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Risk associated with toxic blooms of marine phytoplankton functional groups on *Artemia franciscana*

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PEER REVIEW

Peer reviewer

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Comments

This is a valuable research work in which authors have demonstrated the lethal effect of 3 harmful algae on model organism *A. franciscana*. Furtheremore, the report provided readers the antagonistic effects of harmful algae on mortality of *A. nauplii* during both ternary mixture and the binary combinations. It has ecological significance when evaluate the risk of harmful blooms in natural environment.

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ABSTRACT

Objective: To study mortality of copepod *Artemia franciscana* against the occurrence of harmful marine algae and possible toxicological changes exhibited by binary and tertiary combinations of these harmful algae toxins.

Methods: Tweenty four hours acute toxicity assays were performed with selected concentrations of *Alexandrium minutum*, *Prorocentrum lima* and *Nitzschia* N1c1 living cells. Additionally, the results were analyzed using the median-effect/combination index (CI)-isobologram equation to assess possible changes in the toxic effect induced by phytoplankton functional groups.

Results: Biotoxin equivalent values obtained by immunodetection were (2.12±0.10), (8.60±1.30) and (4.32±1.67) pg/cell for saxitoxin, okadaic acid and domoic acid, respectively. The 24-h LC_{50} values estimated to saxitoxin and okadaic acid equivalents were 4.06 and 6.27 μg/L, significantly below the value obtained for *Nitzschia* N1c1, which was established at 467.33 μg/L. CI analysis applied on phytoplankton assemblages showed that both ternary mixture as the binary combinations exhibited antagonic action on toxic effects in *Artemia nauplii*, which were significantly lower than the toxic effect exhibited by each species studied.

Conclusions: These results show that, although these harmful algae represent a serious risk to estuarine zooplankton community, the presence of phytoplankton functional groups within the same bloom can reduce the potential risk compared to the expected risk when each of the phytoplankton groups are evaluated individually.

KEYWORDS

Harmful algae blooms, *Artemia franciscana*, Phytoplankton functional groups, Combination index, Toxicity

1. Introduction

Hypersaline environments are important natural assets of considerable economic, ecological, scientific and natural value. These ecosystems span large areas worldwide, not only in salt production areas (solar salt works, salterns or salinas) but also in natural lakes and lagoons, and tidal ponds[1]. Hypersaline ecosystems of both marine and continental inputs are essential, integral and dynamic part of the biosphere while the biogeochemical processes occurring in their unique ecosystems have considerable environmental, social and economic values[2].

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Zooplankton is an important link between algae and higher consumers in estuarine trophic chains. On one side, zooplankton can deliver algal toxins along the food chain by accumulating the toxins, on the other side, harmful algae can directly inhibit the cell growth in certain zooplankton species[3,4].

Some marine microalgae exhibit a diversity of chemical defenses (phycotoxins) that could affect the feeding and fitness of zooplankton communities. Although selective grazing can avoid certain harmful zooplankton, which is well documented in the microalgae literatures[5], some grazing populations feed indiscriminately. In the midst of toxic blooms, these indiscriminate grazers also feed on the toxic cells and they might be killed by their phycotoxins[6]. Thus, the effects of toxic algal cells on zooplankton could have severe implications for aquaculture and the local marine environment in areas prone to recurrent toxic algal blooms[7].

Alexandrium minutum (*A. minutum*) is a widely distributed marine, planktonic dinoflagellate species associated with paralytic shellfish poisoning blooms in coastal regions[8]. *Prorocentrum lima* (Ehrenberg) Dodge (*P. lima*) is a highly toxic dinoflagellate that produces okadaic acid (OA) and dinophysis toxins[9], which are implicated in diarrheic shellfish poisoning[10]. Since 1994, species of the toxic genus *Nitzschia* and *Pseudo-nitzschia* have been identified as the toxic producers of domoic acid (DA) outbreaks in northwest Spain[11,12].

Artemia franciscana (*A. franciscana*) belongs to Branchiopoda crustacean, is one of the standard experimental organisms used for toxicity testing of marine contamination[13], and can directly feed and filter the toxic algae. *A. franciscana* is also an ideal experimental organism used for researching the toxicity of toxic algae.

The number of reports on harmful algal blooms has significantly increased in the last three decades[14]. Monitoring phytoplankton populations evidenced that the phytoplankton community was heterogeneous with a wide range of species. Among the potentially harmful algal bloom forming genera, *Alexandrium* spp. and *Pseudo-nitzschia* spp. were the most abundant, especially *Alexandrium minutum* (*A. minutum*) which was detected every year during winter and spring. At the same time, contamination of shellfish species by paralytic shellfish poisoning toxins was confirmed by chemical analysis[15]. Other genera were also monitored, among which *Prorocentrum* spp. were frequently observed throughout the year[16].

Phytoplankton functional groups, non-phylogenetic associations of organisms that are based on physiological, morphological or other features that respond to recurrent patterns or factors similarly[17], of harmful algae often play a role in riskiness of a bloom, even though toxic phytoplankton studies available in the literature are limited to the study of some specific bloom-forming species.

Because very few laboratory studies have verified the potential toxicity of these marine microalgae to the grazing community, the objective of the present work is to study the relationship between the two basic biological components *i.e.* harmful microalgae and *Artemia* in estuarine environments in order to provide a better understanding of the dynamics of this unique ecosystem. Additionally, the toxic risk on *A. franciscana* nauplii derivate of the coexistence between bloom-forming dinoflagellates and diatoms has been analyzed by means of multivariate statistical techniques.

2. Materials and methods

2.1. Algal culture

Cultured samples of *A. minutum*, *P. lima* and *Nitzschia* N1c1 were prepared from algal culture collection of Genetics, Faculty of Veterinary, Complutense University, Madrid, Spain. Cells were axenically grown in cell-culture flasks with 20 mL of artificial seawater enriched with F-2 medium (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), at 20 °C and a photon irradiance of 60 μmol/(m^2·s) over the waveband 400-700 nm, in a 16:8 h light-dark photoperiod.

Cells were maintained in mid-log exponential growth by serial cell transfers to fresh medium. Prior to the experiments, the culture cells were recloned (by isolating a single cell) to assure genetic homogeneity in all the cultures.

2.2. Extraction from algal samples and enzyme-linked immunosorbent assays (ELISAs)

The cell count was determined by mean Neubauer chamber before the cells were lysed. Homogenates corresponding to 20×10^6 cells/mL of each microalgal species were obtained by means of freezing and thawing procedure (three times) and subsequent incubated in a 40 W ultrasonic water bath (Sonicor SC52, New York, USA) for 30 min. Finally, these homogenates were filtered with a 0.2 μm micron polycarbonate filters to remove the cell debris. The filtrate, containing all the extracellular and intracellular toxins content in addition to the disrupted cell components, was diluted with 10% methanol in PBS-Tween prior to analysis in order to avoid unspecific matrix effects.

Detection of saxitoxin (STX), OA and DA toxins in culture media was conducted for ELISA kits (Biosense® Laboratories, Bergen, Norway). Assays were performed according to the kit user manuals. STX and OA tests are a direct competitive ELISA based on the recognition of the appropriate biotoxin by specific antibodies, while DA test is in a direct competition format, where free DA in the sample competes with DA-conjugated protein coated on plastic wells for binding to

anti-DA antibodies free in the solution.

The spectrophotometric analysis of selected biotoxins was performed by measuring the absorbance at 450 nm with a Tecan microplate reader GENios (Tecan UK Ltd, Reading, UK). Calculation of results was done manually using a semilogarithmic function by plotting the results of calibration and the negative control.

2.3. Hatching of the Artemia cysts and collection of the nauplii

The *Artemia* cysts were purchased from Argent (Argentemia Silver Grade, Argent Chemical Lab., Washington, USA). The method of Persoone *et al*.[18] to obtain *Artemia* for the test was modified according to the following procedure. Encysted *A. franciscana* were hydrated in distilled water at 4 °C for 12 h, followed by washing to separate the cysts that float from those that sink. The sinkers were collected on a Buchner funnel and washed with cold distilled water followed by synthetic seawater. Synthetic seawater was prepared by mixing 35‰ synthetic sea salts (Waterlife Research Ltd., UK) with distilled and deionized (Milli-Q) water, stirring for 24 h with suitable aeration and filtering through 30 μm Millipore cellulose filters.

Cysts were incubated in a graduated glass cylinder in 100 mL of seawater medium. The temperature was 25 °C, pH 8.6, photon irradiance of 16 μmol/(m^2·s) over the waveband 400-700 nm, and a slight aeration was maintained by a small tube in contact with the bottom of the cylinder. Under these conditions, the time required for the cysts to hatch was approximately 24 h.

2.4. Assessment of toxicity assays

Standard environmental conditions for acute toxicity assays were: temperature 25 °C, salinity 35‰, pH 8.6 and darkness. Assays were conducted in sterile 24-well polystyrene tissue culture plates.

The acute toxicity assays were performed to define the lethal effect on *A. franciscana* larvae, induced by selected concentrations of *P. lima*, *A. minutum* and *Nitzschia* N1c1 living cells by mean 24-h exposures. At least eight replicate test series were performed to each cell or homogenate concentration. The cell concentrations tested for each of the microalgal species are summarized in Table 1. Assay plates were placed in an incubator under standard conditions for a period of 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during 10 s of observation.

Table 1

Living cells concentrations tested by standardized concentration-dependent (1-5) model type, and performed to define the acute lethal effect of selected harmful algae populations on *A. franciscana* nauplii.

Specie	Living cell concentration (cell/mL)					
	control	[1]	[2]	[3]	[4]	[5]
P. lima	–	250	500	1 000	2 000	4 000
A. minutum	–	250	500	1 000	2 000	4 000
Nitzschia N1c1	–	5 000	10 000	100 000	25 000	500 000

2.5. Combination index (CI) for determining combined biotoxins interactions

The results were analyzed using the CI-isobologram equation which is based on the median-effect principle that demonstrates that there is an univocal relationship between dose and effect independently of the number of substrates or products and of the mechanism of action or inhibition[19-21].

This method involved plotting the dose-effect curves for each compound and their combinations in multiple diluted concentrations. These parameters were used to calculate doses of the marine biotoxins and their combinations required to produce various effect levels. CI<1, =1 and >1 indicates synergism, additive effect and antagonism, respectively.

2.6. Data analysis

Lethality was expressed as $LC_{50(24)}$ estimates with 95% confidence limits, implemented in the Pharmacologic Calculation System (PCS version 4.0, New York). The $LC_{50(24)}$ estimates were subjected to a Two-way analysis of variance with replication within the subgroups (ANOVA) followed by *post hoc* contrast with Duncan test. Differences were considered significant with a probability level of $P<0.05$. The highest cell density (cells/mL) demonstrating no effect [24-h non observable effect (NOEC)] as compared to the controls was estimated by Dunnet's test for statistical significance ($P>0.05$). Computer program CompuSyn (Compusyn Inc, USA) was used for calculation of CI values corresponding to the selected biotoxin combinations. Statistical calculations were performed using the computer software package GraphPad Prism v5.0 (Graph-Pad Software Inc., USA).

3. Results

Biotoxin equivalent values obtained by immunodetection for *A. minutum*, *P. lima* and *Nitzschia* N1c1 are shown in Table 2.

Table 2

Biotoxins equivalent concentration values obtained by using ELSA. (mean±SD, n=4).

Toxin equivalent	Equivalent concentration (pg/cell)
STX	2.12±0.10
OA	8.60±1.30
DA	4.32±1.67

Acute toxicity assays performed in *A. franciscana*, exposed to living cells from *A. minutum*, *P. lima* and *Nitzschia* N1c1, show that all of them exhibited a high toxicity in the nauplii exposed to selected cell densities. The 24-h LC_{50} values estimated to STX and OA equivalents were 4.06 and 6.27 μg/L, significantly below the value obtained for *Nitzschia N1c1*, which was established at 467.33 μg/L (Table 3). Lineal regression analysis showed no significant differences between

STX and OA, yet both showed significant differences respect to DA (Figure 1).

Table 3

24 h-NOEC and 24 h-median lethal concentration values $LC_{50(24)}$ (CL 95%) obtained in *A. franciscana* nauplii exposed to selected harmful algae living cells (n=8).

biotoxin	24 h-NOEC (μg/L)	24 h-NOEC (cell/mL)	$LC_{50(24)}$ (CL 95%) (μg/L))	$LC_{50(24)}$ (CL 95%) (cell/mL)
STX	0.50	236	4.06 (3.53-4.74)	1915 (1665-2236)
OA	1.20	140	6.27 (4.57-8.18)	729 (531-951)
DA	18.00	4170	467.73[a,b] (400.87-550.81)	108271 (92794-127502)

Results are expressed as biotoxin equivalents (μg/L) and living cell concentrations (cell/mL). [a,b]: Significant differences (P<0.001) respect to STX and OA equivalent $LC_{50(24)}$ values, respectively.

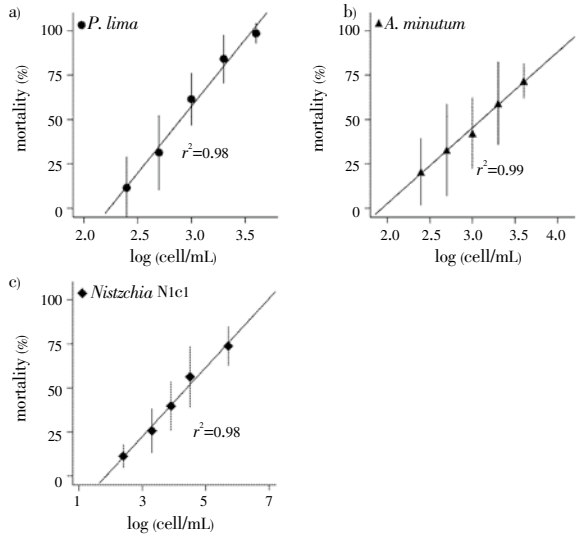

Figure 1. Linear regressions corresponding to inhibitory response induced on *A. franciscana* nauplii exposed to increasing concentrations of three selected algae. a: *P. lima* living cells; b: *A. minutum*living cells; c: *Nitzschia* N1c1 living cells. Points represent means with vertical lines showing standard deviation (n=8).

A relationship between 24-h NOEC values, obtained for each of the three biotoxins studied and their equivalence in the number of cells showed that the ecotoxicological risk, induced by *A. minutum* (236 cell/mL) and *P. lima* (140 cell/ mL) blooms on *A. franciscana* populations, is significantly greater than that induced by *Nitzschia* N1c1 blooms (4170 cell/mL).

Table 4 shows the mean combination index (CI) values of biotoxin combinations obtained in the range of fraction affected (Fa) 0.1-0.9. The results indicated that in all cases, combinations of the selected marine biotoxins induced a significant antagonic effect.

Table 4

CI associated with affected fraction (Fa) obtained in binary and ternary combinations among selected equivalent biotoxins (n=8).

Fraction affected	STX/OA	STX/DA	OA/DA	STX/OA/DA
0.10	2.12±0.48	1.28±0.32	1.57±0.36	2.20±0.31
0.25	2.12±0.36	1.41±0.32	1.70±0.34	2.42±0.38
0.50	2.14±0.35	1.83±0.24	2.00±0.35	2.96±0.35
0.75	2.14±0.27	2.15±0.28	2.39±1.37	3.43±0.33
0.90	2.15±0.38	2.47±0.37	2.57±0.31	3.86±0.31

Binary combination between STX and OA showed antagonic effect in all Fa values. However, binary combinations between STX-DA and OA-DA showed a slight antagonism at low effect levels but the antagonism increased at the highest Fa levels. The ternary combination among STX, OA and DA showed a moderate antagonism at low Fa values, which increased at higher Fa values.

Comparison between all comparisons analyzed showed that the antagonic effect exhibited by the ternary combination was significantly higher than those observed in binary combinations (Figure 2).

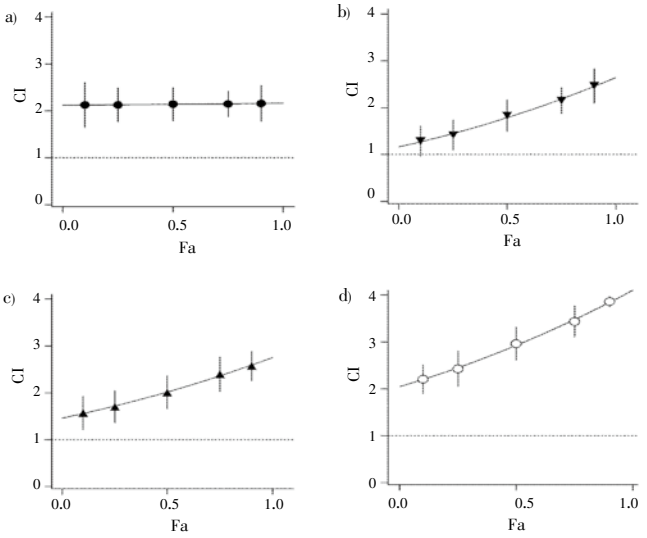

Figure 2. Computer-generated graphical presentation of the combination index (CI) with respect to fraction affected (Fa) for the antagonic effect induced by the binary combinations.
a: Saxitoxin and okadaic acid; b: Saxitoxin and DA; c: Okadaic acid and DA; d: The ternary mixture conformed by saxitoxin, okadaic acid and DA. CI<1, =1 and >1 represent synergistic, additive and antagonistic effects, respectively. Points represent mean±SD of 8 replicates.

4. Discussion

The genus *Artemia* is the dominant macrozooplankton present in many hypersaline environments. This crustacean often dominates food web dynamics in hypersaline environments and its grazing activities control water clarity[22], and consequently they are often introduced into salt production facilities to minimize algal blooms[23].

It seems that algal composition as the main food source of *Artemia* in both natural habitat and culture media has significant effect on *Artemia* growth and reproduction rates. For example, D'Agostino noted that the growth of *Artemia* was influenced by both the species of phytoplankton in the diet and the culture media used to grow the phytoplankton[24]. In the present study, *A. minutum*, *P. lima* and *Nitzschia* N1c1 live multiseries cells were found to be harmful to *A. francsicana* nauplii at compatible cell densities with those observed in natural blooms.

Alexandrium blooms occur when there are weak spring tides. These blooms develop as a consequence of a favorable

balance between in situ growth rate and tidal dilution[25,26], and the dominant *Alexandrium* species found was the toxin producing *A. minutum*[27].

Although the toxin content of *A. minutum* can vary on a global scale, MacKenzie and Berkett[28] reported a toxin content of (1.29±0.58) pg STX equivalent/cell. These levels are comparable with the toxicity of *A. minutum* in culture of 1.6-2.6 pg STX equivalent/cell obtained by Touzet *et al*[29]. However, the value of 2.12 ng STX equivalent/cell obtained in this work is significantly lower than what have been reported by these authors. These differences could be related to the cell growth phase. It is known as very little (<5%) of the toxin is released from the cells during exponential growth, and however the toxin is highly released during senescence, which would coincide in natural conditions with the induction of sexuality during bloom decline[30]. These circumstances could justify differences in toxin levels into the single cell.

Okadaic acid is produced by dinoflagellates of the genus *Prorocentrum*. However, rather large differences between the nominal and measured OA concentrations were detected by different authors. Throughout two bloomless years of monitoring, Takahashi *et al*.[31] have isolated a maximum of 0.2 μg OA per litre from the water column near North Stradbroke Island (Australia). MacKenzie *et al*.[32] however made report of dissolved OA concentrations up to 67 μg/L during a dinoflagellates bloom.

Since *P. lima* prefers epibiotic growth, pelagic cell counts are rare and difficult to compare to lab conditions. Bravo *et al*.[9] found total intracellular toxin productions from 2.0 to 28.6 pg/cell. By adopting the same protocols, the intracellular OA of our lab-grown *P. lima* culture was determined at 8.60 pg equivalent OA/cell. This is well within the limits of the range determined by Bravo *et al*.[9], as well as the range of 0.4-17.1 pg/cell found by Nascimento *et al*.[33].

The ability to produce the marine toxin DA is shared by several species of the diatom genera *Pseudo-nitzschia* and *Nitzschia*. Mixed blooms of *Pseudo-nitzschia* or *Nitzschia* spp. have been reported to release significant levels of DA as well[34]. These organisms have been frequently observed to cause blooms with cellular densities over 10^3 cells/mL[35].

Although several of these species are widespread, the level of DA production appears subject to large geographical variation as well as variation between strains of the same species[36]. The *Nitzschia* N1c1 strain used in the presented experiments was found to release 4.32 pg equivalent DA/cell into its growth medium. This is consistent with the results of Lelong *et al*.[37], which showed dissolved DA levels between 1 to 5 pg/cell. Intracellular DA production was determined at 1.5 (0.08-3.45) pg DA/cell by Hagström *et al*[38]. Considering these data, the value reported in this study appears reasonable.

In the present study, CI was applied on the short-term toxicity results obtained in the selected harmful marine phytoplankton. The CI is a fractional approach method used in pharmacology to analyze the combined effect of drugs[39], it was developed and mathematically derived from enzyme-substrate kinetics by Chou and Talalay and their definition of additivity is essentially derived from the classical definition of isobologram[19].

This method is independent of any consideration on the mode of action of the substances under analysis. It can be used to analyze and quantify the degree of the deviation from additivity (synergism or antagonism) of two to *n* substances with similar, dissimilar or unknown mode of action at any level of effect exerted on any organism[19].

In this work, the CI method was applied to study the interaction exhibited by several mixtures of harmful phytoplankton populations in *A. franciscana* nauplii. Both in the ternary mixture as in the binary mixtures analyzed, CI values between 1 and 2 were obtained. These results showed that all mixtures analyzed causing a weak antagonic phenomenon. Some recently reviews about this issue state that deviations from additivity lesser than a factor of two should not be considered as a significant deviation from additivity for risk assessment purposes, but it is still a matter of discussion[40-42].

Another aspect to consider is the fact that the nature of the interaction of the pollutants also varied with the test organism. Even in similar organisms sharing common physiological functions (as green algae and cyanobacteria), an opposite behavior can be found in the interaction of the same chemicals. Rosal *et al*.[43] found that 2,4,6-trichlorophenol/triclosan mixtures were strongly antagonic for the green algae and mainly synergistic for *Anabaena* CPB4337. Similar findings have been reported by other authors[44-46]. So that in general, it would be very risky to catalog them as synergistic, antagonic or additive mixture in an absolute way since the "interaction" is, as bioavailability, a biological function and not an intrinsic property of the chemicals.

The results of this research indicate that the phycotoxin levels of saxitoxin, okadaic acid and DA, which are currently reported for the natural environment in estuarine ecosystems, are of high concern for the survival of brine shrimp *A. franciscana*. Yet it must be emphasized that these results are only based on the first 24 hours of the larval development. The analysis of phytoplankton assemblages using multivariate analysis techniques showed that both ternary mixture as the binary combinations exhibited antagonic action on toxic effects in *A. franciscana* nauplii, which were significantly lower than the toxic effect exhibited by each species studied.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study has been supported by Spanish Government (CTM2012-34757) and the Mexican UMSNH University (CIC/11/301). The technical support of Juan José Garcia and Lara de Miguel is kindly acknowledged.

Comments

Background

The increased algal blooms and it possible effects on zooplankton or ecosystem. I think the authors provide the readers a good understanding between harmful microalgae and its grazers (*Artemia*) in estuarine environments.

Research frontiers

The present research work depicts the lethal effects of 3 harmful algae species on zooplankton *A. nauplii*. Furthermore, CI analysis applying on phytoplankton assemblages showed that both ternary mixture as the binary combinations exhibited antagonic action on toxic effects in *A. nauplii*, which were significantly lower than the toxic effect exhibited by each species studied.

Related reports

The lethal effects of the mention harmful algae had been reported. However, it is interesting that the existence of antagonic action action during both ternary mixture and the binary combinations.

Innovations and breakthroughs

There were no new methods used in this paper, but the antagonistic effects of harmful algae on mortality of *A. nauplii* during both ternary mixture and the binary combinations were an attractive results for the readers.

Applications

Although these harmful algae represent a serious risk to estuarine zooplankton community, the presence of PFGs within the same bloom can reduce the potential risk compared to the expected risk when each of the phytoplankton groups is evaluated individually.

Peer review

This is a valuable research work in which authors have demonstrated the lethal effect of 3 harmful algae on model organism *A. franciscana*. Furtheremore, the report provided readers the antagonistic effects of harmful algae on mortality of *A. nauplii* during both ternary mixture and the binary combinations. It has ecological significance when evaluate the risk of harmful blooms in natural environment.

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