



Evaluation of marine phytoplankton toxicity by application of marine invertebrate bioassays

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Summary: The dinoflagellate *Alexandrium minutum* and the haptophyte *Prymnesium parvum* are well known for their toxin production and negative effects in marine coastal environments. *A. minutum* produces toxins which cause paralytic shellfish poisoning in humans and can affect copepods, shellfish and other marine organisms. Toxins of *P. parvum* are associated with massive fish mortalities resulting in negative impacts on the marine ecosystem and large economic losses in commercial aquaculture. The aim of this work is to improve our knowledge about the reliability of the use of marine invertebrate bioassays to detect microalgae toxicity, by performing: (i) a 24- to 48-h test with the brine shrimp *Artemia franciscana*; (ii) a 48-hour embryo-larval toxicity test with the sea urchin *Paracentrotus lividus*; and (iii) a 72-h test with the amphipod *Corophium multisetosum*. The results indicate that *A. franciscana* and *P. lividus* larvae are sensitive to the toxicity of *A. minutum* and *P. parvum*. LC₅₀ comparison analysis between the tested organisms reveals that *A. franciscana* is the most sensitive organism for *A. minutum*. These findings suggest that the use of different organizational biological level bioassays appears to be a suitable tool for *A. minutum* and *P. parvum* toxicity assessment.

Keywords: *Alexandrium minutum*; *Prymnesium parvum*; *Paracentrotus lividus*; *Artemia franciscana*; *Corophium multisetosum*; toxicity bioassays.

Evaluación de la toxicidad de fitoplancton marino mediante la aplicación de bioensayos con invertebrados marinos

Resumen: Las microalgas *Alexandrium minutum* y *Prymnesium parvum* son bien conocidas por la producción de toxinas y sus efectos negativos en nuestras costas. *A. minutum* produce la toxina PSP (Paralytic Shellfish Poisoning) que afecta a humanos y una gran variedad de organismos marinos como copépodos y marisco. Las toxinas de *P. parvum* producen grandes mortalidades de peces ocasionando graves impactos negativos en el ecosistema marino e importantes pérdidas económicas en acuicultura. Este trabajo tiene como objetivo mejorar el conocimiento del uso de bioensayos con invertebrados marinos en la detección de la toxicidad de microalgas, mediante: (i) un test de 24-48 horas con *Artemia franciscana*; (ii) un test de 48 horas con embriones y larvas del erizo de mar *Paracentrotus lividus*; y (iii) un test de 72 horas con el anfípodo *Corophium multisetosum*. Los resultados muestran que las larvas de *A. franciscana* y *P. lividus* son sensibles a la toxicidad de *A. minutum* y *P. parvum*. Los análisis LC₅₀ revelan que *A. franciscana* es el organismo más sensible a *A. minutum*. Estos resultados indican que los bioensayos con diferentes niveles de organización biológica parecen ser una herramienta apropiada para la evaluación de la toxicidad de *A. minutum* y *P. parvum*.

Palabras clave: *Alexandrium minutum*; *Prymnesium parvum*; *Paracentrotus lividus*; *Artemia franciscana*; *Corophium multisetosum*; bioensayos de toxicidad.

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INTRODUCTION

Over the last few decades, the incidence of toxic phytoplankton blooms has apparently been increasing in coastal waters worldwide (GEOHAB 2012). Toxic

blooms cause negative impacts and economic losses in many parts of the world and their consequences and mechanisms depend on the species involved. Broadly, there are four categories of deleterious effects: risks to human health; loss of natural or cultured seafood re-

sources; impairment of tourism and recreational activities; and damage to non-commercial marine resources and wildlife (GEOHAB 2001, Garcés and Camp 2011, Anderson et al. 2012). Several taxonomic groups of microalgae are known as harmful marine species: dinoflagellates, diatoms, raphidophyceans, cyanophytes and haptophytes (Moestrup 1994).

Among the genera responsible for harmful algal blooms (HABs), the genus *Alexandrium* is one of the most important, in terms of severity, diversity and distribution of bloom impacts (Anderson et al. 2012). *Alexandrium minutum* Halim is well known for the production of paralytic shellfish poisoning toxins and its presence can affect copepods, shellfish, and other marine organisms (Zhenxing et al. 2006). *A. minutum* was first described in the Mediterranean Sea forming a red water discoloration in Alexandria Harbour, Egypt (Halim 1960); it has since then been reported widely on the Mediterranean and Atlantic Spanish coast (Delgado et al. 1990, Franco et al. 1994, Frangópulos et al. 2000, Figueroa et al. 2007, Penna et al. 2008 and ICES 2011).

The haptophyte alga *Prymnesium parvum* N. Carter has been responsible for toxic events, with severe ecological impacts, in many parts of the world (Moestrup 1994). This organism produces a set of highly potent exotoxins, commonly called prymensins, that have been shown to have several biological effects, including ichthyotoxic, neurotoxic, cytotoxic, hepatotoxic and hemolytic activity towards a range of marine organisms (Igarashi et al. 1996, Manning and La Claire 2010). *P. parvum* was described for the first time in England (Carter 1937) and has been responsible for massive fish mortalities around the world (Granéli and Turner 2006, Johnsen et al. 2010).

Because of these deleterious effects, several methods for determining the toxicity of marine microalgae have been used to date. The most common is the mouse bioassay developed by the Japanese Ministry of Health and Welfare (Yasumoto et al. 1978). This method is characterized by being accurate and of short-term response, but the controversy in the use of mammals for bioassays has led to the search for and practice of new alternative bioassays (FAO 2005). In this research field, phytoplankton toxicity has been tested in marine organisms, including fish and invertebrates; the tests include mortality tests with crustaceans of the genus *Artemia* (Houdan et al. 2004, Zhenxing et al. 2006, Faimali et al. 2012). Moreover, toxicity of extracts from different diatoms has been evaluated in sea urchin embryos (Buttino et al. 1999, Hansen et al. 2003). It should be noted that the toxic capacity of some toxic microalgae is still unknown and that toxicity induction depending on the environmental conditions, mainly nutrient availability, has led to some controversy (Granéli and Johanson 2003, Rimmel et al. 2011).

Due to the high toxicity reported for *A. minutum* and other dinoflagellates, its toxicity level should be fully evaluated to prevent the risk of bloom episodes in coastal areas. In addition, the involvement of the genus *Prymnesium* in fish mortalities makes its toxicity evaluation important in order to ensure the good quality of areas with fish aquaculture and shellfish exploitation.

Toxicity may be variable between strains of potentially toxic species (e.g. Ichimi et al. 2002, Montoya et al. 2010, Varela et al. 2012). It is therefore necessary to evaluate the toxicity of local strains of known potentially toxic species, such as *Alexandrium minutum* and *Prymnesium parvum*.

In the present study, bioassays with three different marine invertebrates (with different levels of biological organization) were applied as a detection tool to assess the toxic capacity of these marine microalgae species. A set of ecotoxicological tests was carried out to investigate the effects of *A. minutum* and *P. parvum* using different treatments on larvae of the sea urchin *Paracentrotus lividus* (Lamarck 1816), the branchiopod *Artemia franciscana* (Leach 1819) and adults of the amphipod *Corophium multisetosum* (Stock 1952).

The branchiopod *A. franciscana* is used routinely as a test organism for screening in ecotoxicological studies (Sorgeloos et al. 1978). Its life cycle begins with the hatching of dormant cysts that, once rehydrated in salt water, start the development as nauplii larvae, which are the material for the bioassays. These crustaceans are characterized by their adaptability to a wide range of salinity and temperature conditions; their short life cycle; their high adaptability to adverse environmental conditions; their high fecundity; their bisexual/parthenogenetic reproduction strategy (with nauplii or cysts production); their small body size; and their adaptability to variable food resources. They are also non-selective filter feeders sensitive to toxic substances (Sarabia 2002, Faimali et al. 2012).

The sea urchin *Paracentrotus lividus* is also used widely for testing particular contaminants (Fernández 2002) and determining phytotoxicity (Buttino et al. 1999, Hansen et al. 2003, Privitera et al. 2012). *P. lividus* has a wide geographic distribution, is abundant, and is easily harvested and maintained in the laboratory (Garmendia et al. 2009). Moreover, obtaining gametes and in vitro fecundation do not involve difficulties, while the embryonic development is short, thus allowing viable larvae to be obtained in a short time (Garmendia et al. 2009).

Marine and estuarine amphipods are regarded as one of the most suitable model species for integrating the effects of multiple toxic compounds (USEPA 2001, OSPAR Commission 2005). However, *C. multisetosum* has not been used previously for determining microalgae toxicity.

The bioassays were applied with the aim of (i) evaluating the sensitivity of the abovementioned species to the toxicity of *A. minutum* and *P. parvum* in the presence and absence of cells; and (ii) comparing the sensitivity of the three organisms to the toxicity of the mentioned phytoplankton species.

MATERIALS AND METHODS

Algae species

Strains origin

Microalgae samples were collected in the spring and summer of 2004 in the Abra of Bilbao, a bay at the sea-

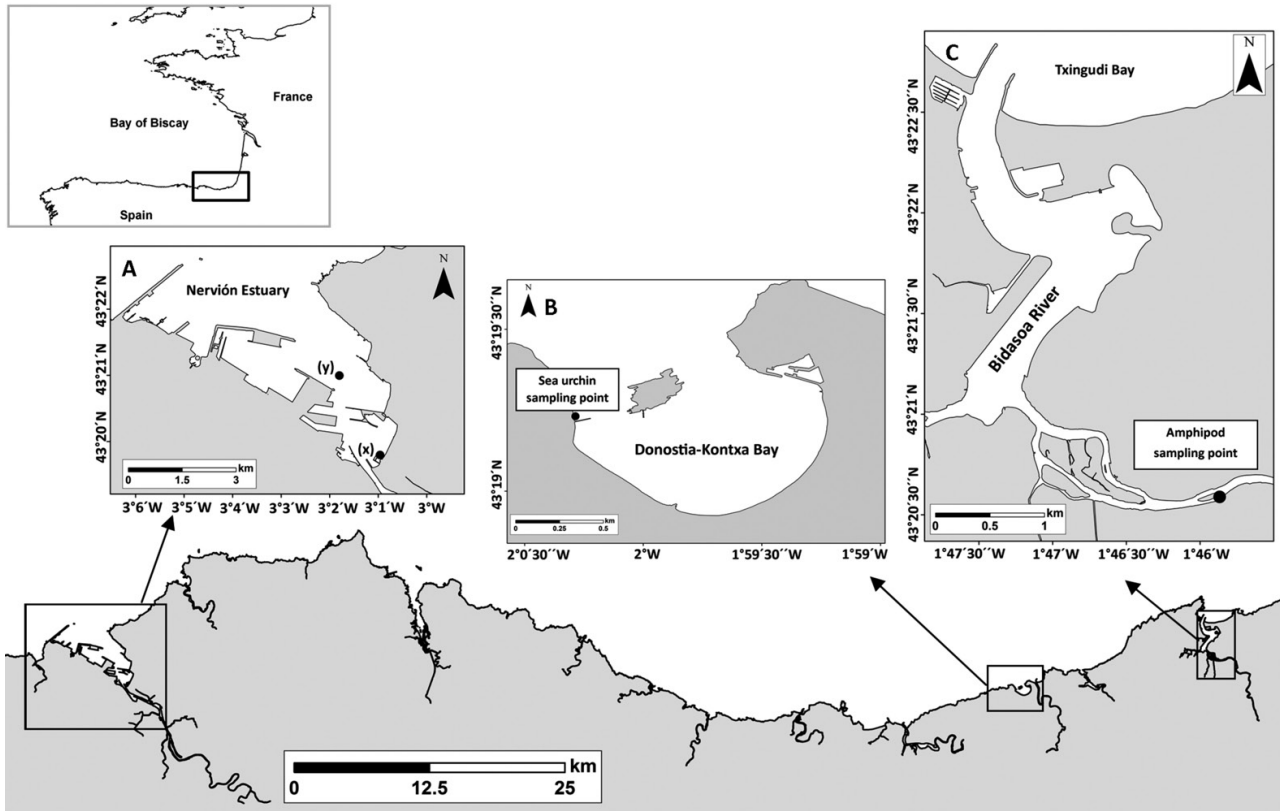


Fig. 1. – The Basque Coast, showing the sampling localities. Microalgae sampling points: A, *Alexandrium minutum* (x) and *Prymnesium parvum* (y); B, sea urchin *Paracentrotus lividus*; C, amphipod *Corophium multisetosum*.

ward end of the Nervión estuary, in the Bay of Biscay. The clonal strain Dn4EHU of *Alexandrium minutum* was isolated from a sample collected in May 2004 in the marina of Getxo (inner Abra of Bilbao) (Fig. 1A). For strain isolation, a single cell was captured with a glass capillary under an inverted microscope (Nikon Diaphot TMD). Before being inoculated into the culture plate, the cell was transferred to a sterile culture medium drop and captured again, to eliminate possible contaminants. For the taxonomic identification, cells were stained with calcofluor (0.15 mg mL^{-1}) and thecal plates were observed using a LeicaTM DMRB light microscope.

The *Prymnesium parvum* strain (Hp13EHU) was isolated from a plankton sample collected in August 2004 in the outer Abra of Bilbao (Fig. 1A). In order to obtain unialgal cultures, serial dilution cultures were made from the field sample. Taxonomic identification was performed through cellular scale characterization, using transmission electron microscopy (Seoane et al. 2009).

Culture conditions

A. minutum and *P. parvum* were grown in 1 L Erlenmeyer flasks containing 0.5 L of culture medium and maintained at 20°C , at an approximate irradiance of $100 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in a 14:10 light:dark period. Cultures were grown in f/2-Si (Guillard and Ryther 1962) prepared with filtered and autoclave-sterilized seawater. To obtain the growth curve, fluorescence

was measured daily with a Turner Designs 10-100R fluorometer as a proxy of cell density. Toxicity tests were performed with both cultures (*A. minutum* and *P. parvum*) in their early stationary growth stage in order to obtain high start-point cell densities.

Before being used for toxicological testing, the cell density was determined with a Neubauer chamber. Cultures were diluted with marine filtered water ($0.22 \mu\text{m}$) (MFW) to obtain different algal concentrations.

Test organisms

Paracentrotus lividus

Fourteen *P. lividus* individuals with a test diameter (excluding spines) greater than 40 mm were collected from a rocky shore located on the west coast of Donostia-San Sebastián (Fig. 1B) in May and June 2012. Specimens were transported alive in a container with seawater and processed immediately in the laboratory.

Artemia franciscana

Artemia franciscana tests were performed following the standard operational procedure (Artoxkit 1990). Cysts (MicroBioTests Inc., Nazareth, Belgium) were hatched in 10 mL of standard seawater at 25°C under continuous light for 30 hours. After that time, all larvae had moulted to instar II-III stages and the hatched nauplii were separated from remaining cysts using a Pasteur pipette.

Corophium multisetosum

Six hundred specimens of *C. multisetosum* were collected from sediments of the Bidasoa estuary (Fig. 1C) in June 2012 during low tide and transported to the laboratory. Individuals were maintained for one week following the USEPA (2001) recommendations; by being placed in trays containing water with continuous aeration, at 20°C and a salinity of 25 with an increase of three units per day until it reached salinity of 35.

Toxicity bioassays

The ecotoxicological tests were performed using different cell concentrations and two treatments: test organisms were exposed (i) to the microalgae culture (cells + medium) and (ii) to the medium devoid of cells after eliminating the cells by filtration in order to detect the toxicity caused by the excretion of the toxins.

The bioassays were performed on II-III stage nauplii larvae of *Artemia franciscana*, on newly-fertilized sea urchin embryos (*Paracentrotus lividus*), and on adults of the amphipod *Corophium multisetosum*.

In order to define the accurate microalgae concentration range of *Alexandrium minutum* and *Prymnesium parvum* for the bioassay with *P. lividus* and *A. franciscana*, an initial test was undertaken. The mortality observed in this initial experiment helped us to define a more precise concentration range for a definitive test (see Table 1 for concentration ranges).

P. lividus: toxicity bioassay

The procedure followed in this study to carry out the *Paracentrotus lividus* embryo-larval bioassay, was taken from the protocol of Garmendia et al. (2009). After equatorial dissection on the sea urchins (Fig. 2A), oocytes (500 µL) were transferred to a 100-mL flask filled previously with MFW, and a small quantity of sperms was then added. After 5-10 min, egg density and fecundation success were determined by taking four 20-µL replicates and counting the total eggs in the aforementioned volume and the number of eggs that contained the fertilization membrane (Fig. 2B). A minimum fecundation success of 90% was established as being indicative of the good quality of the model organisms (Garmendia et al. 2009).

The bioassay was based on the exposure of the newly-fertilized eggs to different concentrations of cultured and filtered microalgae for 48 hours (Table 1).

After fertilization, approximately 500 eggs were transferred to propylene glasses (6 replicates for each algae concentration and 11 for the control, following Garmendia et al. [2009] and Beiras et al. [2002]) containing 20 mL of toxic algae solution or MFW. The glasses were covered and stored in the incubation camera in darkness for 48 hours at 20°C. After incubation, one replicate per treatment was used to measure oxygen, salinity and temperature in order to ensure normal optimal conditions. In the remainder of the replicates, 40% formaldehyde was added to arrest larva development. Embryonic success was estimated by counting the normal pluteus larvae among the 100 first larvae observed. Larvae were considered normal when the four arms were correctly formed (Garmendia et al. 2009) (Fig. 2C). On the other hand, larvae were considered non-well-developed when no four arms were formed (Fig. 2D). To consider the test as valid, the embryo success in the control must be higher than 90% (Fernández 2002). A negative control was performed with the non-toxic algae *Isochrysis galbana* (strain Hp1EHU).

A. franciscana: toxicity bioassay

The toxicity tests were performed in 24-well polystyrene multiwell plates containing 1 mL of different cell concentrations (Table 1). Marine standard water was used as a control and *I. galbana* as a negative control. Ten nauplii larvae were transferred to each well and the multiwell plates were covered with a parafilm and stored at 20°C in darkness for 48 hours. After 24 and 48 hours, dead larvae were counted. Larvae were considered dead when no movement was detected after 10 seconds of observation and semi-dead when the movement had decreased compared with the non-affected larvae. To consider the test as valid, the mortality in the control must be lower than 10% (Artoxkit 1990). Dead larvae were observed under the microscope in order to determine the presence or absence of algae cells inside the digestive system.

C. multisetosum: toxicity bioassay

The experiment was based on the exposure of adult amphipods to different concentrations of *A. minutum* and *P. parvum* (Table 1). Ten amphipods were placed in the glass flasks containing 100 mL of different microalgae concentrations. Three replicates were prepared for each concentration. Flasks were stored in the incubation cabinet in darkness for 72 hours at 20°C. After that time,

Table 1. – *Alexandrium minutum* and *Prymnesium parvum* concentration ranges in the different bioassays.

| Algae species | Test organism | Bioassay | Concentration (cells mL ⁻¹) |
|----------------------------|------------------------|-----------------|---|
| <i>Alexandrium minutum</i> | <i>P. lividus</i> | Initial test | 0; 100; 500; 1000; 5000; 50000; 100000 |
| | | Definitive test | 0; 50000; 60000; 75000; 90000; 100000 |
| | <i>Artemia</i> sp. | Initial test | 0; 100; 500; 1000; 5000; 50000; 100000 |
| | | Definitive test | 0; 1000; 5000; 10000; 50000; 100000 |
| | <i>C. multisetosum</i> | - | 0; 40000; 60000; 75000; 100000 |
| <i>Prymnesium parvum</i> | <i>P. lividus</i> | Initial test | 0; 1000; 5000; 10000; 100000; 230000 |
| | | Definitive test | 0; 100000; 150000; 180000; 200000; 250000; 300000 |
| | <i>Artemia</i> sp. | Initial test | 0; 1000; 5000; 10000; 100000; 230000 |
| | | Definitive test | 0; 5000; 10000; 100000; 200000; 250000 |
| | <i>C. multisetosum</i> | - | 0; 50000; 100000; 150000; 205000 |

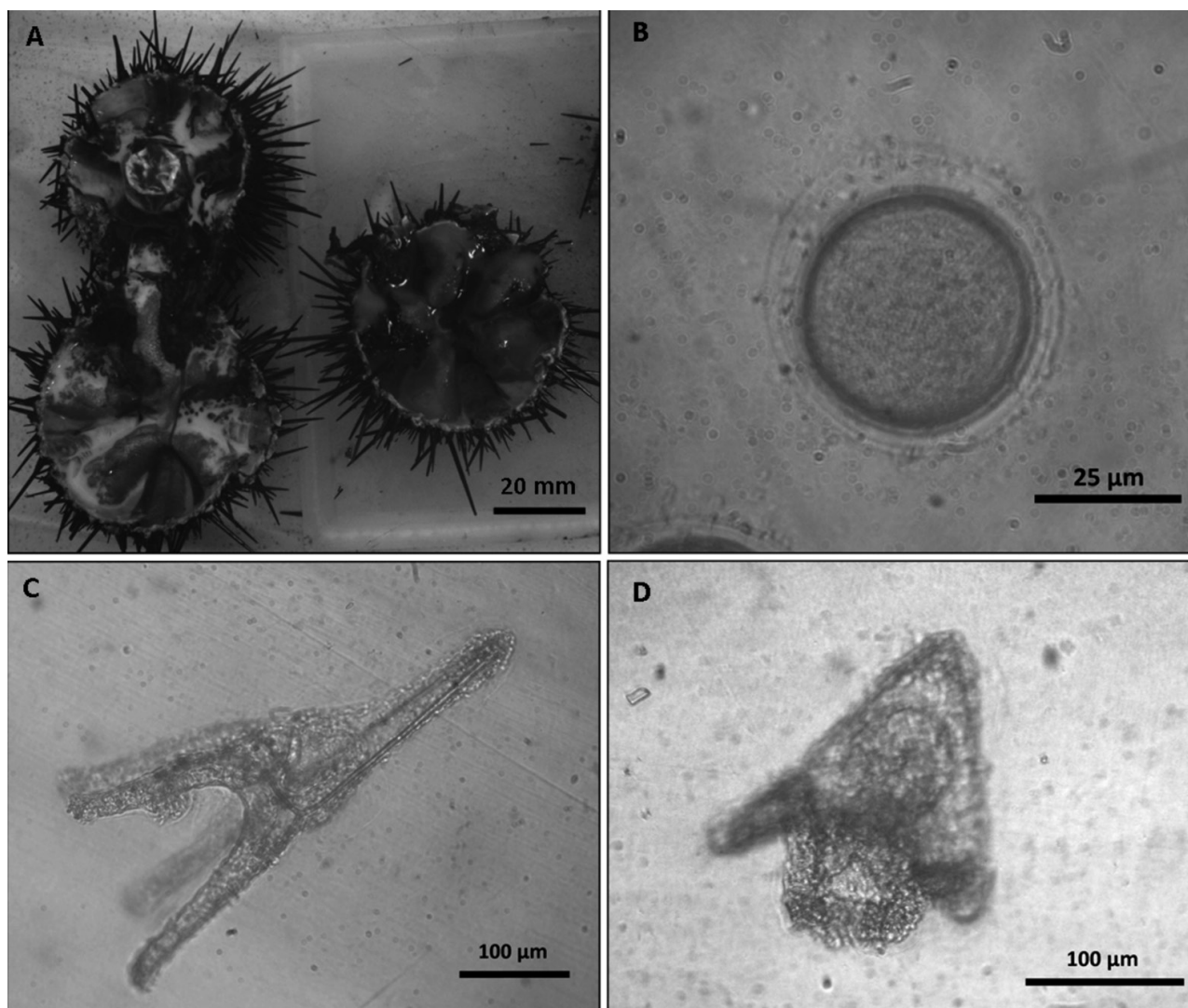


Fig. 2. – *Paracentrotus lividus* bioassay. A, equatorial dissection of the sea urchin before fertilization; B, oocyte with the fertilization membrane after fertilization; C, sea urchin larva completely developed with the four arms correctly separate; D, poorly developed sea urchin larva resulting after exposure to toxic sample.

amphipods were considered dead when no movements were registered within 60 seconds. To consider the test as valid, the mortality in the control must not be higher than 10% (Casado-Martínez et al. 2006).

Reference toxicant tests

The quality and sensitivity of the model organisms used in the different bioassays was evaluated by the use of reference substances. Sodium dodecyl sulphate (SDS) was used in a range from 2 to 8 mg L⁻¹ for *P. lividus* and ammonium in a range from 4 to 128 mg L⁻¹ for *C. multisetosum*.

Statistical analysis

The LC₅₀ and LC₁₀ values (the concentrations causing, respectively, 50 and 10% mortality or non-normal development), together with their 95% confidence intervals were calculated by fitting the survival data to a regression curve by a Probit analysis (Fernández 2002),

with the statistical package Statgraphics® Plus 5.0. The “goodness of fit” was checked using the χ^2 test. Following the methodology described by the United States Environmental Protection Agency (USEPA 2002), when data did not fit Probit requirements (normality and homoscedasticity) a graphical method was used to determine LC₅₀ value.

Following Pérez and Beiras (2010), the NOEC (no effective observed concentration) and LOEC (low effective concentration) values were calculated with the Kruskal-Wallis and the Mann-Whitney U non-parametric test. Differences were considered as significant when *p* was lower than 0.05.

RESULTS

The abiotic parameters in all microalgae culture flasks (oxygen, salinity and temperature) are reported in Table 2. The LC₁₀ and LC₅₀ values for SDS in *P. lividus* and the LC₅₀ value for NH₄⁺ in *Corophium* are reported in Table 3.

Table 2. – Physico-chemical parameters measured in flasks of the different microalgae cultures.

| Species | Experiment | Oxygen (%) | Salinity | Temperature (°C) |
|----------------------------|---|------------|----------|------------------|
| <i>Isochrysis galbana</i> | Negative control test <i>P. lividus</i> | 119 | - | - |
| <i>Isochrysis galbana</i> | Negative control test <i>Artemia</i> | 113.5 | 33.1 | 23.6 |
| <i>Alexandrium minutum</i> | Initial test | 100.3 | 35.1 | 20.4 |
| <i>Prymnesium parvum</i> | Initial test | 101 | 35.4 | 21 |
| <i>Alexandrium minutum</i> | Definitive test | 92.7 | 34.6 | 21.2 |
| <i>Prymnesium parvum</i> | Definitive test | 92.8 | 35.4 | 21 |

Table 3. – Lethal concentration (mg L⁻¹) for SDS in *Paracentrotus lividus* and for NH₄⁺ in *Corophium multisetosum*, their respective confidence intervals (95%) and NOEC (mg L⁻¹) and LOEC (mg L⁻¹) values.

| Toxicant reference | Test organism | Bioassay | LC ₁₀ (mg L ⁻¹) | LC ₅₀ (mg L ⁻¹) | NOEC (mg L ⁻¹) | LOEC (mg L ⁻¹) | Reference |
|------------------------------|-------------------------------|-----------------|--|--|----------------------------|----------------------------|---------------|
| SDS | <i>Paracentrotus lividus</i> | Initial test | 3.02 (2.91 – 3.12) | 4.05 (3.98 – 4.11) | 2.1 | 3.15 | Present study |
| | | Definitive test | 1.99 (1.91 – 2.06) | 2.65 (2.61 – 2.70) | - | 2.03 | Present study |
| | | - | - | 3.95 (3.34 – 4.56) | - | - | Menchaca 2010 |
| NH ₄ ⁺ | <i>Corophium multisetosum</i> | - | - | 25.47 (20.98 – 31.15) | 8 | 16 | Present study |
| | | - | - | 55 (26 – 114.9) | - | - | Pérez 2006 |

The LC₅₀-SDS for *P. lividus* in the initial test was 4.05 mg L⁻¹; it was 2.65 mg L⁻¹ in the definitive test. Furthermore, NOEC and LOEC values for the initial test were 2.1 and 3.15 mg L⁻¹, respectively. LOEC for the definitive test was 2.03 mg L⁻¹ (Table 3). The LC₅₀-NH₄⁺ for *C. multisetosum* was 25.47 mg L⁻¹. Moreover, NOEC and LOEC values were 8 and 16 mg L⁻¹, respectively.

Paracentrotus lividus bioassay

The highest concentration of the dinoflagellate, 100000 cell mL⁻¹, caused the inhibition of the embryonic development in all of the sea urchin larvae while from the lowest concentration tested (100 cell mL⁻¹, to 1000 cell mL⁻¹) no significant differences from the control were detected (Fig. 3A). A toxic effect was observed at

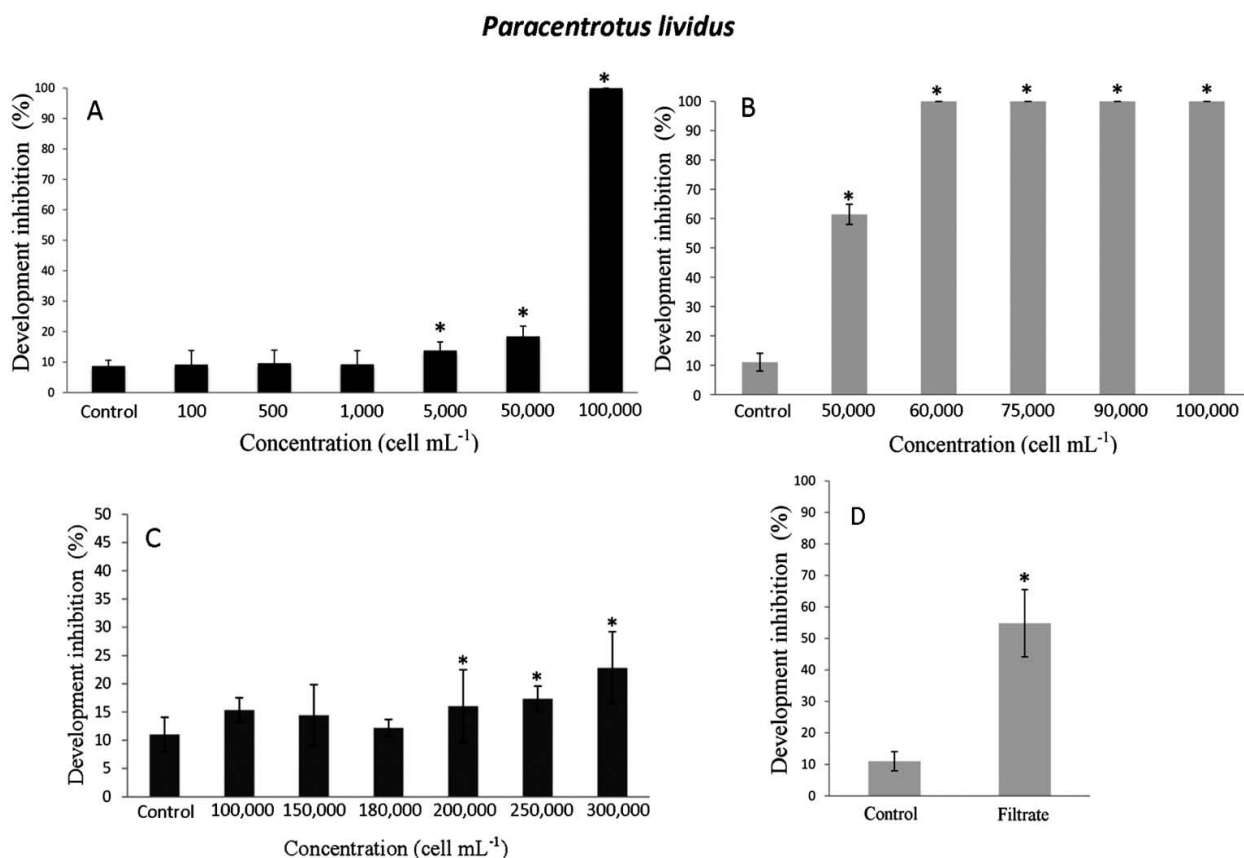


Fig. 3. – Percentage of *Paracentrotus lividus* embryonic development inhibition as a function of algal cell concentration with standard error of the mean for *Alexandrium minutum* culture of the initial (A) and definitive (B) tests and for *Prymnesium parvum* culture (C) and filtrate (D). *: Significant differences ($p < 0.05$).

Table 4. – Lethal concentration (mg L⁻¹) for *Alexandrium minutum* and *Prymnesium parvum* (LC₁₀ and LC₅₀) and their respective confidence intervals (95%), NOEC (mg L⁻¹) and LOEC (mg L⁻¹) values.

| Algae species | Test organism | Bioassay | LC10 (cell mL ⁻¹) | LC50 (cell mL ⁻¹) | NOEC (cell mL ⁻¹) | LOEC (cell mL ⁻¹) | Reference | |
|-------------------|------------------------|------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|---------------------------------------|---------------|
| <i>A. minutum</i> | <i>P. lividus</i> | Initial test | - | 65381 | 1000 | 5000 | Present study | |
| | | Definitive test | - | <50000 | - | 50000 | Present study | |
| | <i>Artemia</i> sp. | 24 h | - | 150629 (83816 -) | 1000 | 5000 | Present study | |
| | | 48 h | 645 (107.2-1659.6) | 16218 (9332.6-30199.5) | 1000 | 5000 | Present study | |
| | | <i>C. multisetosum</i> | - | - | 100000 | - | Zhenxing et al. 2006 Present study | |
| <i>P. parvum</i> | <i>P. lividus</i> | Initial test | - | - | 100000 | 230000 | Present study | |
| | | Definitive test | - | - | 180000 | 200000 | Present study | |
| | <i>S. granularis</i> | - | - | 3-400000 | - | - | Hansen et al. 2003 | |
| | | <i>Artemia</i> sp. | 24 h | - | - | 250000 | - | Present study |
| | | 48 h | - | - | 250000 | - | Present study | |
| | | 24 h | - | 500000 | - | - | Houdan et al. 2004 | |
| | 48 h | - | <100000 | - | - | - | | |
| | <i>C. multisetosum</i> | - | - | 26500 | - | - | Granéli and Johansson 2003 | |
| - | | - | - | 205000 | - | Present study | | |

5000 cell mL⁻¹, with approximately 15% of embryonic inhibition. At 50000 cell mL⁻¹ the inhibition reached 20%. Considering these observations, a new range was applied in a definitive test: from 50000, to 100000 cell mL⁻¹ (Table 1). The results showed a marked toxic effect at 50000 cell mL⁻¹, while 100% of inhibition was achieved above 60000 cell mL⁻¹ (Fig. 3B). For the initial and definitive tests, LOEC values were 5000 and 50000 cell mL⁻¹, respectively (Table 4). On the other hand, the filtrate of *A. minutum* at the maximum concentration did not show any toxic effect on the sea urchin's embryonic development (data not shown).

For the initial test of *Prymnesium parvum*, significant differences from the control in the development inhibition of sea urchin larvae ($p < 0.05$) were observed in a concentration of 230000 cell mL⁻¹. For the definitive test, the concentration range was established at between 100000 and 300000 cell mL⁻¹. From 100000 to 180000 cell mL⁻¹, development inhibition was similar to the control (Fig. 3C); at and above 200000 cell mL⁻¹, significant differences from the control were observed ($p < 0.05$). The highest development inhibition was reached at 300000 cell mL⁻¹ (22.8%), so LC₅₀ could not be calculated. NOEC and LOEC values were 180000 and 200000 cell mL⁻¹, respectively (Table 4). Considering the filtrate of *P. parvum*, inhibition of more than 50% of embryo development was obtained at a concentration of 300000 cell mL⁻¹, showing significant differences from the control sample ($p < 0.05$) (Fig. 3D).

Artemia franciscana bioassay

The mortality of the 15% of nauplii population was induced at a concentration of 5000 cells mL⁻¹ of the dinoflagellate *A. minutum* after 24 hours of exposure; however, 50% of mortality was not achieved with the initial concentrations. Higher cell densities were therefore used to perform the definitive bioassay (Table 1). In addition, the exposure time was increased from 24 to 48 hours. Incubation with the dinoflagellate significantly affected nauplii mortality during both exposure periods ($p < 0.05$). After 24 hours of exposure, significant differences from the control were observed from

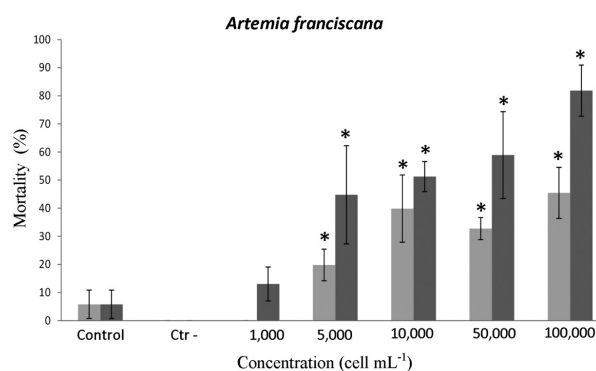


Fig. 4. – Mortality after 24 h (dark grey bar) and 48 h (light grey bar) of nauplii larvae of *Artemia franciscana* exposed to *Alexandrium minutum* culture. Two controls were used: standard seawater (0.22 µm) and *Isochrysis galbana* as a negative control. * significant differences ($p < 0.05$).

5000 cell mL⁻¹ (19 % of mortality) ($p < 0.05$), increasing with the concentration until 45% mortality at 100000 cell mL⁻¹ (Fig. 4). After 48 hours, at 50000 and 100000 cell mL⁻¹, the mortality in the nauplii population was 58% and 81%, respectively. The effect of exposure time can be observed with LC₅₀ values at 24 and 48 h: 150629 and 16218 cell mL⁻¹, respectively (Table 4). In addition, the minimum concentration with significant mortality was 5000 cell mL⁻¹ at 24 and 48 hours of exposure. Related to the filtrate of *A. minutum*, the maximum algae concentration (100000 cell mL⁻¹) showed no significant differences from the control sample ($p > 0.05$) (data not shown). On the other hand, *A. franciscana* nauplii survival was not affected by the presence of *Prymnesium parvum* cells or filtrate at maximum exposure concentration, i.e. 250000 cell mL⁻¹ ($p > 0.05$) (data not shown). However, a sub-lethal effect was detected along the concentration gradient, i.e. the movement of the nauplii was reduced at maximum exposure concentration (250000 cell mL⁻¹), without reaching a mortality effect.

Due to logistic difficulties, the presence of cell content inside the alimentary canal of *A. franciscana* could not be identified.

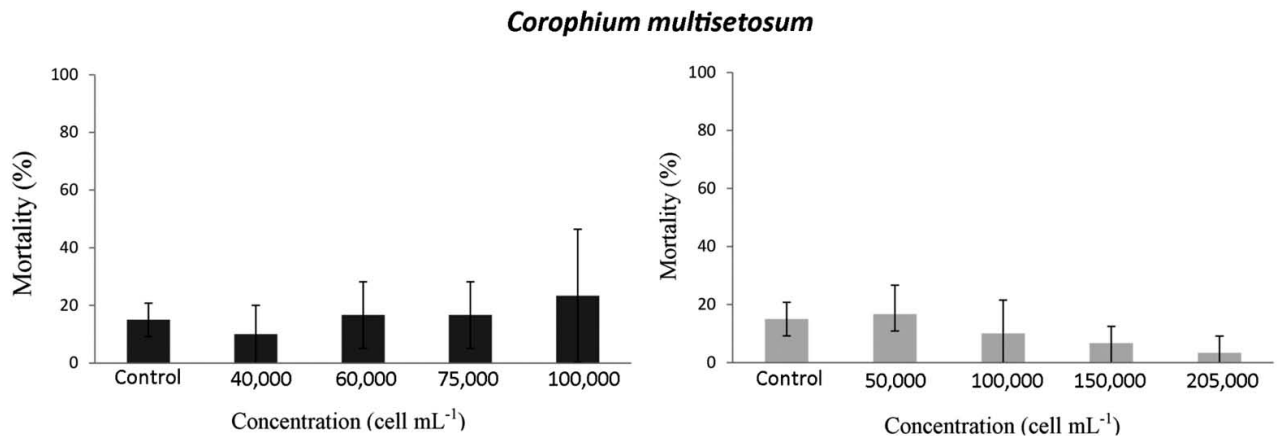


Fig. 5. – Mortality after 72 h of *Corophium multisetosum* exposed to culture of *Alexandrium minutum* (left) and *Prymnesium parvum* (right). No significant differences in mortality between control and samples were observed.

Corophium multisetosum bioassay

In relation to *C. multisetosum* mortality results, after 72 hours of exposure to both microalgae, significant differences from the control were not observed for any concentration ($p > 0.05$). However, an increase in mortality was observed throughout the concentration range of *Alexandrium minutum* (Fig. 5).

DISCUSSION

Lethal concentrations (mg L^{-1}) for SDS in *P. lividus* and for NH_4^+ in *C. multisetosum*, as calculated in this study, lie within the range of those obtained previously by Menchaca (2010) and Pérez (2006), respectively (Table 3). Our results have therefore confirmed the good quality of the model organisms (differences in the quality of gonad material were observed in the two different periods where sea urchins were collected, as the LC_{50} value for SDS in *P. lividus* obtained in May (the initial test) was higher than the value obtained in June (the definitive test). This observation is in accordance with Garmendia et al. (2010), who also detected variability in the quality of the gonad material throughout the year. Therefore, this assumption must be considered when interpreting the differences found in results of the two sets of trials performed with *P. lividus* exposed to *A. minutum*.

The dinoflagellate *Alexandrium minutum* was toxic for *A. franciscana* nauplii at $5000 \text{ cell mL}^{-1}$, while the LC_{50} value increased with exposure time. The 48-hour exposure proved to be much more harmful, indicating that both concentration and exposure time contributed to the toxic response in the nauplii population. The toxic effect of *A. minutum* on *A. franciscana* nauplii survival has rarely been examined. Our results agree with those of Zhenxing et al. (2006), who found that four strains of *Alexandrium* spp. were lethal for *A. salina* at a density of $2000 \text{ cell mL}^{-1}$. Regarding the filtrate of *A. minutum*, the maximum concentration ($100000 \text{ cell mL}^{-1}$) showed no toxic effect in the *A. franciscana* nauplii, which is explained by the fact that their toxicity is linked to the intact cell, as has been reported for *Ostreopsis cf. ovata* (Faimali et al. 2012)

In contrast, *A. franciscana* nauplii survival was not affected by the presence of *P. parvum* culture or filtrate at the maximum exposure concentration ($250000 \text{ cell mL}^{-1}$). These results contrast with those of Houdan et al. (2004), who found an LC_{50} value of a *P. parvum* culture (in the stationary phase) lower than $100000 \text{ cell mL}^{-1}$ for *A. salina*. Some authors have suggested that this sensitivity disagreement could be related to differences in microalgae culture conditions (Granéli and Johanson 2003). According to the aforementioned authors, excretion of toxic metabolites might be a general mechanism among toxic haptophytes when exposed to stress events, e.g. nutrient-deficient conditions. However, recent studies hypothesize that the majority of *Prymnesium* toxicity is cellular-based rather than related to exotoxins production (Rommel et al. 2011). Further, reduction of *A. franciscana* movement with exposure time, as shown in the present study at the maximum exposure concentration of *P. parvum*, is in accordance with the results of Rommel and Hambright (2012). These investigators observed that *Prymnesium* cells were attached to most exterior surfaces, to fish gills and to feeding and respiratory appendages in zooplankton, which contributed to the movement depletion of individuals. Moreover, they found that the number of attached cells increased with exposure time and density of *Prymnesium*.

The toxicity of *A. minutum* and *P. parvum* had not been evaluated previously using sea urchin embryo bioassays. However, a dose-dependent toxic effect of several marine microalgae on sea urchin cell division has been recorded in several studies (Paul and Fenical 1984, Lemée et al. 1993, Pedrotti et al. 1996, Buttino et al. 1999). In the present study, *A. minutum* was toxic to sea urchin embryos at $5000 \text{ cell mL}^{-1}$ (LOEC value). These results are in accordance with those of Privitera et al. (2012), who demonstrated the toxic effect in competent sea urchin larvae when exposed to the dinoflagellate *Ostreopsis cf. ovata*. On the other hand, filtrate of the dinoflagellate showed no toxic effect on the development of the sea urchin. This scenario is also in agreement with Faimali et al. (2012), who demonstrated that dinoflagellates do not show significant toxicity with growth medium devoid of cells.

By contrast, the culture and filtrate of *P. parvum* significantly inhibited embryo development at 200000 and 300000 cell mL⁻¹. These effective concentrations are within the range of those calculated by Hansen et al. (2003), who reported a cell division reduction of newly-fertilizing eggs of the sea urchin *Sphaerechinus granularis* exposed to a haptophyte. However, Rimmel and Hambright (2012) suggested that the presence of toxins in the filtrate could be an artefact caused by the vacuum pressure used in the filtration process, which can cause cell disruption and the subsequent release of intracellular toxins. The result of the filtrate reported in the present study should therefore be considered carefully.

In the present study, adult amphipods were not sensitive to the toxicity of *A. minutum* and *P. parvum* at the concentrations tested. This finding contrasts with those of other authors who have routinely used adult zooplankton organisms (*Euterpina acutifrons* and *Acartia grani*) to evaluate the toxicity of *A. minutum* (Frangópulos et al. 2000, Costa and Fernández 2002). However, Romano et al. (2003) observed that secondary metabolites, such as unsaturated aldehydes produced by some algae during stress events, such as depredation (Pohnert 2000), were not toxic in adult copepods, while they inhibited the development of oocytes and embryos. Romano et al. (2003) hypothesized that this behaviour could be a defence mechanism of microalgae, designed to regulate the populations of herbivorous zooplankton.

Assuming similar test conditions (photoperiod, temperature, salinity and oxygen) for all the bioassays performed, among the tested organisms *A. franciscana* nauplii showed the highest sensitivity for *Alexandrium minutum* according to LC₅₀-48h. These results are in agreement with those of Faimali et al. (2012) and Privitera et al. (2012), who suggested that the development stages could affect the sensitivity of the organisms, increasing the mortality with the ingestion rate. Moreover, Zhenxing et al. (2006) confirmed a decrease in the survival of *Artemia salina* nauplii with the feeding rates of *Alexandrium* spp. However, in the present study, owing to logistic difficulties, the presence of cell content inside the alimentary canal of *A. franciscana* could not be identified. As a result, it cannot be concluded that the higher sensitivity of *A. franciscana* nauplii is related to the ingestion of the microalgae. In the case of *P. parvum*, difficulties in the LC₅₀ calculation did not permit comparison of the sensitivity between the tested organisms.

The presence of toxic microalgae on the coasts of the Basque Country is monitored every year, within the context of the EU Water Framework Directive (WFD) (e.g. Borja et al. 2009). In 2008 the cell density of *Alexandrium minutum* was lower than 100 cell L⁻¹, much less than the value considered as a limit for toxicity risk found in the present study. *Prymnesium parvum* has not been reported specifically in the WFD reports. The inverted light-microscope technique based upon fixed samples used in this monitoring programme for phytoplankton identification and counting does not usually allow species or even genus-level identification, of

small naked flagellates such as *Prymnesium* spp. The species level identification of prymnesiophyceans requires the analysis of the body scales through electron microscopy (Seoane et al. 2009) or molecular methods.

Regarding toxicity of *Alexandrium minutum*, it must be considered that the long culture maintenance of the dinoflagellate in the present work may have led to the decrease in toxin production. Martins et al. (2004) demonstrated that saxitoxin production is not a constitutive, stable characteristic of *Alexandrium*, but can be lost during routine culture maintenance over a prolonged interval.

CONCLUSION

Only larvae of *Paracentrotus lividus* and *Artemia franciscana* have been demonstrated to be sensitive to the toxicity of the two marine microalgae *Alexandrium minutum* and *Prymnesium parvum*. The algae strains tested were isolated much in advance compared with the performed test. Therefore, the minimum toxic concentration values established in the present study for the model organisms cannot be considered as a real toxic value. We suggest performing the bioassays of the present study with the model organisms, using recent isolated *A. minutum* or *P. parvum* during toxic blooms and combining different parameters (nutrients, light and temperature) to study the potential toxicity in different conditions. Nevertheless, our results place *P. lividus* and *A. franciscana* bioassays as a viable alternative for the detection of *A. minutum* and *P. parvum* toxicity in the context of routine monitoring. Other standardized toxicological assays using more distant taxonomic groups are also available and have been applied extensively (Ribo and Kaiser 1987, Zon and Peterson 2005). Therefore, besides working on the use of the bioassays described in the present work, we also suggest the use of alternative methodologies in order to explore a wider range of tests for the detection of toxic events.

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