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# Development of a qPCR assay to detect and quantify ichthyotoxic flagellates along the Norwegian coast, and the first Norwegian record of *Fibrocapsa japonica* (Raphidophyceae)

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ABSTRACT

Blooms of ichthyotoxic microalgae pose a great challenge to the aquaculture industry world-wide, and there is a need for fast and specific methods for their detection and quantification in monitoring programs. In this study, quantitative real-time PCR (qPCR) assays for the detection and enumeration of three ichthyotoxic flagellates: the dinoflagellate *Karenia mikimotoi* (Miyake & Kominami ex Oda) Hansen & Moestrup and the two raphidophytes *Heterosigma akashiwo* (Hada) Hada ex Hara & Chihara and *Fibrocapsa japonica* Toriumi & Takano were developed. Further, a previously published qPCR assay for the dinoflagellate *Karlodinium veneficum* (Ballantine) Larsen was used. Monthly samples collected for three years (Aug 2009–Jun 2012) in outer Oslofjorden, Norway were analysed, and the results compared with light microscopy cell counts. The results indicate a higher sensitivity and a lower detection limit (down to 1 cell $L^{-1}$) for both qPCR assays. Qualitative and semi-quantitative results were further compared with those obtained by environmental 454 high throughput sequencing (HTS, metabarcoding) and scanning electron microscopy (SEM) examination from the same samplings. All four species were detected by qPCR and HTS and/or SEM in outer Oslofjorden (Aug 2009–Jun 2012); *Karlodinium veneficum* was present year-round, whereas *Karenia mikimotoi, Heterosigma akashiwo* and *Fibrocapsa japonica* appeared mainly during the autumn in all three years. This is the first observation of *Fibrocapsa japonica* in Norwegian coastal waters. This species has previously been recorded off the Swedish west coast and German Bight, which may suggest a northward dispersal.

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## 1. Introduction

During the last few decades, significant attention has been paid to harmful algal bloom (HAB) events. Most coastal regions in the world are affected, and the number of described causative species and the toxins they produce are increasing (Tillmann et al., 2009). Over the past few decades HABs have increased in frequency (Anderson et al., 2012), which imposes financial constraints on the aquaculture industry. To reduce financial losses and improve seafood safety, most countries that trade in seafood have an algal monitoring system in place (Medlin, 2013).

The current standard method in monitoring of microalgae is based on the counting technique described by Utermöhl (1958), where a volume of water sample (usually 5–50 mL) is preserved with a fixative, such as Lugol's solution, and left to settle in a sedimentation chamber before enumeration in an inverted microscope. The accuracy of this method is dependent on several factors, such as the sampling procedure, the fixative chosen, and the taxonomic expertise of the researcher conducting the survey (Bott et al., 2010). Another factor is the morphology of the species of interest e.g. small size, lack of hard cell components, and fixative induced changes to the morphology can make many flagellates difficult to detect and enumerate correctly under a light microscope (LM). Recent investigations indicate that the species

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diversity is larger than observed by microscopy for dinoflagellates (Nézan et al., 2014), haptophytes (Egge et al., 2015) and protists in general (de Vargas et al., 2015). Time- and financial restraints make it desirable to develop molecular methods to compliment LM cell counts in monitoring programs (Medlin, 2013). Implementing molecular methods in monitoring for certain ichthyotoxic species may lower detection limits, increase sensitivity and accuracy, and reduce both costs and processing time per sample. Several molecular techniques have been developed for the detection and quantification of microalgae, such as fluorescent in situ hybridization (FISH-probes) or microarrays with molecular probes and high throughput sequencing, but quantitative real-time PCR (qPCR) is currently considered the most advantageous for detection and quantification of a restricted number of target species (Ebenezer et al., 2012). The initial tasks of designing, testing, and validating qPCR primers and hydrolysis probes is a major effort, but once an assay is established, it is highly sensitive, specific, and cost-effective. It can also be applied to preserved environmental samples (Bott et al., 2010; Eckford-Soper and Daugbjerg, 2015a). Recently qPCR has been utilized in monitoring of ichthyotoxic *Prymnesium parvum* Carter in USA (Zamor et al., 2012) and several toxic species in New Zealand, e.g. *Alexandrium catenella* (Whedon & Kofoid) Balech (Rhodes et al., 2013; Smith et al., 2014), and the results are promising. One of the drawbacks of qPCR is that it only detects targets actively being searched for. Consequently, untargeted and invasive species will go unnoticed, and it will not give information about the phytoplankton community as a whole. There are other molecular techniques, viz., microarrays and environmental high throughput sequencing (HTS) of marker genes, also called metabarcoding (Dittami et al., 2013; Kegel et al., 2016, de Vargas et al., 2015), which used in combination with qPCR could facilitate and improve monitoring in the future.

The present study focused on four ichthyotoxic flagellates, known- or suspected to occur in Norwegian coastal waters: *Karenia mikimotoi, Karlodinium veneficum, Heterosigma akashiwo* and *Fibrocapsa japonica*. The two dinoflagellates, *K. mikimotoi* and *K. veneficum,* have formed recurrent blooms in the Oslofjorden (Throndsen et al., 2003). One of the first major algal blooms to cause public attention in Norway was caused by the raphidophyte, *H. akashiwo,* in 1964 (Braarud and Nygaard, 1967), and since then, it has been reported regularly (Naustvoll et al., 2002). The other targeted raphidophyte, *F. japonica,* has not previously been reported in Norwegian coastal waters. It has, however, been reported from several other European locations in the North Sea (Elbrächter, 1999) and from the Swedish west coast (www.smhi.se/klimatdata/oceanografi/havsmiljodata).

The aim of this study was to develop a rapid detection and enumeration method for these ichthyotoxic species, which can be utilized in algal monitoring as a compliment to LM. We further wanted to improve our knowledge about the seasonal distribution of ichthyotoxic species present in the Skagerrak.

## 2. Material and methods

### 2.1. Field sampling

Field sampling was carried out monthly using the University of Oslo's research vessel R/V Trygve Braarud over the course of three years (Aug 2009–Jun 2012) at station OF2 (59.18 N, 10.69 E) in outer Oslofjorden (Fig. 1). Water samples were collected from 1 m depth using Niskin bottles attached to a rosette and used for all samples described below. For LM cell counts, 100 mL samples were collected directly from the Niskin bottles and preserved with 1 mL neutral Lugol's solution (Throndsen, 1978). Samples for DNA-isolation and subsequent qPCR were collected in two ways: during

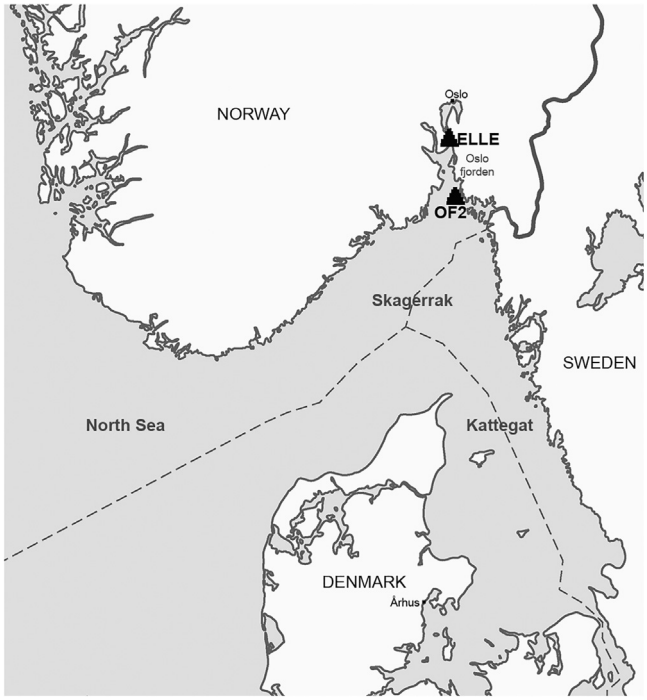


**Fig. 1.** Map of sampling area, with stations OF2 and ELLE in outer Oslofjorden marked.

the first two years (Aug 2009–Jun 2011) 20 L seawater were pre-filtered through a 180 μm mesh to remove large zooplankton, before being filtered by peristaltic pumping (Masterflex 07523-80, ColeParmer, IL, USA), on to 0.45 μm pore size, 142 mm diameter Durapore filters (Polyvinylidene fluoride (PVDF), Millipore, Billerica, MA, USA), placed in a Millipore stainless steel tripod. Filters were cut into four approximately equal pieces on board and frozen separately in liquid nitrogen. They were kept at −80 °C until further processing. In the last year (Aug 2011–Jun 2012), 1 L sea water samples were pre-filtered through a 180 μm mesh and then filtrated down on 25 mm nitrate cellulose filters (Sartorious-stedim, Göttingen, Germany) with 1.2 μm pore size and frozen directly in liquid nitrogen. The filters were kept at −80 °C until further processing.

### 2.2. Algal culturing

All cultures used in this study are listed in Table 1 and were obtained from the following culture collections: The Norwegian Culture Collection of Algae (NORCCA), Roscoff Culture Collection (RCC), CMS Algal Research Collection (ARC), National Institute for Environmental Studies (NIES), National Center for Marine Algae and Microbiota (CCMP), and Microalgae Culture Collection of the Department of Plant Biology and Ecology of the University of the Basque Country (EHU). Culture conditions are detailed in Table S1, and for media recipes, readers are referred to Andersen (2005).

### 2.3. DNA-isolation

All samples were defrosted on ice and sodium phosphate buffer (provided by the MPBio Fast DNA Spin Kit) was added before cell lysis. Filter disruption was performed with a Precellys 24 homogenizer for 2 × 15 s at 6000 rpm (Bertin, Montigny le Bretonneux, France). Two negative controls were employed to ensure no contamination took place during DNA-isolation, negative environmental control (NEC), and negative sample control (NSC). The NEC consisted of a tube with 200 μL molecular

**Table 1**
Algal strains used in this study, with results of specificity testing.

| Strain | Species | Class | Isolator | Location | Specificity: | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *K. mik* | *K. ven* | *H. aka* | *F. jap* |
| UIO019* | *Karenia mikimotoi* | Dinophyceae | K. Tangen | Oslofjorden, Norway | + | – | n/a | n/a |
| SCCAP K-1274 | *Karenia brevis* | Dinophyceae | J. Rogers | Gulf of Mexico, FL, USA | – | – | n/a | n/a |
| UIO254* | *Karlodinium veneficum* | Dinophyceae | K. Tangen | Oslofjorden, Norway | – | + | n/a | n/a |
| UIO297 | *Karlodinium* cf. *veneficum* | Dinophyceae | S. Ota | Oslofjorden, Norway | – | + | n/a | n/a |
| SCCAP K-1471 | *Alexandrium tamarense* | Dinophyceae | A. Godhe | Lysekil, Sweden | – | – | n/a | n/a |
| SCCAP K-0675 | *Levanderina fissa* | Dinophyceae | E. Silva | San Andre lagoon, Portugal | – | – | n/a | n/a |
| SCCAP K-1332 | *Gymnodinium catenatum* | Dinophyceae | S. Ribeiro | Sines, Portugal | – | – | n/a | n/a |
| CCMP2088 | *Polarella glacialis* | Dinophyceae | C. Lovejoy | Baffin Bay, Canada | – | – | – | – |
| UIO296 | *Azadinium* cf. *Spinosum* | Dinophyceae | S. Ota | Oslofjorden, Norway | – | – | n/a | n/a |
| SCCAP K-1137 | *Prorocentrum micans* | Dinophyceae | T. Berge | Flekkefjorden, Norway | – | – | n/a | n/a |
| UIO284 | *Scrippsiella trochoidae* | Dinophyceae | S. Ota | Black Sea, Bulgaria | – | – | n/a | n/a |
| UIO081 | *Amphidinium carterae* | Dinophyceae | K. Tangen | Unknown | – | – | n/a | n/a |
| RCC1502 | *Heterosigma akashiwo* | Raphidophyceae | J. Fresnel | La Rochelle, France | n/a | n/a | + | – |
| ARC HA0309-2 | *Heterosigma akashiwo* | Raphidophyceae | C. Tomas | Offats Bayou, TX, USA | n/a | n/a | + | – |
| SCCAP K-1549* | *Heterosigma akashiwo* | Raphidophyceae | G. Hansen | Århus harbour, Denmark | n/a | n/a | + | – |
| RP02EHU | *Heterosigma akashiwo* | Raphidophyceae | S. Seone | Bay of Biscay, Spain | – | – | + | – |
| RCC1501 | *Fibrocapsa japonica* | Raphidophyceae | I. Probert | English Channel | n/a | n/a | – | + |
| SCCAP-K0542* | *Fibrocapsa japonica* | Raphidophyceae | J.Ø. Nielsen | North Sea, Germany | n/a | n/a | – | + |
| ARC CS0707-1 | *Chattonella subsalsa* | Raphidophyceae | C. Tomas | Inland Bay, DE, USA | n/a | n/a | – | – |
| ARC CA0800 | *Chattonella marina* var. *antiqua* | Raphidophyceae | S. Yoshimatsu | Kagawa Prefecture, Japan | n/a | n/a | – | – |
| ARC HD0110 | *Haramonas dimorpha* | Raphidophyceae | S. Yoshimatsu | Kagawa Prefecture, Japan | n/a | n/a | – | – |
| NIES-15 | *Olisthodiscus luteus* | Incerta sedis | I. Inouye | Seto Inland Sea, Japan | – | – | – | – |
| UIO109 | *Pseudochattonella farcimen* | Dictyochophyceae | B. Edvardsen | Langesund, Norway | – | – | – | – |

*Strains used for primer/probe design, specificity testing, qPCR standards and SEM examination.

grade water (Promega, Madison, WI, USA), which was left open on the bench-top during DNA-isolation. The NSC consisted of a sample where DNA was isolated from molecular grade water.

DNA-isolation from Durapore membranes and algal culture pellets were carried out using MPBio Fast DNA Spin Kit (MP Biomedicals, Santa Ana, USA), following the manufacturer's protocol with the following modifications: after step four, the supernatant was transferred to a fresh tube, and 200 μL of protein precipitation solution (PPS) were added, the sample was then gently mixed by hand before centrifugation (14,000 rpm for 5 min) in an Eppendorf centrifuge 5424 (Hamburg, Germany) to pellet the debris. In step five, the supernatant and binding matrix were mixed in a 15 mL tube to facilitate optimal binding of DNA. After the washing steps, filters were air dried for 5 min at room temperature before elution of DNA.

DNA-isolation from nitrocellulose filters was carried out with MPBio Fast DNA Spin Kit for Soil (MP Biomedicals), following the manufacturer's protocol, with one modification: an additional elution step was included where the samples were incubated for 5 min at 55 °C to increase yield.

### 2.4. Assay design

The DNA sequence of selected strains (Table 1) was obtained from cultured algal cells in the exponential growth phase. DNA was isolated as described in Section 2.3 before PCR, and sequencing were performed following the protocol in Engesmo et al. (2016). The generated sequences are available at GenBank with accession-numbers: *K. mikimotoi* strain UIO019: KU314866, *K. veneficum* strain UIO254: KU314867, *H. akashiwo* strain SCCAP K-1549: KP702879, KP702897 and *F. japonica* strain SCCAP K-0542: KU314865. DNA from the remaining strains listed in Table 1 was used for specificity testing of the qPCR assays.

Species-specific primers and hydrolysis probes were designed manually using Geneious version 7.1.7 (Biomatters, Auckland, New Zealand). Known raphydophyte and dinoflagellate sequences were imported from NCBI (https://www.ncbi.nlm.nih.gov/genbank/) and aligned with sequences generated in this study using MAFFT (Katoh et al., 2002) plugin for Geneious. The alignments were examined by eye and sequences compared to reveal intra-species genetic variation and select potential probe and primer sites. The specificity was then examined in silico by Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), and the suitability of the sequence determined using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html) and OligoAnalyzer 3.1 available from Integrated DNA Technologies (https://eu.idtdna.com/calc/analyzer). All primers and probes designed for this study were located in the 28S rDNA (Table 2). The assay used for *K. veneficum* was published previously by Park et al. (2009) and targeted the ITS1 region. The probe was designed first and primers were designed on both sides to generate an amplicon of 50–150 base pairs (bp). When possible, one primer was placed as close as possible to the probe sequence, without overlap. The probe and primers were designed to be species-specific. Primers were synthesised by Eurofins Genomics (Ebersberg, Germany). All primers and probes are listed in Table 2.

Optimization of qPCR working conditions was established for each assay by running a temperature gradient of 10 °C, starting 3 °C below the lowest primers melting temperature (Tm), and testing four different primer concentrations (125, 250, 500 and 1000 nM) and three probe concentrations (75, 125 and 250 nM) on the dilution series used for standard curves. Assays were then tested for specificity against a matrix of relevant species (Table 1) to determine if they amplified only the desired target.

### 2.5. qPCR

All qPCR reactions were performed on a BioRad CFX96 or CFXTOUCH 96 (Bio-Rad, Hercules, CA, USA) using 96-well plates (blue plates with clear wells) sealed with transparent adhesive. All qPCR reactions consisted of 7.5 μL 2× TaqMan® environmental mastermix (Life Technologies, Carlsbad, CA, USA), 250–500 nM primers (Table 2), 125–200 nM probe (Table 2), 1 μL DNA template (Tables 3, S3), and molecular grade water to a final volume of 15 μL. Distribution of master mix and addition of template DNA was carried out using a Biomek 3000 pipetting robot (Beckman Coulter, Brea, CA, USA). The qPCR assays were run with the cycling conditions: 10 min initial denaturation (hot-start) at 95 °C

**Table 2**
Properties of qPCR primers and probes used in this study: Melting temperature (Tm), proportion of probe or primer sequence rDNA GC content (GC), amplicon length, qPCR annealing temperature (Ta), qPCR primer and probe concentration.

| Name | Sequence (5-3) | Target species | rDNA | Tm | GC | Amplicon | Ta | Primer | Probe |
|---|---|---|---|---|---|---|---|---|---|
| Kmik2-F | CTCGCCTTGCATGTCAACGTCAGTT | *Karenia mikimotoi* | 28S | 62 °C | 52% | 178 bp | 62 °C | 500 nM | 250 nM |
| Kmik4-R | TCT GCT CTG CAT GAA GGT TGT TGG T | *Karenia mikimotoi* | 28S | 61 °C | 48% | 178 bp | | | |
| Kmik1-P | FAM- CAC TGC TTC ATG TGC T –MGB | *Karenia mikimotoi* | 28S | 50 °C | 50% | | | | |
| KVITSF3* | CTGTGAACTTCTTTGTGAGCTCTT | *Karlodinium veneficum* | ITS1 | 55 °C | 42% | 128 bp | 60 °C | 500 nM | 250 nM |
| KVITSR3* | TAGCGATAGCTTCGCAGACA | *Karlodinium veneficum* | ITS1 | 56 °C | 50% | 128 bp | | | |
| KVITSP3* | FAM-AGGTGAATCCCAATGCTGCTCCACTA-TAMRA | *Karlodinium veneficum* | ITS1 | 62 °C | 50% | | | | |
| Haka9-R | TGC AAT CCC AAG CAA CAC | *Heterosigma akashiwo* | 28S | 59 °C | 52% | 161 bp | 62 °C | 250 nM | 125 nM |
| Haka8-F | AGC TTG CTG GCG AAT TGT AGT C | *Heterosigma akashiwo* | 28S | 60 °C | 60% | 161 bp | | | |
| Haka1-P | FAM- AAG GTG CGT GCT CAG TCG TGG TCC –TAMRA | *Heterosigma akashiwo* | 28S | 65 °C | 63% | | | | |
| Fjap7-F | GAAAGGGAAGCGAAGGAAGTCA | *Fibrocapsa japonica* | 28S | 58 °C | 52% | 171 bp | 62 °C | 250 nM | 125 nM |
| Fjap6-R | CACGACATGCCACAGGGTT | *Fibrocapsa japonica* | 28S | 58 °C | 58% | 171 bp | | | |
| Fjap2-P | FAM- CAT ATT TCG TGC CTT –MGB | *Fibrocapsa japonica* | 28S | 50 °C | 44% | | | | |

*Previously published Park et al., 2009

followed by 50 cycles: 15 s at 95 °C and 30 s annealing time at the primer-specific temperatures (Table 2). All samples were run in technical triplicates, two of the three replicates had to amplify for a sample to be considered positive, and all positive results with quantification cycle (Cq) higher than 40 was considered negative. All qPCR results are given as the average of the three technical replicates, with error bars indicating standard deviation. DNA templates were diluted ×10 in molecular grade water (Wilson, 1997) to reduce the influence of natural PCR inhibitors present in seawater and to avoid false negative results or underestimation of cell numbers.

The negative DNA-isolation controls (NEC and NSC) were tested with all primer-probe sets. In addition, a negative template control (NTC) was included with all qPCR runs to ensure that no contamination occurred during preparation of the sample plate.

### 2.5.1. Construction of standard curves

Standard curves were constructed from DNA isolated from 10 mL of culture harvested by filtration during the exponential growth phase (Table 3). The concentration of each species was determined using a hemacytometer (*H. akashiwo* and *K. veneficum*: Fuchs-Rosenthal, *F. japonica* and *K. mikimotoi*: Sedgewick-Rafter) under a Nikon Eclipse TE300 inverted microscope (Nikon, Tokyo, Japan). DNA was isolated as described in Section 2.3. The standard curve was constructed as a 5-step, 4-fold dilution series for all species, except OF2 samples with the *K. veneficum* assay, which were run as a 5-step 10-fold dilution series (Fig. 2). To avoid degradation of DNA during multiple thawing cycles the standards was diluted ×10 in molecular grade water (Promega), aliquoted, and stored at −20 °C. Diluted DNA standards were discarded after the first use.

### 2.5.2. Calibration

The accuracy of the qPCR assays was tested by adding cultures of known cell concentrations of the four target species to a sea water sample collected July 2015 at station Elle (59°37 N, 10°37 E, Fig. 1) in Outer Oslofjorden, following the procedure described in Section 2.1. The spiked sea water samples were filtered onto 25 mm polycarbonate filters with 1 μm pore size (Millipore) and DNA was isolated as described in 2.3. Two samples (500 mL) were processed without the addition of cultured cells and two samples were each spiked with 5 mL cultured cells of each target species to a total volume of 500 mL. At the same time aliquots of each culture were fixed in neutral Lugol's solution, final concentration 1%, and enumerated using a hemacytometer. QPCR was performed on the isolated DNA of the non-spiked samples and a 5-steps, 10-fold dilution series of the spiked samples. Cell concentrations where calculated for each target species in the same manner as the sea water samples (Section 2.5.3).

### 2.5.3. Data analysis

Amplification data were handled in Bio Rad CFX manager v 3.0 (Bio-Rad), with Cq determination mode set to single threshold, and the baseline decided by baseline subtracted curve fit. Unknown cell concentrations were derived directly from the standard calibration curve by Bio Rad CFX manager v 3.0. Raw data were extracted to Microsoft Excel Professional Plus 2010 where they were inspected manually.

## 2.6. Verification

### 2.6.1. Molecular verification

The PCR products from all qPCR reactions were run on a 3% agarose gel in TBE buffer at 5 kV $cm^{-1}$ for 25 min to check that the PCR products were of the expected length. Two PCR products (high and low Cq) were selected for sequencing. The products were diluted ×5 with molecular grade water, purified with ExoSAP-IT (Affymetrix) and Sanger sequenced using the same primers as for qPCR (Table 1) by the GATC sequencing service. The resulting DNA-sequences were inspected manually in Geneious and taxonomically assigned with BLASTn (http://blast.ncbi.nlm.nih.gov).

### 2.6.2. Cell counts in light microscopy

For all sample dates, two replicate 5 mL Lugol's preserved sea water samples were settled for approximately 24 h and subsequently enumerated following the Utermöhl's sedimentation technique (Utermöhl, 1958). The naked dinoflagellates were counted as unidentified naked dinoflagellates (UND). Five selected

**Table 3**
Properties of the standards used to quantify qPCR results.

| Species | Strain | Cells/10 mL | DNA cons (ng/μL) | Purity A260/A280 |
|---|---|---|---|---|
| *Karenia mikimotoi* | UIO019 | 7450 | 53.89 | 1.56 |
| *Karlodinium veneficum* | UIO254 | 227692 | 38.58 | 1.62 |
| *Heterosigma akashiwo* | SCCAP K1549 | 521000 | 22.31 | 1.93 |
| *Fibrocapsa japonica* | SCCAP K0542 | 8440 | 12.54 | 1.96 |

sample dates were re-examined to perform a more thorough LM examination where UND was identified to species level when possible and raphidophytes were searched for especially (Table 4). Aliquots (10 mL) of Lugol's preserved sea water samples were left to settle for approximately 12 h and enumerated following the Utermöhl's technique (a 50 mL sample was used for Oct 2010).

*2.6.3. Scanning electron microscopy*

Samples for scanning electron microscopy (SEM) were pre-filtered through a 45 μm sieve, concentrated with tangential flow filtration (Vivaflow 200; VivaScience, Hannover, Germany) and preserved in 1% osmium tetroxide ($OsO_4$) (Sigma-Aldrich, St. Louise, USA) and diluted in sterile filtered (0.2 μm mesh) natural sea water. The samples were mounted on glass cover slips covered in poly-L-lysin (Sigma-Aldrich) and left to settle overnight in a moist chamber before dehydration, followed by critical point drying and sputter coating in accordance with Engesmo et al. (2016). The samples were examined in a S-4800 Hitachi Field Emission Scanning Electron Microscope (Hitachi, Tokyo Japan). Cultures of *K. mikimotoi*, *K. veneficum* and *H. akashiwo* were prepared and examined as above for comparison. It was necessary to prepare *Fibrocapsa japonica* for SEM separately, because cell morphology became distorted when fixed in $OsO_4$. To prevent the trichocysts from discharging, cells were added to the Lugol's solution (1% final concentration), quickly followed by the addition of glutaraldehyde (GLA, 1% final concentration) and then gently mixed by inverting the tube. Cells were left to sink 1 h before they were collected from the bottom of the tube, mounted on a glass slide and prepared as above.

*2.6.4. 454 High throughput sequencing*

Field sampling (from 1 m depth, cell size fraction 3–45 μm), RNA extraction, reverse transcription to cDNA, PCR, and sequencing followed in large the protocol by Egge et al. (2015), with one modification: PCR amplification targeted the hypervariable V4 region (~380 bp) of the 18S rRNA gene was performed using the universal eukaryote primers TAR454-F3: 5′-CCAGCASCYGCGGTAATTCC-3′ and TAReukREV3: 5′-ACTTTCGTTCTTGATYRA-3′ described by Stoeck et al. (2010). Analyses of 454 reads were carried out as described by Logares et al. (2014) with some modifications: AmpliconNoise v.1.6.0 (Quince et al., 2011) was used to denoise the amplicons (~400 bp), and Perseus was used to remove the putative chimeras (Quince et al., 2011) as implemented in QIIME pipeline v.1.4 (Caporaso et al., 2010). UCLUST v.1.2.22 (Edgar, 2010) was used to cluster the reads at a 98% similarity threshold. All generated OTUs that contained singletons (only one read) or doubletons (only two reads that were both present in the same sample) were removed. Operational taxonomic units (OTUs) assigned to Raphidophyceae were aligned to the Engesmo et al. (2016) Raphidophyceae alignment, using MAFFT-add in v.7.1.9 with the Q-INS-I strategy (http://mafft.cbrc.jp/alignment/server/). The alignment was checked and manually edited in Geneious v.7.1.9. Phylogeny was inferred with MrBayes v. 3.2.2 in Geneious, using the substitution model GTR and invariable gamma rate variation, MCMC settings 2 000 cycles, four heated chains, and subsampling frequency of 500 (Huelsenbeck and Ronquist, 2001). OTUs assigned to Dinophyceae were further taxonomically assigned as far as possible by local Blast in Geneious against a local database consisting of 1593 dinoflagellate reference sequences from PR2 (Guillou et al., 2013), followed by phylogenetic analyses (Gran-Stadniczeñko et al., unpublished). The nucleotide sequences of the OTUs were submitted to ENA and have the accession numbers PRJEB20755 (study) and ERZ407999 (analysis).

## 3. Results

The specificity of the qPCR assays was tested by running the two dinoflagellates assays (targeting *Karenia mikimotoi* and *Karlodinium veneficum*) on cultures of other dinoflagellate strains (Table 1). The two raphidophyte assays (targeting *Heterosigma akashiwo* and *Fibrocapsa japonica*) were tested on other raphidophytes and dictyochophytes (Table 1). No unspecific amplification occurred for any species. All qPCR products were checked by agarose gel electrophoresis and only one clear band of the expected length was visible on the gel, indicating no unspecific binding masked by the probe. Sequences of the qPCR-products were blasted and matched their intended targets. The qPCR properties (efficiency, slope and $r^2$) as calculated from the standard curves are shown in Fig. 2 for all sample runs. The PCR efficiency was generally high (>90%). No bands of DNA were visible in the negative controls.

**Table 4**
Comparison of all results for selected sample dates (2009–2012).

| Karenia mikimotoi | Sep 09 | Oct 10 | Jun 11 | Aug 11 | Jun 12 |
|---|---|---|---|---|---|
| qPCR (cells $L^{-1}$) | 54 | 1665 | 0 | 662511 | 444 |
| SEM (recorded) | X | | | X | |
| LM (cells $L^{-1}$) | 200 | 1000 | 0 | 6800 | 600 |
| LM UND* 25–40 μm (cells $L^{-1}$) | 1888 | 0 | 10500 | 8400 | 1200 |
| Karlodinium veneficum: | | | | | |
| qPCR (cells $L^{-1}$) | 189 | 7604 | 438210 | 82 | 34665 |
| SEM (recorded) | X | | X | | X |
| LM (cells $L^{-1}$) | 600 | 4600 | 9000 | 100 | 6000 |
| LM UND* 8–24 μm (cells $L^{-1}$) | 8800 | 16500 | 110400 | 8400 | 30800 |
| Heterosigma akashiwo: | | | | | |
| qPCR (cells $L^{-1}$) | 581 | 8402 | 0 | 219 | 0 |
| SEM (recorded) | | X | | | |
| LM (cells $L^{-1}$) | 0 | 3800 | 0 | 0 | 0 |
| 454 HTS (recorded) | X | X | | – | – |
| Fibrocapsa japonica | | | | | |
| qPCR (cells $L^{-1}$) | 257 | 27 | 0 | 225 | 0 |
| SEM (recorded) | | | | | |
| LM (cells $L^{-1}$) | 0 | 100 | 0 | 0 | 0 |
| 454 HTS (recorded) | X | | | – | – |

UND = Unidentified naked dinoflagellate, – = No data for this date, X = species recorded, no quantifiable data available, blank space = data analysed but species not recorded.

### 3.1. Calibration experiment

Samples of seawater spiked with known concentrations of the four species were accurately detected using the qPCR assays (Fig. 3). Detection limits were determined based on this experiment and on the results from the field samples. There was only slight variation between the two biological replicates for all species. The detection limit for *Karenia mikimotoi* (spiked with 110,000 cell $L^{-1}$, Fig. 3a), *Karlodinium veneficum* (spiked with 270,000 cells $L^{-1}$, Fig. 3b) and *Fibrocapsa japonica* (spiked with 16,000 cells $L^{-1}$, Fig. 3d) were found to be approximately 1 cell $L^{-1}$. The assay for *Heterosigma akashiwo* (spiked with 110,000 cells $L^{-1}$, Fig. 3c) was not able to detect the lowest concentrations, giving a detection limit of approximately 100 cells $L^{-1}$.

### 3.2. Field samples

The qPCR assays confirmed the presence of all four species in Oslofjorden (Fig. 4). The dinoflagellates appeared frequently in the samples, *Karenia mikimotoi* was recorded during most summer and autumn months in low quantities (<1000 cells $L^{-1}$). There was one peak in August 2011 (665,000 cells $L^{-1}$); the population was then present in low cell numbers throughout 2012 (Fig. 4a). In June 2011 there was a peak of *Karlodinium veneficum* with cell numbers reaching 438,000 cells $L^{-1}$, and it was recorded almost every

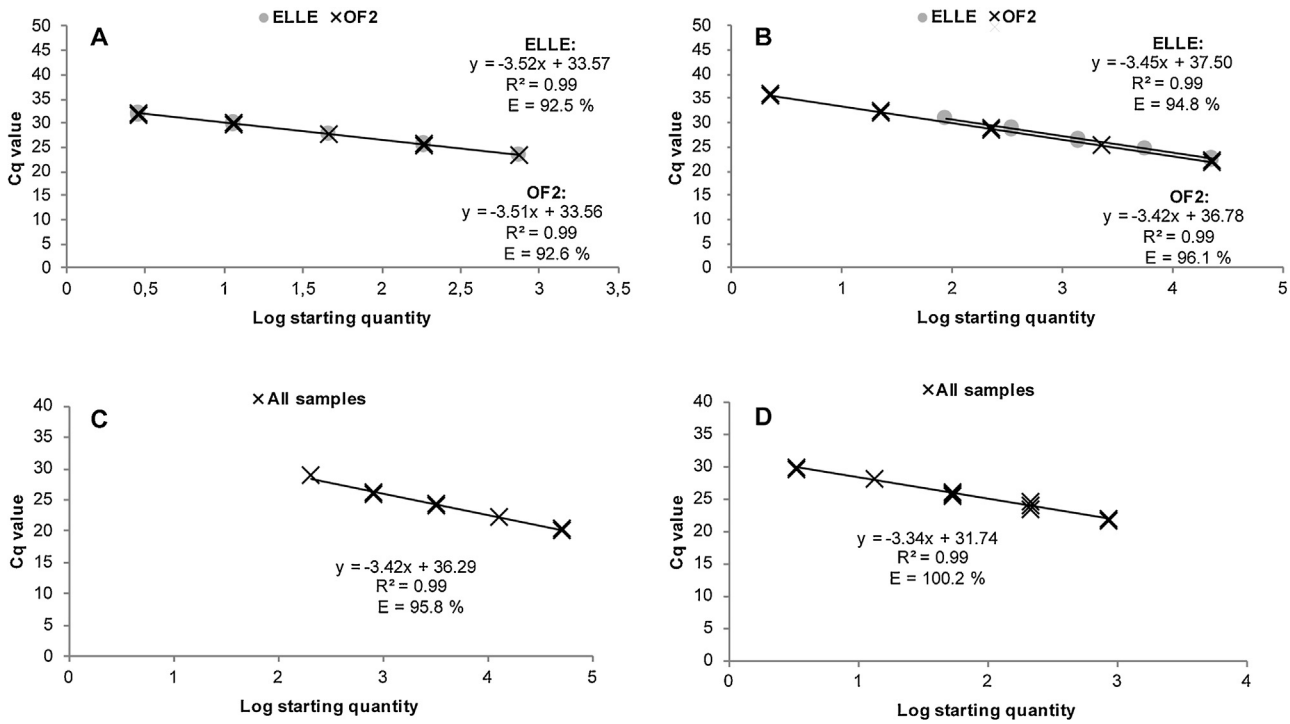


**Fig. 2.** qPCR properties showing efficiency, slope and $r^2$ as calculated from the standard curves for a) *Karenia mikimotoi* b) *Karlodinium veneficum* c) *Heterosigma akashiwo* and d) *Fibrocapsa japonica.*

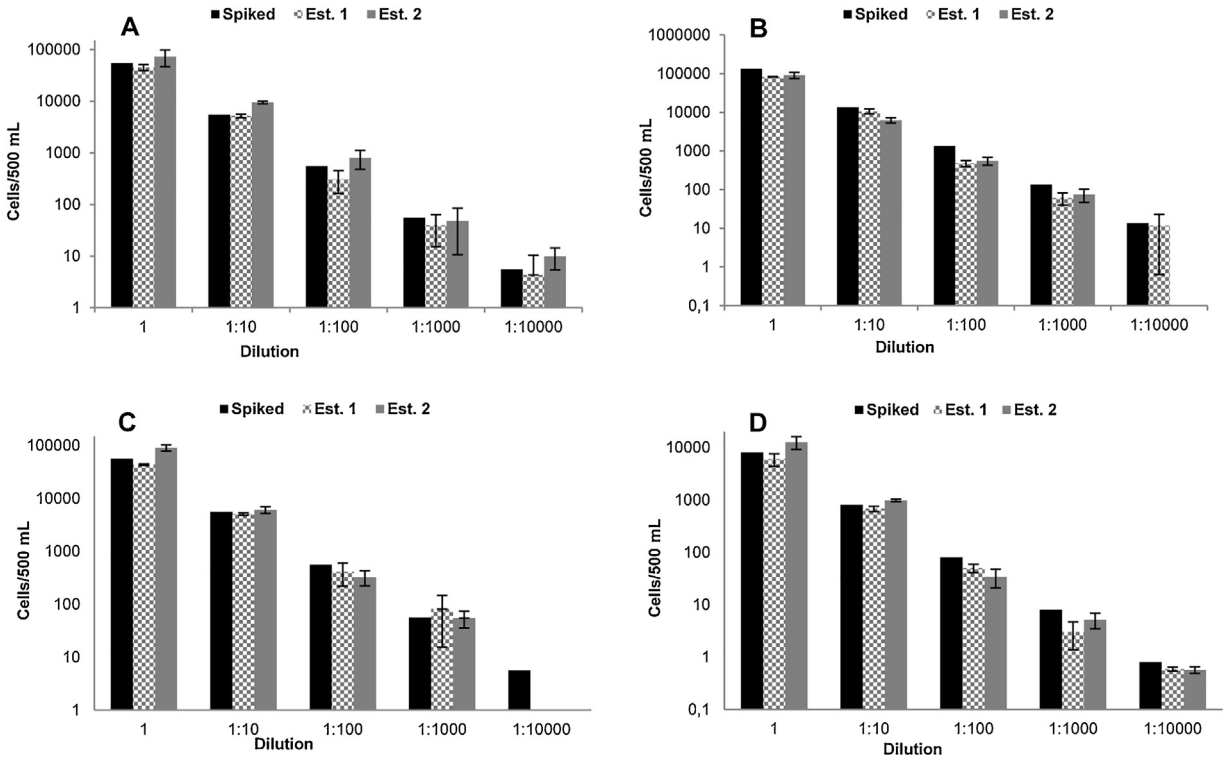


**Fig. 3.** Results from the experiments with spiked samples from ELLE: a) *Karenia mikimotoi* b) *Karlodinium veneficum* c) *Heterosigma akashiwo* and d) *Fibrocapsa japonica*. The left column (spiked) indicates the number of cells added as counted in a hemocytometer. The estimations are two biological replicates of qPCR estimates of the number of cells. All cell estimates are given as the average of three technical replicates, with error bars indicating standard deviation.

month from August 2009 until August 2011 (except Oct 09) in relatively low abundances, 100-10,000 cells $L^{-1}$. During the last year it occurred less frequently except in June 2012 when there was a small peak of 35,000 cells $L^{-1}$ (Fig. 4a).

The raphidophytes appeared less frequently than the dinoflagellates (Fig. 4b), and both *Heterosigma akashiwo* and *Fibrocapsa japonica* was recorded during the autumn of 2009, 2010 and 2011 in low abundances. The highest recorded cell estimate of *H. akashiwo* was in October 2010 with 8400 cells $L^{-1}$, while cell estimates of *F. japonica* never went above 250 cells $L^{-1}$.

### 3.3. Light microscopy cell counts

None of the species in this study were initially registered in the microscopic cell counts (data not shown); however, *Karenia mikimotoi* and *Karlodinium veneficum* were included as UND in their respective size groups. Five samples (Sep 09, Oct 10, Jun 11, Aug 11 and Jun 12) were re-examined in LM (cell counts) with special emphasis on the four species included in this study (Table 4). The two dinoflagellates, *K. mikimotoi* and *K. veneficum*, were identified and counted in LM for all dates that had a positive qPCR signal. There was good correlation between the qPCR and LM cell estimates, with the exceptions of June and August 2011, where the qPCR estimates were two orders of magnitude larger than the LM estimates (Table 4). The only LM registration of *Heterosigma akashiwo* and *Fibrocapsa japonica* were from October 2010 with 3800 cells $L^{-1}$ and 100 cells $L^{-1}$, respectively.

### 3.4. Morphology

Material from the five sampling dates that were chosen and recounted in LM were also examined in SEM. In September 2009 and August 2011 *Karenia mikimotoi* was identified and compared with cultured cells of strain UIO019 (Fig. 5). Cells were dorso-ventrally

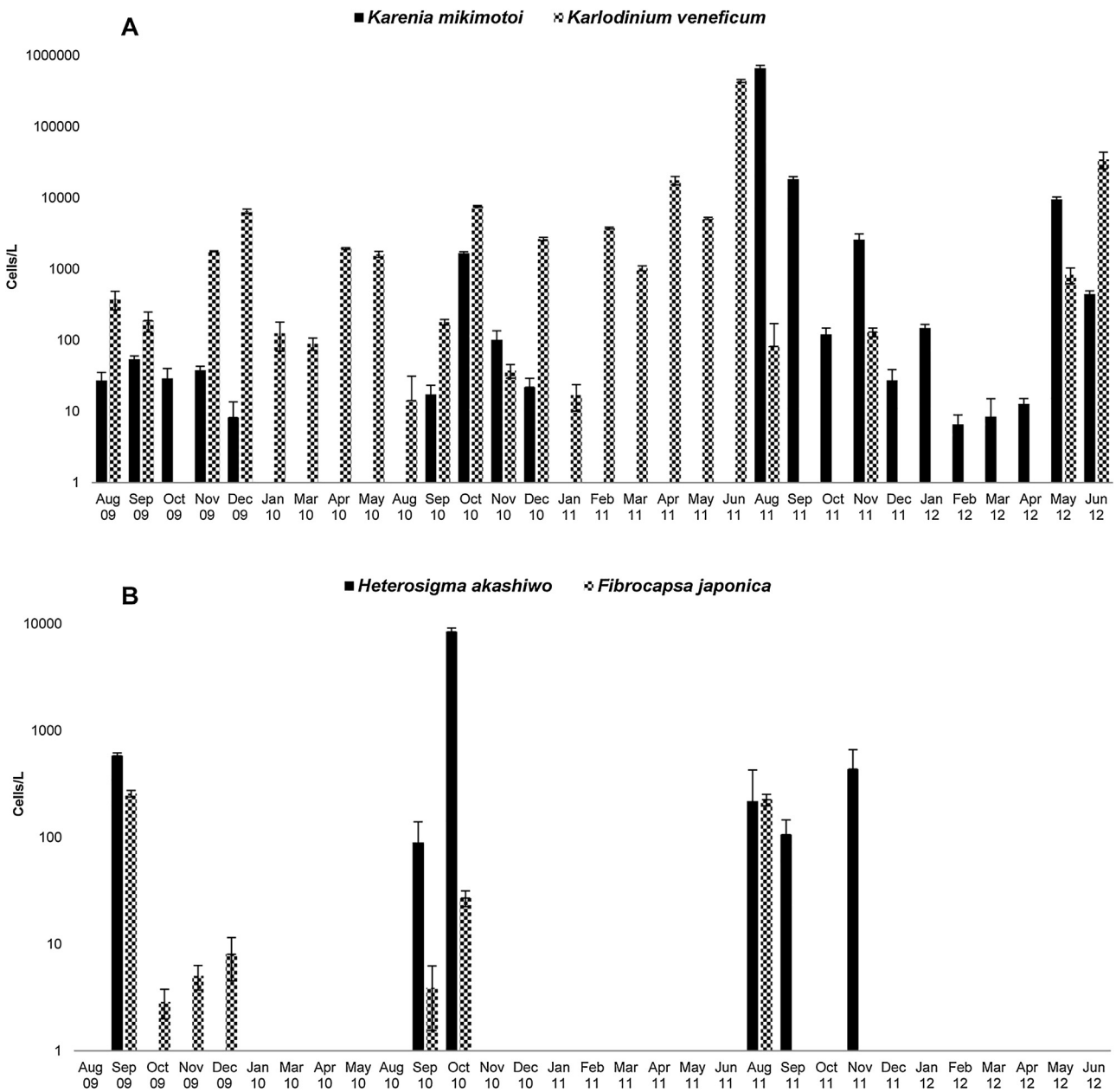


**Fig. 4.** qPCR results from station OF2 in the outer Oslofjorden for a) *Karenia mikimotoi* and *Karlodinium veneficum* and b) *Heterosigma akashiwo* and *Fibrocapsa japonica*. All estimates are given as cells $L^{-1}$ presented as the average of three technical replicates, with error bars indicating standard deviation.

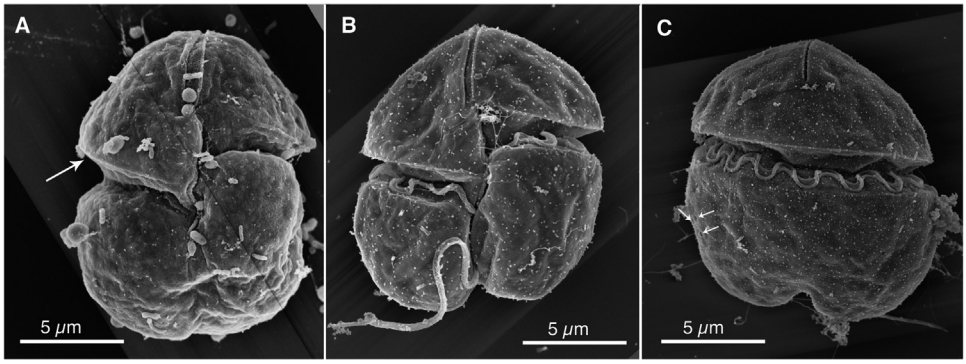


**Fig. 5.** SEM images of *Karenia mikimotoi,* a) ventral view of cell from field material (OF2 Aug 11), note the distinct edge on the epitheca (arrow) which is relatively visible despite the cell not having preserved well. b) Ventral view of strain SCCAP K-0260 with the distinct epithecal edge. c) Dorsal view of strain SCCAP K-0260 with three antapical pores (arrows).

compressed, somewhat taller than wide, and displayed large cingulum displacement. The width:height ratio varied, but cells were always taller than wide. They had a distinct, straight apical groove, extending from the ventral side of the epicone (Fig. 5a–b), over apex and down into the dorsal side of the epicone (Fig. 5c). The epicone had a distinct edge, which is easily recognisable in both LM and SEM. Three antapical pores were seen in left dorsal view (Fig. 5c). The cells from the field samples closely resembled those in culture (Fig. 5b–c).

Cells of *Karlodinium veneficum* were identified from samples collected in September 2009, June 2011 and June 2012 (Table 3) and compared with cultured cells of strain UIO254. Cells had large cingulum displacement, with sulcal intrusion into the epitheca and longitudinal rows or depressions beneath the amphiesma vesicles (Fig. 6). The apical groove was straight, but less pronounced than in *Karenia mikimotoi* (Fig. 5); in dorsal view, the termination of the apical groove was discernible close to the apex (Fig. 6c). Two apical pores were visible (Fig. 6a–b). Cells from the field material closely resembled cells from culture as shown in Fig. 6a, which is from a field sample collected in June 2012.

The morphology of the two Raphidophycean species, *Heterosigma akashiwo* and *Fibrocapsa japonica,* were difficult to detect in field samples with SEM (Figs. 7 and 8); however, *H. akashiwo* was identified from October 2010. The cell was not well preserved, but the heterokont flagella were intact, and it was apparent that it was naked because of the tear in the cell. The cell had a rounded outline with surface structures, discharged mucocysts, and rod-like structures (Fig. 8b). No identification of *F. japonica* was done in SEM (Table 4), but the morphology is depicted by cultured cells. The cells were rounded to oval with a variable outline and two apically inserted flagella. Trichocysts were concentrated in the posterior end of the cells.

### 3.5. 454 high throughput sequencing

The 454 HTS demonstrated the presence of minimum four different genotypes from the dinoflagellate Family Kareniaceae. It also successfully documented the presence of raphidophytes in Oslofjorden: In September 2009 and October 2010 *Heterosigma akashiwo* occurred, and *Fibrocapsa japonica* was detected in September 2009 (Fig. 9 and Table 4). Aligning all Raphidophyceaen 454 HTS generated sequences to a raphidophyte reference alignment also revealed previously undocumented genetic variety within Raphidophyceae (Fig. 9). An unknown genotype, probably representing a novel genus, was recorded during early spring of 2010 and 2011 (Mar 10 and Feb–Apr 11).

## 4. Discussion

In this study, we were able to detect and enumerate the ichthyotoxic flagellates *Karenia mikimotoi*, *Karlodinium veneficum*, *Heterosigma akashiwo,* and *Fibrocapsa japonica* over the course of three years (Aug 2009–Jun 2012) from environmental water samples collected in Outer Oslofjorden using qPCR. The results document the first occurrence of *F. japonica* in Norwegian waters and demonstrate the potential of qPCR as a monitoring method.

The two dinoflagellates, *Karenia mikimotoi* and *Karlodinium veneficum,* are known to be relatively common components of the coastal phytoplankton community along the Norwegian coast (Throndsen et al., 2003). During the autumn of 2009 and 2010, *K.*

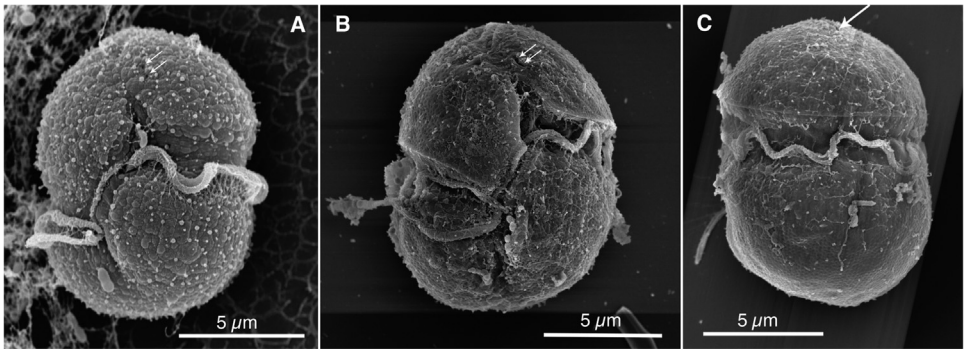


**Fig. 6.** SEM images of *Karlodinium veneficum,* a) ventral view of cell from field material (OF2 June 2012), note the sulcal intrusion into the epitheca and the two apical pores (arrows). b) Ventral view of strain SCCAP K-1640 with sulcal intrusion into the epitheca, two apical pores (arrows) and visible rows of depressions beneath the aphiesmal vesicles. c) Dorsal view of strain SCCAP K-1640 with visible rows of depressions beneath the aphiesmal vesicles, also notice the short apical groove (arrow).

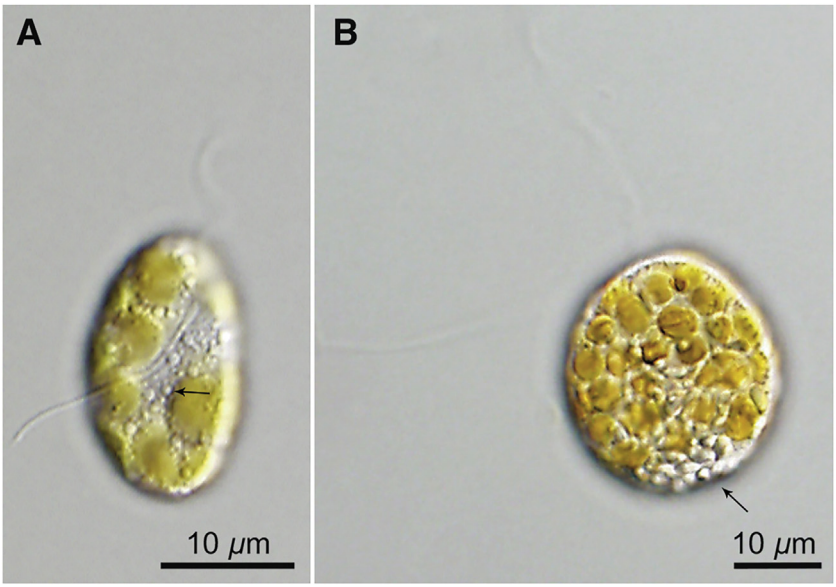


**Fig. 7.** LM of a) *Heterosigma akashiwo* strain SCCAP K-1549 showing both anterior (beating) and posterior (trailing) flagella, peripheral chloroplasts and mucocysts (arrow). The central nucleus is visible as a grey area surrounded by chloroplasts. b) *Fibrocapsa japonica* strain SCCAP K-0542 showing both the anterior (beating) and posterior (trailing) flagella, multiple chloroplasts and the mucocysts located in the posterior end (arrow). Both images kindly provided by Gert Hansen.

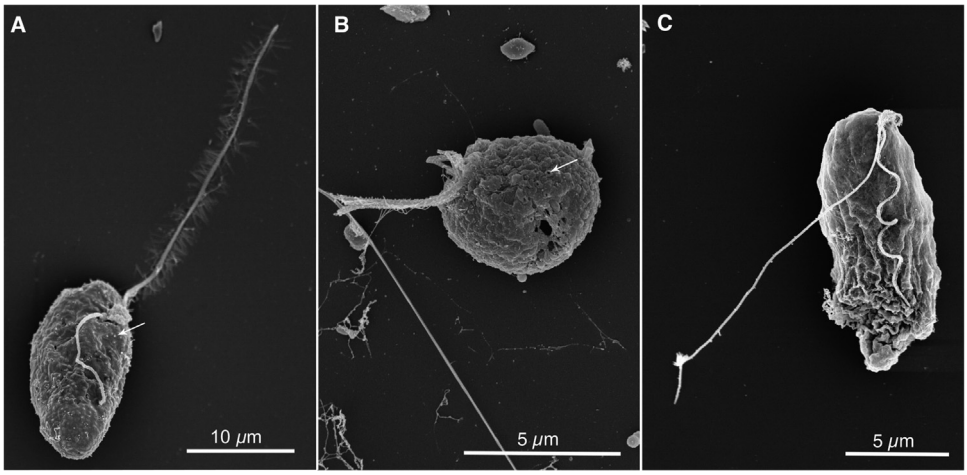


**Fig. 8.** SEM images of a) *Heterosigma akashiwo* strain SCCAP K-1549 with hairy (anterior) flagellum, smooth (posterior) flagellum and unreleased mucocysts (arrow). b) *Heterosigma akashiwo* cell from field material (OF2 Oct 10) with both flagella and discharged mucocysts (arrow). Note the rupture in the cell revealing the fragile plasma membrane. c) *Fibrocapsa japonica* strain SCCAP K-0542 with hairy (anterior) flagellum, smooth (posterior) flagellum and smooth cell surface. Note the "wrinkled" end, which contains the trichocysts.

*mikimotoi* appears in low concentrations, but it was not recorded during winter, spring, or summer. The concentration peaked in August 2011 and appeared to linger until June 2012, which marked the end of the sampling period. The other dinoflagellate, *K. veneficum,* was present year around in the period August 2009 to June 2011 and its concentration peaked in June 2011; however, it was not common from August 2011 to June 2012, when *K. mikimotoi* was frequent. Neither *K. mikimotoi* nor *K. veneficum* were identified from initial LM cell counts because they were included as unidentified naked dinoflagellates (UND) in their respective size groups. Field samples from five sample dates were re-examined in LM, and new cell counts were performed with special emphasis on finding the species targeted in the present qPCR assays. Upon re-examination in LM, cells that complied with the morphology of both *K. mikimotoi* and *K. veneficum* were found in all samples with positive qPCR signals. Results showed a positive correlation between the cell estimates given by qPCR and the LM cell counts. In the two months with the highest qPCR signals (Aug 2011 for *K. mikimotoi* and Jun 2011 for *K. veneficum*), the discrepancy between qPCR and LM estimates were notable, with LM estimates being two orders of magnitude below the qPCR estimates. Both LM examinations of the sample from August 2011 recorded less than 10,000 cells $L^{-1}$ (UND 25–40 μm and *K. mikimotoi*), whereas qPCR estimated 660 000 *K. mikimotoi* cells $L^{-1}$. In June 2012, the discrepancy between the two LM examinations was also large, with the first examination recording approximately 110,000 cells $L^{-1}$ (UND) and the second only 9000 cells $L^{-1}$ of *K. veneficum*. The reason for the observed discrepancy between qPCR and LM cell estimates may be that both species are relatively difficult to detect in LM, and could be overlooked. They are also sensitive to fixation and may have their morphology distorted, rendering them unidentifiable. Another possibility is that there are closely related, novel species present in Oslofjorden, which are picked up by the qPCR assays, but not recorded in LM. Because we

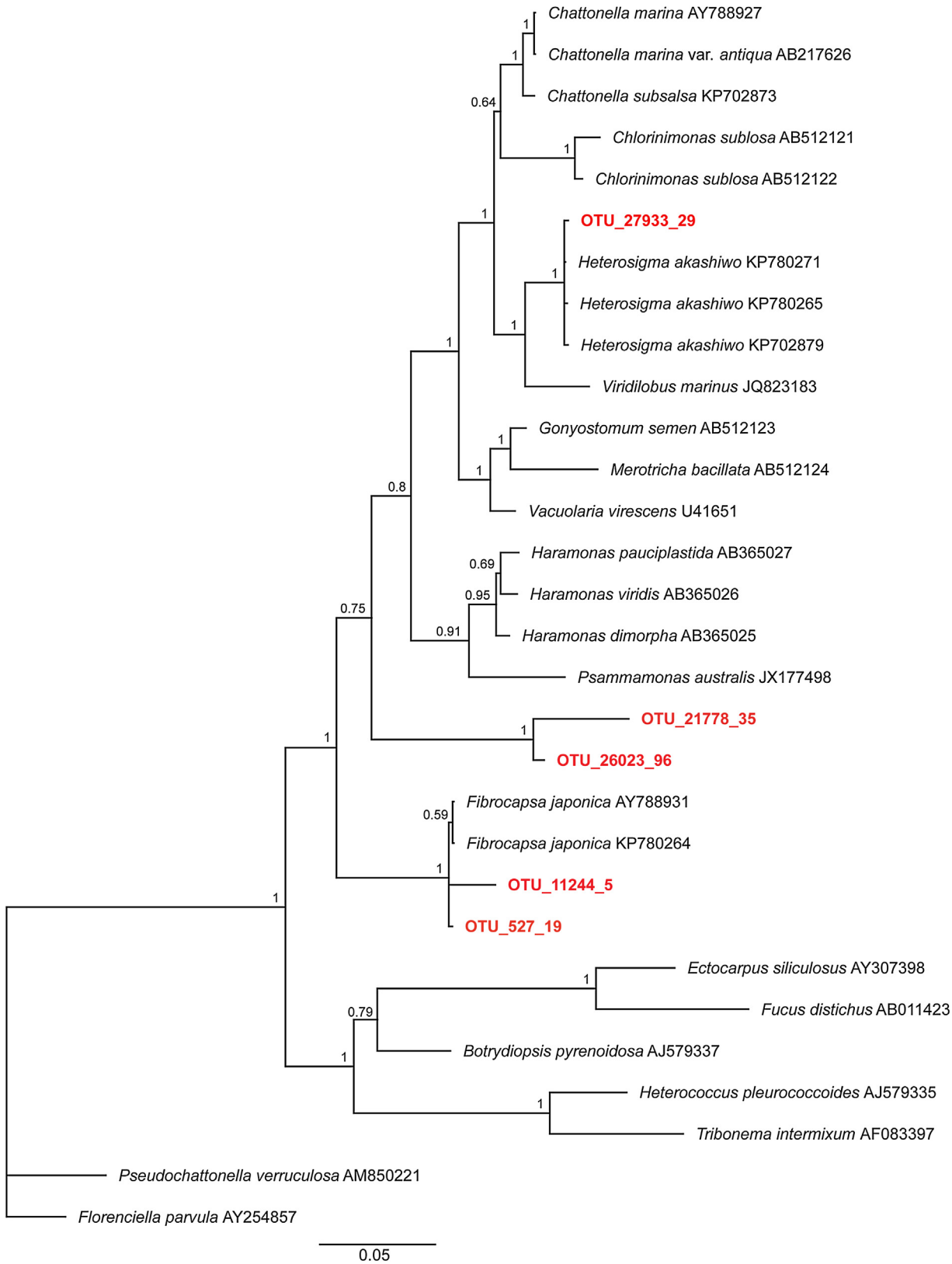


**Fig. 9.** Phylogeny of Raphidophyceae based on 18S rDNA (1988 characters), aligned with 454 environmental sequencing OTUs (371–421 bp in the V4 region) using Bayesian Interference (MrBayes). Supporting values are given as Bayesian posterior probability. OTUs are marked in bold. The nucleotide sequences of the OTUs are available from ENA and have the accession numbers PRJEB20755 (study) and ERZ407999 (analysis).

chose to work with DNA, instead of RNA, there is also the possibility that we are detecting eDNA from cells that are no longer viable, which may contribute to overestimation of qPCR cell estimates and lead to false positive samples (Goldberg et al., 2015).

The raphidophyte, *Heterosigma akashiwo,* was recorded with qPCR from all three sampling years, albeit at very low concentrations, and the highest concentration (8400 cells $L^{-1}$) was recorded in October 2010 (Table 4). It went undetected in the initial LM cell counts, but when the samples were re-examined, 3800 cell $L^{-1}$, possibly corresponding to *H. akashiwo's* morphology were counted from October 2010. One cell of *H. akashiwo* was identified in SEM from October 2010 (Fig. 8b). It was not recorded in LM from any other sampling date. The presence of the dictyochophyte, *Pseudochattonella farcimen* (Eikrem, Edvardsen & Throndsen) Eikrem, was recorded in September 2009 and October 2010, and *H. akashiwo* has previously been reported to appear alongside this species (Edvardsen et al., 2007; Naustvoll et al., 2002). In the present study, *Fibrocapsa japonica* was detected with the qPCR assay in the autumn of 2009, 2010 and 2011, albeit in very low concentrations (the highest concentration was 257 cells $L^{-1}$ in Sep 09). One cell was recorded in LM from October 2010. This is the first time *F. japonica* was recorded in Norwegian waters, but there is a LM record from the Swedish coast at the island of Åstol, which is only 160 km away from the Norwegian border (www.smhi.se/klimatdata/oceanografi/havsmiljodata). Alongside other toxic raphidophytes, *Fibrocapsa japonica* has been observed in French and Dutch coastal waters since 1991 (Billard, 1992; Vrieling et al., 1995). It is now well established in the North Sea and has occurred in bloom concentrations in the German Bight (Rademaker et al., 1998) and in the northern Adriatic Sea (Cucchiari et al., 2008), causing kills of farmed fish, and it has on one occasion been linked, although not unequivocally to the death of seals (Leftley and Hannah, 2009). So far, *Fibrocapsa japonica,* has not been detected by the Norwegian Surveillance Programme (www.algeinfo.imr.no) that conducts light microscopy examinations of water samples collected along the Norwegian coast. A possible introduction and establishment of *F. japonica* in Norwegian waters may pose a future challenge to fish-farmers and wild life. Verifying the presence of *F. japonica* with microscopy proved difficult, therefore we used available 454 HTS data (Gran-Stadniczeňko et al., unpublished) for the first two sampling years (Aug 09–Jun 11), in which *F. japonica* was recorded from September 2009. The 454 HTS data did not detect *F. japonica* from any other positive sample, suggesting that the qPCR detection limit is lower than 454 HTS. The 454 HTS also confirmed the presence of *H. akashiwo,* and it suggested the presence of a novel Raphidophyceae genus in Oslofjorden. Interestingly, this unknown, novel taxon, expected to represent a novel genus appeared during early spring (Mar 10, Feb–Apr 11), unlike the two other raphidophytes, which only occurred in the autumn (data not shown).

The concentrated samples of small phytoplankton (<45 μm) were examined in SEM, which allowed the morphology of cells from field samples to be compared with cells from cultures (Figs. 5–8). The morphology of the *K. mikimotoi* cells found in field samples was compared to the morphology of strain UIO019, which was isolated from Oslofjorden in 1977. Both cultured cells and cells from field material clearly conformed to previous descriptions of *K. mikimotoi* (Daugbjerg et al., 2000; Haywood et al., 2004). Certain cells, both in culture and field material exhibited the morphology of *K. mikimotoi,* but were smaller than the previously published size range (18–40 μm in length and 13–35 μm in width). This indicates that the currently published size range for *K. mikimotoi* should probably be amended to include smaller cells. The field material also contained cells as small as 10 μm fitting *Karenia* morphology from sample dates that were negative for *K. mikimotoi*, indicating that there is a novel *Karenia*-species commonly present in Oslofjorden. A recent investigation from French coastal waters indicate that the diversity of the dinoflagellate family Kareniaceae is much larger than previously recorded (Nézan et al., 2014). The *Karlodinium veneficum* strain UIO254 was isolated from Oslofjorden in 1977 and was used here to compare morphology with the cells found in the field samples. The cultured cells conform to previous descriptions of the species (Daugbjerg et al., 2000; Bergholtz et al., 2006). Although it was outside the scope of this project to identify and describe novel species, it was clear from LM, SEM and 454 HTS that the diversity of naked dinoflagellates in Oslofjorden was greater than currently recognized.

No identification of *Fibrocapsa japonica* were obtained from field samples in SEM, which could be due to its low cell concentrations or because *F. japonica* does not preserve well in $OsO_4$-fixation. Strain SSCAP K-0542 isolated from the North Sea (Helgoland, Germany) was examined in SEM, and provided some further insights into why SEM detection of this species is particularly difficult. Satisfactory results were not obtained with $OsO_4$ fixation (Fig. S2), therefore a separate procedure had to be followed for *F. japonica* (see Section 2.6.3). The "wrinkled" end of *F. japonica* is where the trichocysts are located. When disturbed, they readily discharge, causing disruption to the cell, covering it in mucus and discharge as seen in Fig. S2.

Absolute quantification of microalgal targets in qPCR assays are usually achieved using standard curves. The curve is generated using a serially diluted DNA standard of a known quantity, which creates a linear relationship between the threshold cycle (Cq) and the logarithm of the starting quantity of DNA in the standard (Heid et al., 1996). Two types of standards are typically used: DNA from cultured cells of the targeted species (Park et al., 2007; Handy et al., 2008; Park et al., 2009; Eckford-Soper and Daugbjerg, 2015b) or a cloned plasmid of the targeted gene (Galluzzi et al., 2008; Galluzzi et al., 2010; Yuan et al., 2012; Zamor et al., 2012). The plasmid approach will generate the copy number of the targeted gene; it is therefore essential to know how many copies of the targeted gene are present per cell, and if this number is constant. In a eukaryote, nuclear genome rDNA will typically consist of hundreds of tandemly repeated copies, but it can consist of as few as one copy or up to several thousand (Hillis and Dixon, 1991). It is also previously documented that the amount of rRNA can vary between both strains and life stages (Galluzzi et al., 2010), growth conditions (Dittami and Edvardsen, 2013) and for dinoflagellates especially, biovolume (Godhe et al., 2008). In this study, standards with known quantity of cultured cells were used, therefore bypassing the problem of determining the gene copy number per cell. Using cultured cells as a standard also has its shortcomings. DNA in whole cells is much less stable than in cloned plasmids, meaning new standards must be prepared from live and fresh cells. Keeping live cultures is time consuming, and so is enumeration and DNA isolation. In the present study, DNA degradation of the cultured standard appeared as a major challenge. If a dilution series was thawed twice, the lower concentrations would start to degrade, resulting in lower qPCR efficiencies (E), which results in over-estimation of cell numbers. Subsequently, TE buffer was used for diluting samples for the standard curves and for storing standards.

A guideline for minimum information of publication of qPCR results (MIQE) was published in 2009 (Bustin et al., 2009). Several different qPCR assays have been published since the early 2000's, including the micro algal species of the present study (i.e. Coyne et al., 2005; Bowers et al., 2006; Yuan et al., 2012). In order to comply with the MIQE guidelines, new primers and probes were designed for all species except *K. veneficum*, where an assay developed by Park and co-workers (Park et al., 2009) was used. The region of a recently published assay for *K. mikimotoi* (Smith et al.,

2014) is located slightly downstream of the assay presented herein, but targeting largely the same region of 28S rDNA. The detection limit reported by Smith et al. is lower (0,007 cells $L^{-1}$) than for the present assay (1 cell $L^{-1}$), however there is no reason to believe the assay presented herein could not detect lower concentrations, if applied.

The results demonstrate that qPCR is a sensitive tool for the quantification of the four species targeted in the present study and would be suitable and valuable as a compliment to LM-based monitoring as the qPCR assays had a higher sensitivity and lower detection limit than LM cell counts.

## Acknowledgements

The authors are very grateful to colleagues at the University of Oslo, mainly Sissel Brubak for assistance with molecular biological lab work, Vladyslava Hostyeva for the initial LM cell counts and Elianne Egge for allowing us to use her DNA and HTS reads for the first two sampling years. We also thank the curator of SCCAP culture collection, Gert Hansen, for allowing us the use of his LM picture of strain SCCAP K-1549 and SCCAP K-0542 (Fig. 7). The Norwegian Research Council is thanked for funding project 196702/E40 Toxic Algae. [SS]

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.hal.2018.04.007.

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