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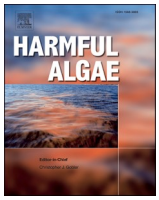

Quantitative Polymerase Chain Reaction for the estimation of toxigenic microalgae abundance in shellfish production waters

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ABSTRACT

Certain species of marine microalgae produce potent biotoxins that pose a risk to human health if contaminated seafood is consumed, particularly filter feeding bivalve shellfish. In regions where this is likely to occur water and seafood produce are regularly monitored for the presence of harmful algal cells and their associated toxins, but the current approach is flawed by a lengthy delay before results are available to local authorities. Quantitative Polymerase Chain Reaction (qPCR) can be used to measure phytoplankton DNA sequences in a shorter timeframe, however it is not currently used in official testing practices. In this study, samples were collected almost weekly over six months from three sites within a known HAB hotspot, St Austell Bay in Cornwall, England. The abundance of algal cells in water was measured using microscopy and qPCR, and lipophilic toxins were quantified in mussel flesh using LC-MS/MS, focusing on the okadaic acid group. An increase in algal cell abundance occurred alongside an increase in the concentration of okadaic acid group toxins in mussel tissue at all three study sites, during September and October 2021. This event corresponded to an increase in the measured levels of *Dinophysis accuminata* DNA, measured using qPCR. In the following spring, the qPCR detected an increase in *D. accuminata* DNA levels in water samples, which was not detected by microscopy. Harmful algal species belonging to *Alexandrium* spp. and *Pseudo-nitzschia* spp. were also measured using qPCR, finding a similar increase in abundance in Autumn and Spring. The results are discussed with consideration of the potential merits and limitations of the qPCR technique versus conventional microscopy analysis, and its potential future role in phytoplankton surveillance under the Official Controls Regulations pertaining to shellfish.

1. Introduction

Harmful Algal Blooms (HABs) occur when the expansion of marine or freshwater phytoplankton populations causes widespread ecological disruption, economic loss, and risk to public health (Anderson et al., 2012, 2021; Karlson et al., 2021). Although HABs are natural events, they may be influenced by climate change and other human factors including the discharge of nutrients into natural water systems (Gobler, 2020), albeit the relationship between human expansion and HABs remains unclear. Globally, a perceived increase in the frequency and severity of HABs may only reflect an increase in awareness and monitoring (Hallegraeff et al., 2021), nonetheless the impacts of HABs are likely to become more severe with increasing use of waters for food production by aquaculture. The risk to public health comes from exposure to phytoplankton species that biosynthesise toxins, causing a range of debilitating syndromes, some of which can be fatal (Dionysiou, 2010; Neves et al., 2021; Sobel and Painter, 2005). The predominant route of exposure to algal toxins is through the consumption of finfish and filter feeding bi-valve shellfish species (including oysters, clams and mussels) which accumulate algal toxins in their flesh. The toxin compounds are not readily destroyed by cooking or freezing the product prior to consumption (Reguera et al., 2014).

Statutory surveillance of HAB species and shellfish toxins within regulated shellfisheries waters is undertaken for the protection of public

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health (Anderson, 2009). Local authorities routinely collect water and shellfish samples and deliver them to centralised laboratories where efforts are made to both identify and enumerate algal cells in seawater and quantify regulated toxins in shellfish tissues. In standard practice, water samples are fixed on-site with Lugol's solution and 'settled' for several hours before the identification and enumeration of HAB species by specialist taxonomists using light microscopy (Hasle G, 1978). Toxins are measured in shellfish tissue extracts using liquid chromatography linked to fluorescence detection (LC-FLD), ultraviolet detection (LC-UV) or tandem mass spectrometry (LC-MS/MS), for Paralytic Shellfish Toxins (PST), Amnesic Shellfish Toxins (AST) and Lipophilic Toxins (LT), respectively (Turner et al., 2019). These official reference methods require expensive apparatus and highly trained personnel, and local authorities await results for up to 48 h after sample collection. When a regulatory threshold level (Maximum Permitted Limit (MPL)) of a toxin group is breached the official harvesting areas are closed until toxin levels fall beneath the MPL for two successive analyses taken at least 48 h apart. If, however, food business operators have harvested shellfish from a closed area, without waiting for official control test results, or without conducting their own end product testing (EPT), there needs to be an investigation into the number of shellfish harvested since the sample was taken, which might result in the closure of shellfish beds and a full-scale food chain product recall. In some instances, the products have already been consumed (Young et al., 2013). This is a risk for shellfish harvesters, a burden for business, and risks public health and consumer confidence. Microscopy methods also struggle to detect very low algal cell counts per litre accurately, making it challenging to identify trends that could signal the early stages of a harmful bloom and thus preventing more proactive mitigation of HAB impacts.

Improvements to the efficiency of current practices for HAB surveillance will come from the development and implementation of rapid analytical methods that can provide an early warning of HAB events, enabling authorities to manage the collection and processing of samples for laboratory analysis. Quantitative Real Time Polymerase Chain Reaction (qPCR) is a molecular-biological method of nucleic acid (DNA) sequence amplification that can be used to identify algal cells based upon genetic information rather than morphology (Pearson et al., 2021; Penna and Galluzzi, 2013). To do this, genomic DNA is extracted and purified from a sample, and a short DNA sequence unique to the target group is amplified and detected in real-time using a fluorescent reporter. A comparison between the number of amplification cycles required to achieve a threshold level of fluorescence with a series of DNA standards, containing known quantities of target sequence copies, is used to calculate the amount of the target sequence in the original sample, and estimate the number of cells. There are several potential advantages to using qPCR. (1) The process is very rapid, and results can be achieved within an hour using up-to-date instrumentation and reagents. (2) With careful assay design the analysis can be tailored towards potentially any species or taxonomic group with high selectivity. (3) The methods are particularly sensitive, enabling the detection of a small quantity of cells per litre of sample; important when algal toxins in seafood can reach harmful levels when cell densities in the surrounding water are very low (Dyhrman et al., 2010). (4) Modern qPCR workflows are typically undertaken using commercially available user kits, which can be used in tandem with automated processing apparatus and equipment that supports multiplexed or parallel assays. This reduces the level of molecular-biological training required and has improved throughput and accuracy whilst also reducing costs for routine measurements.

Methods using qPCR for the detection of HAB taxa are now included in the research and surveillance programs of international marine sciences institutes. Most qPCR HAB assays measure 'universal' taxonomic marker sequences, most commonly within the ribosomal RNA (rRNA) genes encoding either the large or small ribosomal subunits (LSU or SSU) (Gao et al., 2015; Hatfield et al., 2019; Howard et al., 2012; Smith et al., 2014; Toebe et al., 2013) or the Internally Transcribed Spacer (ITS) sequence that resides between them (Barkallah et al., 2020; Elleuch et al., 2021; Kim et al., 2017; Yuan et al., 2012). These assays can be used to target different algal taxa, and generally have very low limits of detection because the genes are present at very high copy number per cell. However, variability in copy number between species, and different life cycle stages reduces the accuracy of quantification. Less commonly employed gene targets include *rbcL* (encoding Ribulose-1, 5-bisphosphate carboxylase/oxygenase; RubisCO) (Gray et al., 2003; La Du et al., 2002), *cob* (encoding Cytochrome B) (Zhang and Lin, 2005) and the mitochondrial sequence COX1 (Song et al., 2021). Alternatively, qPCR can be used to selectively measure toxin-producing cells by targeting genes that encode the enzymes required for toxin biosynthesis. These could be a better indicator of risk if they enable the exclusion of non-toxic variants within a population that may share morphometric features used for microscopic identification. Examples include assays that selectively target the *sxt* gene clusters necessary for Saxitoxin biosynthesis, a cause of Paralytic Shellfish Poisoning (PSP) produced primarily by the *Alexandrium* spp., or the *dab* gene cluster in *Pseudo-nitzschia* spp, necessary to produce domoic acid, a cause of Amnesic Shellfish Poisoning (ASP) (Brunson et al., 2018; Harðardóttir et al., 2019; Mendoza-Flores et al., 2018; Murray et al., 2019; Penna et al., 2015).

This study was carried out to investigate the utility of qPCR as an early warning of increases in phytoplankton numbers for the surveillance of select HAB taxa in waters belonging to the county of Cornwall, in Southwest England, a region known for producing fine seafoods including Mussels, Oysters and Razor clams. The local authority (LA), Cornwall Port Health Authority, take responsibility for the classification of shellfish beds in this region under the Directives of the European Commission Official Control Regulations 2017/625 and 2019/627 and undertake at least monthly (winter) and fortnightly (summer) sampling for algal biotoxin analysis. This is undertaken at an accredited testing laboratory (UKAS accreditation using methods that conform to ISO17025) operated by the Centre for Environmental, Fisheries and Aquaculture Sciences (CEFAS), on behalf of the UK Food Standards Agency (FSA). During September 2021 to March 2022, additional sampling of water and shellfish (mussels) was carried out at three sites within the LA jurisdiction. The sample analysis focused on the measurement of the *Dinophyceae* which are historically problematic within the study area. Measurements were made for cell density (from water) and toxins (from mussel flesh) including okadaic acid and other lipophilic toxins commonly associated with the condition diarrhetic Shellfish Poisoning (DSP). In tandem, water samples were filtered to collect suspended cells and the filters were processed for qPCR analysis to identify and quantify gene sequences specific for *Dinophysis accuminata, Alexandrium* spp. and *Pseudo-nitzschia* spp.

2. Materials and methods

2.1. Sampling and sample preparation

Water and Shellfish (mussel) samples were collected by boat, from three offshore locations in St Austell Bay, Southern England, UK. These were Ropehaven Outer (grid reference SX05744972), Porthallow North (grid reference SW80212383) and South Mevagissey Bottom (grid reference SX05214698). Sampling took place between the 13th of September 2021 and the 28th of March 2022, but was suspended through December and Early January due to anticipated low temperatures and low levels of phytoplankton in the waters. Not all sites were sampled each week. Full details of the sampling routine are provided in Table S1 in the supporting information.

A total of 46 filtered water samples were collected over the course of the study for qPCR analysis. Water was collected by Pole sampling, and approximately 500 mL was immediately filtered using a Sterivex filtration Unit (Millipore, USA) with a 0.22-micron pore size polyethersulfone membrane. Sea water was forced through the filter using a disposable 50 mL plastic syringe with a Luer lock and chased through

with air to dry the membrane. Filter units were sealed, cooled, and immediately returned to shore, where they were frozen (−80 °C) until further processing.

For microscopy analysis, water samples were collected at precisely the same time and location as the qPCR samples but were not filtered. A volume of 500 mL was mixed with Lugol's iodine solution in a sealed container. Mussels were collected at approximately the same time and location as the water and filter samples. These were recovered from moored containers filled with live mussels, positioned at the centre of each site. Enough Mussels were collected to ensure at least 100 g of flesh for analysis; a range of sizes were selected to represent the population. The mussels were placed in a polythene sample bag. The fixed water samples and mussels were transported in an insulated box, packed with ice and protective foam.

2.2. Enumeration of Dinophysis spp

Microscopy was used to count the total number of *Dinophysis* spp. cells in each fixed water sample, speciating each individual as far as possible. Each sample was slowly and gently inverted 10 times to mix, then immediately poured into a 'settling' chamber until the chamber just began to overflow. A glass cover was immediately slid onto the top to seal the chamber. After a few hours, the 25 mL chamber was viewed using an inverted microscope to determine if the sample volume was appropriate for analysis, or if a smaller chamber volume was appropriate; 25 mL, 10 mL and 5 mL chambers were used as required, in each case settling occurred for at least 12, 8 or 4 h respectively before mounting onto a Perspex stage holder on an inverted microscope. Focus and illumination was configured to ensure optimal conditions for resolving morphological features of settled phytoplankton cells. Two enumeration methods were used, depending on the cell density of the reportable taxa. When the cell concentration was low (less than approximately 4 cells per field of view (FOV) the whole base of the chamber was scanned for the presence of the cells at a magnification of 200X. This allowed a minimum detection level of 40 cells per litre for a 25 mL chamber. When the cells were too numerous to ensure accurate counting over the whole base of the chamber (greater than approximately 4 cells per FOV), random FOV were counted. Empty cells were not included in the analysis. Where a cell could be identified and contained visible cell contents (whether a half cell or damaged), it was included in the analysis count. The concentrations of algal cells in terms of cells per litre was subsequently calculated by multiplying the cell count by the raising factor (1000/subsample size).

2.3. Biotoxin analysis

Mussels were rinsed to remove debris, drained and the edible flesh removed and collected. Once a minimum of ten animals and 50 g flesh had been collected the tissue was homogenised prior to extraction and analysis following the EU Reference Laboratory (EURL) reference method for Lipophilic Toxins (EURLMB, 2015). 2.0 ± 0.01 g of each homogenate was added to a 50 mL polypropylene tube with 9 mL of methanol, vortex mixed for 3 min and centrifuged at 3500 rpm for 8 min. Supernatants were recovered, and the remaining pellets were subjected to another two extraction steps as above. The supernatants from the 3 extractions were combined and made to a final volume of 20 mL with methanol using a Volumetric flask prior to filtering with a 0.2 μm pore size Nylon filter (Phenomenex, Manchester, UK). For each batch of samples analysed, a negative control procedural blank and positive control laboratory reference material (LRM) was co-extracted for quality control assessment. Hydrolysis of methanolic extracts was achieved based on the procedure described by (Mountfort et al., 2001) whereby 125 μL of 2.5 M sodium hydroxide was added to 1 mL of extract, vortex mixed for 5 s and heated for 40 min at 76 °C. Samples were cooled to ambient temperature, neutralised using 125 μL 2.5 M hydrochloric acid and vortex mixed for 5 s. Hydrolysed samples were analysed alongside a crude aliquot of filtered unhydrolysed extract using LC–MS/MS as follows.

Two UHPLC systems (Acquity and Acquity I-class) were coupled to a Xevo TQ and Xevo TQ-S triple quadrupole mass spectrometer respectively (Waters Ltd., Manchester, UK). The alkaline (pH 11) LC method described by Gerssen et al. (2009) was adopted with modifications and previously subjected to in-house validation prior to implementation into routine biotoxin monitoring programmes. Mobile phase A comprised of deionised water adjusted to pH 10.7 +/- 0.2 with 0.1% ammonium hydroxide. Mobile phase B was 90% acetonitrile with 0.1% ammonium hydroxide. Chromatographic and mass spectrometry conditions were as detailed by Dhanji-Rapkova et al. (2018). Instrument data was analysed using MassLynx™ v.4.1 (Waters Ltd., Manchester, UK). LC-MS/MS performance was checked by applying quality control (QC) criteria outlined in internal Standard Operating Procedures (SOPs) and the EURLMB SOP (EURLMB, 2015). External calibrations generated from analysis of calibrant solutions were used for quantification of all target compounds, in this case Individual okadaic acid (OA)-group toxin concentrations were calculated with post-hydrolysis concentrations equating to concentrations of free toxins and esterified toxins combined. Toxicity Equivalent Factors (TEFs) recommended by the European Food Safety Authority (EFSA) were applied and toxin concentrations were subsequently summed into representative groups as stipulated by EU legislation (Regulation (EC) 853/2004). The OA group included OA, DTX1, DTX2, PTX1 and PTX2 and the combined concentration was expressed in μg OA eq./kg. Non-hydrolysed extracts were used for quantification of all toxin compounds, including OA, DTX1 and DTX2 in their free form. Total (free form plus fatty acid esters) concentrations of these toxins were assessed using hydrolysed samples only.

2.4. DNA extraction from filters

DNA was recovered from the Sterivex filter units using the Power Water DNA isolation Kit (Qiagen Ltd.), working to the manufacturer's recommended protocol. DNA was eluted into Tris-EDTA (pH 8.0); the final volume of eluent obtained from each Sterivex unit was 100μL. Prior to qPCR analysis, each extracted DNA sample was analysed using a Nanodrop spectrophotometer and if necessary additional DNA purification was carried out using the QIAquick DNA Purification Kit (Qiagen Ltd.) according to the manufacturer's recommended protocol. The DNA samples were stored at −20 °C until use.

2.5. Quantitative polymerase chain reaction

Each DNA sample was analysed using 3 different qPCR assays, targeting either *Alexandrium* spp., *Pseudo-nitzschia* spp. or *Dinophysis accuminata*. The assays for *Alexandrium* spp. and *Pseudo-nitzschia* spp. were based upon protocols originally described by Galluzzi et al. (2004) and Fitzpatrick et al. (2010) respectively, with oligonucleotide sequences provided in Table 1. For *Dinophysis accuminata* we used a novel qPCR assay that was developed and tested using primer sequences designed to amplify a short fragment of the 5.8 s rRNA gene sequence for this species based on a consensus derived from multiple alignment of 328 unique accessions available within the Genbank (NCBI) sequence database.

Each qPCR reaction was prepared in a dedicated PCR workstation with air filtration and ultraviolet decontamination, using nuclease free and DNA free plastic consumables and reagents. Reactions were prepared on ice, using micro-pipettors with aerosol barrier tips. Each qPCR reaction was set-up to contain 12.5μL of Sso Fast EvaGreen Supermix (Biorad Ltd, UK), 1 μL of each primer (final concentration of 400 nM), and 9 μL of PCR-grade water; template DNA (1 μL) was added to a total reaction volume of 25 μL. The qPCR reactions were carried out using a LightCycler 96 real-time PCR instrument (Roche) according to the manufacturer's recommended protocol. Each reaction was run for 40 cycles, followed by a high-resolution dissociation (melting) analysis.

Table 1
Oligonucleotide Sequences used for qPCR.

Species	Target	Forward Primer	Reverse Primer	Reference
Alexandrium spp.	ITS1–5.8S-ITS2	YGATGAAGAATGCAGCAAMATG	CAAGCAHACCTTCAAGMATATCC	(Galluzzi et al., 2004)
Dinophysis accuminata	5.8S	GCATGCTGTATGTATCACA	AATGAGGCCATACAGACA	This Study
Pseudo-nitzschia spp.	18S	CTGTGTAGTGCTTCTTAGAGG	AGGTAGAACTCGTTGAATGC	(Fitzpatrick et al., 2010)

The thermal cycling parameters were as follows. For the *Alexandrium* spp. assay: 95 °C for 5 min, then 95 °C for 15 s and 60 °C for 60 s per cycle. For the *D. accuminata* assay: 95 °C for 5 min, then 95 °C for 15 s, 57 °C for 15 s and 72 °C for 45 s per cycle. For the *Pseudo-nitzschia* spp. assay: 95 °C for 5 min, then 95 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s per cycle. Each reaction was prepared in triplicate, and the average quantification cycle (C_q) value for each replicate was used for analysis and quantification. Cell number was calculated as cells per 100 mL of sampled water after considering the amount of DNA sample added to each reaction, and the quantity of water passed through the Sterivex filter unit when the sample was collected. Further information regarding the qPCR methodology can be found in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) checklist included in the supporting information, Table S2.

2.6. Estimation of cell number by qPCR

For *Alexandrium* spp. and *Dinophysis accuminata* the number of cells in the samples was estimated by calculating the number of target sequence copies, and then converting this into an estimate of cell number. To do this, standard curves were prepared by amplifying a 10-fold dilution series of synthetically produced double-stranded-DNA (dsDNA) fragments containing the target sequence. The dsDNA fragments were synthesised by Integrated DNA Technologies Ltd, purified by HPLC, and diluted in PCR-grade water immediately prior to use. The manufacturer provided the molecular weight and mass of DNA produced, enabling the calculation of the exact number of dsDNA molecules in a given volume. The number of target sequence copies in each standard was plotted against the mean C_q value from 5 replicate qPCR reactions, fitted using a simple linear regression line of best fit. The number of target sequence copies in each field sample was extrapolated from the standard curves, and used to estimate the number of cells using an assumption of the number of target sequence copies per cell. For *Alexandrium*, this was 1084, based on the calculation made by Galluzzi et al. (2004). For *Dinophysis* this was 49, based on the calculation made by Ajani et al. (2021).

Quantification of *Pseudo-nitzschia* spp. was achieved using cell number standards. These were prepared from a culture of *Pseudo-nitzschia multistriata* (SZN-B954, originally recovered from the Gulf of Naples, Italy) in f/2 growth medium, maintained at 18 °C with a 12-hour photoperiod. An exponentially dividing culture was enumerated using a Sedgwick–Rafter cell counting slide (PYSER-SGI) and a compound light microscope (Carl-Zeiss). Cell counts were carried out according to recommendations described by Woelkerling et al. (1976). A threshold of 50 to 200 cells per field of view (FOV) was established for replicate sub-samples, and cell counts were repeated for each sub-sample until the Standard Error of the Mean (SEM) was less than 10%. Dilutions were prepared in f/2 medium. DNA was extracted using the DNeasy Power Water kit and the DNA samples were used as template for a series of qPCR reactions, which were used to construct the standard curve. Each DNA sample was measured in triplicate, using 3–5 replicate qPCR reactions to derive a mean value for each measurement.

2.7. Statistical comparison between qPCR and microscopy

A penalised regression approach was used to quantify the difference between microscopy and qPCR measurements of *Dinophysis* cell abundance. This was done by fitting Generalized Additive Models to data from each study site independently of other sites. To do this we modelled *Dinophysis acuminata* cell count *y_i* taken on day *t* at the log-scale through the combination of smooth functions of time, the effect of the cell count methodology used, and a Gaussian (Normal) error term:

$$\log(y_i(t)) = \beta_0 + f(t) + g(t)x_i + \in_i$$

$$\in_i \sim Normal\left(0, \sigma^2\right)$$

The term *f(t)* captured smooth variation over time in the microscopy measurements, tracking increases and decreases in cell abundance. The variable *x_i* equals 0 if measurement *i* is a microscopy result and equals 1 if measurement *i* is a qPCR result. The term *g(t) x_i* therefore captures smooth variation over time in the difference (at the log-scale) between laboratory measurements and qPCR measurements. The term *g(t)* is the 'qPCR coefficient' as for any given *t* it tells you what value a qPCR result must be divided by to obtain a result on the same scale of microscopy results.

3. Results

In this study, Shellfish production areas were monitored for harmful algae by measuring phytoplankton cells, biotoxins, and DNA, focusing on three coastal sites in Southwest England (Fig. 1). The region is frequently impacted by HABs, and Local Authority (LA) patrols regularly collect water and shellfish (mussel) samples for determination of phytoplankton cell densities and biotoxins respectively. During the study period of September to March 2021/22, additional sampling was undertaken to include the collection of suspended cells by passing seawater through specialist filtration units. The filter membranes were processed to extract DNA, which was analysed by qPCR to estimate the levels of *Dinophysis accuminata, Alexandrium* spp. and *Pseudo-nitzschia* spp.

3.1. Dinophyceae cell densities and DSP biotoxin analysis

Direct phytoplankton counts were carried out by microscopy, analysing water samples that had been fixed in situ. The analysis focused exclusively on the identification of *Dinophyceae* spp., a group that has historically been a problem within the study location (Dhanji-Rapkova et al., 2018), speciating each cell as far as possible. Most samples contained undetectable or low cell densities that fell below the threshold 'trigger level' of 100 cells per litre for this group. This is the minimum cell number at which it is required to initiate an increase in the frequency of shellfish flesh sampling for biotoxin analysis set out in Regulation (EC) 2017/625 and 2019/627 (formerly 854/2004). However, samples collected in September and October often breached trigger levels, containing various *Dinophysis* species including most commonly *D. acuminata* complex. This occurred on multiple occasions at each location. Other species detected were *D. ovum* and *D. fortii*, together with rare occurrences of *D. sacculus, D. acuta, D. dens, D. caudata*, as well as *Phalacroma rotunda* and *Phalacroma nasturtium* (formerly classified as *Dinophysis* sp.). The full set of microscopy results is given in Table 2.

OA-group toxins from *Dinophysis* were determined in mussel flesh extracts regardless of whether microscopy indicated the presence of *Dinophysis* cells. The analysis measured freely extractable and total okadaic acid (after alkaline hydrolysis incorporating both free and esterified toxins) and OA analogues dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2). The LC-MS/MS analysis included PTX toxins,

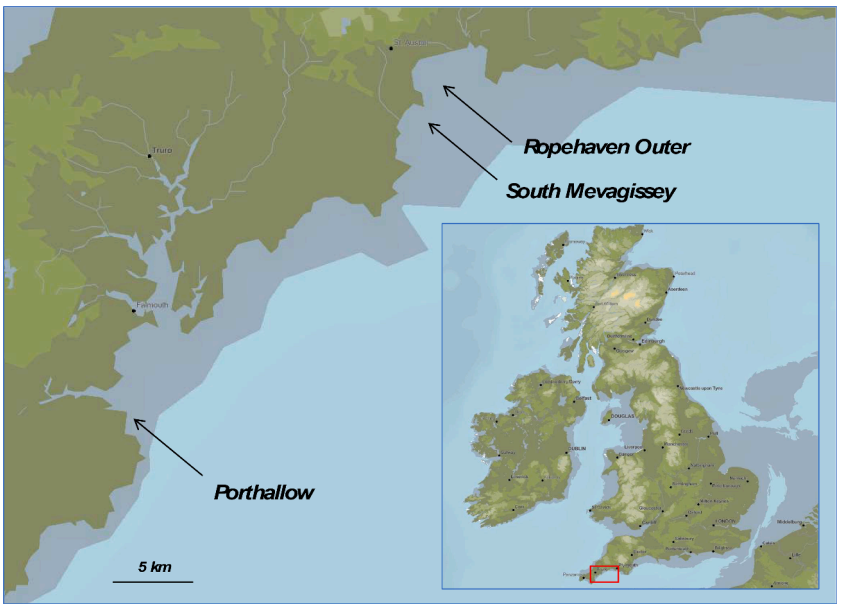

Fig. 1. Study Sites off Southwest England, County of Cornwall. Samples were collected from three shellfish production areas within St Austell Bay, a known HAB hotspot, between September 2021 and March 2022.

but none were detected, as well as other Lipophilic Toxins (LTs) such as Azaspriacids and Yessotoxins, but these are not reported as they have no relevance to *Dinophysis* spp. As expected, levels of total OA in mussel flesh coincided with elevated levels of *Dinophysis* cells in the water, with results shown in Table 3. The dominant toxin detected and quantified in the mussel tissues was OA, with only trace levels of DTX2 and very occasionally trace DTX1. On average 97% ± 8% of the total DSP toxin concentrations consisted of OA. The results indicated that the majority of OA was present in ester form, with on average only 15% ± 17% freely extractable. This means that alkaline hydrolysis is essential to avoid significantly underestimating total OA-group toxicity in mussel samples from this region.

3.2. Estimation of HAB cell numbers by qPCR

This study focused on the *Dinophysis* group, of which *D. accuminata* is typically the most abundant species, however the qPCR analysis was expanded to include additional HAB taxa *Alexandrium* spp. and *Pseudo-nitzschia* spp, which also bloom within each sampling location (Fig. 1). DNA was extracted from filter membranes prepared on-site, and amplified using primer sets that were specific for each group. For *D. accuminata* and *Alexandrium* spp. cell number was estimated by quantifying the number of target gene copies in each sample, and considering the number of copies expected per cell. For *Pseudo-nitzschia* spp. cell number was determined using cell number standards prepared from living cultures of *Pseudo-nitzschia* multistriata,. Details are given in the materials and methods section.

Fig. 2 shows the results of qPCR-based quantification of each HAB taxa from September 2021 to March 2022 for the 3 sites, with the results expressed as estimated cells per litre. Each HAB taxa was detected (a positive amplification versus no DNA controls) in all samples, throughout the study. *D. accuminata* levels ranged from as low as ~10 estimated cells/L in samples collected between January and March, and up to >10,000 estimated cells/L recorded at the Porthallow site at the end of September. Estimated *D. accuminata* cell numbers were highest in the early phase of the study (September-November), when the microscopy and LC-MS/MS analysis indicated a similar increase in *Dinophysis* cells and elevated levels of lipophilic toxins in mussel flesh. Therefore, the qPCR analysis was successful in recording this event, which occurred across all 3 study sites. The trigger level for *Dinophysis* phytoplankton is set at 100 cells/L, at which point the local authority would implement an increase in the frequency of mussel sampling for biotoxin analysis. Although microscopy data indicates that this was breached on 12 occasions, all within September and October, the qPCR method indicated a breach on 31 occasions throughout study period. Furthermore, there was a clear increase in qPCR-estimated *D. accuminata* cell abundance towards the end of the study (late March), where estimated cell numbers at each site were more than 200 cells/L, but this was generally not detected in the microscopy or biotoxin screening data, with the exception that one sample taken from Porthallow in mid-March contained 160 *Dinophysis* spp. cells/L (120 cells/L speciated as *D. accuminata*). This, however, did not lead to contamination of local mussels with lipophilic toxins, as evident from the biotoxin screening for which all samples from mid-November were below the reporting limit of 16 μg OA eq/kg.

Microscopy cell counts and biotoxin data were only collected for *D. accuminata*, however the DNA extracts prepared for qPCR could be used to estimate the abundance of other HAB taxa co-occurring within the study window. Using the same DNA extracts, *Alexandrium* spp. and *Pseudo-nitzschia* spp. were also detected in every sample. Levels of both groups generally followed the same trend as for *D. accuminata*, increasing towards the beginning and end of the study, when sea temperatures and sunlight intensity would have been higher. There was an upwards trend in estimated cell numbers for all taxa and sites, starting from late February, potentially indicating the beginning of a 'spring' bloom. In the locality, the trigger level for *Alexandrium* spp. is set at >40 cells/L, which therefore (according to qPCR-based estimations) was breached frequently (in 25/44 samples). The highest levels occurred at the South Mevagissey site, where estimated cell numbers reached ~1000 cells/L on 2 occasions. Although the estimated levels of *Pseudo-nitzschia* spp. were generally far higher than for both other groups, never falling below and estimated ~1000 cells/L, and reaching more than 100,000 estimated cells/L at the Outer Ropehaven site during March, a trigger level of 150,000 cells/L was never breached.

3.3. Statistical comparison between microscopy and qPCR

There were discrepancies between the estimated *Dinophysis acuminata* cell number obtained by qPCR and the actual cell counts obtained using microscopy. To investigate this, a statistical comparison was made between each set of results over the study period. This was challenging

Table 2
Microscopy Cell Counts for *Dinophysis* spp.

Study Site	Grid Reference	Sample Collection Method	Date Sample Collected	Total *Dinophysiaceae* cells	*Dinophysis* sp	*Dinophysis acuminata* complex	*Dinophysis ovum*	*Dinophysis fortii*	*Dinophysis sacculus*	*Dinophysis acuta*	*Dinophysis dens*	*Phalacroma rotundata*	*Dinophysis caudata*	*Phalacroma nastutum*
Ropehaven Outer	SX05744972	POLE	13/09/2021	**1080**	**40**	**840**	**80**	**120**	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	15/09/2021	**1720**	**40**	**1480**	**120**	**80**	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	15/09/2021	**160**	ND	**160**	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	SURFACE	20/09/2021	**920**	**120**	**480**	**160**	**120**	**40**	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	20/09/2021	**1000**	ND	**1000**	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	22/09/2021	**800**	**80**	**440**	**200**	ND	ND	ND	**40**	**40**	ND	ND
Porthallow North	SW80212383	POLE	22/09/2021	**760**	**80**	**480**	**120**	ND	ND	**40**	**40**	ND	ND	ND
Porthallow North	SW80212383	POLE	27/09/2021	**80**	**40**	**40**	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	27/09/2021	**160**	**40**	**120**	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	29/09/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	29/09/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	04/10/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	06/10/2021	**40**	ND	ND	**40**	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	11/10/2021	**40**	ND	**40**	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	11/10/2021	**120**	**40**	ND	ND	ND	ND	**40**	ND	**40**	ND	ND
Ropehaven Outer	SX05744972	POLE	11/10/2021	**80**	ND	**80**	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	13/10/2021	**120**	ND	**80**	ND	ND	ND	**40**	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	10/13/2021	**160**	ND	**120**	ND	ND	**40**	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	18/10/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	17/10/2021	**200**	**40**	**120**	ND	ND	ND	ND	ND	ND	**40**	ND
South Mevagissey Bottom	SX05214698	POLE	21/10/2021	**40**	ND	**40**	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	25/10/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	10/20/2021	**80**	ND	ND	ND	ND	ND	**40**	ND	**40**	ND	ND

(*continued on next page*)

Table 2 (*continued*)

Study Site	Grid Reference	Sample Collection Method	Date Sample Collected	Total *Dinophysiaceae* cells	*Dinophysis* sp	*Dinophysis acuminata* complex	*Dinophysis ovum*	*Dinophysis fortii*	*Dinophysis sacculus*	*Dinophysis acuta*	*Dinophysis dens*	*Phalacroma rotundata*	*Dinophysis caudata*	*Phalacroma nastutum*
Ropehaven Outer	SX05744972	POLE	27/10/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	02/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	02/11/2021	**40**	ND	ND	ND	ND	ND	ND	ND	ND	ND	**40**
Ropehaven Outer	SX05744972	POLE	03/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	08/11/2021	**40**	ND	**40**	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	10/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	17/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	17/11/2021	**40**	ND	**40**	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	17/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	24/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	24/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	01/12/2021	**40**	ND	ND	ND	**40**	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	29/11/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	06/12/2021	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	17/01/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	18/01/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	24/01/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	26/01/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	01/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	02/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	02/07/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	02/09/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	14/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

(*continued on next page*)

Table 2 (*continued*)

Study Site	Grid Reference	Sample Collection Method	Date Sample Collected	Total *Dinophysiaceae* cells	*Dinophysis* sp	*Dinophysis acuminata* complex	*Dinophysis ovum*	*Dinophysis fortii*	*Dinophysis sacculus*	*Dinophysis acuta*	*Dinophysis dens*	*Phalacroma rotundata*	*Dinophysis caudata*	*Phalacroma nastutum*
South Mevagissey Bottom	SX05214698	POLE	14/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	22/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
porthallow North	SW80212383	POLE	23/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	03/02/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	02/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	07/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	SURFACE	10/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	14/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	24/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ropehaven Outer	SX05744972	POLE	24/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
South Mevagissey Bottom	SX05214698	POLE	29/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Porthallow North	SW80212383	POLE	30/03/2022	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Dark shading indicates a breach in Trigger level. Light shading indicates a positive result that did not breach Trigger level.

Table 3
Okadaic acid group biotoxin analysis results.

Date	Site	Free OA	Free DTX1	Free DTX2	OA total	DTX1 total	DTX2 total	Total OA/DTXs/PTXs (μg OA eq/kg) Low value calculated from MU	Total OA/DTXs/PTXs (μg OA eq/kg) Actual calculated value	Total OA/DTXs/PTXs (μg OA eq/kg) High value calculated from MU
20/09/2021	Ropehaven outer	65.5			188.8			107	189	271
20/09/2021	Mevagissey	26.1			77.3			44	77	111
20/09/2021	Porthallow	2.4			22.8			13	23	33
22/09/2021	Porthallow	18.3		1.9	54		1.9	30	54	78
22/09/2021	Mevagissey	25.4			83.5			47	84	120
27/09/2021	Ropehaven outer	42.5		1.6	127.7		2.9	72	128	183
27/09/2021	Mevagissey	30.9			99.3		1.2	56	99	143
27/09/2021	Porthallow	14.4			45.3			26	45	65
29/09/2021	Porthallow North	15			46.1			23	46	69
29/09/2021	Ropehaven	42.2			121.1			68	121	174
04/10/2021	South Mevagissey Bottom	26.4		1.4	61		2.5	31	61	91
04/10/2021	Ropehaven Outer	35.5		0.9	85.2		2.2	43	85	127
04/10/2021	Porthallow	1.4			10.7			<RL	<RL	<RL
06/10/2021	Ropehaven Outer	39.9	1.8		67.6	1.8	3.6	34	68	101
06/10/2021	Porthallow North	4	2.1		12.5	3.6		<RL	<RL	<RL
06/10/2021	Mevagissey	35			68.3			39	68	98
11/10/2021	Ropehaven Outer	22.1			54.3			31	54	78
11/10/2021	Porthallow				2.9			<RL	<RL	<RL
13/10/2021	South Mevagissey Bottom	23.9			58.7			33	59	84
13/10/2021	Porthallow North				5.1			<RL	<RL	<RL
13/10/2021	Ropehaven	8.6			37.6			21	38	54
18/10/2021	Ropehaven Outer	15.2			29.8			17	30	43
18/10/2021	South Mevagissey Bottom	8.8		0.5	31.3		1.6	18	31	45
18/10/2021	Porthallow				8.8			<RL	<RL	<RL
20/10/2021	Porthallow North	2.1			7.9		1.5	<RL	<RL	<RL
21/10/2021	Mevagissey	1.7			19.3			11	19	28

(*continued on next page*)

Table 3 (*continued*)

Date	Site	Free OA	Free DTX1	Free DTX2	OA total	DTX1 total	DTX2 total	Total OA/DTXs/PTXs (μg OA eq/kg) Low value calculated from MU	Total OA/DTXs/PTXs (μg OA eq/kg) Actual calculated value	Total OA/DTXs/PTXs (μg OA eq/kg) High value calculated from MU
25/10/2021	Ropehaven Outer	10.2		2.5	23.2		3.5	13	23	33
25/10/2021	Porthallow	1.6			9.9			<RL	<RL	<RL
27/10/2021	Ropehaven	8		0.7	25.3		1.3	13	25	38
01/11/2021	Ropehaven Outer	7.1		1.1	17.3		2.2	10	17	25
01/11/2021	Porthallow	1.4			5.1			<RL	<RL	<RL
03/11/2021	Ropehaven	10.3		0.8	28.5		0.8	14	29	43
08/11/2021	Ropehaven Outer	15.4			38.7			20	39	58
10/11/2021	Ropehaven	3.9		0.4	17.3		0.4	9	17	26
15/11/2021	South Mevagissey Bottom			2.3	7.3		2.3	<RL	<RL	<RL
15/11/2021	Ropehaven Outer				6.7			<RL	<RL	<RL
17/11/2021	Porthallow North				3.1			<RL	<RL	<RL
17/11/2021	Porthallow	1.8		0.2	7.3		0.2	<RL	<RL	<RL
17/11/2021	Mevagissey	2.6		0.5	9.5		1.9	<RL	<RL	<RL
22/11/2021	Mevagissey Bay							<RL	<RL	<RL
22/11/2021	St. Austell Bay							<RL	<RL	<RL
24/11/2021	Ropehaven	2		0.3	11.5		0.2	<RL	<RL	<RL
24/11/2021	Porthallow	1.2			8.3			<RL	<RL	<RL
29/11/2021	Ropehaven Outer				6.3		4	<RL	<RL	<RL
29/11/2021	South Mevagissey Bottom				5		3.8	<RL	<RL	<RL
29/11/2021	Porthallow				6.3			<RL	<RL	<RL
01/12/2021	Mevagissey	2.4		0.3	9.2		0.1	<RL	<RL	<RL
06/12/2021	South Mevagissey Bottom				5.6			<RL	<RL	<RL
06/12/2021	Ropehaven Outer				6.1		0.8	<RL	<RL	<RL
14/12/2021	Porthallow North				4.3			<RL	<RL	<RL
05/01/2022	Ropehaven Outer	0.9		0.4	8.6		0.4	<RL	<RL	<RL
05/01/2022	South Mevagissey Bottom	0.3			4.6			<RL	<RL	<RL
13/01/2022	Porthallow North				0.1			<RL	<RL	<RL

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Table 3 (*continued*)

Date	Site	Free OA	Free DTX1	Free DTX2	OA total	DTX1 total	DTX2 total	Total OA/DTXs/PTXs (μg OA eq/kg) Low value calculated from MU	Total OA/DTXs/PTXs (μg OA eq/kg) Actual calculated value	Total OA/DTXs/PTXs (μg OA eq/kg) High value calculated from MU
17/01/2022	Porthallow				3.4			<RL	<RL	<RL
18/01/2022	Mevagissey				4.9			<RL	<RL	<RL
22/01/2022	Porthallow				4.5			<RL	<RL	<RL
24/01/2022	Ropehaven				4.8			<RL	<RL	<RL
31/01/2022	Mevagissey				5			<RL	<RL	<RL
02/02/2022	Porthallow				4.8			<RL	<RL	<RL
07/02/2022	South Mevagissey Bottom				8.2			<RL	<RL	<RL
07/02/2022	Ropehaven Outer				5			<RL	<RL	<RL
07/02/2022	Porthallow				4.8			<RL	<RL	<RL
09/02/2022	Ropehaven				7			<RL	<RL	<RL
14/02/2022	Mevagissey				6.8			<RL	<RL	<RL
14/02/2022	Porthallow				4.2			<RL	<RL	<RL
22/02/2022	Ropehaven				6.7			<RL	<RL	<RL
23/02/2022	Porthallow North							<RL	<RL	<RL
23/02/2022	Porthallow				6.5			<RL	<RL	<RL
02/03/2022	Mevagissey				10.4			<RL	<RL	<RL
03/03/2022	Porthallow				4.1			<RL	<RL	<RL
07/03/2022	Ropehaven				7			<RL	<RL	<RL
10/03/2022	Porthallow				5.5			<RL	<RL	<RL
11/03/2022	Porthallow North				0.8			<RL	<RL	<RL
14/03/2022	Porthallow				5.4			<RL	<RL	<RL
17/03/2022	Mevagissey				6.3			<RL	<RL	<RL
18/03/2022	Ropehaven Outer				6.7			<RL	<RL	<RL
18/03/2022	South Mevagissey Bottom				4			<RL	<RL	<RL
24/03/2022	Ropehaven				6.7			<RL	<RL	<RL
24/03/2022	Porthallow				6.5			<RL	<RL	<RL
29/03/2022	Mevagissey				7			<RL	<RL	<RL

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Table 3 (*continued*)

Date	Site	Free OA	Free DTX1	Free DTX2	OA total	DTX1 total	DTX2 total	Total OA/DTXs/PTXs (µg OA eq/kg) Low value calculated from MU	Total OA/DTXs/PTXs (µg OA eq/kg) Actual calculated value	Total OA/DTXs/PTXs (µg OA eq/kg) High value calculated from MU
30/03/2022	Porthallow				8.8			<RL	<RL	<RL

Dark shading indicates values above permissible limit. <RL = less than reporting limit of 16 µg OA eq/kg.

because the microscopy data contained a very high proportion of non-detects (74%), therefore, to enable quantitative comparison with the qPCR results, all non-detects were replaced with the value 10 cells/L (i.e. 25% of the minimum detection limit for the microscopy method, which was 40 cells.). A different value could have been chosen, meaning all results in this statistical comparison were conditional on that choice. Cell counts obtained using both methodologies over the project duration, for the three study sites are shown in Fig. 3. The qPCR measurements were around one order of magnitude higher than the microscopy measurements, both capturing a decrease in cell abundance in the early phase of the study (September to November 2021), likely following the end of the 2021 summer/autumn bloom season. Towards the end of the project (March 2022) in Mevagissey Bay, there was a noteworthy increase in the estimated *D. accuminata* levels from the qPCR results that was not captured in the microscopy results. This may have indicated the very beginnings of a spring bloom. Using Offical Controls (OC) data it was possible to confirm that the trend in the qPCR results corresponded with the results of OC microscopy data collected after our study was terminated; in this case the qPCR provided an earlier indication of a *D. accuminata* bloom by approximately 4 weeks .

A penalised regression approach was used to quantify the difference between microscopy and qPCR measurements of *Dinophysis* cell abundance more formally, fitting Generalized Additive Models to data from each site independently of other sites. The term *g(t)*, Fig. 4, is the 'qPCR coefficient' as for any given *t* it tells you what value a qPCR result must be divided by to obtain a result on the same scale of microscopy results. The coefficient fluctuated considerably over the study period, for instance in the Porthallow site the qPCR measurements were on average over 30 times higher than microscopy results in September, reducing to under 10 times in March.

4. Discussion

Consumption of seafood contaminated with okadaic acid group toxins, produced by the *Dinophysiaceae,* can cause a diarrhoetic shellfish poisoning syndrome characterised by abdominal pain and severe diarrhoea caused by disruption to intestinal regulation and function (Lloyd et al., 2013). In severe cases this can be fatal. Therefore the development and evaluation of new approaches towards advanced early warning and surveillance of algal populations is required to maintain standards in seafood production world-wide. Breaches in permissible levels of toxigenic algal cells and toxins within the overall study location, St Austell Bay, are common. Particularly, the Ropehaven site has experienced repeated HAB events since the site opened in 2010 (Ross Brown et al., 2022). Mean HAB frequencies in 2010–2017 (expressed as% of weeks throughout May-August, in which cell counts in surface water (2 m depth) exceeded advisory trigger levels) were: *Dinophysis* spp. (10%), *Prorocentrum cordatum* (7%), *Alexandrium* spp. (3%); *Pseudo-nitzschia* spp. (4%). In 2018, during a summer heatwave, *Dinophysis* spp. bloomed and OA-group toxin concentrations in shellfish meat exceeded EU regulatory action levels (160 µg kg^{-1} OA eq), causing the site to close for 18 consecutive weeks from spring to early autumn. This study provides evidence that the contamination of local seafood with OA remains an issue in 2021. However, there have been no toxin breaches in relation to other HAB species at Ropehaven.

Quantitative PCR could provide early warning of these events because the analytical process is significantly faster than cell counting by microscopy. However, there is a paucity of data for real-world applications of qPCR in HAB surveillance programs, and to our knowledge the technique has not before been adopted for a time-series measurement of HAB species in this region. Marine phytoplankton are abundant, even outside of bloom events and the qPCR methodology is highly sensitive. Therefore, and as anticipated, each sample taken during the study contained detectable levels of algal DNA. However, the full significance of these results is unclear because, for reasons that are discussed below, the use of qPCR data for the estimation of cell number is

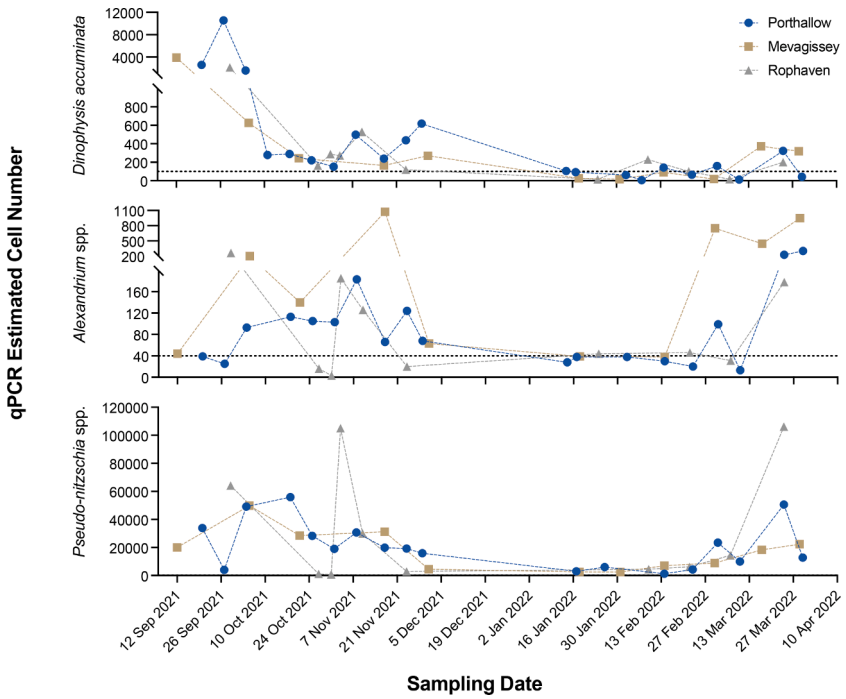

Fig. 2. qPCR-estimated phytoplankton cell number at each site over the duration of the study. Water samples were collected from 3 shellfish production areas and filtered to recover suspended cells. Filter membranes were transported back to the laboratory where DNA was isolated, purified and analysed by qPCR to estimate the numbers of *D. accuminata, Alexandrium* spp. and *Pseudo-nitzschia* spp, expressed as estimated number of cells per litre of seawater.

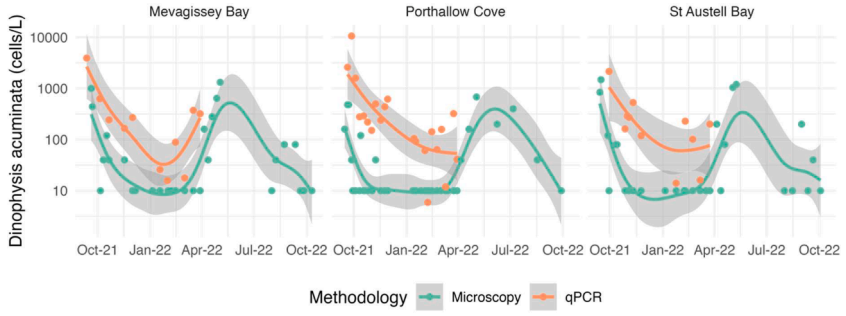

Fig. 3. A comparison of microscopy and qPCR measurements of *Dinophysis acuminata* cell at 3 study sties between September 2021 and March 2022. The data points represent measurements, with non-detects (microscopy only) assigned a value of 10 cells/L, with fitted smooth lines of best fit with 95% confidence intervals. Microscopy data points shown after the end of March 2022 were taken from Official Controls data and were not generated as part of this study.

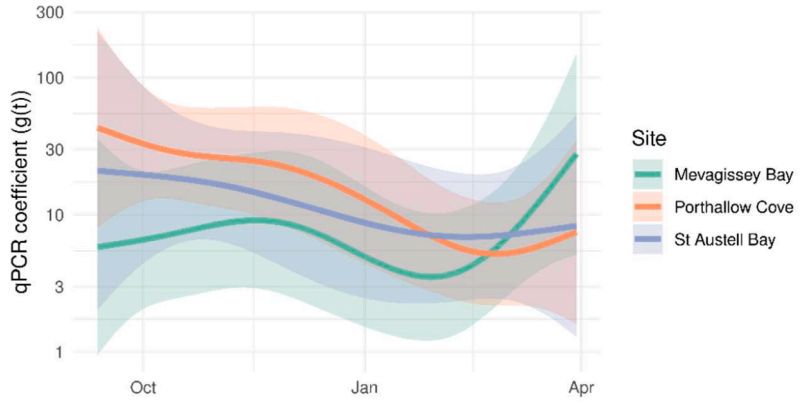

Fig. 4. Comparisson of qPCR and Microscopy Cell Counts for *D. accuminata*. Estimates of the qPCR coefficient g(t) as the value you should divide a qPCR cell count by to obtain a result on the same scale of contemporary microscopy-based counts, with 95% confidence intervals.

subject to a number of assumptions, and a full consideration of these limitations is necessary to fully interpret the potential utility of the technique, including for other environmental monitoring applications beyond HABs. However, crucially, the measurement of the comparative levels of target DNA sequences between samples (e.g. the identification of an upward or downwards trend in phytoplankton cell number) is not subject to these assumptions and can be interpreted with greater confidence.

In this study the qPCR estimation of *D. accuminata* was consistently higher than cell counts taken by microscopy, and we suggest two reasons for this. First, the qPCR method cannot unequivocally discriminate between DNA obtained from living and dead cells; all DNA collected on the filter membranes will have contributed to the PCR amplification. In contrast, the microscopy method actively excludes cell fragments and cells which appear non-viable at the discretion of the microscopist. It is possible to exclude extracellular DNA from the qPCR analysis by treating samples to preferentially degrade or digest nucleic acids that are not protected by an in-tact cell membrane (Champlot et al., 2010; Nocker et al., 2006), however this adds complexity, cost, delay and additional sources of error to the workflow. The DNA overestimation bias is reduced if Reverse Transcription qPCR (RT-qPCR) is used to quantify RNA, a labile analogue of DNA that is bio-synthesised only by living and metabolically active cells and has a short environmental 'half-life' (Li et al., 2017). This is particularly relevant in scenarios where only the viable cell fraction is important, for example in the detection of infectious agents, however as the RNA component of a cell can vary under changing conditions it can be difficult to interpret quantitative results without a detailed understanding of how cellular RNA levels are regulated in the target group.

A second source of discrepancy between qPCR and microscopy is the difficulty in accurately translating the qPCR result, which reports DNA target sequence copies, into a cell count. The phytoplankton assays used in this study target ribosomal subunit encoding gene sequences, for which the number of copies in a cell can vary considerably between species and populations, and the genomes of many important HAB species have not yet been made available. This characteristic is potentially one of the greatest challenges when using 'molecular' methods such as qPCR to achieve a cell count. Our quantification of *D. accuminata* and *Alexandrium* spp. was achieved by assuming the number of copies per cell based on information provided by others (Ajani et al., 2021; Galluzzi et al., 2004) and represents an estimate only; our prediction on cell number is entirely dependant on these assumptions. The same limitation applies to all qPCR assays that target species for which there is uncertainty in target gene copy number. However, importantly, this does not impact the potential benefit of qPCR as an advanced early warning technique because the relative levels of target sequence between samples collected through time are not impacted by gene copy variation. Therefore, trends in algal abundance can be interpreted with some confidence including, for example, sudden increases that may indicate the beginning of a bloom or a decline in a population indicating a return to safe levels. This information could guide the frequency of microscopy analysis in order to confirm actual cell numbers required for compliance with advisory trigger levels (abundance thresholds).

Our estimation of *Pseudo-nitzschia* spp. cell number did not assume a gene copy number, rather a cell count was determined from a standard curve produced from cultured *Pseudo-nitzschia* sp. cells (*P. multistriata*). This approach could improve the accuracy of cell counts if the cell standards contain the same genomic composition as the environmental populations, but this was unclear in this case. Altogether, the issue of absolute quantification for HAB assays that measure ribosomal gene sequences requires more information on the genetic composition and variability of populations within the study area, which could be achieved with the preparation of genome sequencing datasets to assess the relationship between cell number and rDNA gene copies. Recently, a digital PCR (dPCR) method was adopted for the prediction of large subunit (18S) sequence copy number in a range of eukaryotic single cells, including *Dinophysis fortii* (Yarimizu et al., 2021). In future a similar approach could be used to characterise the populations impacting the St Austell Bay study area. Alternatively, the selection of low copy number gene targets would improve confidence in cell estimations. Low copy number gene sequences that are associated with toxin biosynthesis make attractive candidates for HAB surveillance, particularly if this approach could be used to discriminate between toxic and non-toxic blooms. However, there remains uncertainty in the relationship between the presence of 'toxin-gene' sequences and the actual capacity of a cell to biosynthesise the corresponding compounds.

Other sources of error (e.g. DNA overestimation) could be addressed by considering the qPCR-based cell estimates in tandem with those obtained by microscopy, using a statistical approach for calibration between the two methods. The statistical comparison described in Fig. 3 and Fig. 4 has two main limitations. The first is the high proportion of non-detects in the laboratory data. The second is that the project period, occurring over winter, mostly coincided with periods of low cell abundance. Taken together, these limitations make it challenging to draw firm conclusions on the discrepancies seen between either method. For example, the uptick in cell abundance seen in Mevagissey Bay might suggest increased sensitivity of the qPCR methods in detecting the early stages of the spring bloom, which would significantly improve prospects for regulatory monitoring using qPCR for early warning. This is because there may only be a brief time between when increased cell abundance is detected by microscopy and toxin concentrations in shellfish flesh breach safe harvesting levels. A key advantage of the qPCR approach is the provision of quantitative estimates even when HAB cell counts are low, such that more proactive measures can be taken to reduce HAB impacts. Nonetheless, as already stated the relative HAB cell abundance between samples is unaffected by these assumptions, and therefore the current qPCR methodology could be used reliably to indicate trends in phytoplankton populations between sampling sites and through a monitoring time series. The potential of qPCR for early warning in population fluxes is unaffected by these limitations once a baseline level has been established from which any increase above that baseline may indicate an upwards trend in HAB cells.

In conclusion, we have shown that qPCR analysis has considerable utility in the early warning of HAB events. Our results indicate that qPCR methods are more rapid, time- and cost- efficient and sensitive for early detection of increasing trends in HAB cell abundance (compared to traditional microscopic analysis). Studies like ours are important first steps towards building a case for including qPCR in OC methods for HAB early warning. Before replacing microscopy methods longer-term intercalibration between qPCR and microscopic methods and trigger levels (cell abundance thresholds) are likely to be needed and demonstrated for more sites and for a more comprehensive set of HAB species. Trigger levels are 'advisory' only, offering some flexibility to embrace newly adopted methodology. Training and ring-testing will also be required to ensure capability and consistency between analytical laboratories, possible including the standardisation of qPCR methodology.

CRediT authorship contribution statement

Jonathan S. McQuillan: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization, Supervision, Funding acquisition, Project administration. **Ahmed Alrefaey:** Methodology, Validation, Investigation. **Andrew D. Turner:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization, Supervision, Funding acquisition, Project administration. **Nadine Morrell:** Writing – original draft, Project administration, Investigation. **Oliver Stoner:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization, Supervision, Funding acquisition, Project administration. **Ross Brown:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization, Supervision, Funding acquisition, Project administration. **Suzanne Kay:** Methodology, Validation, Investigation. **Simon Cooke:**

Methodology, Validation, Investigation. **Timothy Bage:** Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Visualization, Supervision, Funding acquisition, Project administration.

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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hal.2023.102497.

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