APPLIED IN VITRO TOXICOLOGY
Volume 8, Number 3, 2022

DOI: 10.1089/aivt.2022.0008

An Integrated *In Vitro* Approach for Human Health and Environmental Risk Assessment of Mediterranean *Ostreopsis* cf. *ovata*, *Prorocentrum lima*, and *Coolia monotis* Strains

Francesco Misurale,[1,2] Cristina Scarone,[2] Laura Pezzolesi,[3] Rossella Pistocchi,[3] Anna Maria Bassi,[2,4] Antonio Novellino,[1] Matilde Calderoni,[2] Aldo Pagano,[2] Valentina Giussani,[5] and Susanna Alloisio[1,6]

Abstract

Introduction: Harmful algal blooms are becoming a serious issue in the Mediterranean Sea (MS); dinoflagellate blooms are among the most worrying, as some representatives of this group are capable of producing potent marine toxins. Among these, the *Ostreopsis* genus are well known for the production of palytoxin-like compounds. Blooms of *Ostreopsis* cf. *ovata* have caused health issues, and damages to the economy and the environment. *Ostreopsis* cf. *ovata* often co-occurs with other benthic dinoflagellates such as *Prorocentrum lima* and *Coolia monotis*, in bloom events in the MS. Algae from the genus *Prorocentrum* are able to produce diverse toxins responsible for severe diarrhetic shellfish poisoning, while *C. monotis* is not included in the UNESCO-Intergovernmental Oceanographic Commission toxic species list.

Materials and Methods: In this study, an integration of *in vitro* techniques has been applied for the first time to investigate the potential toxicity of the natural mixture of toxins produced by each of three dinoflagellates mentioned above. The proposed approach allowed to evaluate (1) skin and eye irritation potential on human three-dimensional reconstructed tissues; (2) alteration of neuronal activity by means of microelectrode array (MEA) electrophysiology on mouse neuronal networks; and (3) environmental toxicity by lethal toxicity test on *Artemia franciscana*.

Results: Results revealed no significant effect on human skin and eye irritation tests for all the tested species. Interestingly, MEA analyses on mean firing rate and mean bursting rate revealed strong inhibition of functional activity by *Ostreopsis* cf. *ovata* and *P. lima*. The same species showed an important ecotoxicological effect after 48 hours of exposure to *A. franciscana*.

Conclusion: Our approach was found to be suitable for the assessment of the whole algal toxicity potential, also accounting for the potential synergic effects of the mixture of toxins produced by each species.

Keywords: *Coolia monotis*, harmful algal bloom, *in vitro* toxicology, *Ostreopsis ovata*, *Prorocentrum lima*, toxin mixture

[1]ETT SpA, Genoa, Italy.
[2]Department of Experimental Medicine, University of Genoa, Genoa, Italy.
[3]Department of Biological, Geological, and Environmental Sciences, University of Bologna, Bologna, Italy.
[4]Inter-University Center for the Promotion of the 3Rs Principles in Teaching and Research (Centro 3R), Genoa, Italy.
[5]ARPAL Liguria, Genoa, Italy.
[6]National Research Council, Institute of Biophysics, Genoa, Italy.

Introduction

THE UNCONTROLLED GROWTH of microalgae or *harmful algal bloom (HAB)* is a phenomenon that occurs more and more frequently along both coastal and fresh water all over the world as the result of a combination of environmental factors, including available nutrients, temperature, sunlight, ecosystem condition (e.g., turbidity), hydrology (e.g., river flow), and the water chemistry (e.g., pH, conductivity, salinity). At these conditions, microalgae that normally proliferate in tropical and subtropical areas have been able to spread in temperate environments.[1] However, the combination of factors that triggers and sustains an algal bloom is not well understood at present and it is not possible to attribute algal blooms to any specific factor. These phenomena have led to heightened scientific and regulatory attention, especially because some microalgae species produce toxins that have impacts on aquatic ecosystems and human health.[2]

The causative marine HAB organisms are primarily dinoflagellates. In the Mediterranean Sea (MS), dinoflagellate blooms have been reported to cause severe illnesses to humans after consumption of contaminated seafood or exposure to marine aerosol during bloom events.[3] Most notably, the benthic dinoflagellate *Ostreopsis* cf. *ovata* (Fukuyo, 1981) produces various palytoxin-like compounds that are considered one of the most potent marine biotoxins.[4,5] Acute toxicity *Ostreopsis* cf. *ovata* has been described widely, demonstrating a strong link between inhalation of marine aerosol or contaminated water contact and toxic response.[6–9] These characteristics, alongside reported severe intoxication events, that is, along the coasts of Genoa in 2005,[10] made *Ostreopsis* cf. *ovata* one of the most worrying species of the MS.

Palytoxin absorption from gastrointestinal tract has been demonstrated to cause tissue damage in organs, including kidney, lung, and intestines.[11] Systemic symptoms have been reported in humans after accidental dermal contact with palytoxins, suggesting that absorption may occur also from this route.[12,13] Also, neurotoxic effects have been reported for palytoxin and okadaic acid (OA).[14]

Furthermore, it has been observed that two dinoflagellates commonly co-occur during *Ostreopsis* cf. *ovata* blooms in the MS, that is, *Prorocentrum lima* (F. Stein, 1878) and *Coolia monotis* (Meunier, 1919).[15–18] *P. lima* is able to produce, among the other toxins, OA, the main representative diarrheic shellfish poisoning toxin.[19,20] Despite the wide distribution of *P. lima* in the MS, no toxicity event has been related with its presence.[3] Differently, although not formally included in the UNESCO-Intergovernmental Oceanographic Commission toxic species list, *C. monotis* is of great interest because of its co-presence in blooms of the previously cited species.[21] UNESCO lists *Scrippsiella trochoidea* as harmful algal bloom because it is able to reach high densities, especially in stratified waters, but have no previously reported link with toxicity.[22,23]

Human exposure to biotoxins generally refers only to acute and short-term events for single toxic compounds, so that acute reference doses have been established. Differently, ecotoxicological tests are generally performed exposing marine invertebrate or vertebrate organisms to the entire or sonicated algal cells that include their natural mixture of biotoxins.[24,25]

This study aims to evaluate the human and ecotoxicological risk of four dinoflagellate species present in MS: *Ostreopsis* cf. *ovata*, *P. lima*, *C. monotis*, and *S. trochoidea*. By the integration of different assays, we examine human skin and eye irritation potential, mammalian neuronal functional alteration, and ecological acute toxic effects. To take in to account the natural mixture of toxins produced by each single species, a cell lysis-based approach was chosen to prepare experimental treatments.

Materials and Methods

Reagents and chemicals

Polyethyleneimine (PEI) and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA (Darmstadt, Germany). Neurobasal medium (NB), B27 supplement, L-glutamine, Dulbecco's Modified Eagle Medium, Dulbecco's Phosphate-Buffered Saline (DPBS) and Ca++- and Mg++ -free DPBS were purchased from Thermo Fisher (Waltham, MA). For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays, reagents were provided inside the testing kits purchased from MatTek (Bratislava, Slovak Republic).

Algal cultures

Algal monocultures of *Ostreopsis* cf. *ovata* (Fukuyo, 1981), *Prorocentrum lima* (F. Stein, 1878), *C. monotis* (Meunier, 1919), and *S. trochoidea* (A.R. Loeblich III, 1976) were obtained by isolation of single cells from environmental samples collected in the Adriatic Sea.[16,26,27]

Monocultures were maintained in 200 mL sterile vented plastic flasks for cell culture (SARSTEDT, Germany) in sterilized (autoclaved 20 minutes at 120°C) natural sea water (FNSW) supplemented with Guillard's (F/2) Marine Water Enrichment Solution halved to F/4 (Merck, Germany). All flasks were maintained at 20 ± 0.5°C with a 16-h light:8-h dark cycle (light intensity 85–135 μE m^{-2} s^{-1}).

Treatment preparation

Once the algal growth stationary phase was reached, a sample was collected and concentrated by filtration with nylon filters (6 μm mesh) and resuspension in the cell medium specific for the model used for testing (i.e., NB for neurons, Dulbecco's Modified Eagle Medium for eye and skin models and FNSW for brine shrimp). A cell count was performed to determine the algal cell concentration. The preparation was then sonicated in ice for 12 minutes with an immersion sonicator at 50 Hz (Branson Sonifier SFX250, USA) to lyse the cells and collect the cytosolic content in the solution. Finally, the sample was filtered with nylon filters (6 μm mesh) to eliminate the cell debris and obtain the mother treatment for each single species, from which to realize the successive dilutions.

Primary neuron cultures

Cerebral cortices were isolated from mouse on fetal day 15. The cortices were dissociated through mechanical dissociation in culture media containing NB, 2% B27 supplement, and 1% L-glutamine. Cells were then cryopreserved in liquid nitrogen until the day of seeding following the procedure described by Rahman et al. (2010).[28]

Neurons were thawed, suspended in NB media and counted using a hemocytometer. A drop of cell suspension containing 50,000–70,000 cells was placed at the center of the 0.1% PEI precoated standard 60-electrode microelectrode array (MEA) chips (Multi Channel Systems GmbH, Reutlingen, Germany) according to previously described procedures[29] and into wells of 96 well-plates prefilled with 150 μL of cell suspension. Cultures were maintained in NB medium supplemented with 2% B27 supplement and 1% L-glutamine in a humidified incubator at 37°C in a 5% CO_2-enriched atmosphere. Half the medium volume was replaced with fresh medium thrice a week. Experiments on MEA were carried out when neuronal networks were mature after 4–6 weeks, while experiments on 96-well plates were carried out after 7–11 days *in vitro*.

Skin irritation assay

Human epidermal model EpiDerm™ (EPI-200; MatTek In Vitro Life Science Laboratories) was used for the skin irritation test.[30]

In brief, each EpiDerm tissue (surface 0.6 cm^2) cultured on cell culture inserts was exposed to a single concentration of algal treatments. Final concentrations used wereas follows: 1×10^1, 5×10^1, 1×10^2, 5×10^2, and 1×10^3 cells*mL^{-1} for *Ostreopsis* cf. *ovata*; 1×10^2, 1×10^3, 5×10^3, 1×10^4, and 1×10^5 cells*mL^{-1} for *P. lima*; and 1×10^3 and 1×10^4 cells*mL^{-1} for both *C. monotis* and *S. trochoidea*. Sterile DPBS was used as negative control and 5% sodium dodecyl sulfate solution as positive control, both present in the commercial kit. After incubation, the treatment was removed and tissues were exposed to 300 μL of MTT solution (1 mg/mL). The incubation times of treatment were as follows: 1 hour, according to the OECD TG 439, and 4 hours, in agreement with the mean time to onset of symptoms from exposure to *O. ovata*.

After a 3-hour MTT incubation, the blue formazan salt formed by cellular mitochondria was extracted with 2.0 mL/tissue of isopropanol (extractant solution, part no. MTT-100-EXT) and the optical density (OD) of the extracted formazan was determined using the spectrophotometer at 550 nm (Molecular Devices Vmax, USA). Relative cell viability is calculated for each tissue as % of the mean of the negative control tissues treated with sterile DPBS. Three replicates were made for each concentration. As indicated in the manufacturer's protocol, >50% reduction in cell viability is used to indicate the presence of a significant biological effect.

Eye irritation assay

Reconstructed human cornea-like epithelium (NHCE), EpiOcular™ (OCL-200-EIT; MatTek In Vitro Life Science Laboratories), was exposed topically to 50 μL of different concentrations of algal treatment for 30 minutes[31]: 1×10^1, 5×10^1, 1×10^2, 5×10^2, and 1×10^3 cells*mL^{-1} of *Ostreopsis* cf. *ovata*; 1×10^2, 1×10^3, 5×10^3, 1×10^4, and 1×10^5 cells*mL^{-1} of *P. lima*; and 1×10^3 and 1×10^4 cells*mL^{-1} of *C. monotis* and *S. trochoidea*. Sterile deionized H_2O was used as negative control and methyl acetate (CAS No. 79-20-9) as positive control, both present in the commercial kit. Specifically, for *Ostreopsis* cf. *ovata* treatments, we also considered a 4-hour exposure.

After incubation, the tissues were extensively rinsed with Ca++ an-d Mg++ -free DPBS, incubated for 12 minutes in culture medium followed by 120 minutes in new culture medium before the MTT assay. Then, the tissues were exposed to 300 μL of MTT solution (1 mg/mL). The formazan crystals derived by the conversion of MTT by living cells was then solubilized with isopropanol and measured at 550 nm using a spectrofluorimetric reader (Molecular Devices Vmax). Three replicates were tested for each concentration. For each test chemical, the mean OD of treated tissues was determined and expressed as relative percentage of viability of the negative control. According to the manufacturer's protocol, eye irritation potential of the test compound is predicted if the remaining relative cell viability is below 60%.

Neuron function alteration

Five increasing concentrations of *Ostreopsis* cf. *ovata* treatment (1, 2.5, 5, 1×10^1, and 2.5×10^1 cells*mL^{-1}) and six increasing concentrations of *P. lima* treatment (2.5, 5, 1×10^1, 2.5×10^1, 5×10^1, and 1×10^2 cells*mL^{-1}) were cumulatively administered and tested on MEA chips.[32] After an initial 40-minute equilibration period, the neuronal network spontaneous electrical activity was recorded for 20 minutes in control conditions followed by at least 20 minutes of recording for each concentration.

The neuronal network electrical activity was recorded by the USB MEA 120 INV 2 BC System from MultiChannel Systems (MCS GmbH). A MEA amplifier (Gain 1000×) was used and, to record data, the MC_Rack software (MCS GmbH, version 4.4.1.0) was set to sample at a 10 kHz rate.[32] Raw signals were added with a band pass digital filter (60–4000 Hz) to remove electrical background noise. A detection threshold was applied for spike train extraction (i.e., 5.5 times the standard deviation of the mean square root noise), only electrical signals that overcome the threshold are identified and recorded by the MC_Rack spike detection.

A temperature controller (TC02, MCS GmbH) was allowed to maintain the cell culture at 37°C during experiment and a controlled humidified atmosphere (9% CO_2, 19% O_2 and 72% N_2) was maintained to balance the pH of the supernatant (pH was 7.1 ± 0.1). All analyses were conducted on binned data with bin size of 60 seconds. Data were averaged considering only the exposition period, except a ''buffer'' period of 2 minutes before and 3 minutes after each sample addition.

This makes sure that only the last 15 minutes of each recording (which is the most stable timeframe for stable exposure effects) are used for the analysis. This procedure is well established and typically used when it is verified that the exposure effect of the new compound is stable and not just transient by comparing the results of different timeframes (e.g., the first 10 minutes against the last 10 minutes). Moreover, in a 15-minute time window, a sufficiently large number of spikes and bursts can be measured to reliably calculate any change in activity. Furthermore, only channels with >2 bursts/min were considered for the analysis.

Averaged over all the baseline recordings, there were 35.3 ± 4.4 active electrodes/MEA and 8.2 ± 2.3 spikes/s and 20.5 ± 3.4 bursts/min (mean ± standard error of the mean [SEM], $n=18$). The analysis was conducted by importing

data (for MCS software, *.mcd files) into NeuroExplorer software (Nex Technologies) by which the burst analysis tool was applied, where the following burst definition parameters were set: bin size = 1 second; maximum interval of starting a burst = 0.01 second; maximum interval of ending a burst = 0.075 seconds; minimum burst interval = 0.1 second; minimum burst duration = 0.02 seconds; and minimum of number of spikes in burst = 4. Burst parameters were determined for each individual electrode on a MEA, and then data from all bursting electrodes in a network were averaged.

The analysis considered the network mean firing rate (MFR; number of spikes/s) and bursting behavior represented by the parameter mean burst rate (MBR; number of bursts/min). The data were exported to Excel spreadsheets and bursting parameters at each concentration for each chip were determined by calculating the averages of bursting channels.

Neuronal viability

The 96-well plates were used to expose the neuronal networks for 4 hours to *Ostreopsis* cf. *ovata* and *P. lima* treatments. The concentrations tested for each algal species were as follows: 1, 1×10^1, 1×10^2, and 5×10^2 cells*mL^{-1} for *Ostreopsis* cf. *ovata* and 1, 1×10^1, 1×10^2, and 1×10^3 cells*mL^{-1} for *P. lima*. After the exposure time, the cell viability MTT assay was performed adding 200 μL of MTT solution (1 mg/mL) to each well and incubating for 3 hours at 37°C. The solution was then removed and 200 μL of DMSO was added to each well. Finally, plates were read at 550 nm with a spectrofluorometer (Molecular Devices Vmax). Six replicates were made for each concentration.

Acute toxicity on A. franciscana

A. franciscana (Leach, 1819) cysts were hatched in FNSW with a salinity of 33% at 25 ± 1°C during 24 hours before the test was set up as described by Garaventa et al (2010). About 15–20 nauplii were isolated and put in 24-well plates with 1 mL of sterilized natural seawater containing algal treatments at different concentrations. The concentrations tested for each alga were as follows: 1×10^1, 5×10^1, 1×10^2, 5×10^2, and 1×10^3 cells*mL^{-1} for *Ostreopsis* cf. *ovata*; 1×10^2, 1×10^3, 5×10^3, 1×10^4, and 1×10^5 cells*mL^{-1} for *P. lima*; and 1×10^3, 1×10^4 cells*mL^{-1} for *C. monotis* and *S. trochoidea*.[32,33]

After 48 hours of exposure in dark conditions at the temperature of 25°C, mortality evaluation was carried out with a stereomicroscope by eye: nauplii were considered dead if they did not show any movement after a 10-second observation. Data were expressed as % control mortality. Four replicates were made for each concentration.

Statistical analysis

The results were expressed as mean ± SEM. The data collected were analyzed using the statistical software R version 3.6.1 (R Core Team 2021).[34] The analysis of variance was carried out performing Shapiro–Wilk normality test, Levene homogeneity test, and one-way analysis of variance (ANOVA) test followed by the Dunnett *t*-test. Data that could not be analyzed by ANOVA due to the distribution of data were analyzed with the *post hoc* tests Kruskal–Wallis and Mann–Whitney. The statistical significance was considered if $p<0.05$ and indicated with the symbol *. To obtain the estimated IC_{50} values (half-maximal inhibitory concentration) and LC_{50} (half-maximal lethal concentration), the normalized concentration–response curves of single treatments were interpolated by a four-parameter logistic function using GraphPad Prism version 8.2.1, which has the following formula:

$$f(x) = Max + (Min - Max)/(1 + (\varepsilon/x)\beta)$$

where the variable x is the concentration of the compound; the parameter *Min* is the minimum effect; the parameter *Max* is the maximum effect; the parameter e is the concentration at the inflection point of the concentration–response curve, that is, the concentration at which the effect is reduced by 50% (IC_{50}); and β is a parameter related to the maximum slope of the curve, which occurs at concentration ε.

Results

Skin irritation potential

To evaluate if the selected algae were irritant to human skin, MTT ET-50 assay was performed according to the supplier's recommendations. Increasing concentrations of algal treatment were separately administered to the tissues. All algal treatments produced nonsignificant (i.e., $p>0.05$) reductions of viability at any concentration tested after 1 (data not shown) and 4 hours of exposure (Fig. 1).

Eye irritation potential

Similar results to skin irritation test were observed in eye irritation potential test. In this case, tissues were exposed for 30 minutes to all algal treatments at the same concentrations used for irritation skin test. An additional 4-hour exposure test was performed with treatments of *Ostreopsis* cf. *ovata* at the concentrations of 1×10^1, 5×10^1, 1×10^2, and 1×10^3 cells*mL^{-1}. As shown in Figure 2, no significant viability reduction was observed in any of the tested tissues after 30 minutes of exposure. The same result was observed in a 4-hour-long *Ostreopsis* cf. *ovata* treatment exposure (Fig. 2A).

Neuron function alteration

To evaluate whether algal treatments were able to alter the neuronal function, an electrophysiological test was performed using the MEA-based system. Neuronal cultures were subjected to cumulative exposure of increasing concentrations of algal treatments.

As shown in Figure 3 *Ostreopsis* cf. *ovata* and *P. lima* induced a strong dose-dependent inhibition of the spontaneous electrical activity. In particular, *Ostreopsis* cf. *ovata* was the most effective to inhibit electrical activity and showed a significant inhibition of functionality already at the concentration of 5 cells*mL^{-1}, with the complete inhibition of the network electrical activity at the maximum tested concentration (25 cells*mL^{-1}) in both the evaluated parameters (MFR and MBR). The exposure of cortical neuronal network to increasing concentration of *P. lima* treatments induced a significant dose-dependent inhibition of neuronal functionality from the concentration of 25 cells*mL^{-1} for MFR and 10 cells*mL^{-1} for MBR. The total absence of neuronal activity

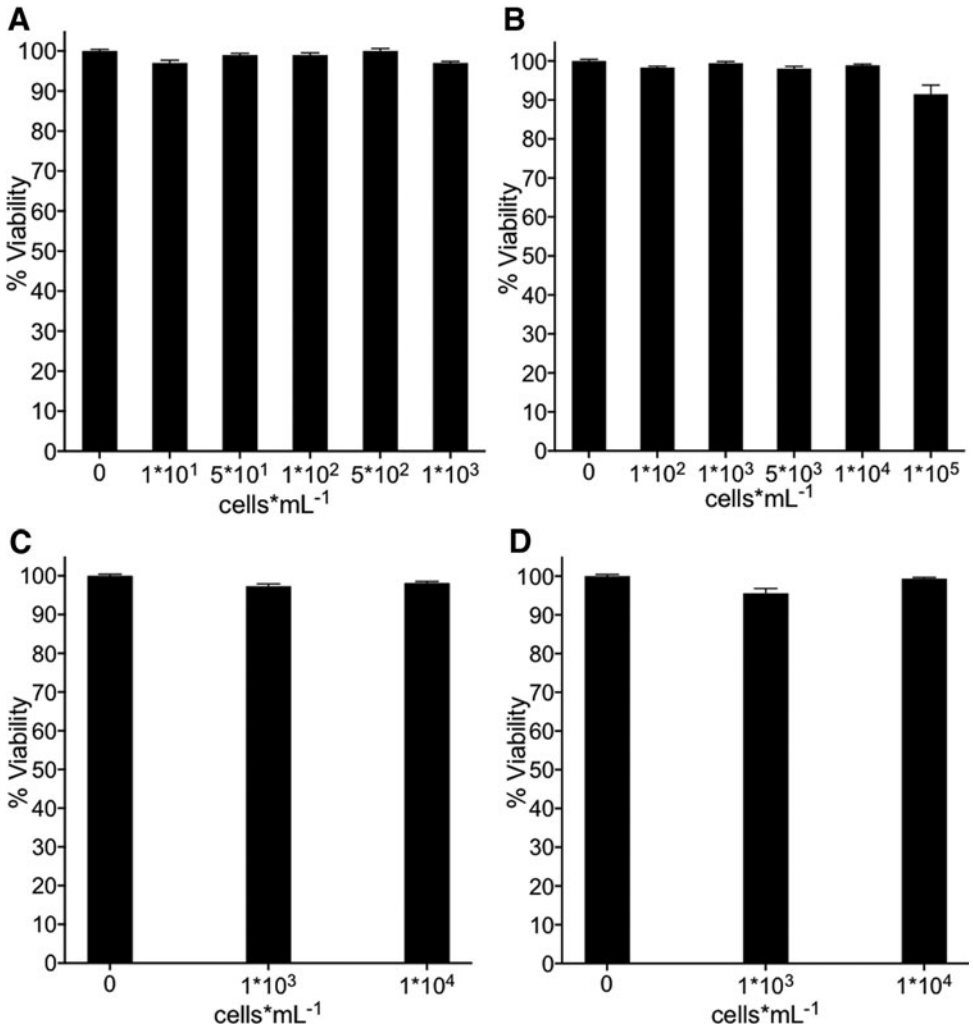

FIG. 1. Percentage of viability of reconstructed human epiderm (EpiSkin) exposed for 4 hours to treatments of *Ostreopsis* cf. *ovata* **(A)**, *Prorocentrum lima* **(B)**, *Coolia monotis* **(C)**, and *Scrippsiella trochoidea* **(D)**. Data are reported as % of control and are expressed as the mean ± SEM of four independent experiments performed in triplicate. SEM, standard error of the mean.

was observed for both parameters (MFR and MBR) at the maximum concentration tested (1×10^2 cells*mL^{-1}).

As shown in Figure 3, the concentration–response curve of MFR and MBR parameters represent the spontaneous electrical activity developed from cortical neuronal networks exposed to *Ostreopsis* cf. *ovata* and *P. lima* treatments. *Ostreopsis* cf. *ovata* (1; 2.5; 5; 1×10^1; 2.5×10^1 cells*mL^{-1}) generated a concentration-dependent inhibition for both MFR (A) and MBR (B), with IC_{50} values of 4.38 ± 0.9 cells*mL^{-1} and 3.26 ± 0.7 cells*mL^{-1}, respectively ($n=6$). *P. lima* (2.5; 5; 1×10^1; 2.5×10^1; 5×10^1; and 1×10^2 cells*mL^{-1}) produced similar, but less strong effects on MFR (C) and MBR (D), with IC_{50} values of 20.02 ± 6.73 cells*mL^{-1} and 10.03 ± 2.2 cells*mL^{-1}, respectively ($n=5$). Data are expressed as normalized mean ± SEM ($^*p<0.05$).

Neuronal viability

The exposure to *Ostreopsis* cf. *ovata* treatments induced a significant reduction of neuronal viability from the 1×10^1 cells*mL^{-1} after 4 hours of exposure, as shown in Figure 4A. Although significantly reduced, the viability did not reach 0% even after exposure to the maximum concentration used (34 ± 3% viability at 5×10^2 cells*mL^{-1}).

Results obtained by exposing neurons to *P. lima* treatments (Fig. 4B) show a dose–response pattern, but a significant reduction of viability only at the highest concentration tested (66 ± 1% viability at 1×10^3 cells*mL^{-1}).

Acute toxicity on A. franciscana

To evaluate ecotoxicity of our algal treatments, tests were performed using *A. franciscana*, a biological model used widely due to its advantages of rapid hatching, easy accessibility of nauplii hatched, and low cost-efficiency.

A. franciscana nauplii contained in 24-well plates were exposed to different concentrations of algal treatments and evaluated the LC_{50} after 48 hours of exposure.

Ostreopsis cf. *ovata* treatments caused a significant mortality from 1×10^2 cells*mL^{-1} with 100% of mortality at 5×10^2 and 1×10^3 cells*mL^{-1} (Fig. 5A). A LC_{50} value of 84 ± 7 cells*mL^{-1} was calculated. *P. lima* treatments had a similar effect, even though at a higher concentration, with an LC_{50} value of 2253 ± 111 cells*mL^{-1} (Fig. 5B).

Differently, *C. monotis* and *S. trochoidea* treatments did not have significant effect, not even at the highest concentrations tested (Fig. 5C, D).

The overall results obtained from the different assays are resumed in Table 1.

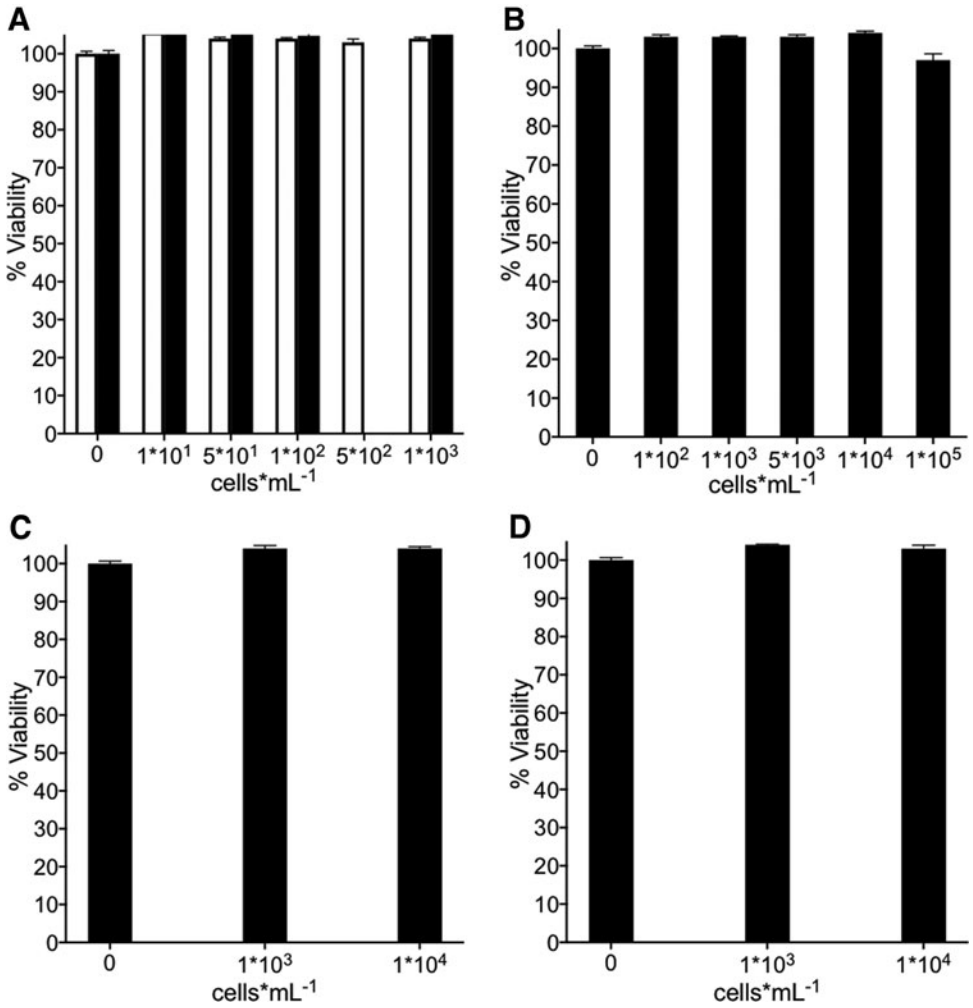

FIG. 2. Percentage of viability of reconstructed human corneal tissue (EpiOcular™) exposed for 30 minutes (white bar) and 4 hours (black bar) to treatments obtained from *Ostreopsis* cf. *ovata* **(A)** and for 30 minutes to treatments of *Prorocentrum lima* **(B)**, *Coolia monotis* **(C)**, and *Scrippsiella trochoidea* **(D)**. Data are reported as % of control and are expressed as the mean ± SEM of four independent experiments performed in triplicate.

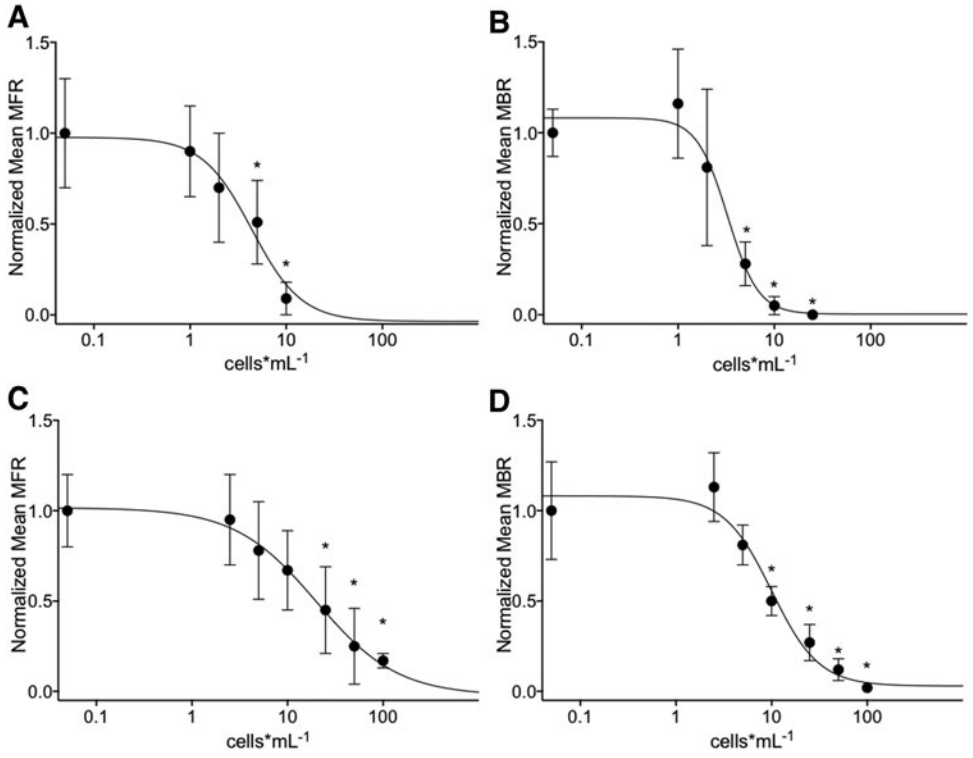

FIG. 3. Dose–response curves of neuronal networks exposed to treatments of **(A)** *Ostreopsis* cf. *ovata* MFR, **(B)** *Ostreopsis* cf. *ovata* MBR, **(C)** *Prorocentrum lima* MFR, and **(D)** *P. lima* MBR. Statistical differences: $*p < 0.05$. MBR, mean burst rate; MFR, mean firing rate.

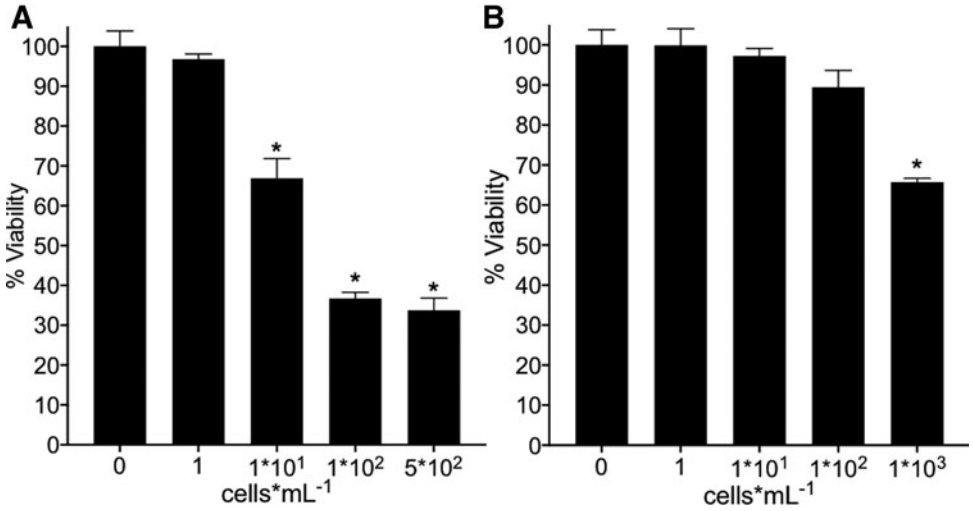

FIG. 4. Percentage of viability of embryonic mouse neuronal networks exposed to *Ostreopsis* cf. *ovata* **(A)** and *Prorocentrum lima* **(B)** treatments for 4 hours. Data are reported as % of control and are the mean ± SEM of two independent experiments performed in triplicate. Statistical differences: $*p < 0.05$.

Discussion

The objective of this study was to investigate the potential toxic effects of three dinoflagellates on different target organs: skin, eye, and brain. The organs were modeled by integrating four *in vitro* tests, that is, skin irritation test, eye irritation test, neuronal viability, and functional assay for neurotoxicity assessment. Moreover, an *in vivo* ecotoxicity assay was performed to evaluate the possible adverse effects of the selected algal species on the aquatic environment. The studied algae species were chosen because of their widespread presence in the MS and their ability to produce various toxic compounds.

Symptoms commonly reported after *Ostreopsis* cf. *ovata* blooms are eye irritation and dermatitis[36–38]; the link

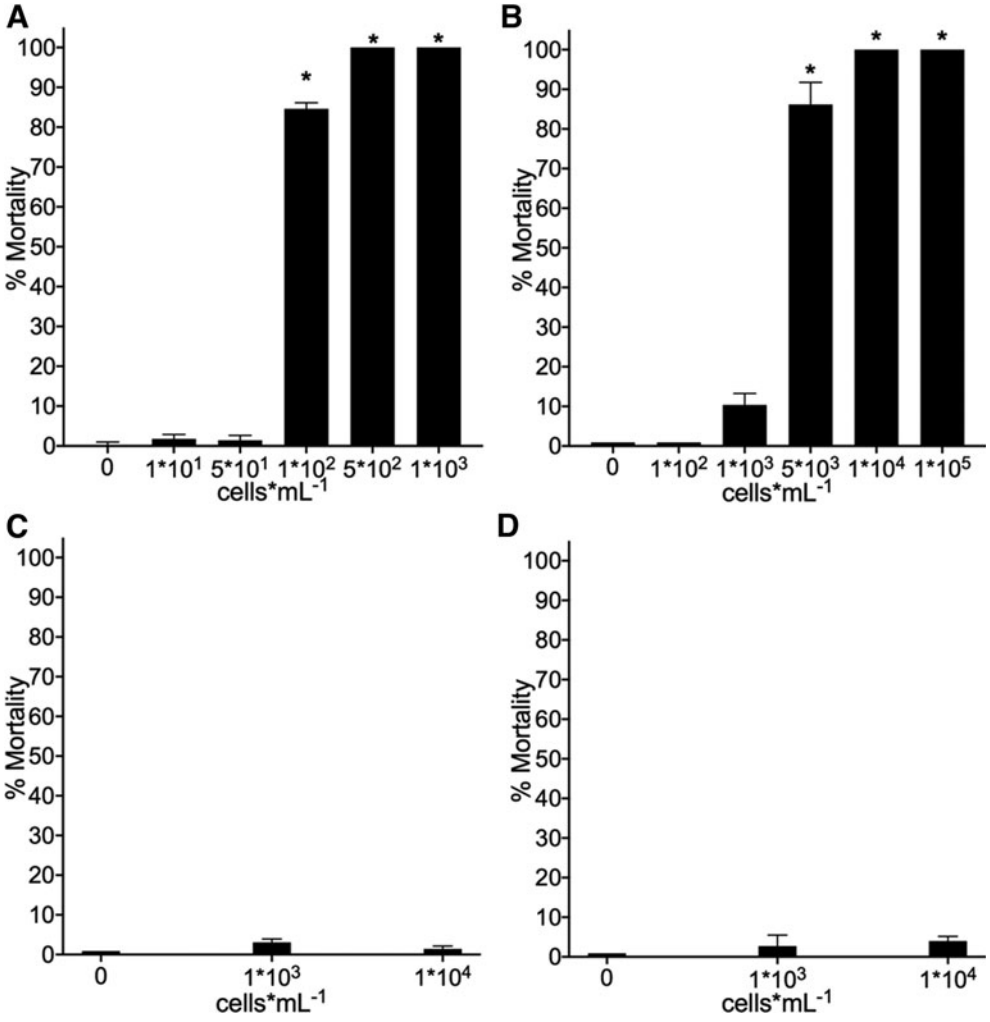

FIG. 5. Percentage of mortality of *Artemia franciscana* nauplii I exposed to treatments of *Ostreopsis* cf. *ovata* **(A)**, *Prorocentrum lima* **(B)**, *Coolia monotis* **(C)**, and *Scrippsiella trochoidea* **(D)** for 48 hours. Data are reported as % of control and are the mean ± SEM of four independent experiments performed in quadruplicate. Statistical differences: $*p < 0.05$.

TABLE 1. SUMMARIZED RESULTS OBTAINED BY DIFFERENT ASSAYS PERFORMED FOR TOXICITY EVALUATION OF *OSTREOPSIS* CF. *OVATA*, *PROROCENTRUM LIMA*, *COOLIA MONOTIS*, AND *SCRIPPSIELLA TROCHOIDEA*

	Endpoint				
	Eye irritation	*Skin irritation*	*Neuronal functionality*	*Neuronal viability*	Artemia franciscana *acute mortality*
Ostreopsis cf. *ovata*	NE	NE	IC_{50} (MFR) = 4 cells*mL^{-1} IC_{50} (MBR) = 3 cells*mL^{-1}	SE	LC_{50} = 84 cells*mL^{-1}
Prorocentrum lima	NE	NE	IC_{50} (MFR) = 10 cells*mL^{-1} IC_{50} (MBR) = 10 cells*mL^{-1}	LSE	LC_{50} = 2253 cells*mL^{-1}
Coolia monotis	NE	NE	NE	NE	NE
Scrippsiella trochoidea	NE	NE	NE	NE	NE

IC_{50}, half-maximal inhibitory concentration; LC_{50}, half-maximal lethal concentration; LSE, low significant effect (at concentration of 1×10^3 cells*mL^{-1}); MBR, mean burst rate; MFR, mean firing rate; NE, no effect; SE, significant effect.

between these symptoms and *Ostreopsis* cf. *ovata* toxins are also supported by experiment by Poli et al on mice.[7] For full replacement for the *in vivo* testing (OECD Test Nos. 404 and 405), this study adopts an OECD-approved stand-alone *in vitro* test method based on standard three-dimensional (3D) reconstructed skin and eye *in vitro* models (EpiDerm and EpiOcular Test Guideline 439 and 492, respectively), applied in cosmetic and chemical development.[39]

According to the Test Guidelines 439 and 492, the viability of the reconstructed tissues following, respectively, 60 or 30 minutes of exposure to a test chemical is determined in comparison to tissues treated with the negative control substance (>50% or >60.0% viability, respectively), and is then used to predict the skin or eye hazard potential of the test chemical (https://www.mattek.com/application/eye-irritation-test-oecd-492/; https://www.mattek.com/application/skin-irritation-test-oecd-439/).

In our experiments, any significant effect was not observed for algal treatments in both skin and eye models. For this reason, the experiment was replicated with a treatment exposure extended to 4 hours, but anyway, the observed cell viability was more than 90% of negative control. In particular, *Ostreopsis* cf. *ovata* treatments did not cause any significant effect both in skin and ocular irritation test. This applied also at the highest concentration used (1×10^6 cells*L^{-1}), which is similar to that found during the marine sampling in Genoa (Italy) in the summer of 2005 (1.8×10^6 cells*L^{-1}; Ciminiello et al.[41]). In that circumstance, about 200 people who spent time on or near beaches sought medical treatment for symptoms such as rhinorrhea, cough, fever, bronchoconstriction with mild breathing difficulties, wheezing, and, in a few cases, conjunctivitis.

The absence of any effect in our skin and eye irritation assays could be due to a possible low capability of EpiDerm and EpiOcular models to predict *in vivo* effect of algal toxins or to a different toxin composition present in cultured algae compared to the environmental algae. Concerning the model capacity to predict *in vivo* effects, it would be advisable to investigate more complex cellular mechanisms, for example, the allergy-linked inflammation,.[42] The second consideration highlights the need to identify algal toxin concentration by liquid chromatography coupled to tandem mass spectrometry present in algal treatments, which are responsible for the experimental results.

A MEA-based approach to assess the neuronal toxicity of chemicals has been studied before.[29,43,44] This approach was used for *Ostreopsis* cf. *ovata* toxicity evaluation in a previous study, showing its valuable sensitivity[35] with a particular regard to two neuronal network electrophysiological activity parameters: the network MFR and the MBR. The *Ostreopsis* cf. *ovata* treatments show a significant reduction of the activity starting at low concentrations with an IC_{50} of 4.38 ± 0.9 cells*mL^{-1} for MFR and 3.26 ± 0.7 cells*mL^{-1} for MBR.

The *Ostreopsis* cf. *ovata* toxicity assessments are comparable with what was observed in a previous study that highlighted an IC_{50} of 1.5 cells*mL^{-1},[35] and both the studies show that the *Ostreopsis* cf. *ovata* toxins have a higher effect on the reduction of burst per minute (MBR) than on the general spike distribution (MFR), meaning that these toxins primarily corrupt the network activity (i.e., the capacity of the neurons to act as a system), and at higher concentration, they affect the single neuron electrophysiology.

P. lima treatments show a significant reduction of neuronal activity, resulting in the IC_{50} values of 20.02 ± 6.73 cells*mL^{-1} for MFR and 10.03 ± 2.2 cells*mL^{-1} for MBR. This result confirms that the MEA-based approach is sensitive and suitable for the evaluation of toxic potential of different toxic algae species. No MEA-based neuronal alteration tests have been performed before using neither natural mixture of toxins produced by algae, nor OA, one of the most potent toxins produced by this species.[19,20] However, the neurotoxic effect of OA has been already demonstrated.[44]

Nevertheless, to verify whether the observed alteration in cell functionality was due to neuronal cell death, the reduction of viability of neuronal networks was investigated by MTT assay. *Ostreopsis* cf. *ovata* treatments highlights a significant reduction of viability starting from low concentrations, even though after a 4-hour exposure, a 100% viability reduction was not observed. Differently, *P. lima* treatments caused a viability reduction with a significant effect observed at the highest tested concentration (1×10^3 cells*mL^{-1}). The MTT assay confirms that the alteration of the neuronal functionality was not due to cell death, as significant neuronal activity reduction was recorded at lower concentrations and a much shorter exposure time (20 minutes vs. 4 hours); hence the algae toxins interact and corrupt the neuronal signaling exchange.

To have an integrated vision of the toxic potential of these algae species and to confirm the reliability of the

experimental protocol for treatment preparation, the study also considered a complementary acute ecotoxicity assay that was performed with *A. franciscana* nauplii (48 hours). Although widely utilized in sediments, chemicals, and water routine controls and indicated by Italian National Institute for Environmental Protection and U.S. Environmental Protection Agency, this test is not internationally validated yet.

Ostreopsis cf. *ovata* treatments caused the death of all individuals after 48 hours at 5×10^2 cells*mL^{-1}, following a dose-dependent effect (LC_{50} value: 84 ± 7 cells*mL^{-1}). In our knowledge, there are only two studies that investigated the effects of *Ostreopsis* cf. *ovata* lysed cells by sonication. In the first study, an LC_{50} value of 96 cells*mL^{-1} was reported,[45] which is highly matching our results.

In the second study, an LC_{50} value of 1281 cells*mL^{-1} was reported, that is about 15 times the value we recorded (84 ± 7 cells*mL^{-1}). This discrepancy could be due to a substantial difference in toxin production, which may occur in distinct *Ostreopsis* cf. *ovata* strains.

P. lima treatments caused similar effects to *Ostreopsis* cf. *ovata*, but at higher concentrations. Specifically, after 48 hours of 1×10^4 cells*mL^{-1} *P. lima* treatment, all *A. franciscana* nauplii died. The results are in line with the closest comparable study that utilized the algal culture supernatant as toxic compound on *Artemia* sp.[46] No mortality in *A. franciscana* nauplii was observed administering *S. trochoidea* and *C. monotis* treatments.

Conclusions

This study proposes an integrated alternative method testing strategy to assess the toxicity of harmful algae. It integrated *in vitro* models of three major systems: skin, eye, and brain.

Skin and eye modeled by 3D reconstructed human cell cultures (EpiDerm and EpiOcular) and brain modeled by two-dimensional mouse cortical neuronal networks.

The results show the suitability and sensitivity of the proposed approach and can be summarized as follows: *Ostreopsis* cf. *ovata* exerts strong toxicity on both neuronal and ecological models. In particular, *Ostreopsis* cf. *ovata* toxins interact with the neuronal signaling by corrupting the network physiology and eventually cell viability. *P. lima* exerts lower, but significant toxicity on the same models. No irritation effect has been observed in human reconstructed skin and eye models at any algal concentration. The absence of toxicity of *C. monotis* and *S. trochoidea* species in overall assays confirms their absence of toxicity.

In conclusion, the proposed integrated *in vitro* assay testing strategy allows to obtain valuable and comprehensive information about HAB toxic effects for humans and environment. The same approach can be used also for investigating the potential synergistic effects of toxins produced by *multiple species*. Moreover, the results demonstrate the reliability of the proposed algal treatment preparation protocol, encouraging its use in future investigations.

Acknowledgments

The authors greatly appreciate the technical support of Dr. Chiara Gambardella (CNR-Institute for the study of anthropogenic impacts and sustainability in the marine environment) and Dr. Elisa Costa (CNR-Institute for the study of anthropogenic impacts and sustainability in the marine environment). Thanks are also due to Dr. Federica Colombo (ETT SpA) for her assistance in proofreading the article.

Authors' Contributions

F.M.: writing—original draft (equal); formal analysis (equal); and investigation (lead). C.S.: writing—original draft (equal); investigation (equal); and formal analysis (supporting). L.P.: conceptualization (equal) and writing—review and editing (supporting). R.P.: conceptualization (equal). A.M.B.: funding acquisition (equal) and writing—review and editing (supporting). A.N.: funding acquisition (equal); conceptualization (equal); and writing—review and editing (supporting). M.C.: investigation (supporting). A.P.: conceptualization (equal) and validation (equal). V.G.: conceptualization (equal) and writing—review and editing (equal). S.A.: conceptualization (lead); writing—original draft (lead); formal analysis (lead); investigation (equal); validation (lead); writing—review and editing (lead); formal analysis (lead); supervision (lead); and funding acquisition (equal).

Author Disclosure Statement

The authors declare that they have no relevant or material financial interest that relates to the research described in this study.

Funding Information

This publication has been produced with the financial assistance of the European Union under the Euro Trans-Bio (ETB) program (ETB-2017-028), H2020 NAUTILOS project (No. 101000825) and H2020 JERICO S3 project (No. 871153).

References

1. Trainer VL, Moore SK, Hallegraeff G, et al. Pelagic harmful algal blooms and climate change: Lessons from nature's experiments with extremes. Harmful Algae 2020;91(May 2019):101591; doi: 10.1016/j.hal.2019.03.009.
2. Van Dolah FM. Marine algal toxins: Origins, health effects, and their increased occurrence. Environ Health Perspect 2000;108(Suppl. 1):133–141.
3. Zingone A, Escalera L, Aligizaki K, et al. Toxic marine microalgae and noxious blooms in the Mediterranean Sea: A contribution to the global HAB status report. Harmful Algae 2021; 102(December 2019); doi: 10.1016/j.hal.2020.101843.
4. Yasuwo F. Taxonomical Study on Benthic Dinoflagellates Collected in Coral Reefs. Bulletin of the Japanese Society for the Science of Fish. 47(8). https://doi.org/10.2331/suisan.47.967
5. Vilariño N, Louzao MC, Abal P, et al. Human poisoning from marine toxins: Unknowns for optimal consumer protection. Toxins (Basel) 2018;10(8):1–38; doi: 10.3390/toxins10080324.
6. Ciminiello P, Dell'Aversano C, Dello IE, et al. First finding of *Ostreopsis* cf. *ovata* toxins in marine aerosols. Environ Sci Technol 2014;48(6):3532–3540; doi: 10.1021/es405617d.
7. Pelin M, Florio C, Ponti C, et al. Pro-inflammatory effects of palytoxin: An: In vitro study on human keratinocytes and

inflammatory cells. Toxicol Res (Camb) 2016;5(4):1172–1181; doi: 10.1039/c6tx00084c.
8. Poli M, Ruiz-Olvera P, Nalca A, et al. Toxicity and pathophysiology of palytoxin congeners after intraperitoneal and aerosol administration in rats. Toxicon 2018;150(June): 235–250; doi: 10.1016/j.toxicon.2018.06.067.
9. Tubaro A, Durando P, Del Favero G, et al. Case definitions for human poisonings postulated to palytoxins exposure. Toxicon 2011;57(3):478–495; doi: 10.1016/j.toxicon.2011.01.005.
10. Durando P, Ansaldi F, Oreste P, et al. *Ostreopsis ovata* and human health: Epidemiological and clinical features of respiratory syndrome outbreaks from a two-year syndromic surveillance, 2005-06, in North-West Italy. Euro Surveill 2007;12(6):E070607.1; doi: 10.2807/esw.12.23.03212-en.
11. Sosa S, Del Favero G, De Bortoli M, et al. Palytoxin toxicity after acute oral administration in mice. Toxicol Lett 2009; 191(2–3):253–259; doi: 10.1016/j.toxlet.2009.09.009.
12. Murphy LT, Charlton NP. Prevalence and characteristics of inhalational and dermal palytoxin exposures reported to the National Poison Data System in the U.S. Environ Toxicol Pharmacol 2017;55(August):107–109; doi: 10.1016/j.etap.2017.08.010.
13. Ruiz Y, Fuchs J, Beuschel R, et al. Dangerous reef aquaristics: Palytoxin of a brown encrusting anemone causes toxic corneal reactions. Toxicon 2015;106:42–45; doi: 10.1016/j.toxicon.2015.09.001.
14. Patocka J, Gupta RC, Wu QH, et al. Toxic potential of palytoxin. J Huazhong Univ Sci Technol Med Sci 2015;35(5): 773–780; doi: 10.1007/s11596-015-1506-3.
15. Geohab. GEOHAB CORE RESEARCH PROJECT: HABs IN BENTHIC SYSTEMS. Organization 2012;64.
16. STEIN, Friedrich A., et al. Jacob Sturm, Stettmeister von Strassburg. 1878.
17. Ninčević Gladan Ž, Arapov J, Casabianca S, et al. Massive Occurrence of the Harmful Benthic. 2019.
18. Vila M, Garcés E, Masó M. Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the NW Mediterranean. Aquat Microb Ecol 2001;26(1):51–60; doi: 10.3354/ame026051.
19. Li J, Li M, Pan J, et al. Identification of the okadaic acid-based toxin profile of a marine dinoflagellate strain *Prorocentrum lima* by LC-MS/MS and NMR spectroscopic data. J Sep Sci 2012;35(7):782–789; doi: 10.1002/jssc.201100914.
20. Murata M, Shimatani M, Sugitani H, et al. Isolation and structural elucidation of the causative toxin of the diarrhetic shellfish poisoning. Nippon Suisan Gakkaishi 1982;48(4): 549–552; doi: 10.2331/suisan.48.549.
21. Ben-Gharbia H, Yahia OKD, Amzil Z, et al. Toxicity and growth assessments of three thermophilic benthic dinoflagellates (*Ostreopsis* cf. *ovata*, *Prorocentrum lima* and *Coolia monotis*) developing in the Southern Mediterranean Basin. 2016;8(10):297; doi: 10.3390/toxins8100297.
22. Hallegraeff GM. Harmful algal blooms in the australian region. Mar Pollut Bull 1992;25(5–8):186–190; doi: 10.1016/0025-326X(92)90223-S.
23. Schmidt RJ, Loeblich III AR. Discussion of the systematics of toxic gonyaulax species containing paralytic shellfish poison. In: Developments in Marine Biology. 1979.
24. Pavaux AS, Ternon E, Dufour L, et al. Efficient, fast and inexpensive bioassay to monitor benthic microalgae toxicity: Application to *Ostreopsis* species. Aquat Toxicol 2020;223(April):105485; doi: 10.1016/j.aquatox.2020.105485.
25. Faimali M, Giussani V, Piazza V, et al. Toxic effects of harmful benthic dinoflagellate *Ostreopsis ovata* on invertebrate and vertebrate marine organisms. Mar Environ Res 2012;76:97–107; doi: 10.1016/j.marenvres.2011.09.010.
26. Meunier, A. (1919). Microplankton de la Mer Flamande: 3. Les Péridiniens. Mémoires du Musée Royal d'Histoire Naturelle de Belgique = Verhandelingen van het Koninklijk Natuurhistorisch Museum van België, VIII(1). Hayez, imprimeur de l'Académie royale de Belgique: Bruxelles. 111, 7 plates pp.
27. Loeblich, A.R. III, 1976: Dinoflagellate evolution: speculation and evidence. Journal of Protozoology, v.23, p.13–28.
28. Rahman AS, et al. Cryopreservation of cortical tissue blocks for the generation of highly enriched neuronal cultures. JoVE (Journal of Visualized Experiments), 2010, 45: e2384.
29. Hogberg HT, Sobanski T, Novellino A, et al. Application of micro-electrode arrays (MEAs) as an emerging technology for developmental neurotoxicity: Evaluation of domoic acid-induced effects in primary cultures of rat cortical neurons. Neurotoxicology 2011;32:158–168; doi: 10.1016/j.neuro.2010.10.007.
30. Ocde. Test No. 439: In Vitro Skin Irritation—Reconstructed Human Epidermis Test Method. 2013; doi: 10.1787/9789264203884-en.
31. Ocde. Test No. 492: Reconstructed Human Cornea-like Epithelium (RhCE) Test Method for Identifying Chemicals Not Requiring Classification and Labelling for Eye Irritation or Serious Eye Damage. 2019; doi: 10.1787/20745788.
32. WoRMS (2022). Artemia Leach, 1819. Available from: https://www.marinespecies.org/aphia.php?p=taxdetails&id=480245 Accessed August 29, 2022.
33. Garaventa F, Gambardella C, Di Fino A, Pittore M, Faimali M. Swimming speed alteration of Artemia sp. and Brachionus plicatilis as a sub-lethal behavioural end-point for ecotoxicological surveys. Ecotoxicology. 2010;19(3):512–519. doi: 10.1007/s10646-010-0461-8.
34. R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.
35. Alloisio S, Giussani V, Nobile M, et al. Microelectrode array (MEA) platform as a sensitive tool to detect and evaluate *Ostreopsis* cf. *ovata* toxicity. Harmful Algae 2016;55: 230–237; doi: 10.1016/j.hal.2016.03.001.
36. Ciminiello P, Dell'Aversano C, Fattorusso E, et al. Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the ligurian coasts during the 2006 toxic outbreak. J Am Soc Mass Spectrom 2008;19(1):111–120; doi: 10.1016/j.jasms.2007.11.001.
37. Tichadou L, Glaizal M, Armengaud A, et al. Health impact of unicellular algae of the *Ostreopsis* genus blooms in the Mediterranean Sea: Experience of the French Mediterranean Coast Surveillance Network from 2006 to 2009. Clin Toxicol 2010; 48(8):839–844; doi: 10.3109/15563650.2010.513687.
38. Vila M, Abós-Herràndiz R, Isern-Fontanet J, et al. Establishing the link between *Ostreopsis* cf. *ovata* blooms and human health impacts using ecology and epidemiology. Sci Mar 2016;80(September):107–115; doi: 10.3989/scimar.04395.08A.
39. Walters RM, Gandolfi L, Mack MC, et al. In vitro assessment of skin irritation potential of surfactant-based formulations by using a 3-D skin reconstructed tissue model and cytokine response. ATLA Altern to Lab Anim 2016;44(6): 523–532; doi: 10.1177/026119291604400611.

40. Ciminiello P, Dell'Aversano C, Fattorusso E, et al. The Genoa 2005 Outbreak. Determination of putative palytoxin in Mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method. Anal Chem 2006;78(17):6153–6159; doi: 10.1021/ac060250j.
41. Crinelli R, Carloni E, Giacomini E, et al. Palytoxin and an ostreopsis toxin extract increase the levels of MRNAs encoding inflammation-related proteins in human macrophages via P38 MAPK and NF-KB. PLoS One 2012;7(6): e38139; doi: 10.1371/journal.pone.0038139.
42. Alloisio S, Nobile M, Novellino A. Multiparametric characterisation of neuronal network activity for in vitro agrochemical neurotoxicity assessment. Neurotoxicology 2015; 48:152–165; doi: 10.1016/j.neuro.2015.03.013.
43. Scelfo B, Politi M, Reniero F, et al. Application of multielectrode array (MEA) chips for the evaluation of mixtures neurotoxicity. Toxicology 2012;299(2–3):172–183; doi: 10.1016/j.tox.2012.05.020.
44. Tapia R, Peña F, Arias C. Neurotoxic and synaptic effects of okadaic acid, an inhibitor of protein phosphatases. Neurochem Res 1999;24(11):1423–1430; doi: 10.1023/A: 1022588808260.
45. Pezzolesi L, Guerrini F, Ciminiello P, et al. Influence of temperature and salinity on *Ostreopsis* cf. *ovata* growth and evaluation of toxin content through HR LC-MS and biological assays. Water Res 2012;46(1):82–92; doi: 10.1016/j.watres.2011.10.029.
46. Ajuzie CC. Palatability and fatality of the dinoflagellate *Prorocentrum lima* to artemia salina. J Appl Phycol 2007; 19(5):513–519; doi: 10.1007/s10811-007-9164-9.

Address correspondence to:
Dr. Francesco Misurale
ETT SpA
Via Sestri 37
Genova 16154
Italy

E-mail: francesco.misurale@ettsolutions.com