, FQs are used for cattle, pigs, poultry, cats, fish and dogs (Frade et al., 2014). FQs residues can be removed and disseminate into the environment (Watkinson et al. 2007).

FQs toxicity to microorganisms limits the biological decomposition of antibiotics in soil and water. Norfloxacin belongs to the third generation of quinolones; the main source of pollution in aquatic life with norfloxacin is its use in aquaculture (Bartoskova et al., 2014).

Tylosin is an antibiotic used worldwide as a preventive medication and growth promotor in veterinary medicine (Boxall et al., 2003). Studies have demonstrated the presence of tylosin in many aquatic systems and it is toxic to some freshwater phytoplankton in concentrations somewhat similar the environment concentrations (Pinckney et al., 2013). Such as Norfloxacin, Tylosin is also has the affinity for sedimentation of particle with active survival period (100 days) which refers to existence in the active form for long period which is enough for affecting the benthic communities (Hagenbuch & Pinckney, 2012).

Lincomycin is an antibiotic used in human and veterinary medicine, it affect a wide range of organisms (Ding & He, 2010). Lincomycin prevent D1 protein synthesis in photosystem II that decrease the microalgae ability for recovery from inhibition of light (Bachmann et al., 2004).

Unlike norfloxacin and tylosin, lincomycin is considered as potential environmental toxin (Kim et al., 2008).

The aim of this study is to investigate the effect of 3 antibiotics (tylosin, lincomycin and norfloxacin) as single exposure or in form of mixtures on two marine microalgae.

MATERIALS AND METHODS

Culture of *N. acicularis* and *B. paradoxa Gmelin* were kindly obtained from the Suez Gulf. The two strains were chosen according to their presence in red Sea and their ecological role in coastal areas (Di Dato et al., 2015).

Maintenance of strains cultures were done using 500 ml acid-washed. Guillard's f/2 + Si growth media were prepared then autoclaved, natural seawater (salinity-adjusted) was collected from red sea, Egypt. Cultures and experimental plates were incubated at 23 ^{o}C and 75 µmol/m2/s photon flux for photoperiod = 12:12.

Antibiotics preparation

Antibiotics were acquired from Misr Chemical Industries Co. (MCI). The Tylosin and Lincomycin salts were highly soluble in water while norfloxacin solubility in water is pH dependent as it increase sharply at pH >10 or pH<5 (O'Neil et al., 2001). For each antibiotic, the maximum solubility in sterilized water was determined and hydrochloric acid (0.1 N) was estimated and setup maximum limit for the single compound experiments.

Experiment structure

Experiments were carried out in 24-well tissue culture plates (sterile), the assay were performed in laminar flow. Tenfold serial dilution were done in each row. Each well was loaded with 4.0 µl of concentrated antibiotics then growth medium (2 ml) then microalgae culture (25µl) were added. The aliquot were obtained from cultures that increasingly grew and containing fixed cell numbers which is examined by the microscope (variation coefficient <0.25).

Vortexing of aliquots were done prior to adding them to the wells. The same treatments have been done for control wells except for 4.0 µl aliquot containing either 0.1 N HCl or sterile high purity water

Algal quantification

Microscopic population estimates were used quantitation of the populations and growth rates of Algae. The growth rates of algae were specified using least-squares nonlinear regression of the next formula (Hagenbuch & Pinckney, 2012):

$$N\ (t) = N_0 e^{rt}$$

N: Algal cells Number

t: The time (days).

N_0: Cells initial number.

r: Algeal cell growth rate (d^{-1}).

Estimation of the toxic effect of each studied antibiotic had been done through determination of IC_{50}, which is depend on the inhibition percentage of (r) (Hagenbuch & Pinckney, 2012):

$$inhibation\ percentage\ = 100 - (1 - \frac{r\ treatment}{rcontrol})$$

Tests continued till cell densities be uncountable for 4-5 days until stationary-phase was obtained or absence of fluorescent cells.

(r) Values were quantified by cell counting (daily) using the inverted microscope with magnifications ranging (100x-400x). In each well, the mean of 5 random grids count was used for estimation of total population., *N. acicularis and B. paradoxa Gmelin* (as benthic species) exhibited 99% sinking rate within 1 hr. of being assayed that allowed the quantification of populations by area till the individuals were attached to the wall of the wells.

Quantification of the antibiotic effect

The toxic unit approach was used for examination of the activity and toxicity of the antibiotic mixture for comparing the sensitivity of antibiotic in the two species (Wilkinson et al, 2015). IC_{50} for each dilution was estimated using setting four parameter sigmoidal curve (GraphPad Software). ANOVA was used for estimation of treatments effects.

The antibiotic mixtures toxicity were obtained through replacement of single compound with mixture of antibiotics with range 3.0 - 0.13 TU (1.0 TU equivalent to 1.0 IC_{50}). Mixture compounds interaction was estimated by IC_{50} value of the mixture (Wilkinson et al, 2015). IC_{50} more than 1.0 is considered antagonistic, the IC_{50} = one is considered additive and IC_{50} less than one is considered synergistic.

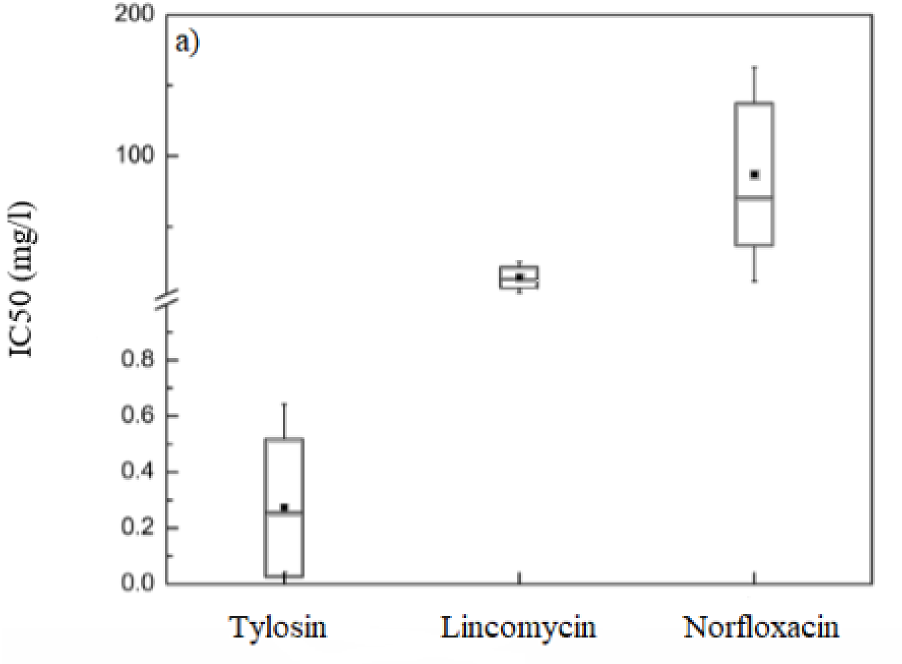
Single tests (SPSS 19) were used for determination the deviation from 1.0

RESULTS

Responses of single compound

The $IC_{50's}$ two-factor ANOVA revealed that antibiotic treatment has statistically significant effect with species ($p < 0.0001$). The results showed a significant interactions between and species, which suggest that the species gave different response in each treatment ($p < 0.0001$). Variance was not uniform.

N. acicularis and *B. paradoxa Gmelin* did not show a great difference in response to the treatment with single antibiotic (Figure1).



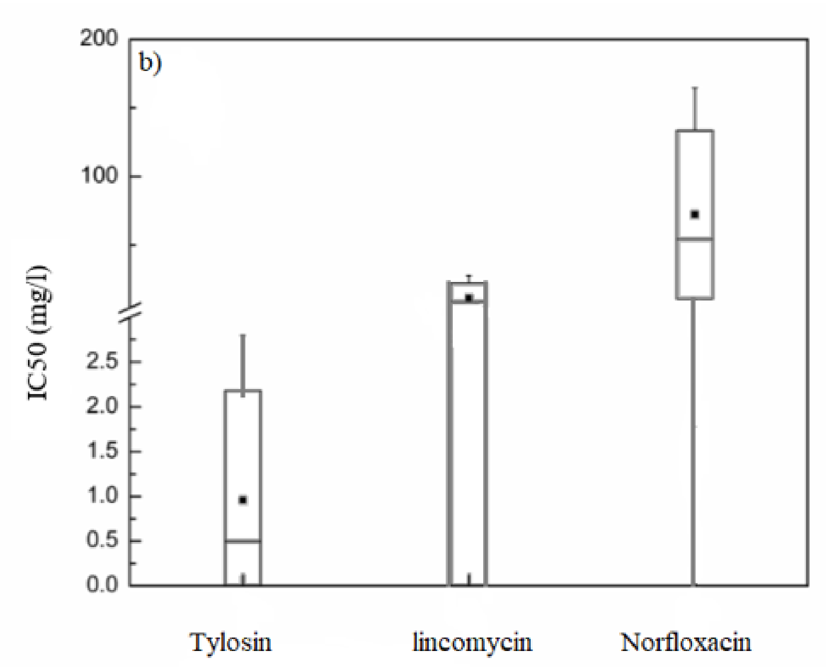


Figure 1: Shows IC$_{50's}$ of single antibiotic for *Nitzschia acicularis* and *Bacillaria paradoxa Gmelin*. a) Response of *Nitzschia acicularis* to tylosin, lincomycin, and norfloxacin. Bars refers to standard deviation, the center line refers to median, ■ refers to mean, and line refers to 0.5 SD. b) Response of *Bacillaria paradoxa Gmelin* to tylosin, lincomycin, and norfloxacin. The break in Y-axis is used due to large difference between tylosin and norfloxacin.

Tylosin showed the most negative effect, where IC$_{50}$ < 1.0 mg/l in the two studied algeal species. Two volumes of size were needed to see the same effect when using lincomycin. Norofloxacin was the least toxic among the three antibiotics used in the study, with variation coefficients 0.35 and 0.87 for *N. acicularis and B. paradoxa Gmelin,* respectively. However, tylosin and lincomycin ranged from 0.51 - 1.28 with *B. paradoxa Gmelin.* Tylosin and lincomycin raised typical sigmoidal dose-response due to 10-fold concentration change, as Norofloxacin concentrations were changed by 10 fold, both tylosin and lincomycin extracted typical sigmoidal dose-response.

On the other hand, Norofloxacin showed very low gradient effect that showed break-point extending only one magnitude order ranging from 14 - 140 mg/l (Figure 2).

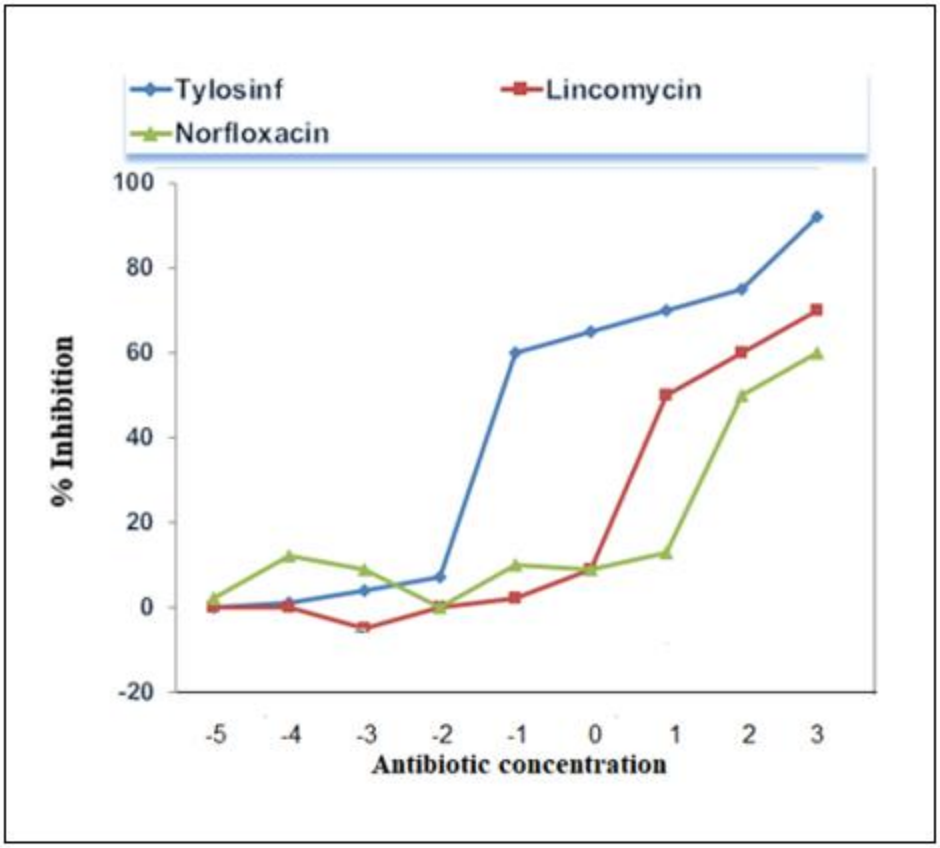


Figure 2. Representative *Nitzschia acicularis* dose-response screening exposed to the three studied antibiotics. Curves of tylosin and lincomycin represent single N equal to four assays. The norfloxacin curve represents 2N equal to 3 assays (1 high concentration and 1 low).

Responses of antibiotic mixture

ANOVA is used for antibiotic mixtures and single-compound. Treatments, algeal species and species-treatment showed significant effects (p less than 0.0001, OP = 1). Toxic units (TU) were used to express mixture concentrations, where 1TU = 1 IC_{50}.

For binary mixtures, 2 TU treatment contain one IC_{50} (mg/l) for each compound and one toxic unit treatment contain 0.5 IC_{50} for each antibiotic.

A mixture IC_{50} = one is considered additive, < 1.0 is considered synergistic and >1.0 is considered antagonistic. One-sample t-tests are used for determining of deviations from 1.0.

The results showed evident differences in the response in the studied species. Out of four antibiotic mixtures of *N. acicularis* three mixtures (tylosin + lincomycin, lincomycin + norfloxacin, tylosin + lincomycin + norofloxacin) showed toxic effect, which is synergistic (p less than 0.001). Fourth mixture effect (tylosin + norofloxacin) was an additive (p = 0.350). However *B. paradoxa Gmelin* response was at contrast as all mixtures took the behavior of additive, as p value ranging from 0.084 - 0.419.

In regarding to variance, it is found that there is marked difference between the studied species, *B. paradoxa Gmelin* showed close to high values and large variation coefficients (0.59 - 1.23). The high variability of *B. paradoxa Gmelin* in the single and mixed compounds is considered spatial distribution artifact.

However, the two diatom species are motile, *B. paradoxa Gmelin* did not spread as thin as *N. acicularis*. *N. acicularis* showed high motility and showed tendency to separate and forming uniform mono-cellular layer at the bottom of the wells. On the contrary, *B. paradoxa Gmelin* slowly spread and had tendency for forming clumps.

DISCUSSION

The most negative effect has been shown by tylosin (IC50 of 0.25 mg/l and 0.93 mg/l in *N. acicularis* and *B. paradoxa Gmelin*, respectively). Lincomycin effect was 14.2 for *N. acicularis* and 11.1 mg/l for *B. paradoxa Gmelin*. *B. paradoxa Gmelin* showed the lesser effect and it was consistent in all treatments except lincomycin. The researcher did not do post-hoc analyses, thus it is not possible confirm that the difference between the two studied diatom species was statistically significant. However it is possible, it is possible to conclude from *B. paradoxa Gmelin* IC_{50} (CV equal to 1) wide standard deviation that the responses has no difference statistically. When norfloxacin applied alone, it showed that it had the less toxic effect for the benthic diatoms than the other compounds (Figure 1). Norfloxacin effect may come from its action mechanism (inhibition of DNA synthesis) that is supposed to have the most obvious effect occur during diatoms division. On the contrary, inhibitors of protein synthesis can be highly active at any time as diatoms, mitochondrial ribosomes or chloroplast are assembling the proteins.

It is believed that antibiotics adversely affect eukaryotes through interference with mitochondria and chloroplasts function (Liu et al, 2011). The toxicity of ciprofloxacin could be lessened by the nature of this study assay by excluding the sediment.

In this present study, the reported $IC_{50's}$ of lincomycin and norfloxacin differ from the EC_{50} previously reported among freshwater microbes. Lincomycin $IC_{50's}$ are 10.87 and 13.15 mg/l which are higher for both algeal species than that obtained by Andreozzi et al. (2006). Norfloxacin IC_{50} values are 54.39 and 71.58 mg/l which are also many times greater than that reported by González (2017).

The reported differences may not simply resulted from differences in algae species, but may be due to the modified ionic sea-water used in the present assays. Some quinolones are exposed to the antagonism of calcium and magnesium ions, which reduces their effectiveness against bacteria (Drlica et al., 2008), the antagonism could raise the minimum inhibitory concentration for some quinolones including norfloxacin (comparing to fresh water) to some pathogenic microbes (Chen & Chu, 2012)

This antagonism mechanism had not been well described although it is reported that the chelation of metal ions happens in 1:1 ratio, which reduces the antibiotic accumulation in cytoplasm (Lunestad & Samuelsen, 2001). It has been reported that not all antibiotics in the same class act similarly, however, there is no data about tylosin and lincomycin chemistry in seawater (Martinsen and Horseberg, 1995). Neither tylosin (such as macrolide) nor lincomycin (as lincosimide) are susceptible to photolysis, but there has been no studies on their chemical interaction in seawater and their decomposition is improbable to be active under upper millimeter of various sediments (Andreozzi et al, 2006).

It is obvious from this study result of the antibiotic mixture assays that confirmed that the differences in compounds reactions are due to algae species (Figure 3).

N. acicularis multiplication took place in all antibiotic mixtures (except T + C) as mixtures worked together for production of negative synergistic response. However in *B. paradoxa Gmelin* response all antibiotic mixtures work in additional manner. *B. paradoxa Gmelin* responses to (tylosin + lincomycin) and (tylosin + lincomycin + norfloxacin) were variable (CV equal to 1.22 and 1.26) which explain the high IC_{50} for the two treatments (TU 6.89 and 10.07) showing non-significant differences from 1 (1.17 and 1.23). Apart from this, this indicates that the diatom subjected to antibiotic mixtures in their environment might be exposed to selection pressure from such compounds, which is comes at line with some researches related to the investigation of antimicrobial effect on microalgae (Makridis et al., 2006).

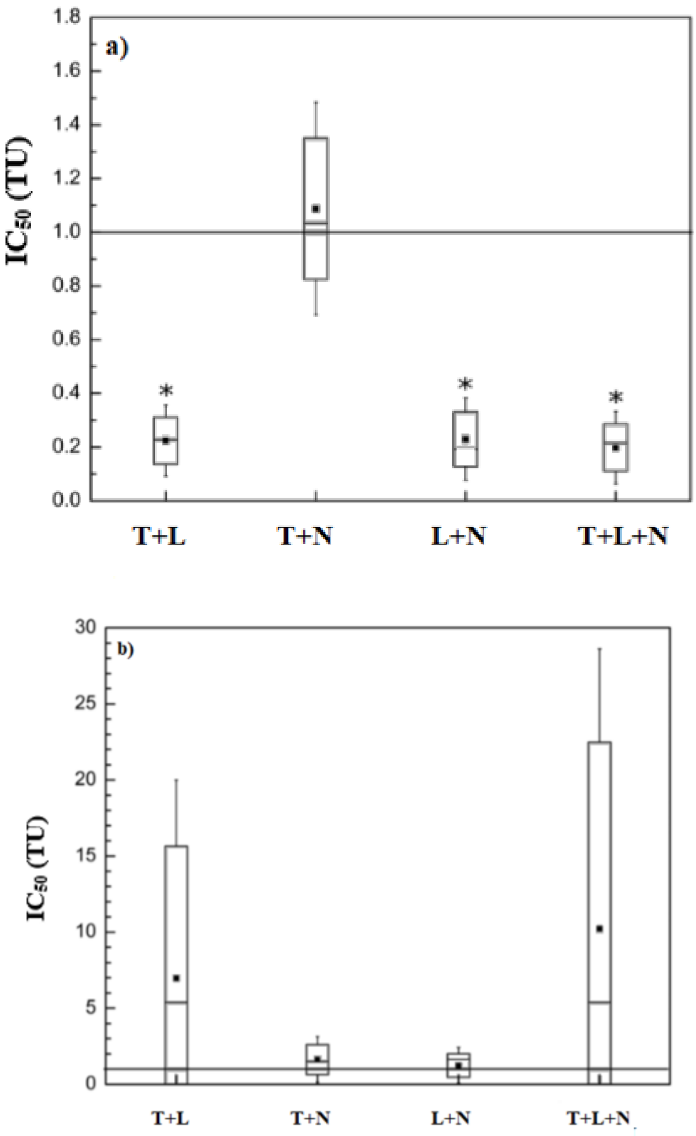


Figure 3: Shows IC$_{50's}$ of mixture of antibiotics for *Nitzschia acicularis* and *Bacillaria paradoxa Gmelin*. a) Response of *Nitzschia acicularis*, b) Response of *Bacillaria paradoxa Gmelin*, to mixture of tylosin, lincomycin, and norfloxacin. Bars refers to standard deviation, the center line refers to median, ■ refers to mean, line refers to 0.5 SD, and * refers to the mean-values (significantly different from 1).

The results of this study suggested that different diatom species possess different responses to different antibiotics. it has been reported that that the benthic consumers completely live on diatom-dominated BMA community that open a path to high nutritional impacts either by reducing available carbon or by changing preferred food abundance (Oakes et al., 2012).

The Microalgae shows unequal productive behavior due to differences in migratory behavior or cell physiology (Cartaxana et al, 2016). Antimicrobial agents used in the present study may act through these paths, the diatoms that exposed to moderate concentrations of antibiotic stopped their motility. Underwood and Kromkamp (1999) stated that even moderate decrease in the benthic diatom motility rate is sufficient to affect primary benthic productivity significantly as a result of narrow association between tidal and benthic migration. It is concluded that biodiversity of algae species improves the quality of water and may give pliability to microalgae community (Cardinale et al., 2009).

More research is needed on the presence of antibiotics and their impact on the environment. Many researchers noted that there was a paucity of data on the antibiotics presence in the environment and their impact on the environment. Because the existing data are few, samples often include water analysis rather than sediment, rather than freshwater instead of marine environments. However, a recent study assured the transfer of antimicrobial agents to coastal environment (Larsson et al., 2018). The differences in studies between the fresh water environment and the marine environment appear to be due to the cost and difficulty of methodological procedures for the study and the extraction and evaluation of antibiotics in benthic organisms. Therefore, the focus is on studying the effect on drinking water.

Antibiotics are found in the environment as mixtures. Various studies examined the toxicity of eukaryotic antibiotics have focused on the responses of single species to single compound. According to biological and chemical heterogeneity of natural water systems, monochromatic methodology is unlikely to give effective ecological predictions or information that can be applied outside the laboratories.

Apart from heterogeneity, the former typical algeal species were more floating marine fish than benthic organisms (González-Pleiter et al., 2013).

Environmental concentrations of antibiotics vary according to the location but are higher in sediments than in overlapping waters due to the convergence of many sediment particles. Contrary to the old belief, it is not true that the absorption of antibiotics reduces their effectiveness (Jiang et al., 2011).

The results of the study showed that algael species of same class were showed the same inhabitation in case of single antibiotic treatment (Fig. 1). Although, single antibiotic responses could not be used for formation of preconceived prediction of the effects of mixtures antibiotic (Figure 3). Algae growth rates inhibition may be selective pressure in the environmental community, which leads to a change in the composition of the community or the spread of antibiotic resistance genes (Halling-Sørensen, 2000). This possibility cannot be underestimated through attracting low antibiotic concentration in the environment due to complex nature of the effects of antibiotic mixtures and the possibility to enhance their effectiveness by the adsorption.

CONCLUSIONS

Globally, micro-algae play an important role in marine environment. Human antibiotics could be found in the surface water that flow to coastal shores. Some of these antibiotics are harmful to algae. Based on that information, it is suggested that antimicrobial agents significantly affect benthic organisms. The results of the study showed that antibiotics are toxic to microalgae and the antibiotics mixture targeting some algael species and the effect of single antibiotic could not be predicted through from single antibiotic effect of microalgae.

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