Dual quantitative PCR assay for identification and enumeration of *Karlodinium veneficum* and *Karlodinium armiger* combined with a simple and rapid DNA extraction method

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Abstract

Karlodinium is a dinoflagellate genus responsible for massive fish mortality events worldwide. It is commonly found in Alfacs Bay (NW Mediterranean Sea), where the presence of two *Karlodinium* species (*K. veneficum* and *K. armiger*), with different toxicity, has been reported. Microscopy analysis is not able to differentiate between these two species. Therefore, new and rapid methods that accurately and specifically detect and differentiate these two species are crucial to facilitate routine monitoring, to provide early warnings and to study population dynamics. In this work, a quantitative real-time PCR (qPCR) method to detect and enumerate *K. veneficum* and *K. armiger* is presented. The ITS1 region of the ribosomal DNA was used to design species-specific primers. The specificity of the primers together with the melting curve profile provided a reliable qualitative identification and discrimination between the two *Karlodinium* species. Additionally, a simple and rapid DNA extraction method was used. Standard curves were constructed from 10-fold dilutions of cultured microalgae cells. Finally, the applicability of the assay was tested with field samples collected from Alfacs Bay. Results showed a significant correlation between qPCR determinations and light microscopy counts ($y = 2.838\ x + 564$; $R^2 = 0.936$). Overall, the qPCR method developed herein is specific, rapid, accurate and promising for the detection of these two *Karlodinium* species in environmental samples.

Keywords: *Karlodinium veneficum*, *Karlodinium armiger*, quantitative PCR, ITS rDNA, DNA extraction.

1. Introduction

Harmful algal blooms (HABs) of dinoflagellates of the genus *Karlodinium* (initially classified as *Gymnodinium* or *Gyrodinium* before the erection of the new genus by Daugbjerg et al. (2000)) have been implicated in numerous fish-killing events around the word, including Europe, Southwest Africa, North America, Australia and East Asia (Garces et al. 2006; Place et al. 2012). In Alfacs Bay (Ebro Delta, NW Mediterranean Sea) winter blooms of *Karlodinium* spp. have been periodically reported since 1994, causing mortality of fish in aquaculture ponds, raft cultures of mussels as well as local wild fauna. Among the 11 *Karlodinium* species reported to date (AlgaeBase), two *Karlodinium* species, identified by morphological and genetic analyses as *K. veneficum* and *K. armiger*, have been described and have become well established in this region (Garces et al. 2006). Both species are known to be mixotrophic, combining photosynthesis with prey feeding (phagotrophy), and produce haemolytic toxins. These toxins function as prey immobilization agents before ingestion (Berge et al. 2012). Recently, karlotoxins from *K. veneficum* (Van Wagoner et al. 2008) and karmitoxins from *K. armiger* (Rasmussen et al. 2017) have been isolated and chemically characterised. It seems that *K. armiger* has a higher level of ichthyotoxicity in comparison to *K. veneficum* (Garces et al. 2006), which may be attributed to the higher toxicity of karmitoxins (Berge et al. 2012).

In Alfacs Bay, *Karlodinium* spp. outbreaks tend to concentrate in calm and low turbulence areas and can attain very high densities (above 4,000,000 cells L^{-1}). Toxicity studies with *Karlodinium* spp. populations have set 366,000 cells L^{-1} as the No Observed Effect Concentration (NOEC) in a fish mortality assay (Fernandez-Tejedor et al. 2004). Accordingly, a level of 200,000 cells L^{-1} has been established as a warning level for *Karlodinium* spp. in this geographic area. The implementation of a monitoring program that provides adequate early warnings of possible imminent blooms is essential to mitigate adverse economic, health and environmental effects caused by HABs. Current monitoring programs use light microscopy to detect and enumerate toxic microalgae. However, this method is time-consuming, requires a great deal of taxonomic expertise and is based on morphological characteristics, which in some cases are insufficient to identify at genus or species level. Identification of cells of the genera *Karlodinium* is particularly difficult because their unarmoured morphology is poor in distinct features (Shao et al. 2004), and thus they can be easily misidentified with other genera like *Gymnodinium*, *Karenia, Heterocapsa* and *Ansanella* (Bergholtz et al. 2006; Garces et al. 2006; Jeong et al. 2014). Moreover, *Karlodinum* species cannot be differentiated using light microscopy (Bergholtz et al. 2006).

To address these challenges, molecular methods are being developed and employed to study HAB species. Molecular tools have advantages with respect to traditional microscopy techniques: (i) they are faster, which makes possible their use as early warning tools; (ii) they are more accurate, allowing the identification of morphologically similar species or genera; and (iii) fewer hours of training are required to attain a level of expertise sufficient for routine laboratory screening. Among the different molecular methods for microalgae detection, quantitative real-time PCR (qPCR) has been used to identify and quantify HAB species in marine environmental samples (Penna et al. 2013). Several qPCR methods have been reported for different microalgae species including *Alexandrium minutum* (Galluzzi et al. 2004), *Karenia mikimotoi* (Yuan et al. 2012), *Karlodinium veneficum* (Berge et al. 2012; Eckford-Soper and Daugbjerg 2015b; Park et al. 2009; Place et al. 2012), *Prymnesium parvum* (Eckford-Soper and Daugbjerg 2015b; Galluzzi et al. 2008) and *Prorocentrum donghaiense* (Zhang et al. 2016). In addition, various qPCR methods have been designed to differentiate between two species within the same genus: *Ostreopsis* (*O. ovata* and *O. siamensis*) (Battocchi et al. 2010; Perini et al. 2011),

Pseudochattonella (*P. farcimen* and *P. erruculosa*) (Eckford-Soper and Daugbjerg 2016), *Alexandrium* (*A. catanella* and *A. taylori; A. catanella and A. tamarense*) (Galluzzi et al. 2010; Hosoi-Tanabe and Sako 2005) and *Dinophysis* (*D. acuta* and *D. acuminata*) (Kavanagh et al. 2010). Even more challenging is targeting more than two species, as described for *Gambierdiscus* (Nishimura et al. 2016; Vandersea et al. 2012) and *Pseudo-nitzschia* (Andree et al. 2011).

Most molecular assays use primers that are designed to hybridize to ribosomal DNA (rDNA) genes, since they are phylogenetically informative and tandemly repeated in high copy number, and significant databases of homologous sequences exist for interspecies comparisons. Different regions of the rDNA can be employed, including the small subunit (SSU), the large subunit (LSU), the two internal transcribed spacers (ITS1 and ITS2) and the external transcribed spacer (ETS). The different degrees of sequence variability within these regions may be exploited to target genus or species. For instance, ITS sequences are generally more useful for designing primers to distinguish among species of the same genus due to its higher content in non-conserved loci (Andree et al. 2011; Shao et al. 2004). Other molecular markers such as protein-encoding genes have also been exploited although to a lower extent (Penna et al. 2013). With reference to *Karlodinium* spp., primers previously developed for *K. veneficum* are based on the LSU rDNA region (Eckford-Soper and Daugbjerg 2015b), the ITS2 rDNA region (Park et al. 2009; Zhang et al. 2008) and the ferredoxin gene (Zhang et al. 2008).

Although quantitative molecular techniques have been increasingly used for microalgae, there is still a lack of a standardized, efficient and simple method for extracting high-quality DNA from phytoplankton (Yuan et al. 2015). Due to the large amount of polysaccharides and polyphenolics in microalgae (Greco et al. 2014), the isolation of high-purity DNA, free from PCR inhibitors, is not straightforward. At present, DNA extraction/purification methods for microalgae are mostly based on cetyltrimethylammonium bromide (CTAB), phenol/chloroform or column-based commercial kits, and normally a bead-beating step is introduced to disrupt the cells (Erdner et al. 2010; Kamikawa et al. 2007; Yuan et al. 2015; Zhang et al. 2016). These methods require many reagents and instrumentation, and reproducibility and DNA yield are sometimes poor (Zhang et al. 2016). Additionally, these methods are time-consuming, limiting the speed of the whole assay. Although the use of crude extracts (without DNA purification) has been proposed to overcome these limitations, the protocol is tedious (Galluzzi et al. 2004; Park et al. 2009). Therefore, rapid and reliable methods to extract DNA from microalgae are highly desired.

The objective of this study was to develop a qPCR assay for the detection and enumeration of two *Karlodinium* species (*K. veneficum* and *K. armiger*) commonly found in Alfacs Bay, combined with the evaluation of a new, simple and rapid DNA extraction method. Species-specific primer sets were designed within the ITS1 region, and their specificity was tested. The performance of the assay was assessed by testing environmental samples and results were correlated with light microscopy determinations.

2. Materials and methods

2.1. Algal cultures

Clonal cultures of *K. veneficum* (strain IRTA-SMM-00-01) and *K. armiger* (strain K-0668) isolated from Alfacs Bay were obtained from IRTA Culture Collection of Algae (Sant Carles de la Ràpita, Spain) and the Scandinavian Culture Collection of Algae and Protozoa (Copenhagen, Denmark), respectively. *K. veneficum* and *K. armiger* clonal cultures were grown in f/2 medium (Guillard 1975; Guillard and Ryther 1962) and L1 + Urea medium (Guillard and Hargraves 1993), respectively, at a salinity of 36 psu. Cultures were maintained at 18 ± 2 °C under a light intensity of 110 µmol photons m^{-2} s^{-1} on a standard 12:12 h light:dark cycle. Additionally, three microalgae commonly found in the Mediterranean Sea were used as control: *Ostreopsis ovata, Pseudo-nitzschia fraudulenta* and *Ansanella granifera*. The ITS1-5.8S-ITS2 region of *Karlodinium* species and control non-target microalgae was PCR-amplified using primers described in Table 1 and bi-directionally sequenced (Sistemas Genómicos, LLC, Valencia, Spain) to identify species. Sequences were manually edited and aligned using BioEdit v7.0.5.2 (Hall 1999) and deposited in GenBank (Table 1).

Culture samples were fixed with Lugol's iodine (Throndsen 1978) and cells were counted under an inverted light microscope (Leica DMIL) following the Uthermöl method (Utermöhl 1958). Cultures were collected at the exponential phase and harvested by centrifugation (3,700 g; 25 min). For all microalgae, pellets containing 10^4 cells were prepared. Additionally, for *K. veneficum* and *K. armiger*, 10-fold serial dilutions from 10^2 to 10^5/10^6 cells were prepared. Cell pellets were stored at -20 °C until extraction of genomic DNA.

2.2. DNA extraction methods

2.2.1. Biomeme (BIM) method

The Biomeme Sample Prep Kit for DNA (provided with standard columns) was obtained from Biomeme Inc. (Philadelphia, USA). DNA extraction was carried out according to general recommendations provided by the manufacturer. Reagents and the sequence of steps to apply are provided, but no protocol exists for non-standard applications. To assess the applicability of the Biomeme (BIM) method to microalgae samples, results were compared with those obtained by the standard phenol/chloroform/isoamylalcohol (PCI) method (see below). The BIM method was first qualitatively tested by qPCR for *K. veneficum, O. ovata* and *P. fraudulenta* using pellets of 10^4 cells, and the addition of a mechanical disruption step using a bead beater was evaluated. The primers used were: Karlo20/KaV160 (this study) for *K. veneficum*, OvataF/OstreopsisR (Battocchi et al. 2010) for *O. ovata*, and 5.8S/QPfrau (Andree et al. 2011) for *P. fraudulenta.* The BIM method was quantitatively tested constructing qPCR standard curves of *K. veneficum* and *K. armiger* cell dilutions (10^2-10^6 cells) and comparing with the PCI method (10^2-10^5 cells). Additionally, 10-fold serial dilutions of the extracted DNA (1:10; 1:100; 1:1,000) were analysed to evaluate the potential presence of PCR inhibitors.

The protocol for the BIM method after optimisation was as follows: cell pellets were resuspended in 300 µL of lysis buffer (1 M NaCl, 70 mM Tris, 30 mM EDTA, pH 8.6), transferred to 2-mL screw-cap cryotubes containing ~50 µg of 0.5-mm diameter zirconium glass beads (Biospec, USA) and disrupted using a BeadBeater-8 (BioSpec, USA) pulsed for 45 s at full speed. The volume used for each buffer was: Biomeme Lysis Buffer (BLB, 500 µL), Biomeme Protein Wash (BPW, 500 µL),

Biomeme Wash Buffer (BWB, 750 µL) and Biomeme Elution Buffer (BEB, 500 µL). The procedure consisted of mixing 250 µL of the homogenized samples with BLB and pumping the fluid through a syringe with an ion-exchange cartridge attached (10 pumps). This was followed by passing, first BPW, and second BWB, through the cartridge one time each by pumping the fluid with the syringe plunger. After the washing steps, the columns were dried (~50 pumps, passing only air through). Finally, purified DNA was eluted in BEB (5 pumps). Genomic DNA was quantified and checked for its purity by reading the absorbance at 260/280 using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Spain), and stored at -20 °C until qPCR analysis.

2.2.2. Phenol/chloroform/isoamylalcohol (PCI) method

The PCI method was used for qPCR specificity tests and as a reference to evaluate the performance of the BIM method. The PCI method, in brief, included resuspension of cells in 200 µL of lysis buffer, which were then transferred to 2-mL screw-cap cryotubes containing ~50 µg of 0.5-mm diameter zirconium glass beads. Then, 25 µL of 10% dodecyltrimethylammonium bromide (DTAB) and 200 µL of chloroform were added to the cryotubes, and the mixture disrupted using a BeadBeater-8 pulsed for 45 s at full speed. After centrifugation (2,300 g; 5 min), the aqueous phase (300 µL) was transferred to a fresh tube and the standard phenol/chloroform/isoamylalcohol (25/24/1, v/v/v) procedure was applied thereafter (Sambrook, 1989). Precipitation of the DNA from the final aqueous solution (240 µL) was achieved by the addition of 2 volumes (480 µL) of absolute ethanol and 0.1 volume (24 µL) of 3 M sodium acetate (pH 8.0). The DNA pellet was then rinsed with 70% ethanol and dissolved in 50 µL of molecular biology-grade water. Genomic DNA was quantified as stated in section 2.2.1.

2.3. Primer design and specificity verification

Primers for *K. veneficum* and *K. armiger* were designed with the aid of the software Amplify4 (© Bill Engels, University of Wisconsin, USA). The ITS1-5.8S-ITS2 rDNA sequences obtained for *K. veneficum* and *K. armiger* were aligned with 33 other *Karlodinium* species sequences (Online Resource 1) in a multiple sequence alignment using ClustalW implemented in the program BioEdit v7.0.5.2. Variable regions were manually identified from the alignments. One genus-specific (*Karlodinium*) primer was designed at the beginning of the ITS1 region, and two species-specific (*K. veneficum* and *K. armiger*) primers were designed downstream in a more highly variable region within the ITS1 region (Table 2). Primer sets were designed to amplify products of approximately the same size to ensure that both assays had similar amplification efficiencies. Oligonucleotides were purchased from Sigma-Aldrich (Spain). Primer specificity was checked *in silico* by BLAST analysis and using Amplify4, as well as experimentally assessed by qPCR and electrophoretic analysis with 4 ng of DNA from target and non-target microalgae. Additionally, cross-reactivity experiments for the two *Karlodinium* species in both single and mixed DNA samples were conducted.

2.4. Quantitative real-time PCR (qPCR) assay

Optimisation of the qPCR protocol was investigated by testing different reagent concentrations and thermocycling conditions. The qPCR assays were performed with an ABI 7300 thermocycler (Applied Biosystems, Thermo Fisher Scientific, Spain) using the following two-step cycling protocol: 95 °C for 10 min followed by 45 cycles at 95 °C for 20 s and 58 °C for 30 s. Each 20-µL reaction mixture contained 10 µL 2X SYBR Green dye (Applied Biosystems, Thermo Fisher

Scientific, Spain), primers (final concentration 0.5 µM) and 2 µL of DNA extracts from cultured cells (see section 2.1) or field samples (see section 2.5) in triplicate. At the end of each run, a dissociation step was included to evaluate melting curve profiles for the absence of primer dimers or non-specific products. The thermal profile for melting curve determination consists of 1 min at 60 °C with a gradual increase of temperature (1° C/15 s). In each qPCR experimental plate, a non-template control containing molecular biology-grade water was included as well as a positive control for each target.

2.5. Field samples analysis

Seawater samples from Alfacs Bay were collected in March 2017 at 4 different stations (Fig. 1). At each station, samples were collected at the surface (S) and at a depth of 5.5 m (B) (Table 3) using polyethylene bottles (1 L). Seawater samples were fixed with Lugol's iodine solution. For light microscopy counting, a volume of 50 mL was settled in sedimentation chambers for 24 h and counted following the Utermöhl method. For qPCR analysis, 50-mL aliquots were centrifuged (3,700 g; 25 min) and maintained at -20 °C until DNA extraction by the BIM method. The total number of *Karlodinium* cells obtained from the two counting methods (optical microscopy for genus-level detection and the sum of two qPCR assays for species-level) was compared by linear regression analysis using SigmaStat software 3.1 (Systat Software Inc., California, USA).

3. Results

3.1. Primers and qPCR specificity

BLAST analysis against the dataset of GenBank showed that primers for *K. veneficum* and *K. armiger* matched only with their respective ITS1 sequences. Analysis by qPCR showed DNA amplification for target species, while amplification products were not detected using control non-target microalgae. Moreover, *K. veneficum* and *K. armiger* specific primers did not show cross-reactivity neither in single nor in mixed DNA samples. Electrophoresis of the qPCR products using target DNA showed bands at the expected molecular weight, and no other bands were visible. Differences in the melting temperature (Tm) and shape of the melting curve profile were evident from the melting curve analysis of *K. veneficum* and *K. armiger*, with Tm of 83.5 °C and 84.1 °C, respectively (Online Resource 2). Additionally, DNA from another *K. veneficum* strain (strain CCMP 415, taxonomic synonym formerly identified as *K. micrum*) (Online Resource 1) that had a somewhat different genotype in a region encompassed by the *K. veneficum* primers was tested. This region was also amplified and showed a slightly different melting curve profile and Tm of 84.3 °C (Online Resource 2).

3.2. Biomeme (BIM) method evaluation

To assess the performance of the new DNA extraction method, results were compared with those obtained by the PCI method. After reading the absorbance at 260/280 nm, good yield and purity were obtained using both methods. However, a small peak at 230 nm was observed using the BIM method, which was also observed in the BWB solution, and thus certainly due to a component of the buffer. Both methods were tested using three microalgae with different cell morphology and structural composition. DNA extracted by PCI was detected for all microalgae by qPCR (Fig. 2). When the BIM method was applied without bead beating, DNA was extracted from *O. ovata* and *P. fraudulenta*, but no DNA

was extracted from *K. veneficum.* Nevertheless, DNA yields increased (Ct values decreased) for all microalgae when a bead-beating step was incorporated in the protocol.

3.3. Standard curves for qPCR assays

Calibration curves for *K. veneficum* and *K. armiger* were constructed using DNA extracted from serial dilutions of cultured cells. The correlation between Ct values and number of cells is shown in Fig. 3. Using the PCI method, calibration curves showed a slope of -3.261 (R^2 = 0.962) and -3.442 (R^2 = 0.973) for *K. veneficum* and *K. armiger*, respectively. The efficiency of the reaction was calculated using the formula E = ($10^{(-1/m)}$ - 1), where *m* is the slope of the linear regression from the calibration curves, and was 102.6% and 95.2%, respectively. Using the BIM method, slopes were -4.334 (R^2 = 0.994) for *K. veneficum* and -4.389 (R^2 = 0.982) for *K. armiger*, which correspond to efficiencies of 70.1% and 69.0%, respectively. To check if the lower efficiency of the BIM method was due to the potential presence of inhibitors (the above-mentioned component of the BWB), 10-fold serial dilutions of the extracted DNA were tested, but the efficiency did not improve. Although using the BIM method the efficiency of the qPCR assays was lower, a high linearity over 5 orders of magnitude was obtained with this method (R^2 > 0.98), which indicated that it is reliable in the quantification of target cells. On the other hand, using the PCI method high linearity (R^2 > 0.96) was only achieved over 4 orders of magnitude.

3.4. Field sample analysis

To assess the ability of the qPCR assay to detect *Karlodinium* species from the environment, 8 seawater samples were collected from Alfacs Bay. All samples were analysed by light microscopy and qPCR. Cells of *Karlodinium* spp. were observed by light microscopy in all samples, although with low abundances (from 1,000 cells L^{-1} to 18,549 cells L^{-1}), cell densities being higher at the surface than at the bottom (Table 3). Based on light microscopy analysis, all samples also contained significant abundances of other toxic species, *A. minutum* (maximum of 5,038 cells L^{-1}), and potentially toxic species of *Pseudo-nitzschia* (maximum of 106,029 cells L^{-1}) (Online Resource 3). The qPCR assay detected the presence of both *Karlodinium* species in 7 out of 8 samples, *K. veneficum* being always more abundant than *K. armiger* (Table 3). In general, total cell densities of *Karlodinium* species determined by qPCR were higher than cell densities of *Karlodinium* spp. estimated by light microscopy. Nonetheless, a significant correlation (R^2 = 0.936; p < 0.0001) of 1 to 2.8 ratio (y = 2.838 x + 564) was found between techniques (Fig. 4). Only one sample displayed a different result: microscopy analysis revealed the presence of 1,000 cells L^{-1} in sample Station 1 (B), while the same sample analysed by qPCR did not show presence of *Karlodinium* species.

4. Discussion

K. veneficum and *K. armiger* have been reported to co-occur forming blooms in Alfacs Bay (Garces et al. 2006). Given that they show different levels of toxicity to marine organisms, the need for discrimination between these two species has become a serious concern within the framework of the local monitoring program. Current toxic microalgae monitoring is performed via light microscopy using the Uthermöl cell-counting method, which is time consuming and does not enable differentiation among *Karlodinium* species. In this study, a qPCR assay was developed for *K. veneficum* and *K. armiger*

discrimination. Further, we have used a novel method that requires no centrifugation equipment or long incubations in thermal blocks to extract DNA quickly and with sufficient purity to perform qPCR assays.

Primers were designed within the ITS1 because it has been demonstrated to be a good region for species-specific detection due to its high variability (Shao et al. 2004). The design aimed at minimising the number of required primers: one genus-specific (*Karlodinium*) and two species-specific (*K. veneficum* and *K. armiger*) primers. A similar strategy has been described for quantifying *Pseudo-nitzschia* species (Andree et al. 2011) and total microalgae biomass (Godhe et al. 2008). Besides rDNA genes, mitochondrial genes (mtDNA) have also been evaluated for species-level discrimination. Although mtDNA was found to be a suitable marker for *Dinophysis* species (Raho et al. 2013), this is not the case for other microalgae since it is too conserved at an inter-species level (Penna et al. 2014). Hence, rDNA genes remain the target of choice to differentiate microalgae at the species level.

Screening of the primers against a large dinoflagellate rDNA gene database confirmed the specificity of the primers. Specificity tests demonstrated that the qPCR assay was specific for target species and was not affected by non-target microalgae that are sympatric in Alfacs Bay (and elsewhere): *Ostreopsis ovata* and *Pseudo-nitzschia fraudulenta*, as well as *Ansanella granifera*, a species morphologically similar to *Karlodinium* spp. Additionally, melting curve analyses were conducted to ensure that amplification derives from the intended product rather than from non-specific amplifications. *K. veneficum* was identified by a peak Tm at 83.5 °C, while *K. armiger* cells presented a peak Tm at 84.1 °C. Moreover, it was possible to identify the two species by a change in the melting curve profile, since melting curve shapes are a function of GC content, length and sequence of the amplicon (Ririe et al. 1997). The specificity of the primers together with the melting curve profile offer a reliable qualitative discrimination between the two *Karlodinium* species of this work.

Phytoplankton comprise a wide range of microalgae, some of them without a cell wall (e.g. naked dinoflagellates) and some others with fortified cell walls (e.g. diatoms with silica frustules or thecate dinoflagellates). Although different DNA extraction methods have been applied to microalgae (Kamikawa et al. 2007; Nishimura et al. 2016; Zhang et al. 2016), the wide microalgae diversity has hampered the implementation of a standardized, rapid and simple method. In fact, using an appropriate DNA extraction method is decisive in terms of sensitivity and rapidity of the whole assay. Therefore, in this study, the BIM method was tested for subsequent qPCR assays. The BIM method was tested with three microalgae representing different cell morphology and structural composition: *K. veneficum* (as a model of a small unarmoured dinoflagellate), *O. ovata* (as a model of a medium thecate dinoflagellate) and *P. fraudulenta* (as a model diatom, possessing a silica frustule). Before DNA extraction, a mechanical disruption step with a bead beater is commonly used to break microalgae (Eckford-Soper and Daugbjerg 2015a; Erdner et al. 2010; Fawley and Fawley 2004; Yuan et al. 2015). The BIM method was qualitatively tested with and without a bead-beating step, showing that cells of some species (*O. ovata* and *P. fraudulenta*) were easily disrupted with the lysis buffer before any mechanical homogenization. However, DNA yield substantially increased after bead beating, indicating that some cells had not been broken with only the lysis buffer. On the other hand, DNA from *K. veneficum* was successfully extracted only when the BIM method was used in conjunction with bead beating. It is unclear why *K. veneficum* was so difficult to lyse and this issue should be further investigated. Our results concluded that the BIM method, with bead beating included, provided a higher recovery yield over the traditional PCI method. To assess the feasibility of applying the BIM method in downstream quantitative

applications, calibration curves for *K. veneficum* and *K. armiger* were constructed and compared with those obtained with the PCI method. The efficiencies obtained reflect not only the quality of the DNA extraction, but also the performance of the primers. Although efficiencies obtained using the PCI method (~100%) were higher than the BIM method (~70%), the latter provided a strong linear regression over a wide dynamic range of 5 orders of magnitude. Similar efficiencies were observed with *P. fraudulenta* and *O. ovata* using the BIM method (data not shown). Additionally, similar slopes have been reported for *K. veneficum* (Eckford-Soper and Daugbjerg 2015a). As previously mentioned, DNA extracted by the BIM method showed an absorbance peak at 230 nm. This peak could be due to the presence of some slight amount of carry-over from a component of the BWB solution and could affect downstream qPCR assays. In order to assess the potential presence of inhibitors, DNA dilutions were tested. However, efficiency did not increase with increasing dilutions. Despite this limitation, there are several advantages of the BIM method over traditional DNA extraction methods: (i) ease and simplicity (it requires fewer steps), (ii) versatility (it has been successfully applied to different microalgae, and it is likely to work with many other kinds of microalgae), (iii) low exposure to hazardous materials, (iv) no specialised equipment (the exception being a bead beater, although small hand-held versions of such devices do exist), (v) low cost and time requirement (it takes less than 4 min), and (vi) applicability for accurate quantifications.

An objective of this study was to develop a qPCR method for application to environmental samples. Seawater samples collected at four sites of Alfacs Bay during the winter of 2017 were analysed by light microscopy and the qPCR assay. Light microscopy examination of the samples revealed a phytoplankton community with a high species diversity including important toxic or potentially toxic species, dominated by *Pseudo-nitzschia* spp., *A. minutum* and *Karlodinium* spp., in decreasing order of abundance (Online Resource 3). The occurrence of *Karlodinium* spp. was detected in all seawater samples, but at a relatively lower concentration (<20,000 cells L^{-1}) than in blooms, and well below the warning level (200,000 cells L^{-1}). The last *Karlodinium* spp. bloom episode reported by the local monitoring program took place in the winter of 2000 and reached 13,000,000 cells L^{-1} (Fernandez-Tejedor et al. 2004), although high densities (> 1,000,000 cells L^{-1}) were also reported in 2003 and 2007 (ICES-IOC 2004; ICES-IOC 2007). Higher abundances have been regularly found at the bottom layers of the bay, probably due to the adaptation of *Karlodinium* spp. to low-light conditions and/or sedimentation processes (Garces et al. 1999). However, in this work, abundances were higher at surface waters than at 5.5 m depth, which could be explained by the strong wind reported days before the sampling, which favoured water mixing. Based on species-specific qPCR analysis, co-existence of both species was found in all samples where *Karlodinium* spp. was detected. These results agree with those reported by Garces et al. (2006), who documented that both *Karlodinium* species co-occurred in a single bloom in Alfacs Bay.

When comparing the two analytical techniques, although qPCR results were 2.8-fold higher than the Uthermöl counts, a strong correlation ($R^2 = 0.936$; $p < 0.0001$) was observed, which indicated the capacity of the qPCR to detect and quantify *Karlodinium* species in environmental samples. Only one sample displayed a significant difference in the cell densities, which coincided with the lowest abundance value detected by light microscopy. Discrepancies between light microscopy and qPCR techniques to enumerate microalgae have been widely reported in the literature and several explanations have been proposed, the most documented being the rDNA gene copy number variation (Casabianca et al. 2014; Eckford-Soper and Daugbjerg 2016; Galluzzi et al. 2010; Nishimura et al. 2016; Vanderesa et al. 2012). The rDNA gene content has

been observed to change throughout the growth phase of microalgae cultures (Eckford-Soper and Daugbjerg 2016; Galluzzi et al. 2010; Perini et al. 2011) and this phenomenon is also expected to happen in nature during the different stages of a phytoplankton bloom. Different approaches have been used to construct calibration curves for the detection of microalgae using molecular methods, such as the use of dilutions of plasmids containing the cloned target sequence or cells. Although it is easy to work with plasmids (Galluzzi et al. 2008; Galluzzi et al. 2004; Yuan et al. 2012; Zhang et al. 2016), the potential variation in the copy number of rDNA genes could hamper the reliability and accuracy of the quantification method (Galluzzi et al. 2010; Perini et al. 2011). On the other hand, some authors have suggested that the use of cell dilutions is more likely to represent an average copy number per cell, without relying on the determination of the exact copy number per genome (Andree et al. 2011). Although standard curves based on cell dilutions have sometimes provided better accuracy (Andree et al. 2011; Eckford-Soper and Daugbjerg 2015a), other works have pointed out some discordant results (Perini et al. 2011). Some environmental factors, such as the daily light cycle or the type of nutrition, are tightly coupled with the cell cycle and/or the type of reproduction (Garces et al. 1999), and may lead to variation in rDNA content of natural samples when compared with cultured samples (Galluzzi et al. 2004; Perini et al. 2011). Moreover, differences in gene copy number among isolates/strains of the same species have been reported (Galluzzi et al. 2010; Perini et al. 2011). With all these factors taken into account, qPCR is likely to over- or under-estimate abundances depending on whether the population as a whole contains more or fewer copies of rDNA per cell than the cultures used to construct the standard curve. Perini et al. (2011) described the use of environmental samples to construct calibration curves (their 'gold standard') in order to normalize the variability of the rDNA copy number between natural populations and cultures. Although rDNA genes are the most reported markers for species-level differentiation (Shao et al. 2004), the rDNA gene copy number variation has shown to complicate the development of a quantification method (Andree et al. 2011; Casabianca et al. 2014; Galluzzi et al. 2010). One way to overcome this problem could be to use the rDNA gene marker together with a control and invariant gene. However, although feasible, it implies longer and more tedious assay protocols than the one proposed herein.

Another explanation regarding discrepancies between light microscopy and qPCR concerns the presence of species morphologically similar to the target in the phytoplankton community, which can easily lead to misidentification using light microscopy (Galluzzi et al. 2004). Even for a highly trained taxonomist, *Karlodinium* spp. identification is a highly skilled task because it resembles other small dinoflagellates. In this study, *Gymnodinium* spp. and *Heterocapsa* spp. were found in natural samples by light microscopy, and an accurate *Karlodinium* spp. identification was extremely difficult. It is important to mention that ambiguous cells were not counted as *Karlodinium* spp. Therefore, this may have contributed to an underestimation of cell abundance using light microscopy. It is important to note that the presence of non-target microalgae species in the natural samples, even at high densities, did not interfere in the qPCR assay, proving again its high specificity.

Phytoplankton monitoring is regulated by the EU legislation (EC 854/2004), which states that monitoring programs must periodically sample for toxic phytoplankton at shellfish growing areas. The monitoring using microscopy includes the Utermöhl cell-counting method to monitor microalgae presence. The qPCR assay developed in this study offers advantages over the traditional microscopy examination. It provides useful information on the abundance of the individual *Karlodinium* species found in Alfacs Bay. Additionally, it is relatively inexpensive, fast (it takes less than 2 hours,

including the DNA extraction) and allows high-throughput sample analysis. Finally, although it has a lower sensitivity (2,000 cells L^{-1}) compared to the Utermöhl method (60 cells L^{-1} (EN 15204:2006) with an error higher than 100% (Edler and Elbrächter 2010)), it allows the quantification of *Karlodinium* species below the warning threshold of 200,000 cells L^{-1}. It is important to note that this quantification limit could be reduced by centrifuging a larger sample volume or reducing the volume of the elution buffer.

To confirm the applicability of the molecular assay developed herein, more data from field samples are necessary to properly assess the 2.8-fold correspondence between the two techniques. This overestimation is another area for further investigation to understand the effect of several factors present in natural samples that might contribute to gene content variability. Additionally, primer specificity against other recently reported *Karlodinium* species should be examined in detail to apply the assay to areas where *Karlodinium* species other than *K. armiger* and *K. veneficum* may be present. Future work is also required to improve the efficiency of the BIM method. Nevertheless, this method meets the requirements for *in-situ* sampling on a ship or on the shore. Combination of this method together with qPCR mobile devices, such as the one commercialised by Biomeme Inc., or biosensors is highly desirable, and will pave the way towards the deployment of in-field diagnostic tools for microalgae monitoring.

In conclusion, this study describes the first molecular assay for the identification, discrimination and quantification of multiple genotypes of the two *Karlodinium* species, *K. armiger* and *K. veneficum*, commonly co-occurring in Alfacs Bay. Accurate species-specific identification and quantification is important because these two species produce distinct toxins, and this poses different risks to marine organisms and the marine-based economy, and because they cannot be differentiated using light microscopy. Another significant finding of this study is the implementation of a rapid DNA extraction method, which considerably reduces the assay time. The qPCR assay developed in this study is a promising new tool for monitoring the cell abundance and dynamics of these two *Karlodinium* species in Alfacs Bay.

Acknowledgements

The authors acknowledge financial support from the Ministerio de Economía, Industria y Competitividad through the SEASENSING (BIO2014-56024-C2-2-R) and PURGA DE MAR (IPT-2011-1707-310000) projects. The authors also acknowledge support from CERCA Programme/Generalitat de Catalunya. Anna Toldrà acknowledges IRTA-Universitat Rovira i Virgili-Banco Santander for her PhD grant (2015PMF-PIPF-67). Authors would like to thank Núria Orra and Rosa Fibla for the initial development of the qPCR methodology as well as María Rey for field samples counting by optical microscopy and her help on culturing of microalgae, José Luis Costa for collecting *Karlodinium* samples at Alfacs Bay and Josep Fumadó for kindly helping to test the environmental samples by qPCR.

References

AlgaeBase http://www.algaebase.org/search/species/; searched on 26 February 2018

Andree KB, Fernandez-Tejedor M, Elandaloussi LM, Quijano-Scheggia S, Sampedro N, Garces E, Camp J, Diogene J (2011) Quantitative PCR coupled with melt curve analysis for detection of selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the Northwestern Mediterranean Sea. Appl Environ Microbiol 77 (5):1651-1659

Battocchi C, Totti C, Vila M, Maso M, Capellacci S, Accoroni S, Rene A, Scardi M, Penna A (2010) Monitoring toxic microalgae *Ostreopsis* (dinoflagellate) species in coastal waters of the Mediterranean Sea using molecular PCR-based assay combined with light microscopy. Mar Pollut Bull 60 (7):1074-1084

Berge T, Poulsen LK, Moldrup M, Daugbjerg N, Hansen PJ (2012) Marine microalgae attack and feed on metazoans. Isme J 6 (10):1926-1936

Bergholtz T, Daugbjerg N, Moestrup O, Fernandez-Tejedor M (2006) On the identity of *Karlodinium veneficum* and description of *Karlodinium armiger* sp nov (Dinophyceae), based on light and electron microscopy, nuclear-encoded LSU rDNA, and pigment composition. J Phycol 42 (1):170-193

Casabianca S, Perini F, Casabianca A, Battocchi C, Giussani V, Chiantore M, Penna A (2014) Monitoring toxic *Ostreopsis* cf. *ovata* in recreational waters using a qPCR based assay. Mar Pollut Bull 88 (1-2):102-109

Daugbjerg N, Hansen G, Larsen J, Moestrup O (2000) Phylogeny of some of the major genera of dinoflagellates based on ultrastructure and partial LSU rDNA sequence data, including the erection of three new genera of unarmoured dinoflagellates. Phycologia 39 (4):302-317

EC No. 854/2004 of the European Parliament and of the Council laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption

Eckford-Soper LK, Daugbjerg N (2015a) Development of a multiplex real-time qPCR assay for simultaneous enumeration of up to four marine toxic bloom-forming microalgal species. Harmful Algae 48:37-43

Eckford-Soper LK, Daugbjerg N (2015b) Examination of six commonly used laboratory fixatives in HAB monitoring programs for their use in quantitative PCR based on Taqman probe technology. Harmful Algae 42:52-59

Eckford-Soper LK, Daugbjerg N (2016) A quantitative real-time PCR assay for identification and enumeration of the occasionally co-occurring ichthyotoxic *Pseudochattonella farcimen* and *P.verruculosa* (Dictyochophyceae) and analysis of variation in gene copy numbers during the growth phase of single and mixed cultures. J Phycol 52 (2):174-183

Edler L, Elbrächter M (2010) The Utermöhl method for quantitative phytoplankton analysis. In: Karlson B, Cusack C, Bresnan E (eds) Microscopic and molecular methods for quantitative phytoplankton analysis. UNESCO, Paris, pp 13-20

EN 15204:2006, Water quality - Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique)

Erdner DL, Percy L, Keafer B, Lewis J, Anderson DM (2010) A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep-Sea Res Part II-Top Stud Oceanogr 57 (3-4):279-287

Fawley MW, Fawley KP (2004) A simple and rapid technique for the isolation of DNA from microalgae. J Phycol 40 (1):223-225

Fernandez-Tejedor M, Soubrier-Pedreno MA, Furones MD (2004) Acute LD50 of a *Gyrodinium corsicum* natural population for *Sparus aurata* and *Dicentrarchus labrax*. Harmful Algae 3 (1):1-9

Galluzzi L, Bertozzini E, Penna A, Perini F, Garces E, Magnani M (2010) Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. J Appl Phycol 22 (1):1-9

Galluzzi L, Bertozzini E, Penna A, Perini F, Pigalarga A, Graneli E, Magnani M (2008) Detection and quantification of *Prymnesium parvum* (Haptophyceae) by real-time PCR. Lett Appl Microbiol 46 (2):261-266

Galluzzi L, Penna A, Bertozzini E, Vila M, Garces E, Magnani M (2004) Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a dinoflagellate). Appl Environ Microbiol 70 (2):1199-1206

Garces E, Delgado M, Maso M, Camp J (1999) *In situ* growth rate and distribution of the ichthyotoxic dinoflagellate *Gyrodinium corsicum* Paulmier in an estuarine embayment (Alfacs Bay, NW Mediterranean Sea). J Plankton Res 21 (10):1977-1991

Garces E, Fernandez M, Penna A, Van Lenning K, Gutierrez A, Camp J, Zapata M (2006) Characterization of NW Mediterranean *Karlodinium* spp. (Dinophyceae) strains using morphological, molecular, chemical, and physiological methodologies. J Phycol 42 (5):1096-1112

Godhe A, Asplund ME, Harnstrom K, Saravanan V, Tyagi A, Karunasagar I (2008) Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR. Appl Environ Microbiol 74 (23):7174-7182

Greco M, Saez CA, Bitonti MB (2014) A simple and effective method for high quality co-extraction of genomic DNA and total RNA from low biomass *Ectocarpus siliculosus*, the model brown alga. PLoS One 9 (7):1

Guillard RRL (1973) Division rates. In: Stein J (ed) Culture methods and growth measurements. Cambridge University Press, Cambridge, p 289

Guillard RRL, Hargraves PE (1993) *Stichochrysis immobilis* is a diatom, not a chyrsophyte. Phycologia 32 (3):234-236

Guillard RRL, Ryther JH (1962) Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229-239

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symposium Series 41:95-98

Hosoi-Tanabe S, Sako Y (2005) Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by real-time PCR assay. Mar Biotechnol 7 (5):506-514

ICES-IOC Working Group on Harmful Algal Bloom Dynamics (WGHABD) report (2004), ICES Oceanography Committee, Corsica, ICES CM 2004/C:08

ICES-IOC Working Group on Harmful Algal Bloom Dynamics (WGHABD) report (2008). ICES CM 2008/OCC:03, ICES Oceanography Committee, Galway, ICES CM 2008/OCC:03

Jeong HJ, Jang SH, Moestrup O, Kang NS, Lee SY, Potvin E, Noh JH (2014) *Ansanella granifera* gen. et sp nov (Dinophyceae), a new dinoflagellate from the coastal waters of Korea. Algae 29 (2):75-99

Kamikawa R, Nagai S, Hosoi-Tanabe S, Itakura S, Yamaguchi M, Uchida Y, Baba T, Sako Y (2007) Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. Harmful Algae 6 (3):413-420

Kavanagh S, Brennan C, O'Connor L, Moran S, Salas R, Lyons J, Silke J, Maher M (2010) Real-time PCR detection of *Dinophysis* species in Irish coastal waters. Mar Biotechnol 12 (5):534-542

Nishimura T, Hariganeya N, Tawong W, Sakanari H, Yamaguchi H, Adachi M (2016) Quantitative PCR assay for detection and enumeration of ciguatera-causing dinoflagellate *Gambierdiscus* spp. (Gonyaulacales) in coastal areas of Japan. Harmful Algae 52:11-22

Park TG, Park YT, Lee Y (2009) Development of a SYT09 based real-time PCR probe for detection and quantification of toxic dinoflagellate *Karlodinium veneficum* (Dinophyceae) in environmental samples. Phycologia 48 (1):32-43

Penna A, Antonella P, Galluzzi L, Luca G (2013) The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. Environmental science and pollution research international 20 (10):6851-6862

Penna A, Battocchi C, Capellacci S, Fraga S, Aligizaki K, Lemee R, Vernesi C (2014) Mitochondrial, but not rDNA, genes fail to discriminate dinoflagellate species in the genus *Ostreopsis*. Harmful Algae 40:40-50

Perini F, Casabianca A, Battocchi C, Accoroni S, Totti C, Penna A (2011) New approach using the real-time PCR method for estimation of the toxic marine dinoflagellate *Ostreopsis* cf. *ovata* in marine environment. PLoS One 6 (3):9

Place AR, Bowers HA, Bachvaroff TR, Adolf JE, Deeds JR, Sheng J (2012) *Karlodinium veneficum*-The little dinoflagellate with a big bite. Harmful Algae 14:179-195

Raho N, Rodriguez F, Reguera B, Marin I (2013) Are the mitochondrial *cox1* and *cob* genes suitable markers for species of *Dinophysis* Ehrenberg? Harmful Algae 28:64-70

Rasmussen SA, Binzer SB, Hoeck C, Meier S, de Medeiros LS, Andersen NG, Place A, Nielsen KF, Hansen PJ, Larsen TO (2017) Karmitoxin: an amine-containing polyhydroxy-polyene toxin from the marine dinoflagellate *Karlodinium armiger*. J Nat Prod 80 (5):1287-1293

Ririe KM, Rasmussen RP, Wittwer CT (1997) Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Anal Biochem 245 (2):154-160
Sambrook J, Fritsch EF, Maniatis T (eds) (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York
Sato S, Nishimura T, Uehara K, Sakanari H, Tawong W, Hariganeya N, Smith K, Rhodes L, Yasumoto T, Taira Y, Suda S, Yamaguchi H, Adachi M (2011) Phylogeography of *Ostreopsis* along West Pacific coast, with special reference to a novel clade from Japan. PLoS One 6 (12):14
Shao P, Chen YQ, Zhou H, Yuan J, Qu LH, Zhao D, Lin YS (2004) Genetic variability in *Gymnodiniaceae* ITS regions: implications for species identification and phylogenetic analysis. Mar Biol 144 (2):215-224
Throndsen J (1978) Preservation and storage. In: Sournia A (ed) Phytoplankton Manual. UNESCO, Paris, pp 69-74
Utermöhl H (1958) Zur Vervollkomnungder quantitativen Phytoplankton-Methodik. Mitt.Int.Ver.Ther.Angew.Limnol. 9:1-38
Van Wagoner RM, Deeds JR, Satake M, Ribeiro AA, Place AR, Wright JLC (2008) Isolation and characterization of karlotoxin 1, a new amphipathic toxin from *Karlodinium veneficum*. Tetrahedron Lett 49 (45):6457-6461
Vandersea MW, Kibler SR, Holland WC, Tester PA, Schultz TF, Faust MA, Holmes MJ, Chinain M, Litaker RW (2012) Development of semi-quantitative PCR assays for the detection and enumeration of *Gambierdiscus* species (Gonyaulacales, Dinophyceae). J Phycol 48 (4):902-915
Yuan J, Li MZ, Lin SJ (2015) An improved DNA extraction method for efficient and quantitative recovery of phytoplankton diversity in natural assemblages. PLoS One 10 (7):18
Yuan J, Mi TZ, Zhen Y, Yu ZG (2012) Development of a rapid detection and quantification method of *Karenia mikimotoi* by real-time quantitative PCR. Harmful Algae 17:83-91
Zhang CY, Chen GF, Zhou J, Wang YY, Lu DD (2016) Development of a quantitative PCR for detection and quantification of *Prorocentrum donghaiense*. J Appl Phycol 28 (3):1683-1693
Zhang H, Litaker W, Vandersea MW, Tester P, Lin SJ (2008) Geographic distribution of *Karlodinium veneficum* in the US east coast as detected by ITS-ferredoxin real-time PCR assay. J Plankton Res 30 (8):905-922

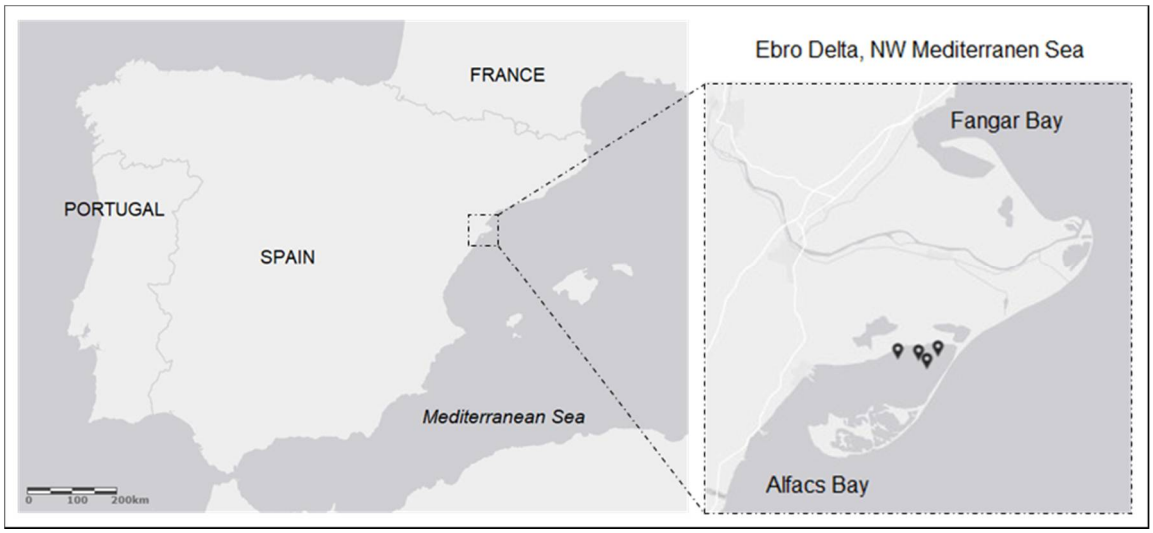


Fig. 1 Locations of the sampling in Alfacs Bay, NW Mediterranean Sea.

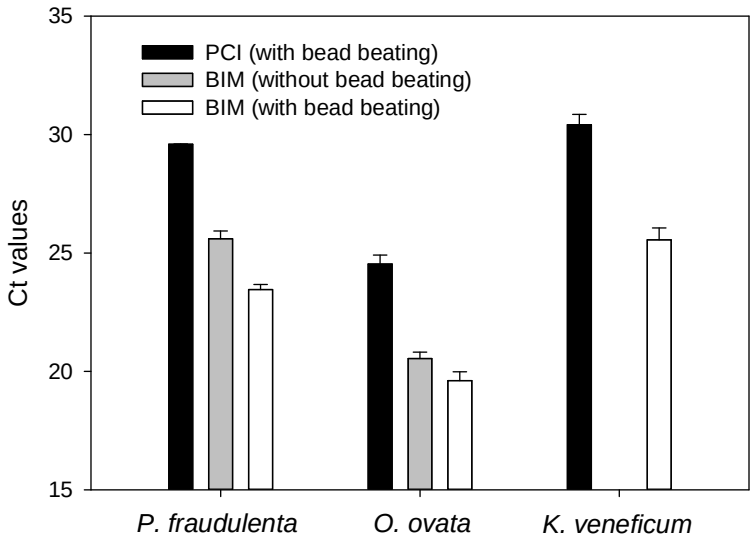


Fig. 2 Comparison of different methods for DNA extraction. Extraction efficiency was assessed by qPCR using 10^4 cells. All results are means of triplicates samples. Error bars represent standard deviation of the Ct values.

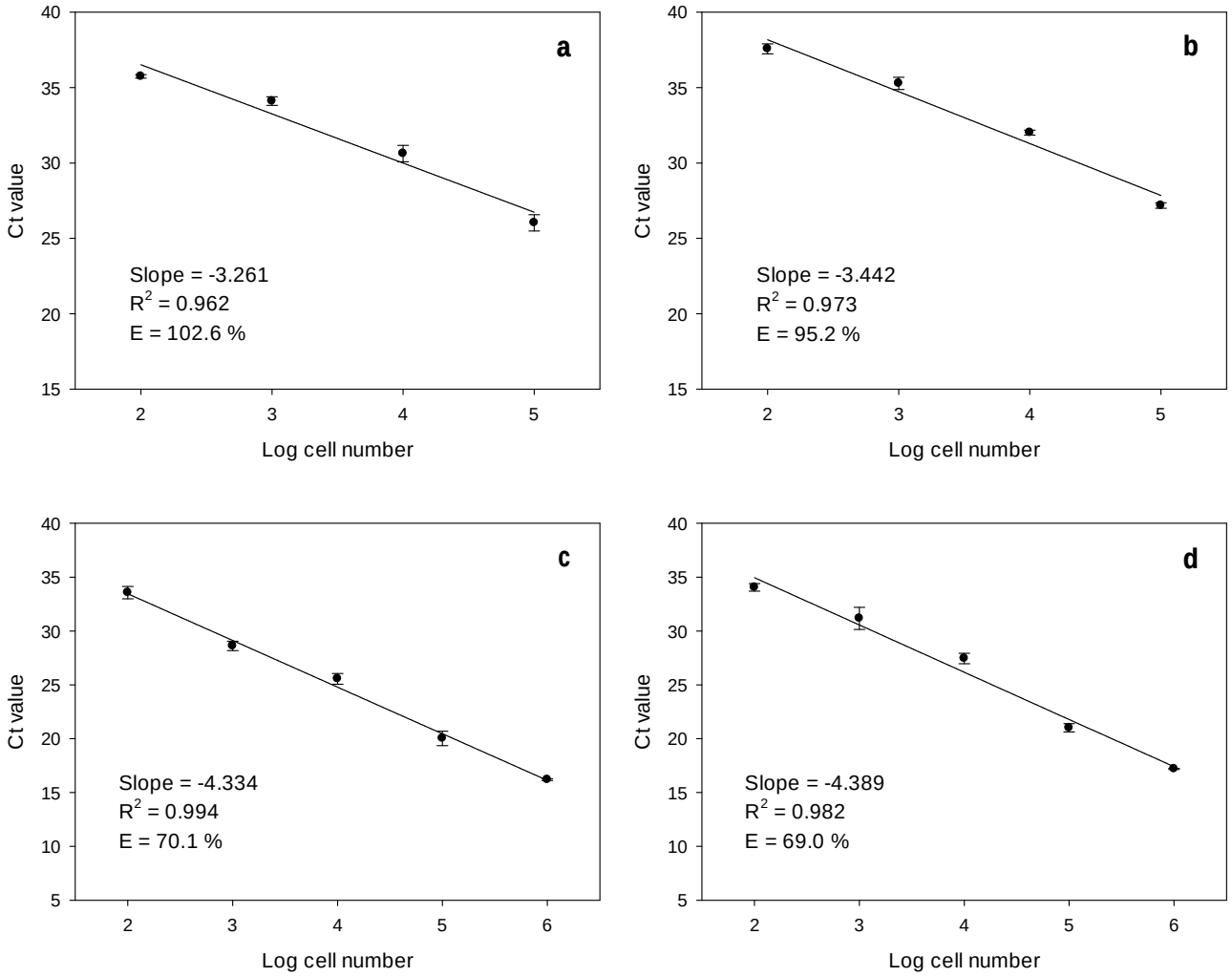

a
Slope = -3.261
R2 = 0.962
E = 102.6 %
b
Slope = -3.442
R2 = 0.973
E = 95.2 %
c
Slope = -4.334
R2 = 0.994
E = 70.1 %
d
Slope = -4.389
R2 = 0.982
E = 69.0 %
Ct value
Log cell number

Fig. 3 Standard curves obtained by the correlation between known cultured cell numbers and Ct values. Standard curves were obtained for each species-specific qPCR assay (**a**, **c**: *K. veneficum*; **b**, **d**: *K. armiger*) and each extraction method (**a**, **b**: PCI method; **c**, **d**: BIM method). All results are means of triplicates samples. Error bars represent the standard deviation of the Ct values.

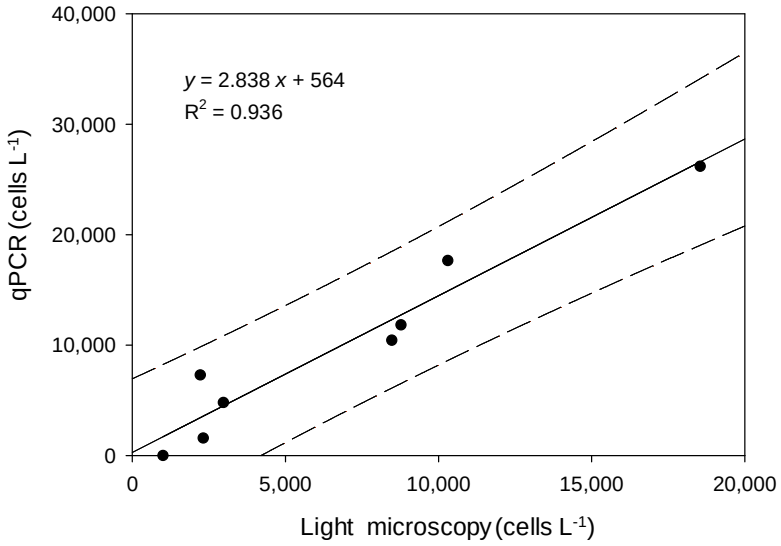


Fig. 4 Comparison of qPCR and light microscopy results for abundances of *Karlodinium* spp. in natural seawater samples. Dashed lines represent the prediction intervals of 95%.

Table 1 Microalgae species used in this study.

Species	Sampling location and year	Source	Strain	GenBank accession number	Primers
Karlodinium veneficum	Alfacs Bay, Spain, 2000	Fernández-Tejedor	IRTA-SMM-00-01	MG642757	MicroSSU/DinoE (Andree et al. 2011)
Karlodinium armiger	Alfacs Bay, Spain, 2000	Fernández-Tejedor	K-0668	MG642758	MicroSSU/DinoE (Andree et al. 2011)
Ostreopsis ovata	Fangar Bay, Spain, 2015	This study	IRTA-SMM-15-13	MG551865	ITSA/ITSB (Sato et al. 2011)
Pseudo-nitzschia fraudulenta	Vilanova, Spain, 2016	This study	IRTA-SMM-16-02	MG551866	MicroSSU/DinoE (Andree et al. 2011)
Ansanella granifera	Alfacs Bay, Spain, 2015	This study	IRTA-SMM-16-43	MG551867	MicroSSU/DinoE (Andree et al. 2011)

Table 2 Oligonucleotide primers targeting the ITS1 rDNA region of genus and species of *Karlodinium*.

Species and/or genus	Primer	Sequence (5'-3')	Amplicon size (bp)	Primer location
Karlodinium spp.	KarIo20	ACATCCAACCATYTCACTGTGAAC	136/149	ITS1 (sense)
Karlodinium veneficum	KaV160	ATAGCTTCGCAGACAAAGGTGAATC	136	ITS1 (antisense)
Karlodinium armiger	KaA160	ATAGCTTCACAGCAGAGGTTACAAC	149	ITS1 (antisense)

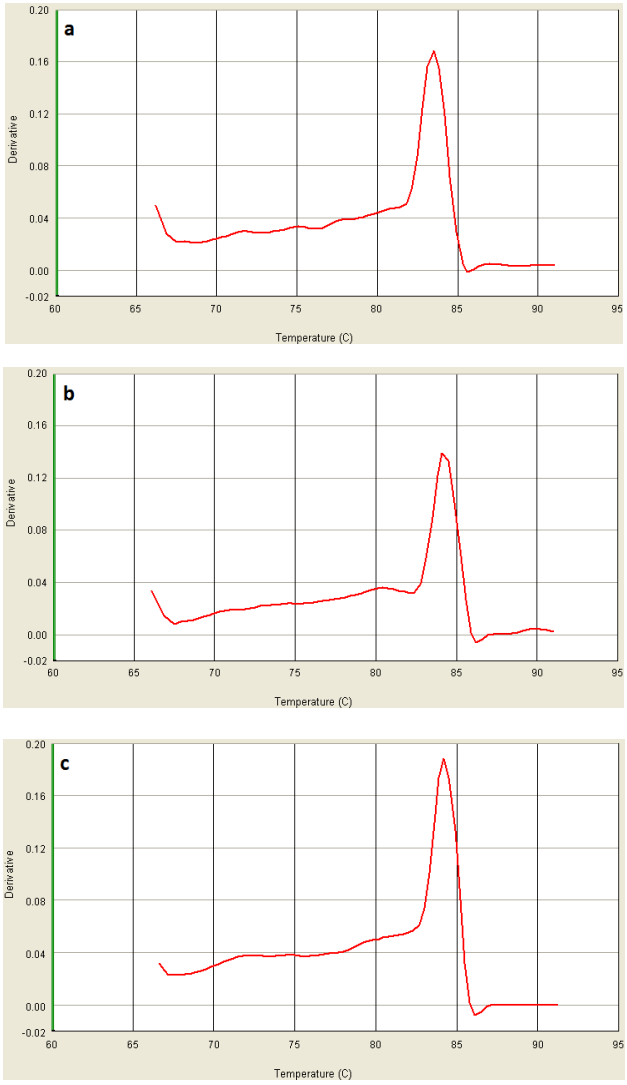
Table 3 Seawater samples (surface and bottom) collected at different stations in Alfacs Bay and results obtained using qPCR and optical microscopy. *Karlodinium* mean abundance was determined in triplicates ± standard deviation. n.d. not detected.

Sample	Geographic coordinates	Total cell number for the two *Karlodinium* species (cells L^{-1})		Number of cells counted using each species-specific assay (cells L^{-1})	
		Microscopy	qPCR	*K. veneficum*	*K. armiger*
Station 1 (S)	40°37'22.9"N 0°42'25.5"E	10,305	35,314	29,505 ± 847	5,809 ± 767
Station 1 (B)	40°37'22.9"N 0°42'25.5"E	1,000	n.d.	n.d.	n.d.
Station 2 (S)	40°37'34.2"N 0°43'31.4"E	8,772	23,647	11,919 ± 623	11,729 ± 2,536
Station 2 (B)	40°37'34.2"N 0°43'31.4"E	2,977	9,582	7,282 ± 1,008	2,301 ± 41
Station 3 (S)	40°36'59.9"N 0°42'55.6"E	8,473	20,881	19,184 ± 3,030	1,697 ± 448
Station 3 (B)	40°36'59.9"N 0°42'55.6"E	2,220	14,574	11,292 ± 1,460	3,282 ± 265
Station 4 (S)	40°37'28.9"N 0°41'07.7"E	18,549	52,390	26,859 ± 4,701	25,532 ± 4,014
Station 4 (B)	40°37'28.9"N 0°41'07.7"E	2,320	3,144	2,415 ± 2,226	729 ± 117

Online Resource 1 ITS1-5.8S-ITS2 rDNA *Karlodinium* sequences used for primers design.

Species	Source	Strain	GenBank accession number
K. veneficum	This study	IRTA-SMM-12-03	MG642759
K. veneficum	This study	IRTA-SMM-12-04	MG642760
K. veneficum	This study	IRTA-SMM-12-05	MG642761
K. veneficum	This study	IRTA-SMM-12-06	MG642762
K. veneficum	This study	IRTA-SMM-12-07	MG642763
K. veneficum	This study	IRTA-SMM-12-08	MG642764
K. veneficum	This study	IRTA-SMM-12-09	MG642765
K. veneficum	This study	IRTA-SMM-12-10	MG642766
K. veneficum	This study	IRTA-SMM-12-12	MG642767
K. veneficum	This study	IRTA-SMM-12-13	MG642768
K. veneficum	This study	IRTA-SMM-12-14	MG642769
K. veneficum	This study	IRTA-SMM-12-15	MG642770
K. veneficum	This study	IRTA-SMM-12-16	MG642771
K. veneficum	This study	IRTA-SMM-12-17	MG642772
K. veneficum	This study	IRTA-SMM-12-20	MG642773
K. veneficum	This study	IRTA-SMM-12-21	MG642774
K. veneficum	This study	IRTA-SMM-12-22	MG642775
K. veneficum	This study	IRTA-SMM-12-23	MG642776
K. veneficum	This study	IRTA-SMM-12-24	MG642777
K. veneficum	This study	IRTA-SMM-12-25	MG642778
K. veneficum	This study	IRTA-SMM-12-26	MG642779
K. veneficum	This study	IRTA-SMM-12-28	MG642780
K. veneficum	This study	IRTA-SMM-12-30	MG642781
K. veneficum	This study	IRTA-SMM-12-31	MG642782
K. veneficum	GenBank	GC-1	AJ534656
K. veneficum	GenBank	GC-5	AJ557028
K. veneficum	GenBank	GC-8	AJ557027
K. veneficum (formerly *K. micrum*)	This study	IRTA-SMM-12-36	MG642783
K. veneficum (formerly *K. micrum*)	GenBank	KM1 CSIC-1	AJ557025
K. veneficum (formerly *K. micrum*)	GenBank	CCMP 415	AJ557026
K. armiger	GenBank	GC-2	AM184204
K. armiger	GenBank	GC-3	AM184205
K. armiger	GenBank	GC-7	AJ557024

Online Resource 2 Melting curves for *Karlodinium* species. **a**: *K. veneficum* (strain IRTA-SMM-00-01); **b**: *K. armiger* (strain K-0668); **c**: *K. veneficum* (strain CCMP 415, formerly *K. micrum*).



Online Resource 3 Microalgae cell abundances (determined by microscopy) of seawater samples (surface and bottom) collected at different stations in Alfacs Bay and results for toxic and potentially toxic species obtained using optical microscopy. Toxic species responsible for paralytic (PSP), diarrhetic (DSP) and amnesic (ASP) shellfish poisoning.

Sample	Geographic coordinates	PSP	DSP	ASP	*Karlodinium* spp. (cells L^{-1})
		Alexandrium minutum (cells L^{-1})	*Dinophysis sacculus* (cells L^{-1})	*Pseudo-nitzchia* spp. (cells L^{-1})	
Station 1 (S)	40°37'22.9"N 0°42'25.5"E	1,832	80	33,507	10,305
Station 1 (B)	40°37'22.9"N 0°42'25.5"E	2,061	20	18,360	2,061
Station 2 (S)	40°37'34.2"N 0°43'31.4"E	4,896	40	1,224	8,772
Station 2 (B)	40°37'34.2"N 0°43'31.4"E	5,038	0	5,967	2,977
Station 3 (S)	40°36'59.9"N 0°42'55.6"E	4,122	0	30,753	8,473
Station 3 (B)	40°36'59.9"N 0°42'55.6"E	1,374	40	11,475	1,374
Station 4 (S)	40°37'28.9"N 0°41'07.7"E	1,374	120	106,029	18,549
Station 4 (B)	40°37'28.9"N 0°41'07.7"E	1,145	80	81,702	1,374