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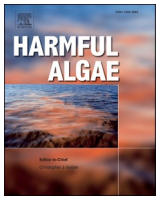

Molecular monitoring of *Dinophysis* species assemblage in mussel farms in the Northwestern Adriatic Sea

Giorgia Ravera [a,b], Monica Cangini [c], Samuela Capellacci [b,d,e], Sonia Dall'Ara [c], Giuseppe Prioli [f], Mauro Marini [e,g], Elena Manini [g], Antonella Penna [b,d,e], Silvia Casabianca [b,d,e,*]

[a] *Department of Pure and Applied Sciences, University of Urbino, Campus Enrico Mattei, Via Ca le Suore, 2/4, 61029, Urbino, Italy*
[b] *CoNISMa, National Inter-University Consortium for Marine Sciences, Piazzale Flaminio, 6, 00184, Roma, Italy*
[c] *Fondazione Centro Ricerche Marine, National Reference Laboratory for Marine Biotoxins, Viale A. Vespucci, 2, 47042 Cesenatico, Italy*
[d] *Department of Biomolecular Sciences, University of Urbino, Campus Enrico Mattei, Via Ca le Suore, 2/4 61029, Urbino, Italy*
[e] *Fano Marine Center, The Inter-Institute Center for Research on Marine Biodiversity, Resources and Biotechnologies (FMC), Viale Adriatico, 1, 61032 Fano, Italy*
[f] *M.A.R.E. S.c. a r.l., Via E. Toti, 2, 47841 Cattolica, Italy*
[g] *National Research Council (CNR), Institute for Biological Resources and Marine Biotechnologies (IRBIM), Via Largo Fiera della Pesca, 2, 60125, Ancona, Italy*

A R T I C L E I N F O

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A B S T R A C T

Several *Dinophysis* species can produce potent lipophilic toxins that pose a risk to human health when contaminated seafood is consumed, especially filter-feeding bivalve mussels. In the mussel farms of the Northwestern Adriatic Sea, seawater and seafood are regularly monitored for the presence of *Dinophysis* species and their associated toxins, but the current methodological approaches, such as light microscopy determinations, require a long time to make results available to local authorities. A molecular qPCR-based assay can be used to quantify various toxic *Dinophysis* species in a shorter timeframe. However, this approach is not currently employed in official testing activities. In this study, field samples were collected monthly or bi-weekly over one year from various mussel farms along the Northwestern Adriatic coast. The abundance of *Dinophysis* species in the seawater was determined using both traditional microscopy and qPCR assays. In addition, the concentration of lipophilic toxins for DSP in mussel flesh was quantified using LC-MS/MS focusing on the okadaic acid group. *Dinophysis* spp. site-specific single cells were isolated and analysed by qPCR yielding a mean rDNA copy number per cell of $1.21 \times 10^4 \pm 1.81 \times 10^3$. The qPCR assay gave an efficiency of 98 % and detected up to 10 copies of the rDNA target gene. The qPCR and light microscopy determinations in environmental samples showed a significant positive correlation (Spearman $r_s = 0.57$, p-value < 0.001) with a ratio of 2.24 between the two quantification methods, indicating that light microscopy estimates were generally 44.6 % lower than those obtained by the qPCR assay. The qPCR approach showed several advantages such as rapidity, sensitivity and efficiency over conventional microscopy analysis, showing its potential future role in phytoplankton monitoring under the Official Controls Regulations for shellfish.

1. Introduction

The marine dinoflagellate *Dinophysis* spp. includes several species responsible for diarrhetic shellfish poisoning (DSP), a human gastrointestinal disease, caused by the consumption of contaminated seafood (Dominguez et al., 2010; Reguera et al., 2014; Farabegoli et al., 2018). Toxins associated with DSP syndrome include okadaic acid (OA) and dinophysistoxins (DTX), both of which are responsible for protein phosphatase inhibition and are associated with diarrheic effects, and pectenotoxins (PTX), another group of non-diarrheic toxins known to cause liver damage in mice (Reguera et al., 2012; Reguera and Blanco, 2019; Gaillard et al., 2021). All these toxins can be accumulated in shellfish tissues by filtering *Dinophysis* spp. in seawater causing human disease with non-fatal symptoms such as diarrhoea, abdominal cramps, nausea and vomiting (Blanco, 2018). To date, ten globally distributed species are known to produce DSP toxins, including *D. fortii, D. acuminata, D. acuta, D. caudata, D. infundibulum, D. miles, D. norvegica, D. ovum, D. sacculus* and *D. tripos* (Reguera et al., 2012, 2014; Simões et al., 2015), and they are all responsible for DSP toxic events worldwide. In relation to this dinoflagellate behaviour, it is known that the maximum

* Corresponding author.
E-mail address: silvia.casabianca@uniurb.it (S. Casabianca).

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abundance of *Dinophysis* spp. in the phytoplankton assemblages seems to be mainly related to the stratification and stabilization of the water column that can be observed between upwelling and downwelling events, allowing cells to accumulate (Reguera et al. 2003, 2012). Moreover, increased abundance of some species has been found in areas characterized by high sea surface temperature, an apparent consequence of climate change. In particular, along the coast, the persistent warming of the seawater surface can cause a prolonged stratification period with an increase in the concentration of different *Dinophysis* species (Ninčević-Gladan et al., 2008, 2020). The ecology and ecophysiology of the genus *Dinophysis* are difficult to study due to its low abundance in marine ecosystems and its mixotrophic/heterotrophic feeding behaviour, which implies the presence of prey in culture systems with difficulties in establishing monoclonal cultures (Park et al., 2006; Reguera et al., 2024). Some authors have postulated an independence between *Dinophysis* occurrences and the concentration of major nutrients, while others reported an increased growth of *Dinophysis* associated with nutrient rich waters, either in phosphate or nitrogen (France and Mozetič, 2006).

In the Adriatic Sea, a region of the Mediterranean Sea, several species of *Dinophysis* associated with DSP events can be found throughout the year. They are most abundant during warmer periods, with peaks of abundance in early summer and early autumn, depending on the species. They also show a marked species succession. Indeed, *D. sacculus* showed high abundance in the spring-summer period, *D. acuminata* can have a long growing season from spring to autumn; *D. caudata* can be considered a summer-autumn species, while *D. fortii* reaches a maximum of concentration in October-November (Mozetič and Obal, 1995; Poletti et al., 1998; Aubry et al., 2000; France and Mozetič, 2006; Reguera et al., 2014). Since the first records of DSP outbreaks (Yasumoto et al., 1978, 1980), interest in *Dinophysis* spp. has increased due to the threat to human health and the socio-economic impacts associated with shellfish harvest closures, including in the Adriatic Sea (Escalera et al., 2012; Pistocchi et al., 2012; Zingone et al., 2021). In June 1989, the first DSP event attributed to the consumption of contaminated shellfish was recorded along the coast of Emilia-Romagna and Marche, in the Northwestern Adriatic Sea (Boni et al., 1992). In a study of 14 years (2006–2019) of chemical monitoring for DSP toxins, these regions had the highest number of records for the analysis of seafood samples (3,502 and 16,859, respectively), with the greatest impact represented by okadaic acid (OA) toxins in 146 and 509 samples, respectively (Accoroni et al., 2024). The presence of these toxins led to repeated closures of shellfish farms due to food safety concerns. As required by European Union legislation (Regulation EC 853/2004), if the accumulation exceeds the regulatory limit (160 μg OA eq/kg shellfish tissue), shellfish harvesting is suspended until toxins levels return to safe concentrations. This poses a serious risk to public health and significant economic losses to the aquaculture sector. It is therefore necessary to implement effective and appropriate monitoring programmes for both seawater and mollusc stocks.

The identification of *Dinophysis* species is based on morphometric characteristics observed by light microscopy (LM), although some species may be difficult to distinguish due to the intraspecific variability in morphological characteristics. For example, in the "*D. acuminata* complex" (consisting of *D. acuminata, D. sacculus* and *D. ovum*), intermediate forms with very similar shape and size may exists (Park et al., 2019; Séchet et al., 2021). Nevertheless, LM is the most commonly used identification and counting method in monitoring programmes (Anon., 2019; Fernández et al., 2019) to control the occurrence and abundance of *Dinophysis* spp. and to describe species dynamics (Ninčević-Gladan et al., 2008; Velo-Suárez et al., 2014). The analysis of DSP toxins in shellfish samples is usually performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; Quilliam et al., 2001) according to the method provided by the European legislation (Regulation EC 2074/2005). Therefore, light microscopy using optical inverted microscopy and LC-MS/MS are the standard monitoring tools to determine the abundance of *Dinophysis* species in seawater and toxin concentrations in shellfish, respectively. Despite accurate estimates of both *Dinophysis* spp. abundance in seawater and toxin concentrations in mussels are carried out, numerous investigations revealed a weak correlation between these two parameters (Alves-de Souza et al., 2014; Bazzoni et al., 2018; Fernández et al., 2019). This can be due to many factors, such as the high variability of intracellular toxins in *Dinophysis* spp., the filtration rates of bivalve molluscs and the availability of alternative food sources for bivalve molluscs (Pizarro et al., 2009; Reguera et al., 2012, 2014; Blanco, 2018). All these factors may influence the time lag between counting and the occurrence of *Dinophysis* spp. in seawater and seafood contamination, making shellfish poisoning difficult to predict. In addition, LM observations typically require time-consuming procedures for target species identification and processing of large numbers of samples, which can be foreseen during monitoring programme activities, resulting in a potential delayed alarm.

It has long been recognized that molecular tools, such as qPCR approaches, demonstrated high sensitivity and provide rapid results for the monitoring and detection of toxic algal species compared to other standard methods providing an early warning for the management of harmful algal proliferations. Many different DNA-based techniques are being developed that combine a nucleic acid amplification step and a detection step, such as standard PCR-based methods and isothermal amplification tools (Loop Mediated Isothermal Amplification, LAMP-; Rolling Circle Amplification, RCA-based and others), coupled with microarray, lateral flow, bot-blot array, electrochemical biosensor and real-time fluorescence (Toldrà et al., 2020). Moreover, the design of specific primers targeting the phytoplankton species of interest allows the molecular identification and enumeration of the target phytoplankton species in field samples and can be applied to different sample matrices such as seawater, sediments, and seafood organisms, which may filter toxic species. Due to their sensitive and rapid applications, the use of molecular tools in multi-species monitoring activities is increasing worldwide (Penna and Galluzzi, 2013; Casabianca et al., 2014; Zhang et al., 2014; Bowers et al., 2017; Toldrà et al., 2018a, 2018b; Murray et al., 2019; Wietkamp et al., 2019, 2020; Casabianca et al., 2020; Pearson et al., 2021). In particular, qPCR methods have become increasingly popular over the years due to their decreasing cost over time, their rapidity, and their ability to be a high-throughput method (Pearson et al., 2021).

The aim of this study was to investigate the qPCR assay as an early warning tool for the occurrence of toxic phytoplankton species in the coastal waters near shellfish farms in the Northwestern Adriatic Sea. A qPCR assay was developed to quantify different *Dinophysis* species cells in the Adriatic Sea. Water and mussel samples for toxin quantification were then collected from May 2022 to July 2023 from several sites along the Northwestern Adriatic coast. The abundance of *Dinophysis* spp. in seawater was determined using both LM and qPCR assays, and the data obtained by the two methods were compared to test the advantages of the applicability of the molecular approach.

2. Materials and methods

2.1. Study area and sampling

The study area was located on the Northwestern Adriatic coast of two Italian regions, Emilia-Romagna and Marche (Fig. S1 and Table S1). Sampling was carried out at a total of 18 mussel farms in Emilia-Romagna and at an additional station (F3000) located in front of the Foglia River transect (43,95,200 N;12,89600E) at 3000 m from the coast of Pesaro in the Marche Region.

The monitoring activity for the mussel production area is regulated by the Health Surveillance Plan on edible lamellibranch molluscs of the Emilia-Romagna Region (Emilia-Romagna, 2020) and surface seawater and mussel samples were collected monthly (winter) and bi-weekly (summer). The study was carried out over approximately one year,

from May 2022 to July 2023. In mussel farms of Emilia-Romagna, seawater column sampling was carried out with hose sampling (Reguera et al., 2016) to the maximum depth of the mussel sock. An amount of 1 L subsamples was harvested per each sampling station. Seawater sampling at station F3000 was carried out monthly, both with hose sampling to the maximum depth of 10 m and with a Niskin bottle at the thermocline level. The Northwestern Adriatic Sea is characterized by a shallow bathymetry but even with the reduced depth, a thermocline appears, mostly in the summer period (Nincevic-Gladan et al., 2015). All samples were immediately fixed with Lugol's iodine solution in dark bottles, and stored at 4 °C until microscopy and molecular analysis. Phytoplankton samples were also collected using a 10 μm mesh net and stored in dark polyethylene bottles for a few hours until single cell isolation.

Various amounts (from 1 to 3 kg) of mussels were gathered from mussel farms in the Emilia-Romagna Region at the same time and location as the water column samples, at three different depths from the sea surface to guarantee complete coverage of the mussel sock. The mussels were placed in a cool box and transferred to the laboratory for chemical analysis.

2.2. *Dinophysis* single cell isolation

Single vegetative cells of *Dinophysis* spp. were isolated from net samples by glass capillary technique using an inverted light microscope (Zeiss Axiovert 40 CFL) at 200x magnification. *Dinophysis* cells were washed twice with sterile artificial seawater, and once with sterile distilled water before being transferred to a qPCR tube containing 2 μL of water volume. Single cells were stored at −80 °C until molecular analysis.

2.3. Microscopy determination

Seawater samples (50 mL each), fixed with neutral Lugol's solution, were analyzed for the enumeration of *Dinophysis* spp. using the Utermöhl's method (Edler and Elbrachter, 2010), as described in the EU reference method (UNI EN 15204:2006), under an inverted Nikon Eclipse Ti2 microscope (Nikon, Japan). Cell counts were carried out at 200x, 400x and 1000x magnifications and the abundance was expressed as cells/L.

2.4. Molecular analysis

2.4.1. DNA extraction from single cells and field samples

Dinophysis spp. single cells were lysed using a freeze/thaw cycle protocol consisting of three cycles of freeze/thaw at −80 °C/+95 °C for 10 min each. To confirm the species identification, the 5.8S-ITS region of rDNA was amplified and sequenced using the universal primers ITSA and ITSB (Adachi et al., 1994) as described in Casabianca et al., (2020).

A total of 27 single cells were used for calculating the mean rDNA copy number per cell following the same freeze/thaw cycle protocol described above and the qPCR reaction as described below.

Three replicates of the same fixed volume of seawater processed by LM (50 mL each) were centrifuged at 4000 rpm for 10 min and the supernatant was discharged. A washing step was carried out with 5 mL of artificial seawater, followed by a second centrifugation. The supernatant was removed, and 1 mL of cell suspension was transferred to a 1.5 mL collection tube for the final centrifugation at 10,000 rpm for 10 min. Pellets were stored at −80 °C until further analysis. Frozen pellets were lysed according to Perini et al. (2011) with minor modifications. They were resuspended in 400 μL of lysis buffer (10 mM Tris–HCl [pH 8.3], 50 mM KCl, 0.5 % Nonidet P-40, 0.5 % Tween 20, 2.5 mM $CaCl_2$, 0.1 mg of proteinase K/mL), and incubated at 60 °C for 3 h with vortexing every 30 min. The samples were then incubated at 100 °C for 5 min to inactivate proteinase K and centrifuged at 12,000 rpm for 1 min. The supernatant, containing total genomic DNA was collected and stored at −80 °C until used in the qPCR assay within 3 - 4 weeks.

2.4.2. Standard curve and qPCR assay

The standard curve for the estimation of the rDNA copy number per cell and total copy number in field samples was generated using scalar dilutions of qPCR product obtained by amplifying two different pools of 5 and 10 *Dinophysis* spp. cells isolated, pooled and lysed as described above. The genus-specific primers Dacu_11F and Dacu_11R, targeting the 28S (large subunit) rDNA region (Ajani et al., 2022; Table 1), were used to amplify of the 133 base pair target sequence of *Dinophysis* spp. The specificity of the primers was checked *in silico* to exclude cross-reactivity with other phytoplankton-related taxa, confirming the specificity results obtained by Ajani et al., (2022).

Primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). After amplification, the qPCR products were visualized on a 1.8 % agarose gel and purified using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). The qPCR product concentration was quantified using a Qubit fluorimeter with a Quant-iT dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA), as recommended by the manufacturer. The corresponding copy number was obtained by using the following formula: number of copies (molecules) $\mu L^{-1} = (A \times 6.022 \times 10^{23}) (660 \times B)^{-1}$, where A is the product concentration (g μL^{-1}), B is the PCR product length, 6.022×10^{23} is Avogadro's number and 660 is the average molecular weight of one base pair. The standard curve for *Dinophysis* spp. was obtained by amplifying 10-fold scalar dilutions with copy numbers ranging from 10^6 to 10^2 (two replicates) and 10 molecules (three replicates) according to the qPCR assay protocol described below. The same standard curve was used to carry out the qPCR analysis to estimate the copy number per cell of *Dinophysis* spp. and to quantify the same genus in environmental samples.

To validate the qPCR assay, additional seawater samples were pooled, resulting in a single sample containing a total of 2254 cells of *Dinophysis* spp. in 46 mL of sample. The following final *Dinophysis* spp. concentrations were considered (final samples of 23 mL, prepared from the original 46 mL): undiluted (1.13×10^3 total cells), 1:2 (5.64×10^2), 1:4 (2.82×10^2), 1:8 (1.41×10^2), 1:40 (2.82×10), 1:80 (1.41×10) and 1:800 (1.41×10^0). All these samples were centrifuged at 4000 rpm for 10 min, and cell pellets were lysed in 800 (for the undiluted sample) or 400 (all the others) μL of lysis buffer as described above and processed by qPCR. The qPCR reactions were performed with three technical replicates in a final volume of 25 μL using the 1X Hot-Rescue Real-time PCR Kit SG containing Sybr Green (Diatheva, Fano, Italy), with primers at a final concentration of 300 nM, 0.625 U of Hot-Rescue Taq DNA polymerase and 2 μL of single cell lysate, undiluted and ten-fold serial dilutions (1:10, 1:100) of crude extracts, respectively. All amplification reactions were performed on a Step-one Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the following thermal conditions 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For each run, a dissociation protocol ranging from 60−95 °C with a resolution of 0.3 °C and melting curve analysis was performed to verify for specific amplification. Positive and negative controls were included in each run.

The qPCR data were acquired and analysed using StepOne software v. 2.3. The standard curve was generated automatically and accepted if the slope was between −3.45 and -3.32 (95–100 % efficiency) and the minimum accepted correlation coefficient (r^2) was 0.98. The efficiency of the assay was calculated according the equation: $E = (10^{(-1/slope)} - 1)$ x100. The rDNA copy number per cell of *Dinophysis* spp. and the total

Table 1
Primers targeting the 28S region of the rDNA used in this study (from Ajani et al., 2022).

Primer name	Direction	Sequence (5′ → 3′)
Dacu_11F	Forward	AAGCAAGCGGGAGCAAGTTT
Dacu_11R	Reverse	GCAGAAGGTTATGCTCATCGC

copy number of the same genus in each field sample were calculated by interpolation of the Ct (threshold cycle) value on the standard curve. The abundance of *Dinophysis* spp. in each seawater sample was obtained by dividing the total copy number by the mean copy number per cell, taking into account the volume of lysis buffer and the dilution factor of the lysates.

2.5. Toxin analysis

OA and DTXs were quantified by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) according to the harmonized protocol EU Harmonised SOP (2015). The sample was first rinsed with fresh water and then minced; at least a 100 g aliquot of mollusc flesh was homogenized. To an aliquot of shellfish tissue (2 g), 10 mL of MeOH was added and extraction was performed using a high-speed homogenizer (Ultraturrax, IKA, Breisgau, Germany) at 15,000 rpm for 1 min. After separation of the supernatants using a centrifuge (5810R; Eppendorf; Germany) at 4000 g for 10 min, the extraction was repeated twice, and the supernatants were collected in 20 mL volumetric flasks and then were made up to 20 mL with MeOH. An aliquot of 5 μL of the extracts was filtered through a 0.2 μm membrane filter (nylon, 13 mm 0.2 μm; Agilent; Santa Clara, California, USA) and analysed by LC-MS/MS. Analysis of OAs and DTXs was performed on a UPLC (Infinity II, Agilent, Santa Clara, USA) coupled to a triple-quadrupole mass spectrometer (6460, Agilent, Santa Clara, USA). Liquid chromatography was performed on a Poroshell120 EC18 (100 × 2.1 mm, 2.7 μm, Agilent, Santa Clara, USA) and separation was achieved by gradient elution using a mobile phase A (2 mM ammonium acetate, 0.1 % v/v acetic acid in 5.2 % v/v MeOH) and mobile phase B (2 mM ammonium acetate in MeOH). The flow rate was 0.4 mL/min, the column temperature was 30 °C and samples were cooled at 4 °C. The total time was 14 min.

At least six-point calibration curves prepared by dilution in MeOH of NRC (National Research Council, Halifax, NS, Canada) reference materials ranging from 0.2 μg/L to 40 μg/L ($R^2 \geq 0.998$) were used to quantify the toxins. The limit of quantification (LOQ) was 20 μg/kg for each toxin. Toxin identification was achieved using the LC-MS/MS set in the DMRM (dynamic multi-reaction monitoring) mode. The ESI interface was operated with the following parameters: gas temperature: 200 °C, gas flow 7 mL/min; nebulizer psi 35; sheat gas heater 400; sheat gas flow 12; capillary (V) 4000; Vcharing 300.

2.6. Statistical analysis

Copy number per cell data were tested for normality using the Shapiro-Wilk test and the mean value for the three species tested was compared using the one-way ANOVA test for equal means. Correlation between LM and qPCR counts and LM or PCR counts and okadaic acid levels was tested using Spearman correlation. Statistical analyses were performed using PAST (v. 4.11, Hammer et al. 2001), with a p-value of < 0.05 determining significance. Graphs were performed using R 4.2.2 statistical and programming software, package "*tidyverse*" (Wickham et al., 2019) and OriginPro (v. 2016, OriginLab Corporation, Northampton, MA, USA).

3. Results

3.1. Amplification and sequencing of the 5.8S-ITS and 28S rDNA genes

The 5.8S-ITS rDNA amplicons from four different single cell specimens identified by light microscopy as belonging to *D. sacculus, D. acuminata, D. acuminata* complex and *D. caudata* (GenBank accession no. from PP270371 to PP270374) were sequenced. The length of the amplified 5.8S-ITS rDNA gene fragment was in the range of 538–566 bp. Due to the highly conserved 5.8S-ITS rDNA regions in *Dinophysis* species, the sequences obtained were only confirmed at the genus level, using the BLAST database (https://blast.ncbi.nlm.nih.gov).

Genus-specific primers targeting the 28S rDNA gene (Ajani et al., 2022) were used to amplify genomic DNA extracted from 27 single cells of *Dinophysis* spp. *In silico* examination confirmed that these primers contain mismatches that prevent them from annealing to the non-target sequence (data not shown). All cells tested were positive for amplification of the target 28S ribosomal DNA region.

3.2. Standard curve, sensitivity, efficiency and reproducibility of the qPCR assay

The standard curve ($y = -3.37x + 38.22$) was generated by using serial dilutions of PCR product from pooled *Dinophysis* spp. cells, as described above, with genus-specific primers. The curve showed a linear relationship over seven orders of magnitude ($r^2 = 0.99$) and an efficiency of 98 %. Based on this curve, up to 10 copies of rDNA were determined. The reproducibility of the qPCR assay was analysed using both the coefficients CV_{Ct} (coefficient of variation of the cycle threshold) and CV_{Cn} (coefficient of variation of the copy number). The mean inter-assay variability for CV_{Ct} ranged from 1.0 % to 2.2 % (mean 1.8 %, range from 1.0 × 10^6 to 10 copies), while the mean values for CV_{Cn} ranged from 17 % to 45 % (mean 32 %, range from 1.0 × 10^6 to 10 copies) (Fig. S2 and Table S2). Melt curve analyses revealed qPCR product with a single peak at ~84 °C, which was used to confirm the specific amplification of the field samples.

3.3. Dinophysis spp. estimation of rDNA copy number

Due to the difficulties in establishing *Dinophysis* spp. monoclonal cultures, we directly used living *Dinophysis* spp. cells isolated from seawater samples and analyzed them by qPCR to obtain the mean rDNA copy number per cell. A total of 27 single cells were isolated by glass capillarity and were identified by light microscopy as *D. sacculus* ($n = 10$), *D. acuminata* ($n = 10$) and *D. caudata* ($n = 7$). The use of genus-specific primers, targeting the 28S rDNA gene, to determine the rDNA copy number of *Dinophysis* spp., revealed intraspecific variability with the mean copies per cell per species ranging from 1.14 × 10^4 to 1.29 × 10^4 with a mean value of 1.21 × 10^4 ± 1.81 × 10^3 (Fig. 1). However, there were no significant differences in the mean copy number per cell among the three *Dinophysis* species (one-way ANOVA test for equal means, $F(2, 24) = 0.06$, p-value > 0.05). The mean value of the copy number per cell obtained was used to estimate the abundance of the target genus in environmental samples using the qPCR assay.

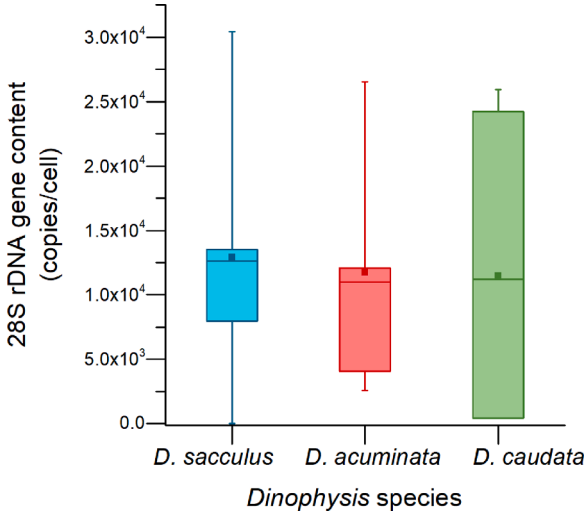

Fig. 1. The rDNA copies per cell of the three *Dinophysis* species, as *D. sacculus, D. acuminata* and *D. caudata*, determined by qPCR assay in the NW Adriatic Sea. Boxplots show the 25th and 75th quartiles (boxes), the median (line), the mean (squares), and the min-max intervals (whiskers).

3.4. Correlation between Dinophysis spp. abundance and rDNA gene copy number

The total gene copy number of *Dinophysis* spp. in the serially diluted combined field sample containing different *Dinophysis* species determined by qPCR assay was plotted against the corresponding cellular serial dilutions of *Dinophysis* spp. Regression analysis between cell concentration (cells/L, determined by light microscopy) and gene copies/L (assessed by qPCR) showed a highly significant linear correlation ($r^2 = 0.97$, p-value < 0.001) with a slope of 2.76×10^4 (Fig. 2). In this validation curve, the comparison between the cell numbers estimated by qPCR and the cell counts by LM provided a ratio of 2.09 (mean cell number by qPCR / mean cell counts by LM).

3.5. Quantification of Dinophysis spp. in environmental samples using light microscopy and qPCR assay

A total of 157 phytoplankton samples was collected and analyzed by both LM and qPCR for *Dinophysis* spp. cell abundance estimation. To exclude the presence of PCR inhibitors, such as humic acids, the reaction efficiency was evaluated. All the dilutions showed Ct differences between 3.3 and 3.4 (corresponding to an optimal efficiency of 100 %) excluding inhibition (data not shown).

During the study period, the highest cell concentrations of *Dinophysis* spp. were recorded in the late spring at the Difo Nord station, with an abundance of $9.35 \times 10^2 \pm 9.88 \times 10^1$ and 3.4×10^2 cells/L by qPCR and LM, respectively. The minimum abundance was found in late spring at the Rica2 station with values of 1.33 ± 0.72 and no cells by qPCR and LM, respectively. The abundance of *Dinophysis* spp. in seawater samples, as determined by LM, was generally lower than the cell concentrations determined by the qPCR assay. The mean cell abundance obtained throughout the study was $4.91 \times 10^1 \pm 7.78$ cells/L and $1.1 \times 10^2 \pm 1.36 \times 10^1$ cells/L determined by LM and qPCR, respectively, giving a difference in mean values of $6.11 \times 10^1 \pm 9.3$ cells/L. Thus, the light microscopy estimates were generally 44.6 % lower than those obtained by the qPCR assay, and comparing these means, a ratio of 2.24 (qPCR/LM ratio) was obtained, which was consistent with the ratio of 2.09 obtained from the validation curve. A total of 93 out of the 157 samples collected were found to contain *Dinophysis* spp. cells by microscopy counting. Quantifiable numbers of cells were successfully detected by qPCR analysis in 139 of the total samples. In 48 samples, the qPCR was able to count cells even when the microscopy was unable to quantify the target species, while in only two cases the opposite was true. However, a significant positive correlation was found between cell abundance determined by LM and qPCR (Spearman $r_s = 0.57$, p-value < 0.001). A comparison was made between the mean cell density of *Dinophysis* spp., obtained by the two quantification methods, for each station over the entire sampling period along the mussel farms distributed along the coast of Emilia-Romagna in the North-western Adriatic Sea (Fig. 3). The blue dots (qPCR estimates) showed larger sizes than the green ones (LM estimates). This meant that the *Dinophysis* spp. abundance determined by qPCR was higher than abundance determined by light microscopy, the only exceptions being the stations AP2 and AP3, where the application of the two approaches resulted in similar mean abundances falling in the same range of abundance. In general, the mean abundance determined by qPCR for the whole monitoring period was higher than that determined by microscopy at 18 stations out of 19 stations, evenly distributed along the entire coast.

Further, both microscopy and qPCR cell abundance estimations in environmental samples showed a similar seasonal trend for *Dinophysis* spp. Higher abundances were found at the beginning of the study period in May 2022, followed by a decrease in autumn, a slight increase in winter and a new increase in late spring (Fig. 4). The okadaic acid levels in mussels never exceeded the national legal limit value (160 μg OA eq/kg, Regulation EC 853/2004). The detected concentrations ranged from values below the limit of quantification (20 μg OA eq/kg) to a maximum of 155 ± 47 μg OA eq/kg, recorded at the Ra2 station on 30 March 2023. No correlation was found between cell abundance estimated by either LM counts or qPCR and okadaic acid levels ($r_s = 0.028$ and 0.064, respectively, $p > 0.05$).

Seawater samples collected during the study period showed the presence of mixed *Dinophysis* species, including *D. acuminata, D. acuminata* complex, *D. sacculus, D. ovum, D. caudata* and *D. fortii*. Since the primer set used for *Dinophysis* spp. was genus-specific, the rDNA gene

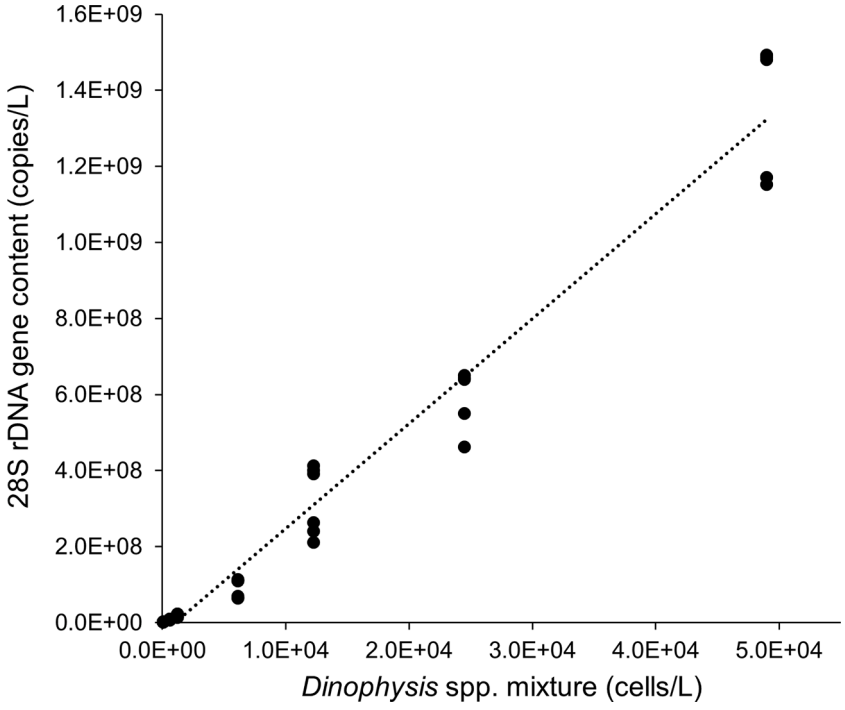

Fig. 2. Linear relationship between *Dinophysis* spp. abundance (cells/L) obtained by light microscopy and 28S rDNA gene content (copies/L) based on qPCR assay. A mixture of seawater samples was used for *Dinophysis* spp. cellular serial dilution calculation and 28S rDNA gene copy number determination.

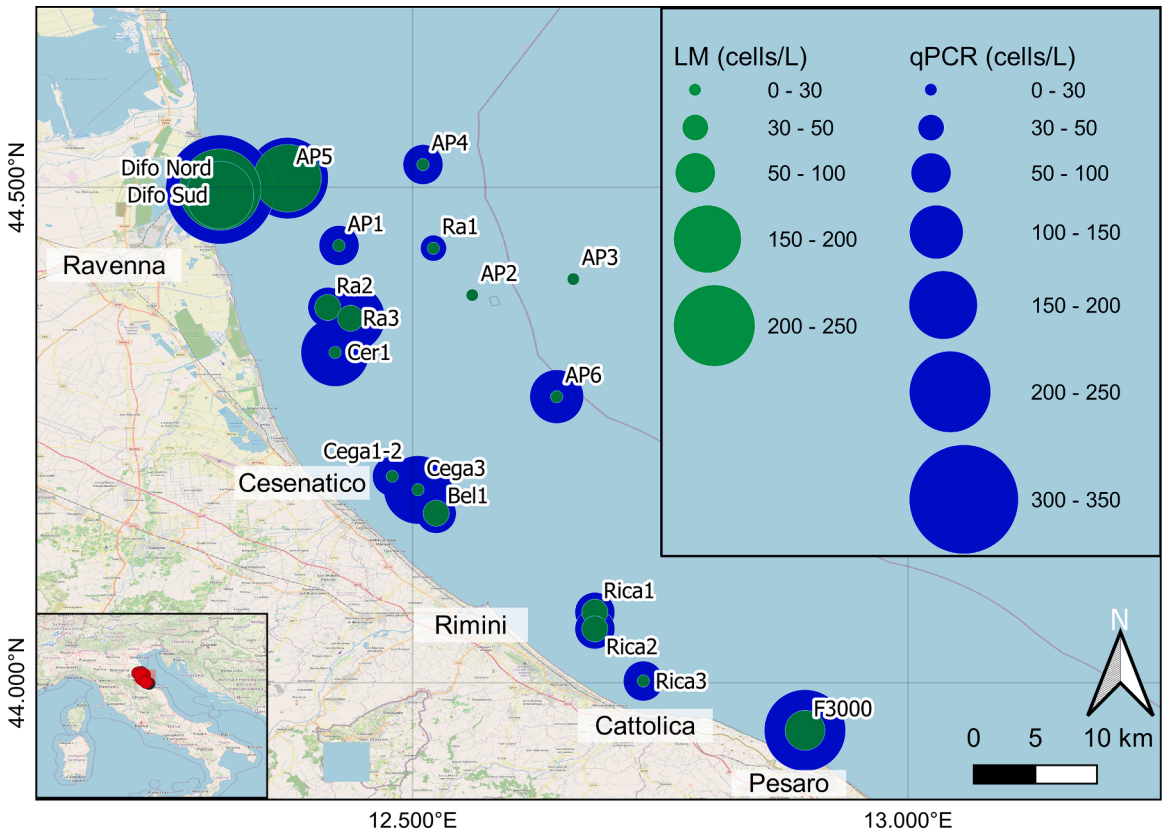

Fig. 3. Map of the north-western Adriatic Sea showing the sampling stations and the mean *Dinophysis* abundances (cells/L) determined by LM and molecular qPCR assay for the entire sampling period. Green circles: LM determination. Blue circles: qPCR determination. LM results are in the upper graphic layer, thus covering the qPCR ones when they fall in the same or a smaller category.

copies in the environmental samples, used for quantification of *Dinophysis* spp., were from a pool of target species. Considering the two most common co-occurring species encountered during the study period, *D. sacculus* and *D. acuminata*, it was possible to estimate the various combinations of a bispecific *Dinophysis* assemblage. The combinations between the two species were not biunivocal, and it was not possible to determine the exact density of each species from the total copy number, but, from the linear relationship between the rDNA gene copy number per cell in the two species and the total copy number, it was possible to estimate a range within which the abundances were likely to fall (Fig. 5 and Table S3).

4. Discussion

In this study, we investigated the use of a qPCR assay for time-series detection and quantification of *Dinophysis* spp. in the Northwestern Adriatic Sea. The *Dinophysis* species found in the analysed samples are all considered harmful as they are associated with the production of toxins responsible for DSP outbreaks (Lundholm et al., 2009). For these reasons, these species are routinely monitored. This molecular assay was characterized by high sample throughput and quantification at low copy number. Indeed, the standard curve used determined 10 rDNA copies/reaction, and showed an efficiency of 98 % and a range of 5 orders of magnitude with realistic reproducibility over the tested dynamic range, confirming the reliability and accuracy over time and over the entire quantification range. Single cells, isolated directly from seawater net samples, were processed in the qPCR assay and copy number variability per cell was found with a mean rDNA value of 1.21×10^4.

4.1. Single cell analysis and copy number variability

In this study, the use of single isolated cells allowed us to i) reduce the difficulties of maintaining *Dinophysis* spp. cultures due to their mixotrophic nature, and ii) study local specimens, which allowed us to account the variability in mean rDNA copy number per cell that is normally present in various protists, such as planktonic foraminifera (Milivojević et al., 2021), ciliates (Wang et al., 2017) and dinoflagellates (Galluzzi et al., 2010; Perini at al., 2011; Casabianca et al., 2014). Despite the difficulty posed by the high variable copy number, ribosomal DNA sequences are typically chosen for qPCR assays because they are largely present in reference databases, are usually able to distinguish target taxa, and are present in multiple copies in eukaryotic cells, allowing high detection sensitivity (Penna and Galluzzi, 2013). Recent studies on various dinoflagellate species revealed important variability in copy number among, and also within, different genera (Table 2). In particular, our results on the three *Dinophysis* species examined showed an intra-specific variation of four orders of magnitude, with copy numbers falling in the range reported for this genus.

In *Alexandrium* species, the rDNA copy number has been found to decrease as the life cycle progresses (Galluzzi et al., 2010), and we can assume that changes in the trophic state of the ecosystem may influence the life cycle and natural population dynamics of *Dinophysis*, with a yet unknown influence on the variation in rDNA copy number per cell due to changes in cell metabolism (Casabianca et al., 2021; 2023). Concerning the environmental conditions favouring *Dinophysis* spp., some studies suggested that eutrophic conditions associated with high levels of dissolved organic and inorganic nutrients could trigger *Dinophysis* blooms, and DSP events, due to increased growth of *Dinophysis* prey *Mesodinium* spp. (Hattenrath et al., 2015; Anschütz et al., 2022). On the other hand, other studies showed that *Dinophysis* grows successfully in

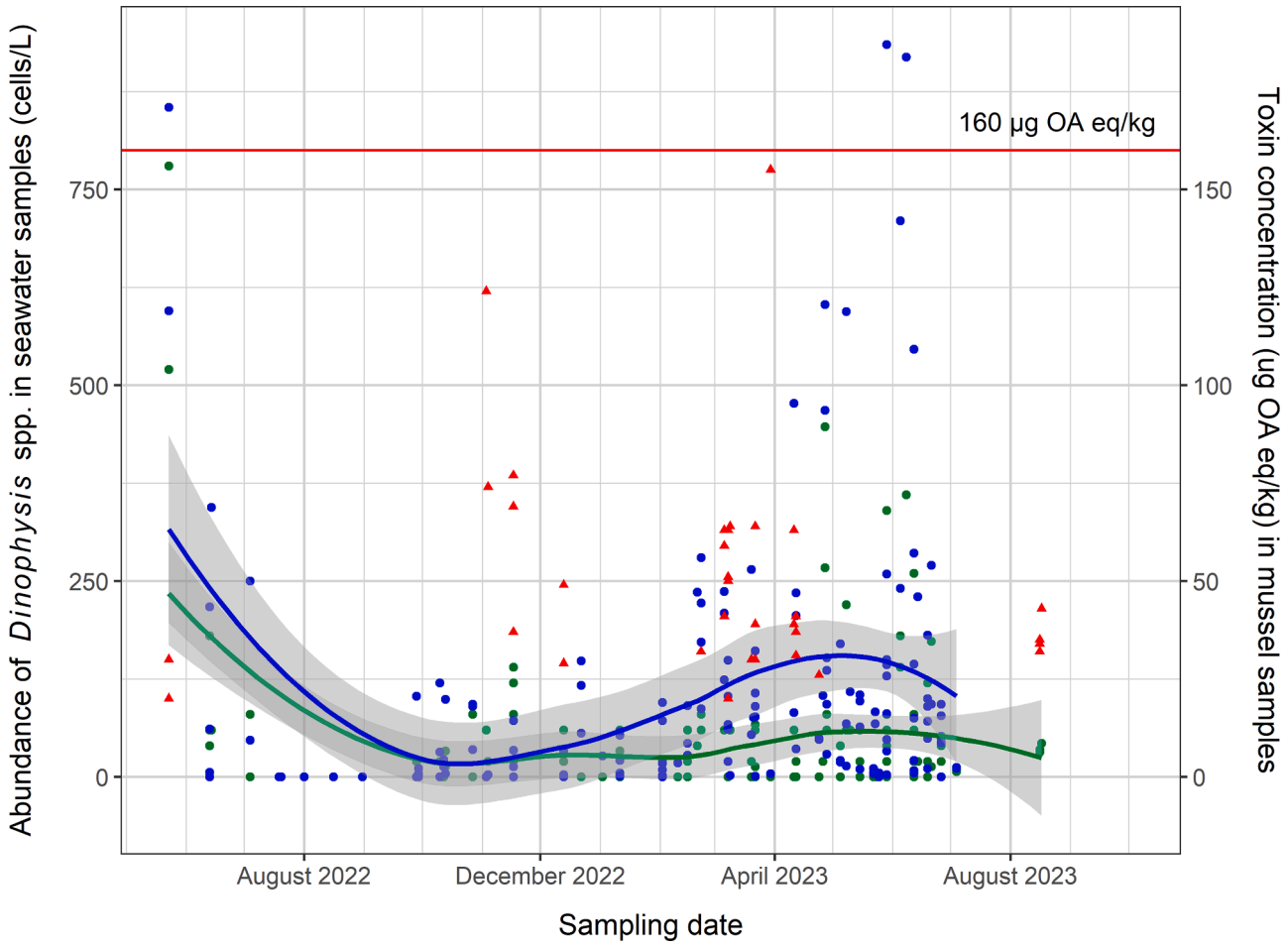

Fig. 4. Data points represent measurements of *Dinophysis* abundance (cells/L) obtained by LM (green) and qPCR (blue), and okadaic acid (OA, red) expressed as µg OA eq/kg at all the sites between May 2022 and July 2023. The points are fitted with smooth lines of best fit, with grey areas representing 95 % confidence intervals. Microscopy data points obtained after June 2023 are not included in the comparative analysis with qPCR data in this study. Red line: legal limit value of 160 µg OA eq/kg according to Reg. EC 853/2004, above which mussel harvesting closure is expected. Only toxin levels above the LOQ (20 µg OA eq/kg) are reported.

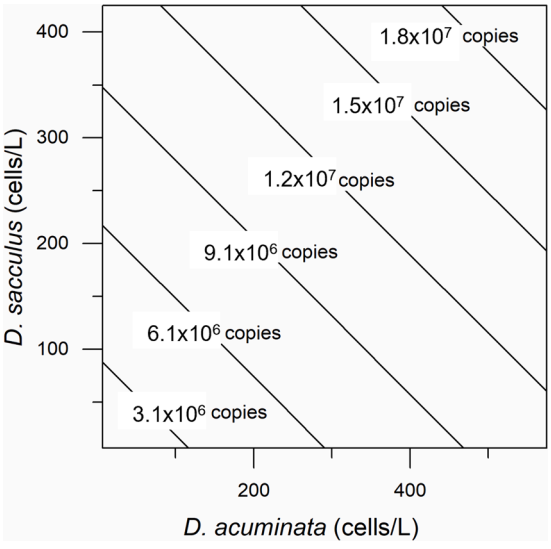

Fig. 5. The rDNA gene copy number isopleths showing possible combinations of coupled *D. sacculus* and *D. acuminata* abundances. After establishing the total mean copy number of rDNA in the sample, the range within which the cell densities of the two *Dinophysis* species can be found.

nitrogen-depleted waters (Seeyave et al., 2009). In addition, higher temperatures may lead to a more pronounced stratification and stabilisation of the water column, which supports the growth of *Dinophysis*, but they may also accelerate the growth and/or decline of *Mesodinium*, leaving less time for *Dinophysis* to be exposed to its prey (Anschütz et al., 2022). Due to these factors, it is difficult to easily and accurately predict the behaviour of *Dinophysis,* either for its bloom or for the management of DSP events. Regarding the study area, the Adriatic Sea, a region of the Mediterranean Sea, a trend towards reduced eutrophic conditions was reported due to a significant decrease in nitrate and phosphate loads in coastal waters recorded from 1997 to 2019 (Cossarini et al., 2012; Grilli et al., 2020; Ricci et al., 2022). The impact of these changes on the trophodynamics and biology of *Dinophysis* was not clear (data not shown; Ravera et al. in contribution).

4.2. The qPCR assay and light microscopy for quantification of Dinophysis species

In the present study, the qPCR assay for the quantification of *Dinophysis* cells showed a strong correlation between LM and qPCR abundances, suggesting that the molecular method could be applied to environmental samples. Thus, the qPCR assay was applied to a study survey along the Emilia-Romagna and Northern Marche coasts and compared with the current official monitoring method, represented by LM counts, to assess the applicability and sensitivity of the developed molecular quantification assay. When qPCR was compared with LM

Table 2
List of Dinoflagellate species and copy number variability.

Dinoflagellate species	Strain	Copy number cell^{-1}	Reference
Alexandrium minutum	RCC4877	2.7×10^{1} [b]	Ruvindy et al., 2023
A. australiense	AT-YC—H	1.2×10^{8} [b]	Ruvindy et al., 2023
A. pacificum	n.r.[a]	1.7×10^{4} [b]	Ruvindy et al., 2023
A. pacificum	n.r.[a]	1.7×10^{7} [b]	Ruvindy et al., 2023
Gambierdiscus sp. ribotype 2	CCMP 1655	4.6×10^{3} [b]	Vandersea et al., 2012;
G. caribaeus	CCMP 1733	2.2×10^{5} [b]	Vandersea et al., 2012;
G. lapillus	HG4	2.2×10^{4} [b]	Kretzschmar et al., 2019
G. lapillus	HG7	5.9×10^{3} [b]	Kretzschmar et al., 2019
Dinophysis fortii	Miya38	2.4×10^{4} 3.4×10^{4} [c]	Yarimizu et al., 2021
D. fortii	Mom13	1.5×10^{4} 1.7×10^{4} [c]	Yarimizu et al., 2021
D. fortii	DF_SAL90	1.3×10^{3} [b]	Ajani et al., 2022
D. caudata	DC_NAG01	8.0×10^{1} [b]	Ajani et al., 2022
D. caudata	Isolate1	4.5×10^{2} [b]	This study
D. caudata	Isolate2	7.1×10^{2} [b]	This study
D. caudata	Isolate3	1.7×10^{4} [b]	This study
D. caudata	Isolate4	2.6×10^{4} [b]	This study
D. caudata	Isolate5	1.1×10^{4} [b]	This study
D. caudata	Isolate6	2.4×10^{4} [b]	This study
D. caudata	Isolate7	4.5×10^{2} [b]	This study
D. acuminata	DA_MOM02	5.4×10^{2} [b]	Ajani et al., 2022
D. acuminata	Isolate1	2.6×10^{3} [b]	This study
D. acuminata	Isolate2	1.1×10^{4} [b]	This study
D. acuminata	Isolate3	1.2×10^{4} [b]	This study
D. acuminata	Isolate4	2.7×10^{4} [b]	This study
D. acuminata	Isolate5	2.5×10^{4} [b]	This study
D. acuminata	Isolate6	2.7×10^{3} [b]	This study
D. acuminata	Isolate7	4.1×10^{3} [b]	This study
D. acuminata	Isolate8	1.1×10^{4} [b]	This study
D. acuminata	Isolate9	1.2×10^{4} [b]	This study
D. acuminata	Isolate10	1.0×10^{4} [b]	This study
D. sacculus	Isolate1	1.0×10^{1} [b]	This study
D. sacculus	Isolate2	8.0×10^{3} [b]	This study
D. sacculus	Isolate3	3.6×10^{1} [b]	This study
D. sacculus	Isolate4	1.2×10^{4} [b]	This study
D. sacculus	Isolate5	1.3×10^{4} [b]	This study
D. sacculus	Isolate6	2.8×10^{4} [b]	This study
D. sacculus	Isolate7	1.4×10^{4} [b]	This study
D. sacculus	Isolate8	3.0×10^{4} [b]	This study
D. sacculus	Isolate9	1.1×10^{4} [b]	This study
D. sacculus	Isolate10	1.3×10^{4} [b]	This study

[a] not reported.
[b] Copy number cell^{-1} determined by qPCR.
[c] Copy number cell^{-1} determined by dPCR.

counts of *Dinophysis* in field samples, qPCR exceeded LM counts by a factor of 2.24. A similar ratio was found between the two techniques for *Karlodinium* spp. cell density (Toldrà et al., 2018a). Due to the variation of copy number per cell of individual *Dinophysis* species in each field sample, different species with different copy number per cell may be present. If *Dinophysis* with a copy number per cell higher than the mean copy number per cell are present in the sample, an overestimation may occur. Moreover, the qPCR showed the potential to detect the presence of up to 10 copies of target rDNA in the samples. This DNA can belong to living, dead or broken cells. When a sample is counted by light microscopy, dead or broken cells are not included in the count, but they contribute to the total amount of DNA which can be amplified by the qPCR assay. This may be another reason for the discrepancy between the two counting methods (McQuillan et al., 2023).

In order to exclude any degradation of phytoplankton and *Dinophysis* cells, the molecular and LM analyses were performed within two months of harvest and Lugol's solution preservation (Eckford-Soper and Daugbjerg, 2015). Indeed, some authors found that sample preservation by Lugol's solution can affect community profiles when next-generation sequencing (NGS) technologies are applied (Mäki et al., 2017). But, the NGS application represents a different issue that could be addressed by showing different purposes with respect to a qPCR application for cell enumeration.

Comparison of the two approaches revealed a similar temporal in occurrence with *Dinophysis* spp. abundances detected earlier by qPCR than by LM. Values showed a decreasing trend in the first part of the study period for both methods, then after December 2022, LM counts oscillate and maintain an almost flat pattern before increasing, while qPCR results start to steadily increase, detecting the *Dinophysis* increase earlier. Winter samples, in particular, showed low/null counts by LM, although *Dinophysis* was detected by qPCR, suggesting that qPCR could be a useful tool as an early warning system. The qPCR assays showed advantages over microscopy, such as speed, sensitivity and efficiency, and have potential for future applications in phytoplankton monitoring programs. Indeed, due to some differences found in cell counts between the two techniques, molecular and LM results, the official monitoring of *Dinophysis* abundance is still based on the LM determinations.

4.3. Determination of Dinophysis species by light microscopy and PCR assays

Some *Dinophysis* species pose a global challenge for species identification due to ambiguities at the morphological and genetic level (Reguera et al., 2012, 2024; Park et al., 2019), such as the "*D. acuminata* complex", which includes *D. acuminata, D. sacculus* and *D. ovum* (Séchet et al., 2021). In the eastern Adriatic Sea, *D. acuminata* and *D. sacculus* occur in close temporal succession, with *D. acuminata* in spring and *D. sacculus* in late spring and summer, with no records of *D. ovum* (Ninčević-Gladan et al., 2008). These reports are consistent with the sequence observed during our study period along the Northwestern Adriatic coast, where *D. acuminata* and *D. sacculus* co-occurred, while *D. ovum* typically appeared earlier in the year. Due to their morphological similarity and co-occurrence, the specimen was labelled as "*D. acuminata* complex" when a clear species assignment was not possible (Fig. S3). Among the identified *Dinophysis* species, *D. sacculus* and *D. acuminata* showed the highest abundances by LM counts and appeared to co-occur frequently, as previously reported. Their morphology is not always straightforward, and even the qPCR assay was unable to quantify them at the species level. Nevertheless, as demonstrated by our model for the molecular estimation of *Dinophysis* spp. based on isopleths, it may be possible to analyse bispecific assemblages based on the total copy number obtained from the qPCR reaction, and to determine a cell concentration range within which the two species are expected to fall. Attempts have been made to apply qPCR assays to both diatoms and dinoflagellates. Species-specific assays have been developed for *Ostreopsis* cf. *ovata* and *O.* cf. *siamensis* (Perini et al., 2011; Casabianca et al., 2013, 2014), various *Alexandrium* species (Galluzzi et al., 2004; Penna et al., 2015; Vandersea et al., 2017), *Karlodinium veneficum* and *K. armiger* (Toldrà et al., 2018a), *Pseudo-nitzschia* spp. (Andree et al., 2011) and many others (Perini et al., 2019; Pearson et al., 2021). In some cases, assays were designed to target a genus or a clade level, such as with the *Pseudo-nitzschia pseudodelicatissima* complex clade I (Ajani et al., 2021), the *Scrippsiella trochoidea* species complex and *Skeletonema* spp. (Casabianca et al., 2020) or *Dinophysis* spp. (Ajani et al., 2022). Identification of *Dinophysis* species using molecular markers is still challenging, and to date no unique marker has been found that can discriminate between all species (Wolny et al., 2020). Raho et al. (2013) proposed the use of the mitochondrial gene cytochrome c oxidase I *(cox1)* sequence, which successfully distinguished *D. acuminata* from the other species in the complex, but failed to distinguish *D. sacculus* from *D. ovum*. As target gene sequences cannot resolve *Dinophysis* species, a multigene phylogenetic analysis strategy has been proposed for species identification (Ott et al., 2022).

4.4. Relationship between Dinophysis spp. and DSP

During our study, DSP toxin levels in mussels never exceeded the aquaculture closure limit of 160 μg OA eq/kg. This was surprising given that closures had occurred in the Emilia-Romagna Region every year from 2012 to 2021, with the exception of 2018 (Accoroni et al., 2024), until the most recent exceedance analysis in March 2022 (data not shown). Data on inter- and intra-species toxicity are scarce, and several studies found a weak relationship between toxin levels in mussel flesh and *Dinophysis* spp. abundance in seawater (Dahl and Johannessen, 2001; Alves-de-Souza et al., 2014). Nonetheless, strains of the same species from different geographical locations were found to have distinct toxin profiles, thus making it necessary to conduct studies on local individuals to gather valid information (Lindahl et al., 2007; Reguera et al., 2014). Moreover, low levels of okadaic acid can be found together with high abundance of toxic DSP phytoplankton and vice versa (Fernández et al., 2019). In our study, to date, no clear conclusion can be drawn as to why there were no toxicity events. The same happened in the year 2018, with the resumption of toxicity events the following year, so it will be important to monitor whether this situation repeats itself in the coming years, indicating a changing dynamic, or whether it remains an isolated event.

However, as several *Dinophysis* species can produce toxins posing a risk to human health through the potential consumption of contaminated mussels, monitoring of these species is fundamental. Despite the molecular assay not being able to genetically separate these species, identification at the genus level was achieved, providing a potential contribution to monitoring activities related to human health safety issues. This qPCR based assay has proven to be an extremely sensitive approach, with results available within a few hours of environmental sampling, avoiding time-consuming of counting and high taxonomic skills.

5. Conclusions

Our study showed how qPCR could be a valuable method for monitoring *Dinophysis* species because it is rapid and sensitive even at low abundance, making it potentially useful for early detection of HAB events even for species with low densities. This study demonstrated the high reproducibility and efficiency of the molecular method in time-series analysis, making it an important first step towards the development of standardized qPCR protocols for the control of *Dinophysis* and other harmful species in the national monitoring programs.

Further steps may include a thorough long-term inter-calibration of qPCR and microscopy counts to validate the method over time. Moreover, additional studies on the copy number variability are needed, as not all *Dinophysis* species were included in this study. The variability of target genes reported in this and other studies highlighted the need to standardize any qPCR assay on local strains of the species of interest. The use of copy number per cell evaluation directly on single cells isolated from the target area, is ideal for determining the target gene variability of the local population, with the added advantage of avoiding the difficulties associated with maintaining mixotrophic species in culture as *Dinophysis* spp. Finally, future studies on specie-specific toxin content should be carried out to analyze the relationship between *Dinophysis* abundance, assessed by both LM and qPCR, and toxin levels to better understand and manage possible future occurrence of DSP outbreaks.

CRediT authorship contribution statement

Giorgia Ravera: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Funding acquisition, Writing – original draft, Writing – review & editing. **Monica Cangini:** Data curation, Writing – review & editing. **Samuela Capellacci:** Formal analysis, Investigation. **Sonia Dall'Ara:** Data curation. **Giuseppe Prioli:** Supervision. **Mauro Marini:** Supervision. **Elena Manini:** Supervision. **Antonella Penna:** Conceptualization, Writing – review & editing. **Silvia Casabianca:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2024.102686.

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