Research in Marine Sciences
Volume 9-Issue 2- 2024
Pages 462-473

Assessment of Caffeine and its metabolites on marine phytoplankton growth

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Received: 2024-05-18 *Accepted: 2024-06-20*

Abstract

This research aims to assess the influence of varying concentrations of caffeine (CA), one of the pharmaceutically active compounds, and its metabolites (i.e., paraxanthine (PX), theophylline (TP), and theobromine (TB)) on the growth of common marine phytoplankton (*Isochrysis galbana* and *Thalassiosira pseudonana)* under controlled laboratory conditions. Additionally, the study estimates the daily discharge of CA into marine ecosystems utilizing a developed model. The most effective metabolite inhibiting the proliferation of *I. galbana* was determined as TP (68.5%) at a concentration of 500 mg/L, while it was CA (65.8%) at the same concentration for *T. pseudonana*. After exposure to CA, the IC_{50} values for *I. galbana* and *T. pseudonana* were calculated as 107.3 mg/L and 136.8 mg/L, respectively after 96 hours. Furthermore, the lowest IC_{50} concentration was 7.4 mg/L in TP treatment in *I. galbana*. Based on a computational model developed in the paper, in a province with about 2 million inhabitants, where all wastewater is treated in wastewater treatment plants (WWTP), the quantity of CA discharged into the sea was computed as 8.11 kg/day. The CA amounts reaching the sea were potentially increased in regions devoid of wastewater treatment infrastructure. Consequently, WWTPs should undergo expansion and enhancement of their capacity, including advanced treatment for chemical removal for sustainable marine life. Additionally, further studies are needed to elucidate the mechanisms underlying the inhibition effects of CA on phytoplankton.

Keywords: Caffeine; Growth inhibition; Paraxanthine; *Isochrysis galbana*; *Thalassiosira pseudonana.*

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1. Introduction

Caffeine is a central nervous system stimulant in the methylxanthine class and the main component of coffee. Besides its wide use as an ingredient of plenty of prescription or over-the-counter medications, and cosmetics, the daily consumption of caffeinated food and beverages (e.g., chocolate, coffee, tea, dairy desserts, and soft drinks), make caffeine (CA) one of the most frequently used drug worldwide (Kosma *et al.,* 2014; Kleywegt *et al.*, 2019). The mean CA consumption in 161 countries calculated as 177 mg/person per day for adults (Korekar *et al.*, 2020).

Caffeine (1,3,7-trimethylxanthine) is metabolized in the human body mainly to paraxanthine (1,7-dimethylxanthine - PX) 84%, theophylline (1,3-dimethylxanthine - TP) 12%, and theobromine (3,7-dimethylxanthine - TB) 4%. Approximately 5% (ranged from 1 to 10%) of ingested CA is excreted in the urine and the remaining is absorbed and metabolized in the liver (Montagner *et al.,* 2014*;* Petrovic *et al.,* 2016; Gracia-Lor *et al.,* 2017). CA and its metabolites can be distributed in all body fluids and pass through all biological membranes (Petrucci *et al.,* 2018). The excreted CA would subsequently reach wastewater treatment plants (WWTPs) and then enter water bodies such as a river or marine water (Beltrame *et al.,* 2018). Therefore, it has been well-accepted that CA is one of the most ubiquitous pharmaceutically active compounds in the natural environment particularly in marine water (Pires *et al.*, 2016; Dafouz *et al.*, 2018). Chemical properties such as high solubility, low octanol/water partition coefficient and insignificant volatility cause this substance to be stable and ideal in most conditions of the environment. Indeed, previous studies on the bioaccumulation of CA residues in organisms indicate potential environmental and ecological risks addressing concentration increase and transfer through food chain (Ali *et al.*, 2018; Zhou *et al.,* 2018; Ondarza *et al.,* 2019). Some literature data on the amounts of CA and its metabolites in aquatic ecosystems is given in the Discussion section.

In aquatic systems, existence of emerging contaminants such as pharmaceutically active compounds have shown growing increase in the last few decades (Leal *et al.,* 2020), and some of them cause acute and chronic effects on aquatic organisms (Aguirre-Martinez *et al.,* 2013). On the other hand, effect of the most pharmaceuticals on aquatic biota is largely unknown.

Phytoplankton is an essential component particularly for primary productivity in aquatic ecosystems and detrimental effects on these organisms may affect the aquatic food chain (Jonsson and Aoyama, 2007; Ergul *et al.*, 2021). Only very limited data is present in open literature about the toxic effects of CA and its metabolites; PX, TP, TB on phytoplankton species (Aguirre-Martinez *et al.,* 2015). The present study provides the first proliferations inhibition rates for CA, PX, TP, and TB on two phytoplankton species, i.e., *Isochrysis galbana* and *Thalassiosira pseudonana*, that have different characteristics and prefer different habitats. Both species are very common in coastal ecosystems including Atlantic and Pacific coasts, and marginal seas (Guiry and Guiry, 2024) and widely used in

aquaculture (Brown, 2002; Spolaore *et al.*, 2006; Zhang *et al.*, 2020).

The main objectives of this research are: 1) to assess the potential ecological risks of CA and its metabolites; PX, TP, and TB by measuring their proliferation inhibitions on marine phytoplankton species (i.e., *Isochrysis galbana* and *Thalassiosira pseudonana*); and 2) to develop a model that predicts the amount of CA reaching the marine ecosystem as a result of anthropogenic usage in a city, where all wastewater is discharged into the sea after being treated in wastewater treatment plants.

2. Material and Method

Test substances: Caffeine (CA), paraxanthine (PX), theophylline (TP) and theobromine (TB) and strains of the *I. galbana* (CCAP 1323) and *T. pseudonana* (CCAP 1335) were purchased (Sigma Aldrich, and National Center for Marine Algae and Microbiota - Bigelow Laboratory, respectively). Growth inhibition test was performed using a modified OECD (2011) method. *I. galbana* and *T. pseudonana* cells were cultivated in F/2 medium (Sigma-Aldrich) within sterilized artificial seawater. After inoculation, phytoplankton species were kept at 15±2°C in a culture room under an illumination intensity of 4000lux with a 14 h/10 h light/dark cycle, 7.8-8.5 pH, and 32.5 salinity. The flasks were gently shaken two times in a day to prevent conglomeration. The incubation was lasted about 3-4 days until log phase growth prevailed. After reaching the exponential growth phase, an intermediate experimental culture was inoculated with the previous one. Ten mL inoculum of homogenized phytoplankton cells, 2 mL F/2 medium and 100 mL artificial marine water were poured into the flasks (250 mL) and mixed. Treatment concentrations of the chemicals (indicated below) were added and mixed. Obtained data was compared with the control (artificial seawater) group. Responses of the phytoplankton to CA and its metabolites were tested for the concentrations ranging from 1 to 500 mg/L.

Both *I. galbana*, and *T. pseudonana* cultures were exposed to concentration of 1, 10, 100, 150, 250, 450, and 500 mg/L for CA, PX, TP, and TB for 96 hours. Cell numbers were determined in every 24 hours using conventionally counting method by Thoma counting chamber under the light microscope (Olympus BX51), and phytoplankton proliferation inhibition concentrations of CA, PX, TB, and TP were recorded. Treatment of CA and its metabolites were performed in triplicate on both species. The average cell number was calculated and using this average value inhibition rates (as percent) and IC_{50} values calculated.

The endpoints were evaluated based on cell count data calculated (0 to 96 h) from the mean cell counts of each test series. The inhibition-concentration curves were used to calculate IC_{50} (50% growth inhibition) values.

The amount of CA discharged into the sea was estimated by a model developed within the scope of the study using literature data regarding excreted CA amount by urine and hot drink residues. In the model, with its over 2 million total inhabitants, a coastal city, Kocaeli Province (Türkiye) where almost all of its wastewater is discharged into the sea (the

Marmara Sea) after being treated by %99 in wastewater treatment plants (URL-1), taken into consideration as a representative sample. In addition, caffeine consumption by adults and the types of caffeinated beverages consumed in the Turkish society (Korekar *et al.*, 2020) were considered. Therefore, the model statement is:

$$CA_{nd} = \frac{\left[\left[\left(CA_{df} * FP\right) + \left(CA_{dm} * MP\right)\right] + \left[\left(CA_{tr} + CA_{cr}\right) * TP\right]\right] * 0.3}{10_6} \quad (1)$$

where;

CA_{nd} = the net discharged amount of caffeine into the marine ecosystem (e.g. the Marmara Sea) in kg per day,

CA_{df} and CA_{dm} = the amounts of caffeine in mg excreted in the urine by an adult female and male, respectively in a province (e.g. Kocaeli).

CA_{cr} and CA_{tr} = the amounts of caffeine in mg that will be discharged to sewerage daily, in the residual coffee grounds and tea residue in the teapot, respectively. To calculate CA_{cr} and CA_{tr}, the amount of CA in the tea residue remaining in the teapot was estimated to be ~2 mg per cup of tea and ~7 mg per cup of coffee. Since, 74.6% of the daily per capita CA consumption in Türkiye (245 mg) comes from tea, 11.8% from coffee, and 13.6% the other sources (Korekar *et al*., 2020), it is estimated that CA_{tr} and CA_{cr} would be 12.18 mg/day and 3.37 mg/day, respectively.

FP and *MP* = the adult female and male populations whose wastewater is discharged into the sea after being treated in the WWTP (e.g. FP is 760.963 people, and MP is 777.550 people for Kocaeli Province) (URL-1).

The value 0.3 is a coefficient in the formula. This coefficient is used to determine the real amount of CA discharged into the sea, since 70% of caffeine is eliminated in WWTP (Camacho-Munoz *et al.,* 2012); accordingly, the amount of 30% (i.e. 0.3) of caffeine discharged in to the sea.

To calculate CA_{df}, and CA_{dm}, a study conducted in Switzerland on urinary CA excretion (Petrovic *et al.* 2016) was utilized. To adapt the study's findings to the Turkish community, average daily CA consumption in both countries (Korekar *et al*., 2020) were used in the following formulas:

$$CA_{df} = CA_{dfS} * \frac{CA_{dcT}}{CA_{dcS}} \quad (2)$$

$$CA_{dm} = CA_{dmS} * \frac{CA_{dcT}}{CA_{dcS}} \quad (3)$$

where,

CA_{dcT} and CA_{dcS} represent 245 and 342 mg, which are the daily CA consumptions in the Turkish and Swiss communities, respectively (Petrovic *et al.*, 2016).

CA_{dfS}, and CA_{dmS} represents daily CA amount discharged by urine as mg per day in Switzerland 2.85 mg for females and 2.76 mg for males, respectively (Petrovic *et al.*, 2016).

This model, given the knowledge of the societal preference ratios for caffeinated beverage consumption and the proportion of female and male populations to the total population, can be applied to cities where wastewaters are discharged into the sea post-treatment.

3. Results

The effects of CA and its metabolites on cell proliferation inhibition and IC_{50} values were determined for each metabolite for *I. galbana* and *T. pseudonana*. Also, the amount of CA reaching the sea daily was estimated with a developed model. Following addition of CA and its metabolites into the cultures at the beginning of the experiments, cell concentrations were decreased for all treatment compared with the control group (Figure 1). For both species showed their highest cell number at 1 mg/L, and their lowest at 500 mg/L. The highest final cell concentration was nearly 4.7 fold that of CA control group in *I. galbana* (Figure 1a).

Growth inhibition rates in both species were determined by comparing the cell numbers at the 96th hour with the control group. The highest inhibition rates considering cell number were measured at 500 mg/L concentrations for CA exposure as 65.0%, and 65.8% on *I. galbana*, and *T. pseudonana*, respectively. These rates were 41.3%, and 15.8%, respectively for both species at 1 mg/L (Table 1). These data indicate that CA remarkably affects *I. galbana* even at low concentrations (i.e., 1 mg/L), whereas after 10 mg/L concentration, it starts to affect *T. pseudonana* in higher rates. Addressing increased concentrations, CA effects on the proliferations of both species is also increased (Table 1).

PX, the most abundant metabolite of caffeine, inhibited *I. galbana* cell proliferations from the initial concentration (i.e. 1 mg/L) and inhibition rate reached 53.0% at its 150 mg/L. After that point, the inhibition rates slightly increased up to 59.1 % at 500 mg/L. These inhibition rates ranged from 11.1% (at 1 mg/L) to 32.8% (at 500 mg/L) for *T. pseudonana*. The obtained data indicate that PX effects on both species are close each other, particularly for the initial concentrations. On the other hand, after 10 mg/L treatment, the number of inhibited *I. galbana* cells rapidly increased with increased concentrations (Table 1).

Table 1**.** Inhibition rate of caffeine and its metabolites by cell number (%) on phytoplankton species

Concentration mg/L	*I. galbana*				*T. pseudonana*			
	CA	PX	TB	TP	CA	PX	TB	TP
1	41.3	6.8	5.8	40.8	15.8	11.1	9.1	7.5
10	46.9	14.1	10.9	52.0	48.0	15.3	16.3	9.7
100	51.3	30.9	15.6	57.8	60.8	23.3	22.1	12.8
150	54.4	53.0	21.9	59.2	61.0	28.4	25.4	18.3
250	57.5	55.3	28.1	64.9	63.3	30.3	29.2	20.2
450	60.6	57.1	29.8	65.7	65.2	31.2	34.2	24.6
500	65.0	59.1	35.5	**68.5**	**65.8**	32.8	37.5	27.2

Inhibition rates in TB-exposed *I. galbana* and *T. pseudonana* cells were ranged from 5.8 % to 35.5% and 9.1% to 37.5% (for 1 to 500 mg/L concentrations), respectively. However, unlike the other metabolites, the initial concentration of TP (i.e., 1 mg/L) caused remarkably higher inhibition as 40.8% in *I. galbana*, quite similar with the CA effect. This rate was only 7.5% in *T. pseudonana*. Similarly, the inhibition rate on *I. galbana* cells by TP increased up to 68.5% at 500 mg/L, higher than that of CA and other metabolites for both species, while this rate remained at 27.2% in *T. pseudonana* (Table 1). As it is seen, except for TB, inhibition rates reached over 59.1% for the CA and its metabolites (e.g., 68.5% for TP) at a concentration of 500 mg/L in *I galbana*, whereas for *T. pseudonana* these rates were below 37.5% except for CA, which inhibited proliferations by 65.8%.

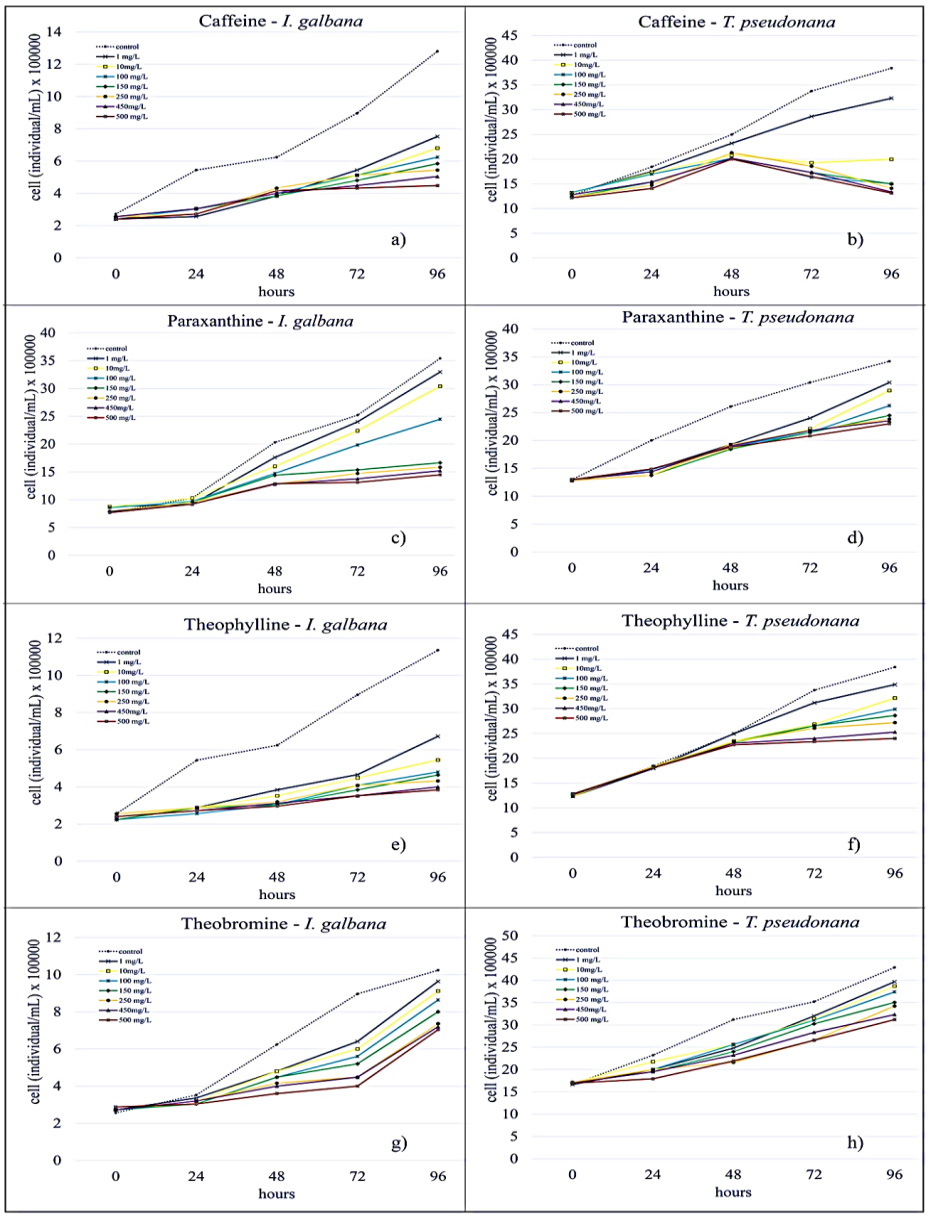

Figure 1. Time-dependent variations in cell numbers of *I. galbana* and *T. pseudonana* exposed to different concentrations of CA, PX, TB and TP.

Furthermore, after exposure to CA, the IC_{50} values for *I. galbana* and *T. pseudonana* were calculated in 96-hour period, as 107.3 mg/L and 136.8 mg/L, respectively (Table 2). The lowest IC_{50} value in *T. pseudonana* was determined in the CA application whereas, in *I. galbana*, the lowest IC_{50} value was obtained in TP treatment as 7 mg/L. The highest IC_{50} value was obtained in the TB treatment on *T. pseudonana* as 1118.2 mg/L. The observations of all IC_{50} values obtained for CA and its metabolites are lower in *I. galbana* compared to *T. pseudonana*. This confirms that *I. galbana*, possessing calcareous plates, is more susceptible to these compounds than *T. pseudonana*, which is characterized by a silica-containing cell wall (Table 2).

Table 2. IC_{50} (96^{th} hours) values of *I. galbana* and *T. pseudonana*

IC_{50} (mg/L)	*I. galbana*	*T. pseudonana*
CA	107.3	136.8
PX	321.9	909.3
TB	857.6	1118.2
TP	7.4	746.4

In the context of the present study, beyond quantifying inhibitions of proliferation and growth rates, a computational model was developed to estimate the quantity of CA released into a marine ecosystem, which concurrently serves as a habitat for the phytoplankton. In the model, the annual CA amount discharged into the Marmara Sea (where *I. galbana* and *T. pseudonana*, used in the present study, also live) from Kocaeli, a province with a population over 2 million and where all domestic wastewaters are treated before being discharged into the sea, has been calculated. According to the model, the amount of CA used by adults and discharged into the sea through the WWTP is calculated to be 8.11 kg/day (i.e., 2.96 tons/year).

4. Discussion

Generally, assessing the data regarding the effects of caffeine and its metabolites on the two phytoplankton species indicates that the compounds that most effectively inhibit the proliferation of *I. galbana* cells are CA, and TP, while the most effective compound inhibiting *T. pseudonana* is CA. For *I. galbana*, particularly CA and TP inhibited the cell proliferations from the initial concentration (i.e., at 1 mg/L), whereas this concentration did not inhibit *T. pseudonana* proliferations remarkably. This situation can be attributed to the easier penetration of CA and its metabolites into the cell in *I. galbana*, a Coccolithophore surrounded by calcareous plates called coccoliths. However, *T. pseudonana*, a diatom species, has greater resistance to CA and its metabolites, especially at low concentrations, and this resistance probably related to its silica-containing cell wall. On the other hand, the cell wall of *T. pseudonana*, is not very thick and contains nanopores (Hilderbrand *et al.*, 2006) and has difficulty to resist increasing doses.

Due to their widespread geographic distribution and significant impacts on several important ecological processes in marine ecosystems, marine diatom *T. pseudonana*, and

marine coccolithophore *I. galbana* were used as representative aquatic organisms for acute toxicity test in this paper. In the traditional toxicity tests, IC_{50}/EC_{50} values are widely used as the endpoint to evaluate the chemicals effect on phytoplankton growth inhibition and compare their sensitivity (Yang *et al.*, 2008). Aguirre-Martinez *et al.* (2015) reported that the half inhibition value (IC_{10}) was calculated as 63.8 mg/L for CA toxicity on *I. galbana.* They also reported the IC_{10} value for a freshwater alga (*Pseudokirchneriella subcapitata*) as 89.7 mg/L. Since there is not enough data in the open literature regarding the IC_{50} values of CA and its metabolites on phytoplankton, the obtained data with studies on macro-organisms beside microalgae below were compared. Diniz *et al., (*2021) reported that in another freshwater alga (*Raphidocelis subcapitata*) for CA treatment, the EC_{50} value was calculated as 154.9 μg/L after 16 days, which is lower than our findings.

In another studies, CA-related, median EC values for a macrophyte (*Lemna gibba*), and a zooplankton (*Dapnia magna*) reported as 1 mg/L and 182 mg/L, respectively (Solomon *et al.*, 1996; Brun *et al.*, 2006). Gray *et al.* (2021) reported that 0.01-0.1 mg/L CA concentrations did not show a significant effect on the growth rate or photosynthetic efficiency of a macroalgae species (*Chondrus crispus*); however, they reported that 100-200 mg/L CA caused acute sublethal effects and mortality in a macroalgae, *Codium fragile* subsp. *fragile*. Therefore, the IC_{50} values obtained herein are thought to be comparable to those scientific studies.

Regional CA consumption is influenced significantly by social culture. In some countries particularly in developing ones, caffeinated beverages consumption per capita is increasing parallel with population growth, and the amount of CA, and its metabolites entering aquatic ecosystems is also rising, often due to the lack of advanced wastewater treatment facilities (Quadra *et al.*, 2020). Therefore, a computational model was formulated to calculate daily amount of CA discharged in Kocaeli Province (Türkiye) which is a coastal city with about 2 million inhabitants and almost all of its wastewater inflows into the sea (the Marmara Sea) after being treated in WWTPs.

As mentioned above, based on the model, the amount of CA discharged by adults into the sea is calculated to be 8.11 kg/day (i.e., 2.96 tons/year). However, the adult population was taken into account in the model, and the CA contribution of young people (under the age of 18) who may consume caffeinated beverages such as cola, iced tea, energy drinks, etc., were not evaluated. In addition, the amount of CA found in cosmetic products such as creams, gels, and shampoos, which could potentially be discharged directly into the sewage system post-washing, was not factored in the model. Consequently, it is thought that the actual quantity of CA, discharged into the sea, may exceed the model's output. Conversely, a portion of the effluent from the WWTPs is reused, thereby not contributing directly to sea discharge. Given the variability of these quantities, assuming the precise volume of CA released into the sea from these facilities is difficult. Therefore, the outcome provided by the model was deemed accurate, addressing these considerations.

The cumulative capacity of wastewater treatment plants in the province of Kocaeli is quantified at 435.000 m^3/day (URL-2). In accordance with our model, a daily discharge of

8.11 kg of CA into the marine environment is also projected. Consequently, based on these data, the concentration of CA in the effluent from wastewater treatment plants within the province being released into the sea is estimated to be average 18.6 µg/L. The reviewed articles demonstrated that the CA concentrations in marine water and WWTPs effluents were ranged from 0.002 µg/L to 303.6 µg/L (Korekar *et al.,* 2020), e.g. 11.0 µg/L found in Australia, 8.20 µg/L in Japan, and 20.4 µg/L in Türkiye (Murakami *et al.,* 2011; Aydin and Talinli, 2013; French *et al.,* 2015). On the other hand, the CA concentrations in untreated wastewater were determined as 3594 µg/L in the Singapore (Tran *et al.,* 2014), 290.0 µg/L in Brazil (Canela *et al.,* 2014), and 159.8 µg/L in Türkiye (Ayman and Işik, 2015). Therefore, according to the developed model's outcomes, it is seen that the predicted quantity of CA discharged into the sea remains within the range of literature data and is at a level comparable to studies conducted in diverse marine ecosystems. Consequently, the model's given knowledge about the societal preference ratios for caffeinated beverage consumption and the proportion of female and male populations to the total population, can be applied to cities where wastewaters are discharged into the sea post-treatment.

Conclusions

In conclusion, it is thought that the obtained results, suggests a potential influence of CA and its metabolites on the phytoplankton species in the context of proliferation and inhibition. These findings could potentially enhance our understanding of circumstances in marine ecosystems and biogeochemical cycles. However, further research is needed to elucidate the mechanisms underlying these effects. The findings of the present study regarding CA consumption, in conjunction with literature, emphasize the continuous discharge of CA and its metabolites into marine ecosystems. Furthermore, the amount of these discharges will increase in parallel with the population growth, particularly in coastal settlements. Despite the discharges being quantified at the µg/L level and having a half-life, they nonetheless pose a significant burden on marine ecosystems. The quantities computed within in the context represent the concentrations of CA discharged into the sea after processing through the WWTPs. Therefore, it is obvious that the amounts reaching the sea will potentially be elevated in regions devoid of wastewater treatment infrastructure. As a result, WWTPs should undergo expansion and enhancement of their capacity. Moreover, these facilities ought to be transformed into advanced treatment facilities with higher capabilities for chemical removal, including CA and its metabolites. These measures will be a crucial step towards the mitigation of environmental impacts and the conservation of marine ecosystems.

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