Mitigation of lethal effects of *Karlodinium veneficum* and *K. armiger* on *Sparus aurata*: changes in haematocrit and plasma osmolality

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ABSTRACT: Between January and April 2000, several experiments were performed during a *Karlodinium* spp. proliferation in Alfacs Bay (Ebro delta, NW Mediterranean) to determine the effects of these dinoflagellates on sea bream *Sparus aurata* cultivated in the area. Moribund fish showed an increase in plasma osmolality together with a decrease in the haematocrit percentage compared to control fish. The efficacy of copper sulphate, hydrogen peroxide, potassium permanganate and formalin against *Karlodinium* spp. was also tested. None of these treatments had mitigation effects when applied in the presence of fish; on the contrary, lethal effects appeared at lower *Karlodinium* spp. densities compared to fish control groups. When a lytic agent, such as copper sulphate, was used as a water pre-treatment, in the absence of fish, *Karlodinium* spp. toxicity was significantly reduced. Protocols for water pre-treatments were studied as a potential tool for combating *Karlodinium* spp. in fish farms.

KEY WORDS: Dinoflagellate · *Karlodinium armiger* · *K. veneficum* · Harmful algal blooms · HABs · Ichthyotoxic · Mitigation

INTRODUCTION

Some phytoplankton species cause fish losses in mariculture. A variety of physiological mechanisms, singly or in combination, may lead to fish mortality from harmful algal blooms (HABs). These can be generally categorised as: (1) physical damage or irritation of gill tissue, (2) toxigenic reactions to ichthyotoxic agents, (3) blood hypoxia from environmental oxygen depletion, or (4) gas bubble trauma from oxygen supersaturation (Anderson et al. 2001).

Mitigation to reduce the impacts of HABs includes strategies for avoiding blooms (prevention), such as moving fish cages from the path of a HAB, or actions that directly target the bloom population (control), such as the direct application of chemicals or other biological control agents that kill or disrupt HAB cells during blooms (Anderson et al. 2001).

Karlodinium armiger and *K. veneficum* (Bergholtz et al. 2006) are small, unarmoured dinoflagellates

(15 to 24 μm in length, 12 to 18 μm in width). *K. veneficum* (syn. *K. micrum*, *Gyrodinium galatheanum* and *G. micrum*; Daugbjerg et al. 2000, IOC 2002, Bergholtz et al. 2006) has been known since the 1950s to be ichthyotoxic in the South Atlantic (Braarud 1957, Pieterse & van der Post 1967) and has also been associated with fish kill events in the Murray River/Estuary, Western Australia (Cosgrove et al. 2000), Chesapeake Bay (Terlizzi et al. 2000), South Carolina (Kempton et al. 2002) and Lebanese coastal waters (Abboud-Abi Saab & El-Bakht 1998). In Alfacs Bay (Ebro delta, NW Mediterranean), these organisms were referred to as *G. corsicum* until recently after a taxonomic study of several strains isolated during fish kill events in 1998 and 2000 (Garcés et al. 2006). For the purpose of this work, we will refer to the 2 species blooming together in Alfacs Bay during the study period as *Karlodinium* spp. *Karlodinium* spp. have been producing winter proliferations in the waters of Alfacs Bay since 1994, and the blooms have been associated with massive fish mortalities

Table 1. *Sparus aurata*. Water treatments used to eliminate the toxicity of *Karlodinium* spp.

Expt number	*Karlodinium* spp. density (cells ml^{-1})	Fish size (mean ± SD)	Treatment	Type of treatment
1	820	53.45 ± 8.14 g, 14.50 ± 0.74 cm	Copper sulphate	Treatment in presence of fish (Time 0)
2	700	53.45 ± 8.14 g, 14.50 ± 0.74 cm	Copper sulphate	Treatment in presence of fish (Time 0)
3	963	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Copper sulphate	Treatment in presence of fish (Time 0)
4	995	53.45 ± 8.14 g, 14.50 ± 0.74 cm	Copper sulphate	Treatment in presence of fish (Time 0)
5	24000	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Copper sulphate	Kinetic assay
6	2197	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Copper sulphate	Kinetic assay
7	2197	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Copper sulphate	Treatment in presence of fish Time 0)
8	2197	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Formalin	Treatment in presence of fish (Time 0)
9	2197	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Hydrogen peroxide	Treatment in presence of fish (Time 0)
10	2197	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Potassium permanganate	Treatment in presence of fish (Time 0)
11	2197	2.23 ± 0.36 g, 5.19 ± 0.23 cm	Copper sulphate + hydrogen peroxide	Treatment in presence of fish (Time 0)

(Delgado et al. 1995). The growth rates measured in Alfacs Bay for *Karlodinium* spp. (0.39 to 0.94 d^{-1}) exceeded the losses by advection (0.12 d^{-1}) during the winter, which generated water discoloration during months when there were no freshwater inputs from the Ebro delta (Garcés et al. 1999).

Lethal effects directly induced by this dinoflagellate have been demonstrated against the co-occurring planktonic copepod *Acartia grani* (Delgado & Alcaraz 1999), although the noxious effects were not attributable to toxin intake through ingestion of the dinoflagellate. The relationships between cell densities of natural *Karlodinium* spp. populations and mortalities of juvenile sea bream *Sparus aurata* and sea bass *Dicentrarchus labrax* are of use in the prevention of fish kills by this dinoflagellate in fish farms (Fernández-Tejedor et al. 2004).

The objectives of the present work were to determine the efficacy of different treatments used in aquaculture for mitigation of *Karlodinium* spp. and, secondly, to monitor the effects on fish during exposure. Mortality, haematocrit (Hc) and plasma osmolality were assessed.

MATERIALS AND METHODS

The main algal killing agents used in aquaculture were assayed and tested against a mixed population of *Karlodinium armiger* and *K. veneficum* in the presence of fish. The agents included copper sulphate, formalin, hydrogen peroxide, potassium permanganate and a combination of copper sulphate and hydrogen peroxide. Copper sulphate was also tested both in the presence of fish and before challenge in 2 time series experiments. For time series experiments, fish were introduced in tanks treated with copper sulphate at different periods after the treatment. In

total, 11 experiments were performed, and the conditions are shown in Tables 1 and 2. For each experiment, 2 controls were used: one was a control using water free of *Karlodinium* spp. that was also treated with the same dose of chemical, the second was a control containing *Karlodinium* spp. but no chemicals.

Water samples containing *Karlodinium* spp. were obtained directly from Alfacs Bay using a pump. The pumped water was used within 1 to 2 h to challenge fish in Expts 1 to 4 and 6 to 11. For Expt 5, a 300 m^3 concrete pond was used to store water from Alfacs Bay. During the day and in the absence of wind, *Karlodinium* spp. aggregated and formed visible patches that contained densities significantly higher than in the water from Alfacs Bay. Water was directly pumped from these clusters and employed for challenge.

The Utermöhl method (Utermöhl 1931) was used to determine phytoplankton densities, using 5 and 10 ml HYDRO-BIOS settling chambers. Each sample was settled in triplicate, and 10 fields in each chamber were counted. The average value of the resulting 30 fields was used. *Karlodinium* spp. were 90 to 99% dominant in cell numbers for all samples (Fernández-Tejedor et al. 2004).

Experimental animals. Two stocks of juvenile gilt-head sea bream *Sparus aurata* (53.45 ± 8.14 g, 14.50 ± 0.74 cm and 2.23 ± 0.36 g, 5.19 ± 0.23 cm) were obtained from 2 different fish farms inside and outside the Ebro delta, respectively. All fish were maintained

Table 2. Water conditions for treatments in Table 1

Expt	Temperature (ºC)	Salinity	Oxygen (% saturation)	pH
1	7.90 ± 1.08	36.15 ± 0.81	84.12 ± 4.03	8.12 ± 0.08
2	8.10 ± 1.05	36.16 ± 0.62	85.14 ± 2.43	8.11 ± 0.11
3, 5–11	18.88 ± 0.17	35.90 ± 0.14	82.5 ± 3.11	8.08 ± 0.09
4	13.90 ± 1.19	36.53 ± 0.92	91.25 ± 2.04	8.14 ± 0.12

for 1 mo before experiments in tanks at a density of 5 kg m^{-3} in running water and free of *Karlodinium* spp.

Gilthead sea bream (57.78 ± 10.86 g, 14.59 ± 1.37 cm) were also sampled for plasma osmolality and haematocrit during a natural mortality episode caused by *Karlodinium* spp. at the same fish farm where the first stock group was obtained 6 wk previously.

Environmental water conditions during challenge. To exclude temperature effects, the water temperature in experimental tanks was maintained at the same level as the temperature in the natural environment from which the *Karlodinium* spp. containing water was received. The temperature of Expts 1, 2 and 4 was the same as the origin of the sample, and unwanted effects related to thermal influences were avoided by maintaining the fish stock at the same temperature measured in the Bay. For Expts 3 and 5 to 11, the temperature of the water containing *Gyrodinium corsicum* was raised to 18.88 ± 0.17°C by leaving the sample in glass beakers until room temperature was reached. Temperature, salinity, percentage oxygen saturation and pH values were monitored every 2 h (Table 2). All treatments were continuously aerated both to keep the fish alive and also to improve cell lyses by the chemicals.

Choice of chemical concentration for water treatment. The ranges of chemical concentrations were based on the doses recommended by Brown (1993). The following doses were chosen for the main experiments: 0.4 mg l^{-1} for copper sulphate, 0.7 mg l^{-1} for potassium permanganate, 8 mg l^{-1} for formaldehyde and 300 mg l^{-1} for hydrogen peroxide.

Chemical application. Two approaches were undertaken: the use of the chemical in the presence of fish and *Karlodinium* spp. at the same time (Time 0) and a kinetic assay for the chemical treatment of the water containing *Karlodinium* spp. before the immersion of fish. Duplicate groups of 5 juvenile gilthead sea bream from the 2 different stocks were placed either in 50 l tanks (Expts 1, 2 and 4) or 2.5 l glass beakers (Expts 3 and 5 to 11). Expts 1 to 4 consisted of duplicate tanks with water containing *Karlodinium* spp. treated with copper sulphate, a sample containing *Karlodinium* spp. without treatment and a control with only copper sulphate. Expt 5 (19 February 2000) and Expt 6 (29 March 2000) consisted of adding copper sulphate to all beakers containing the same density of *Karlodinium* spp. at the same time and adding 5 fish per beaker in duplicate at different times (0, 0.5, 1, 3, 6 and 12 h for Expt 5; 0, 1, 3, 6 and 12 h for Expt 6). Expts 7 to 11 consisted of treatments of copper sulphate, formalin, hydrogen peroxide, potassium permanganate and copper sul-

phate plus hydrogen peroxide, respectively, all of them at the same *Karlodinium* spp. density as Expt 6. Negative controls were performed using filtered Mediterranean seawater free of *Karlodinium* spp.

Fish behaviour was monitored at all times. Fish showing no operculum movement were removed from the beaker, and the time of death was recorded. The experiment lasted 48 h, after which time the final number of dead and live fish was recorded. In addition to the fish mortality results for the different treatments, certain blood parameters were assayed when experimental conditions and fish size allowed. Hc values and mortalities were recorded in Expts 3, 4 and 5. Plasma osmolality values and mortalities were recorded in experiments 1, 2 and 4.

Blood sampling. When fish size (53.45 ± 8.14 g, 14.50 ± 0.74 cm and 57.78 ± 10.86 g, 14.59 ± 1.37 cm) allowed, blood samples were taken from a caudal peduncle puncture, with a heparin-treated disposable plastic syringe, and collected in both heparinised microhaematocrit tubes and heparinised Eppendorf centrifuge tubes. Sodium heparin was used as an anticoagulant. Blood was stored immediately in an ice bath. For smaller sea bream (2.23 ± 0.36 g, 5.19 ± 0.23 cm), the caudal peduncle was cut off for collection directly into a haematocrit tube.

Haematological parameters. Hc values (percent packed cell volume) were determined using a microhaematocrit centrifugation technique (Heraeus Biofuge haemo) immediately after blood collection. Blood samples for plasma chemical studies were centrifuged in Eppendorf centrifuge tubes at $1000 \times g$ for 20 min at 4°C (Eppendorf centrifuge 5415D). The plasma was then separated from the cells and stored at –80°C until it could be analysed. Plasma osmolality was determined by the freezing point method using a microosmometer (Advanced Instruments, 3MO).

Statistics. For comparison of percentage mortalities, a chi-squared test was used. For comparison of osmolality and haematocrit values between groups, a *t*-test, Mann-Whitney and Kruskal-Wallis tests were used. Statistically significant differences between groups were considered when $p < 0.05$.

Table 3. *Sparus aurata*. Summarised mortality results (corresponding to Expts 1 to 4)

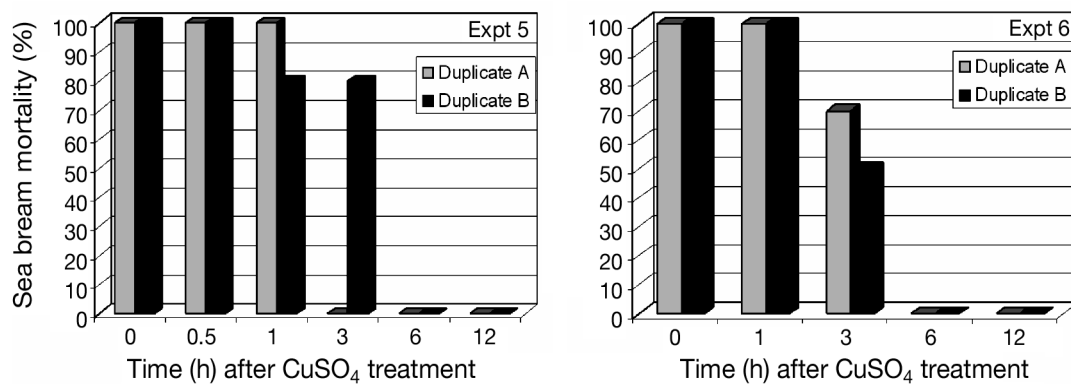| Expt	Mortality (%)			Cells ml^{-1}
	Karlodinium spp. + CuSO$_4$	Control water + CuSO$_4$	*Karlodinium* spp.	
1	100 ± 0	0	0	820
2	80 ± 28	0	0	700
3	100 ± 0	0	0	963
4	70 ± 14	0	0	995

Fig. 1. *Sparus aurata*. Percentage of fish mortalities after exposure to water containing *Karlodinium* spp. pre-treated with copper sulphate for 0 to 12 h: 24 000 cells ml^{-1} for 19 February 2000 (Expt 5) and 2197 cells ml^{-1} for 29 March 2000 (Expt 6)

RESULTS

Treatments

All *Sparus aurata* challenged with *Karlodinium* spp. at densities lower than 1000 cells ml^{-1} survived after 48 h of exposure (Table 3). However, mortalities ranged from 70 to 100% when copper sulphate was added to the tanks at Time 0. There were no significant differences between the percentage mortalities registered in Expts 1 to 4 ($H = 4.5$, p = 0.419).

In Expts 5 and 6, kinetic studies were performed by adding *Sparus aurata* at different times to the tanks containing *Karlodinium* spp. and copper sulphate. Fish mortalities ranged from 100% at Time 0 in both experiment to 0% 6 h after exposure (Fig. 1). Control groups for the copper sulphate treatment had 100% fish survival.

For the different treatment assays, 100% mortality was produced in Expts 7 to 11, when fish were challenged with copper sulphate, formalin, hydrogen peroxide and potassium permanganate, whereas the respective control treatments showed 0% mortality. Only application of copper sulphate plus hydrogen peroxide reduced fish mortality to 0% after 48 h of immersion. These treatments were applied in water containing 2197 *Karlodinium* spp. cells ml^{-1}.

Haematological parameters

After challenge with *Karlodinium* spp., higher osmolality (Fig. 2) values were measured in plasma from moribund fish compared to survivors and control fish (no *Karlodinium* spp. present), both when copper sulphate had been added to the system or in copper sulphate-free water (Expts 1, 2 and 4). The experimental conditions are detailed in Tables 1 & 2.

There was a significant difference between the osmolality medians of moribund and control fish (no *Karlodinium* spp. present) in each experiment (1, 2 and 4) ($t = 2.890$, p = 0.01; $t = 3.532$, p = 0.006; $t = 8.603$ p ≤ 0.001). In Expt 4, there was a significant difference between moribund and surviving fish independently of whether copper sulphate had been added to the glass beakers ($F = 47.281$, p < 0.001). Moreover, in Expt 4, no significant differences in osmolality values were found between any of the 3 surviving fish groups ($F = 0.167$, p = 0.847). No significant differences were found between moribund fish from Expts 1, 2 and 4 ($F = 2.808$, p = 0.080).
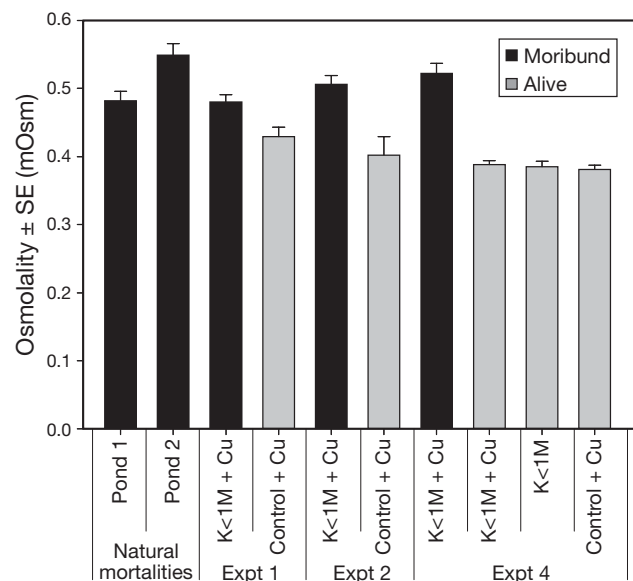

Fig. 2. *Sparus aurata*. Osmolality values (mean and SE) for fish exposed to water treated with copper sulphate (Cu) containing *Karlodinium* spp. (K; <1M: cell density < 1000 cells ml^{-1}) and a control for Expts 1 and 2. For Expt 4, values include a control of non-treated water containing *Karlodinium* spp. n = 10
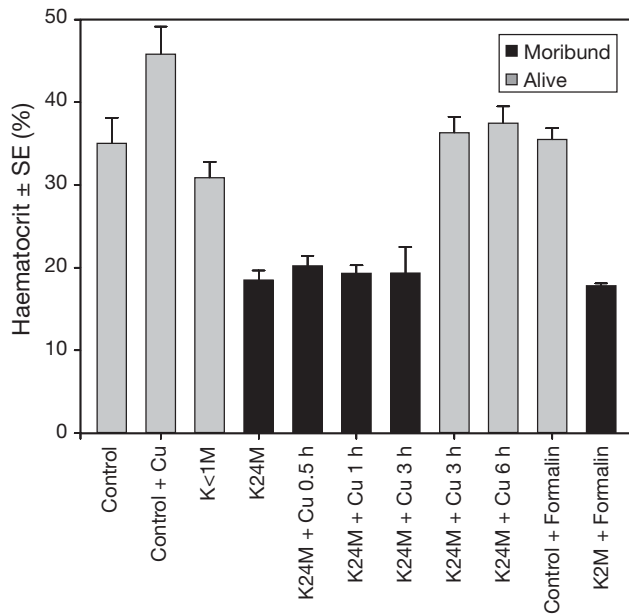
Fig. 3. *Sparus aurata*. Haematocrit values (mean and SE) for moribund, alive and control fish after immersion in copper sulphate (Cu) pre-treated water containing *Karlodinium* spp. (K; <1M: <1000 cells ml^{-1}; 24M: ~24 000 cells ml^{-1}; 2M: ~2000 cells ml^{-1}) and after a formalin treatment of the same water sample

When moribund fish from a nearby aquaculture facility were sampled (see 'Materials and methods') during an episode of mortalities due to *Karlodinium* spp., plasma osmolality values in pond 1 were similar to moribund fish in Expts 1, 2 and 4 ($F = 2.176$, p = 0.110), and values in Pond 2 were similar to moribund fish in Expts 2 and 4 ($F = 2.199$, p = 0.136).

It was not possible to obtain plasma from unexposed fish at the fish farm. However, plasma from the control fish in Expts 1 and 2 could be considered a negative control (no *Karlodinium* spp. present), since both fish stocks and water parameters were equivalent for experimental groups and cultured fish.

Hc was reduced in fish exposed to *Karlodinium* spp. (Fig. 3) compared to the control group. In Expts 5 and 6, copper sulphate produced a non-significant increase in Hc ($45.80 \pm 3.35\%$), while formalin ($35.50 \pm 1.35\%$) did not change the Hc values compared to control values ($35.04 \pm 3.09\%$; $t = -1.954$ p = 0.077; $t = -0.0976$, p = 0.927, 95%). When fish were exposed to *Karlodinium* spp. and copper sulphate was added at different times for water pre-treatment (see 'Materials and methods'), there were no significant differences in the Hc values between groups of moribund fish ($F = 0.338$, p = 0.798, 95%). Hc values from moribund fish in Expt 5 combining all copper sulphate-treated groups together were significantly different from the control ($t = -7.45$, p ≤ 0.001, 95%), the copper sulphate-treated control ($t = 342$, p ≤ 0.001, 95%) and the group exposed to a sub-

lethal density of *Gyrodinium corsicum* ($t = -7.037$, p ≤ 0.001, 95%). There were no significant differences in the Hc values between moribund fish in *Karlodinium* spp. water treated with copper sulphate (Expt 5) or with formalin (Expt 6; $t = -0.515$, p = 0.610, 95%). The highest Hc values recorded in the experiments using *Sparus aurata* were found in the control group treated with copper sulphate ($45.90 \pm 3.35\%$). The lowest values were observed in moribund fish in water containing *Karlodinium* spp. (2197 cells ml^{-1}) treated with formalin ($17.81 \pm 0.27\%$). The Hc values of all moribund fish in Expt 5 formed a homogeneous group, which was significantly different from the survivors ($t = -13.289$, p ≤ 0.001, 95%). In Expt 5, *Karlodinium* spp. produced a decrease in Hc when it was associated with fish mortality, but this reduction in Hc values was not observed at sublethal densities of the dinoflagellate both in comparison to control values ($t = 7.457$, p ≤ 0.001; $t = 1.224$, p = 0.252, respectively, 95%).

DISCUSSION

The recorded *Karlodinium* spp. densities in Alfacs Bay for 20 spring days in 2000 were higher than the lowest experimental LD$_{50}$ (Fernández-Tejedor et al. 2004). As a result, intensive monitoring was established at a fish farm to determine the levels of *Karlodinium* spp. in the water intake every 30 min. However, because this system allowed *Karlodinium* spp. to be detected, high densities entering in clusters could be avoided. For a sustained presence of *Karlodinium* spp. over a long period, a mitigation protocol was required to manage the toxic algae at the fish farms in Alfacs Bay.

We measured higher Hc values in fish immersed in water treated with copper sulphate. The Hc is considered one of the good stress indicators that are variously affected in fish (Soivio & Oikari 1976, Wilson & Taylor 1993, Roche & Bogé 1996). The Hc decreases after intoxication by organic compounds and increased after metallic intoxication. Roche & Bogé (1996) found that the higher values in sea bass intoxicated with metallic compounds resulted from an increased cell volume because no change in blood haemoglobin was shown. Our results agree with these observations. Hc values found in moribund fish after exposure to non-treated and formalin-treated water containing *Karlodinium* spp. (2197 cells ml^{-1}) were lower than those measured in moribund fish after copper sulphate treatment, although the difference was not significant. Control water treated with copper sulphate produced higher Hc values in fish after a 48 h exposure compared to values measured in non-treated and formalin-treated control water.

Changes in osmolality from 429 ± 14 to 480 ± 11 mOsm in Expt 1, from 402 ± 27 to 506 ± 13 mOsm in Expt 2 and from 381 ± 6 to 522 ± 15 mOsm in Expt 4 were similar to those found when juvenile cod *Gadus morhua* were exposed to a culture of *Karlodinium veneficum* (115×10^6 cells l^{-1}); the fish showed an increased plasma osmolality (Nielsen 1993) from 336 ± 6 mOsm (n = 10) to 444 ± 8 mOsm (n = 10) before death. A similar effect on plasma chloride was postulated when exposing Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* to the toxic alga *Chryso-chromulina polylepis* (Leivestad & Serigstad 1989). The toxins may increase the permeability of the fish gill in these cases.

The same effect resulting in greater mortality when a lytic agent was used to treat an ichthyotoxic bloom has been observed for *Karlodinium veneficum* in Chesapeake Bay, USA (Deeds et al. 2002, 2004). These authors discovered a substance that is ichthyotoxic, cytotoxic and haemolytic and appears to be released in small amounts in healthy cultures, but is released in greater amounts upon cell damage or lysis. Deeds et al. (2002) found that the addition of potassium permanganate (4 mg l^{-1}) to *K. veneficum* cells and culture filtrates, as well as to the haemolytic HPLC fractions Tox A (KmTx1) and Tox B, resulted in the complete disappearance of haemolytic activity. Toxicity was eliminated after potassium permanganate (4 mg l^{-1}) treatment. In contrast, in our experiment, potassium permanganate treatment increased mortality. However, we used a much lower concentration of chemical (0.7 mg l^{-1}) because higher concentrations killed sea bream under our test conditions. Potassium permanganate is an oxidant, and the dose used by Deeds et al. (2002) was sufficient to lyse dinoflagellate cells, oxidise the toxin and avoid fish mortalities during the *K. venefi-cum* bloom in Chesapeake Bay. The toxins isolated from *K. veneficum* have been called karlotoxins, and their mode of action seems to be the increase in membrane permeability, resulting in cell death; for example, KmTx2 increases membrane ionic permeability equally for a range of mammalian cell types (Deeds et al. 2006), and KmTx1 causes the release of the cytosolic marker lactate dehydrogenase in rat pituitary tumour cells, suggesting cell lysis (Deeds et al. 2002).

The reduction in fish mortality to 0% using the treatment of copper sulphate plus hydrogen peroxide is probably due to the lysis of dinoflagellate cells by copper sulphate and toxin inactivation by hydrogen peroxide. Nevertheless, our results indicate that a pretreatment with 2 successive applications of hydrogen peroxide or a unique application at a higher dose could probably reduce the waiting time for diminution of its lethal effects before the immersion of fish in the same water.

This study was undertaken with a natural population of *Karlodinium armiger* and *K. veneficum.* Several strains were isolated at different times during a bloom. *K. armiger* strains are more toxic than *K. veneficum* strains (Garcés et al. 2006). Toxic compounds from *K. veneficum* have been isolated and characterised (Deeds et al. 2006), but we do not know if *K. armiger* produces the same toxic compounds. Research will continue to determine the chemical structure of the toxin or toxins implicated in both species present in Alfacs Bay.

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