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*Article*

# Getting the Full Picture of Plankton in the Western Channel

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**Abstract:** Plankton monitoring by microscopy offers long-term ecological perspective of plankton communities but is biased towards those organisms that can be distinguished using the microscope. Genetic identification of marine plankton has become standard but is still not used. This study is a comprehensive study genetically measured taxa in the Western Channel of UK using a small-volume automated water sampler deployed on the CPR platform. The study present one year of high-throughput sequencing data focussing on smaller plankton and separate community to that measured by microscopy that can complement each other for a holistic view of plankton. Quantitative tests of two harmful algae show relatively high abundance of the Pelagophyte Aureococcus anophagefferens during 2011 with low nitrite levels. Three years of Pseudo-nitzschia delicatissima quantitative monitoring also shows a greater abundance of this potentially harmful taxa in 2011. Flow cytometry reveals distinct seasonal cycles with distinct timings.

**Keywords:** Plankton; Monitoring; Harmful algae; microscopic; genetic; Western Channel

## 1. Introduction outline

Ten years of genetic monitoring of marine pelagic environments have revealed extensive diversity particularly in the smaller sized eukaryotic marine microbial community of plankton [1]. Studies have shown they capture unseen or challenging components of oceanic micobes [2] and useful for the development of microbial indicators [3, 4]. Molecular quantitative and qualitative approaches linking are key to understanding microbial responses to ocean change and ecosystem functioning and their inherent resilience to environmental change [5], where existing metrics for ecosystem functionality were missing. Legislature such as the Water Framework Directive and Marine Strategy Framework Directive legally have a requireement to meet Good Environmental Status (GES) [6, 7]. Indicators have been developed for larger planktonic organisms [8] but there is paucity of indicators for smaller microbial components that comprise phytoplankton and mixo- or hetertrophic microbes, due to the technical difficulties in recording them by light microscopy. For example, the smallest marine phytoplankter, Synecococcus is increasing relative to other phytoplankton [9] and yet our understanding of the consequences of this change on marine food webs and functioning is limited because of a lack of biodiversity information at the smallest sized level. It is increasingly recognised that species-level information is required to achieve ecological understanding of key marine health indicators, understand food webs and

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maintain or reduce loss of biodiversity which is reognised internationally and set in policies such as EU marine strategy framework directive and UK and the Aichi Targets for 2020 set out for parties for the United Nations [5, 7, 10]. Thus regulator monitoring of microbes at the species level is essential to develop these indicators.

One example where intensified molecular montioring can be beneficial is the detection of Harmful algae species and pathogens. Harmful algae range of over 200 species of naturally occuring mainly unicellular algae that can cause damage to the ecosystem through a variety of mechanisms. Most notable of these are through toxin production that can poison marine animals and humans by contact or consumption of contaminated organisms or toxins, or through overgrowth resulting in oxygen depletion that can lead to the suffocation of fish [11]. Toxicity can be present in some species or even populations and is modulated through a variety of environmental cues [12]. However, the toxic status of several of these organisms and the envirionmental cues that lead to toxicity or overgrowth are poorly understood because species-level resolution is unobtainable through light microscopy for key potentially toxic species ([13-15]. Much of this is due to a lack of data on harmful algae at sufficient resolution to the species or population level, most regulatory taxonomic monitoring being perfomed using light microscopy noted in those studies. Molecular genetic detection is a useful tool to distinguish species that are similar or identical at the light microscopy level or soft bodied organisms that are destroyed by preservation. For similar reasons long-term information on intracellular pathogens or parasites are are lacking, including potential human or animal pathogens due to their cryptic nature. Whilst multiple populations of hosts and pathogens exist in the environment, their pathogenesis is a combination of environmental and biological interactions and their relationship with each other and the community will be altered due to climate change [16]. Ocean warming is predicted to increase the infectection potential of the Oyster parasite, *Perkinsus marinus* on its host *Crassostrea virginica*. The marine bacteria *Vibrio parahaemolyticus* is taken up by filter-feeding shellfish. Only some forms pathogenic to humans when contaminated shellfish are consumed-however a rapid clonal, pandemic-level expansion of a pathogenic form of *V. parahaemolyticus* emerged in the NW Pacific coast of North America [17]. Further studies reveal that ocean warming is associated with long-term increases in Vibrio populations and increasing possibilities of new pathogenic forms emerging [18]. Thus there is a need to evaluate these organisms alongside other plankton to evaluate their interactions and potential pathogenicity

This paper is a comprehensive case study of the relative merits of detection methods to fully monitor plankton and assess less-represented taxa. The study compares qualitative and quantitative genetic, microscopic diversity combined with flow cytometry from samples taken from an automated water sampler deployed on the Continuous Plankton Recorder (CPR) collection instrument [19] and from CPR and Western Channel Observatory station L4 (www.westernchannelobservatory.org.uk) over a 3 year period

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in the Western Channel (also known as English Channel). We compare the extent that genetic data can overlap or add value to monitoring of less-represented plankton. We used high throughput sequencing methods to determine biodiversity over one year and quantitative PCR (qPCR) over three years to determine the abundance of two smaller sized potential harmful algae that were found to be present by sequencing or microscopic methods: *Pseudo-nitzschia delicatissima* that has varying degrees of toxicity and *Aureococcus anophagefferens*, a phytoplankter less than 5µm that cause brown tides in some marine environments. Finally we compared their presence and abundance to that found by microscopic or flow cytometric methods to show how a combination of these methods together can effectively assess most of the plankton size and taxonomic range.

## 2. Materials and Methods

### 2.1: Sampling

The Water and Microplankton Sampler (WaMS) mechanism is described elsewhere [19]. Approximately monthly sampling was deployed within the payload of a Continuous Plankton Recorder (CPR) instrument on a Ship of Opportunity operated by Brittany Ferries from 2011-2013 in the Western Channel between Roscoff, France and Plymouth, UK (see Fig.1). The WaMS pumps 200-500ml of water directly into ten sampling bag pre-programmed sampling stations along a 122 mile transect and processed immediately after collection. Two sampling bags were chosen to sample one area. They were combined and filtered onto a PALL SUPOR 0.2µM pore membrane filter (VWR, USA), placed into ethanol and stored at -80ºC until DNA extraction. Duplicate 2ML samples were preserved in for flow cytometry were preserved in 1% TEM grade Gluteraldehyde (Sigma) flash-frozen and stored at −80 °C for 24 h before thawing and analysing as described [19]. All samples are listed in supplementary data file 1.

Phytoplankton cell counts were obtained from station L4 phytoplankton time-series [20] contains taxa-specific and total functional group abundance (cells mL-1) and biomass (mgC m-3) data collected from the Western Channel Observatory's Station L4 monitoring site (50 15.0'N; 4 13.0'W), situated off the south-west coast of England, United Kingdom. Samples have been collected weekly (weather permitting) from the 10m depth since October 1992. Paired 200mL water samples were collected using a 10L Niskin bottle attached to a CTD and are fixed in acid Lugol's iodine [21] for enumerating all phytoplankton cells >2µm and neutral formaldehyde for enumerating coccolithophores. Samples are analysed at Plymouth Marine Laboratory according to the Utermöhl counting technique [22] and since 2016, these methods follow the British and European standard document "Water quality – Guidance standard for routine microscopic surveys of phytoplankton using inverted microscopy (Utermöhl technique)" (BS EN 15204:2006). Abundance of each taxa (cells mL-1) are calculated according to the number of individuals per unit volume of sub-sample analysed.

### 2.2: CPR microscopic data

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Plankton sampling using the CPR survey collection is described in detail elsewhere [23, 24] This study used samples collected on a Ship of Opportunity operated by Brittany Ferries from 2011-2012 in the Western Channel between Roscoff, France and Plymouth from 2011-2012. Plankton was counted in a semi-quantitative manner using log-transformed data [24], DOI: https://doi.org/10.17031/1808. Vorticella sp and nematocysts were excluded as non-phytoplankton entities whilst Brown cysts, Black Spine cyst, Filamentous algae and phytoplankton colour) were excluded as too ambiguous.

**2.3 Water Sample DNA Extraction**

Water sampler DNA extraction was completed as follows: The filter was removed with clean forceps and placed into a sterile, 2ml microfuge tube prior to DNA extraction and centrifuged at 13000 x g to collect any remaining cells. Excess ethanol supernatent was removed and the remaining ethanol was evaporated in the fume cupboard. For WaMS samples from February to March 2011, buffer AP1 from DNeasy Plant kit (Qiagen, Valencia, USA) was added using volumes specified in the kit, and DNA extraction proceeded according to the manufacturers protocol and described more fully in Stern et al. (2015).

from thereon was completed by CTAB buffer extraction, followed by phenol-chloroform extraction process adapted from [25] as described by [19] 500µl of CTAB buffer containing 5.6 µg/ml of Proteinase K and 0.2% ß-Mercaptoethanol (Sigma) was added to each sample and incubated for 1h 30 minutes at 55°C. DNA was extracted using equal volumes of phenol (Sigma), followed by chloroform/isoamylalcohol mix (Sigma). DNA was precipitated with isopropanol and washed with ice cold 70% ethanol. The pellet was resuspended in 60µl of TE buffer.

**2.4 QPCR-HRM Assay development**

Algae culture growth, preparation of standards and DNA extractions see Appendix 1. All quantitative assays were assessed through High Resolution Melt-Curve (HRM) quantitative PCR (qPCR) that melts the PCR product at a specific temperature for species detection and simultaneously carrying out qPCR on DNA. *Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia delicatissima* assays were based on previously validated study [26]. Pseudo-nitzschia multiseries was developed as part of this study [27]. Aureococcus anophagefferens assay was based on an earlier study [28]. Table 2 these summarises assays. HRM-qPCR amplifications were performed using a Rotor Gene 6000 (Qiagen, Valencia, USA) in 10µl volumes using a 72 sample rotor using the green channel and three step with HRM option.

For HRM-qPCR assays, reactions were run in duplicate or triplicate for standards (see Table 2) and contained 5µL of SensiFAST HRM Kit (Bioline Reagents Limited, London), 0.4µl of each primers, 2µl (*P. fraudulenta, P. multiseries*) or 4µl (*P. delicatissima*) of DNA in

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a total reaction volume of 10 µl. Additionally, genomic DNA from their correspondinh cultured species were included to determine any differences in outputs from cloned PCR products and to assess copy numbers per cell. Due to a later failure in *P.fraudulenta* CCAP1061/4 or 6,, an additional *P. fraudulenta* qPCR assay was performed on DNA dervied from *P. fraudulenta* culture IO83-07 to convert ITS copy numbers to known cell concentrations. Negative controls with water instead of DNA were used to ensure results were from their respective samples and not external contamination. The optimised thermal cycling conditions were as follows: 1 cycle of 95°C for 3min for polymerase activation, followed by 40 cycles of denaturation at 95°C for 5s, primer annealing at 60°C for 10s and extension at 72°C 20s. Following the cycling phase, HRM melting phase ranged from 70-90 °C, rising in 0.1 °C increments.

Melt curves were inspected to assess contamination and range of curves. HRM standard reports were downloaded from Corbett Rotor Gene 6000 software (Qiagen) All assays revealed species-melt curves whose peak were within 0.5°C of standards. Fig. A1 shows HRM curves of standards and the corresponding genomic DNA positive controls of standards.

**2.4.1 Quantification: HRM-qPCR data treatment**

Quantitative PCR using standard curve was calculated against their respective DNA copy numbers or with DNA concentrations using Corbett Rotor Gene 6000 (Qiagen, Valencia, USA) Rotor Gene Q software under Cycling A Green channel. A full description is in Appendix 1 showing standard curves for *P. fraudulenta* and *P. multiseries* in Fig. A2, *P. delicatissima* in Fig. A3 and *A. anophagefferens* in Fig. A4. Run parameters are shown in Appendix 2 (see Appendix B). Reaction parameters, standard curves and values were reported in standard reports from the software. Table A1 shows qPCR run parameters for P. fraudulenta and P. multiseries which showed low reaction efficiencies and significant inter-run variability for inter-annual comparisons. Thus only presence is recorded for these species.

The target copy numbers per cell for *Pseudo-nitzschia delicatissima* calculated from the qPCR run containing 2011 samples and found to be between 15-20 copies/cell. For the *Aureococcus anophagefferens* assay, it was calculated that *Aureococcus anophagefferens* had 2 copies/cell of the 18S rDNA marker, although standard curves were not identical across all Ct values. Therefore copy numbers for qPCR runs of WaMS samples from 2011-2013 were converted to cell numbers per ml using standard curve formula in Fig. A6 to normalize DNA copy numbers in this study to those of cell numbers from Popel et al. 2003.

**2.4.2 Environmental Sample Testing**

DNA extractions from WaMS samples were subsequently tested for the presence of *P. fraudulenta, P. delicatissima* and *P. multiseries* using the previously optimised protocol. Each HRM-qPCR run included positive control, negative (water), negative (DNA extraction water control) and standard dilution series, run in duplicate. A total of 105 different environmental samples were screened from years 2011 (42 from February 2011-February 2012) and 2013 (35 from March-December, 2012). A selection of products from each 2011 and 2012 was checked for species-specificity by DNA sequencing (Source Bioscience

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(Nottingham, UK) for sequencing according to their protocol (awaiting Genbank accession number).

**2.5: DNA amplification, sequencing and bioinformatic analysis**

Twenty nine samples of genomic DNA were shipped to Molecular Research LP (http://www.mrdnalab.com, Shallowater, TX, USA) where a 500-bp portion of the variable V4 region of the nuclear 18S rRNA was amplifed by PCR using the adaptor-linked barcoded eukaryotic primer pair euk516F (5'-GGAGGGCAAGTCTGGT-3') and euk1055R (5'-CGGCCATGCACCACC-3'). Amplification was performed using HotStarTaq Plus Master Mix Kit (QIAGEN) under the following conditions: initial denaturation at 94 ◦C for 3 minutes, followed by 30 cycles of 94 ◦C for 30 seconds, 53 ◦C for 40 seconds, and 72 ◦C for 1 minute, concluding with an elongation step at 72 ◦C for 5 minutes. Amplicons were pooled in equimolar concentrations and purified using Agencourt AMPure beads (Agencourt Bioscience, MA, USA). Amplicon libraries were pyrosequenced using Roche 454 GS-FLX Titanium chemistry (hereafter called HTS for high throughput sequencing).

**2.5.1 Sequence processing**

Raw sequence data were processed using QIIME v1.9.1 [29]. In the first round of screening (\first pass filtering"), reads were checked for the presence of the forward sequencing primer (euk516F) and a valid barcode and single reads removed (the denoising step). This process was carried out separately for reads from Febraury-April 2011 and for April 2011-February 2012. The output files for both of these were combined to c ontain reads from 42 samples in total were processed. Barcoded reads were imported and had forward primers removed (allowing for a maximum of one mismatch) before being demultiplexed and filtered according to their Phred quality scores. Quality criteria were a minimum read length of 200 bp, a maximum read length of 700 bp, no ambiguous bases, a maximum homopolymer length of 6 (as homopolymer reads are a specific problem to 454 GS-FLX technology used at the time). Additional filtering criteria were also applied with a sliding window quality score of 50 to remove poor quality sequences during denoising steps.

Operational Taxonomic Unit (OTU) clustering on the resulting dataset was carried out at the 97% sequence similarity with a word length of 12 and maximum mismatch range of 20-500bp. Taxonomic assignment was performed using UCLUST [30] and the RDP classifier [31] using the pick OTU command, with a minimum bootstrap threshold of 80%. UCLUST compares the overall sequence similarity of a queried representative sequence against a database of reference sequences whilst RDP classifier compares query sequences to reference sequences using 8bp long strings across the entire length of the sequence and estimates a confidence score for the assignment of each taxonomic level based on 100 bootstrap replicates. For both approaches, the Silva 111 eukaryotic rDNA database was used for identification of taxa. OTU tables were generated from which taxa summary plots and alignments were created. Searches were performed using the BLASTn tool [32] to check the identities and search for Chimeric reads that did not show complete alignment with a

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reference sequence. Chimeras were removed from the alignment using chimera slayer. Sequences were deposited in Genbank (ERP105780)

**2.5.2 Specific species- specific assays**

To confirm presence of amoeba and relationships with Legionella, a partial region of the 18S ribosomal gene was amplified from DNA samples using Jumpstart Redtaq Readymix (Sigma, Dorset, UK) using 0.3µM of JFP (forward) and JRP (reverse) primers from [33] for Legionella at 95˚C for 5 minutes followed by 95˚C for 30 seconds, xx˚C for 30 seconds and 72˚C for 1 minute for 35 cycles, with a final extension at 72˚C for 7 minutes. Partial 18S regions of *Acanthamoeba* and *Hartmanella* were amplified from water samples using JDP1 (forwards) and JDP2 (reverse) primers from Schroeder et al. (2001) and Hv1227F and Hv1728R primers from [34] respectively. Amplification conditions were the same as described above except the annealing temperature was 60˚C for *Acanthamoeba* and X˚C for *Hartmanella* respectively. Amplicons were purified using GeneElute PCR cleanup kit (Sigma, Dorset, UK) and unidirectionally sequenced by Source Bioscience (Nottingham, UK) according to their instructions. Sequences were submitted to Genbank (awaiting accession numbers).

**2.5.3 Phylogenetic analysis**

Selected sequences from the partial 18S read alignment output from Qiime or from Sanger sequencing from WaMS samples were taken, checked for identity by Blastn [32].

For Sanger sequences they were manually edited for ambiguous sequences using BioEdit, version 7.2.5 [35]. All sequences were imported into a new Bioedit file with corresponding species retrieved from public databases and aligned using MAFFT-7 [36] . MEGA 6.06 [37] or MEGA11 [38] was used to carry out phylogenetic analysis. Partial 18S reads were only checked using Neighbour-joining tree using Kimura-2 parameter model with partial-deletion option using 1000 bootstrap as a phylogenetic assessment initially followed by Maximum Likelihood phylogenetic methods for groups of taxa ( see Table S7) in MEGA11.

For phylogenetic analysis of both partial 16S *Legionella* sequences and partial 18S *Amoeba* sequences generated by Sanger sequencing, a Jukes-Cantor model [40] with Gamma distribution with some invariant sites [37, 41] was applied (see Table S7). The tree was drawn to scale. All positions containing gaps and missing data were eliminated.

**2.6 Enumeration of phytoplankton and bacteria by flow cytometry**

Phytoplankton and bacteria were enumerated using a BD Accuri™ C6 flow cytometer equipped with a 50 mW solid state laser providing blue light at 488nm. Samples were analysed at a nominal flow rate of 100 µL $min^{-1}$ and a core of 20 µm for 5 minutes and 1 minute respectively for phytoplankton and bacteria. The flow rate was calibrated on each sampling day using Beckman Coulter™ Flowset™ fluorospheres at a known concentration. Besides counting the cells, the flow cytometer also measured chlorophyll fluorescence (CHL. >675 nm), phycoerythrin fluorescence (PE. 585+/-20 nm), green fluorescence (530+/-

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15 nm), side scatter (SSC. light scattered by particles at 90° to the direction of the laser beam) and forward light scatter (FSC. light scattered by particles at narrow angles in the same direction as the laser beam). Measurements of light scatter and fluorescence, data storage in listmode format and subsequent data analysis were made using a CFlow Plus software (Becton Dickinson, Oxford) with log amplification on a seven-decade scale.

Phytoplankton data acquisition was triggered on both chlorophyll fluorescence, and forward light scatter using prior knowledge of the position of *Synechococcus* sp. cyanobacteria to set the lower limit of analysis. Density plots of FSC vs. CHL fluorescence, PE vs. CHL fluorescence and SSC vs. CHL fluorescence were used to discriminate *Synechococcus* sp. cyanobacteria, picoeukaryote phytoplankton (0.5-3 μm), coccolithophores, cryptophytes and nanophytoplankton (eukaryotes >3 μm, excluding the coccolithophores and cryptophytes). For quantification of bacteria, 500 μL of preserved sample (0.5% gluteraldehyde final concentration) was mixed with 5 μL 1% Sybr Green I DNA dye (1% solution made from adding 1 μL of x10,000 Sybr Green I concentrate to 99 μL 300 μM potassium citrate buffer) and 45 μL 300 mM potassium citrate buffer. Samples were mixed by hand and then placed in the dark at room temperature for one hour before analysis. Data acquisition for bacteria was triggered on both green fluorescence and forward light scatter. Density plots of CHL fluorescence vs. green fluorescence and SSC vs. green fluorescence were used to quantify total heterotrophic bacteria. Data is displayed in Table S11. Only picoeukaryote and nanoeukaryote data was used for further analysis. To increase robustness data from all positions from each cruise was aggregated into mean value.

**2.7 Comparisons between microscopy and genetic findings**

The WCO L4 dataset had a taxonomic hierarchy derived from World Register of Marine Species taxonomy or WoRMS [42] to accompany the data that included diatoms, dinoflagellates, coccolithophorids, flagellates and other (Amoebozoa, Radiolaria). To update class-level taxonomy of diatoms [43] , classification of Bacillariophyceae were altered to their corresponding classes according to the AlgaeBase database [44]. Comparisons were made at Class, Order, Family and Genus level with UCLUST and RDP assigned taxa observed in WCO L4 data with that identified from WaMS partial 18S reads over 2011-2012 period using as close a date as possible to each other. If the dates did not correspond then two nearest dates from WCO L4 data were used for comparisons. All positions of WaMS data were used for comparisons. Any non-taxonomic assignments were ignored. Next, phytoplankton data from the CPR dataset was classified according to WoRMS again using class-level classification according to AlgaeBase and split into the same hierarchical classifications. Only phytoplankton taxa were considered and both the number of taxa and the actual observed taxa in each category was counted. The RDP classifier and UCLUST classification systems for 18S reads from the 2011-2012 WaMS samples differed from WoRMs, and in those cases the nearest equivalent taxa to WoRMS was used for comparison. If a genus name was given, the family and order could be inferred from WoRMs database.

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## 3. Results

Real-time PCR combined with HRM was developed for four harmful algae species *Pseudo-nitzschia delicatissima*, *Pseudo-nitzschia fraudulenta* and *Pseudo-nitzschia multiseries* species as medium sized species (>10µm, <50µm in length per cell) and finally *Aureococcus anophagefferens* as a small sized species (<5µm).

### 3.1 Pseudo-nitzschia assay

HRM curves from plasmid standards and genomic DNA from positive controls revealed a specific peak at 84-84.52°C for *P. fraudulenta*, with 84°C observed for standards at the lowest concentration, although non specific peaks were identified outside its range which may be the reason for reduced reaction efficiency. *P. multiseries* HRM curve showed a specific peak range of 82.47-82.7. P. multiseries contained amplicons of bacterial origin so this assay was deemed to be non-specific. DNA sequencing of positive controls for the P. fraudulenta assay were verified (EBIXXXX). No significant differences in HRM curve range was found between 2011-2012 samples.

The plasmid standard curves for *P. fraudulenta* and *P. multiseries* were generated for each qPCR HRM run (Fig. S2) with good correlation coefficient value ($R^2$>0.99) and consistency between standard Ct value between replicates but their efficiency figures were lower than the recommended 90% on the standard curve qPCR results, ranging from 70-72% for *P. fraudulenta* and 66-70% for *P. multiseries* likely reflected in lowered overall reaction efficiency values of the standard curve slope (M: -4.236 to -4.56) which should be near -3.322. Due to their lowered efficiency the results were not deemed to be reliable for subsequent interpretation of species cell numbers.

HRM analysis of *P. delicatissima* revealed a species specific peak between 82-82.5C (Fig. A1 B, Appendix 1). *P.delicatissima* reaction efficiency (see Table 3) was between 0.8-1.2, 2011 was lower than idea 90% or more efficiency. The copy number calculated of 15-20 copies/cell is similar to average copies/cell reported by other studies for this species: 16 and 26 copies/cell [45], 29 copies per cell [46]. As 2011 efficiency was lower for runs that analysed 2012, 2013 samples, a student's t-test was performed on 2011 and 2012 Ct values revealing no statistical difference between runs (p=0.075).

### 3.2 *Aureococcus anophagefferens* assay

The PCR product of Aureococcus assay developed by Popel et al. [28] had a HRM melt curve peak at 82°C (see Fig A1C, Appendix 1) and was specific to Aureococcus upon sequencing products from environmental samples E4_10_11 (1) or WS32, E1_12_11 or WS34 and E1_2_12 or WS39 (Genbank accession). The reaction efficiencies of 150 samples (tested in four runs) ranged from 0.78-1.04, two runs (2012, 2013 pt1) were under optimal 90% reaction efficiency and may have underestimated cell abundances (Table 3). Other parameters (M, R, $R^2$) were similar to each other. Run AA2011 had very similar reaction parameters to those published in [28] and calculated 2 copies per cell were similar to the

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results from a genome search for 18S. The potential accuracy of mean detection capability is 1.4 cells on average [28] but the minimum standard concentration used in these assay tests 7350-7500 copies $ul^{-1}$ (367-450 cells) so many values fell below this range and actual values may not be accurate as they fall outstand the dynamic range of standards.

**3.3 Environmental analyis of harmful algae species in the Western channel by qPCR**

*Pseudo-nitzschia delicatissima*, considered a nanoeukaryote in size, qPCR results revealed elevated abundances in 2011 compared to 2012 and 2013 (Fig.2B). By contrast microscopy results indicated elevated abundances in 2011 and 2013 (Fig, 2A). Both analyses showed reduced presence in 2012, although samples were missing for 10 out of 36 months that may skew these results and for qPCR measurements. Average levels were used to improve statistical measurements. Inspection of station E5, that is most similar in location to WCO station L4, revealed similar pattern to that of average values over all stations (not shown). Of note cell abundances from the qPCR results were an order higher than microscopic abundances. Microscopy results show increased levels of cells in summer 2011 levels, very few cells throughout 2012 and then in 2013, similar abundance of *P. delicatissima* as 2011 but over a longer period from July-October.

Although quantitative data could not be used for *Pseudo-nitzschia fraudulenta* or *Pseudo-nitzschia multiseries*, their present and absence (Table 1) shows *P. multiseries* was completely absent using this assay. *P. fraudulenta* was present from February to October but showed a seasonality with elevated levels in April and October 2011. The next year it appeared year round in at least one sample from each cruise mostly in mid channel.

Seasonal abundance of *A. anophagefferens* (Fig. 3), a picoeukyote, was dominated by an extremely high cell count in July 2011 which never reappeared. The remaining seasonal abundances were generally low and most of them below the dynamic range of standards but appeared more abundant in Summer-Autumn of 2011 and Spring of 2012. It should be noted this pattern missing data that could have revealed a different patterns. No sampling was carried out for 10 months out of 36 were not sampled due to machine repairs. Furthermore, quality assurance of AA2013 pt 2 run (July-December 2013), many values were discarded, although inspection of raw values showed they were low. For both *A. anophagefferens* and *P. delicatissima*, there was an increased presence in 2011 compared to 2012.

**3.4 Small eukaryotic cells in the Western Channel using Flow cytometry**

Over 2011-2013 smaller photosynthetic aggregated cell types showed different patterns (Fig. 4) with photosynthetic nanoeukaryotes, photosynthetic picoeukaryotes both showing similar seasonal pattern. A bimodal abundance peak in May and October of 2011 and June and September 2012. However, in 2013 a summer-Autumn single abundance peak appeared from July-October for nanoeukaryotes although the picoeukaryotes showed a dip in August. There were an order more photosynthetic picoeukaryotes compared to photosynthetic nanoeukarytes. Microscopic total phytoplankton showed less variations, likely down to more frequent monitoring and elevated abundances over similar periods in 2011 and 2012. The diatoms more or less followed the total phytoplankton pattern, except in 2013 with an autumn peak instead of the spring/summer total phytoplankton peaks. Unfortunately there was no WaMS flow cytometry data for May 2013

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when there was a large spike in microscopic phytoplankton from WCO L4. Microscopic counts were of a similar order to nanoeukaryotes. Both pico- and nanoeukaryotes show relatively lower levels of peak abundance in 2012 compared to 2011. Investigating nutrient conditions over 20 years (see Fig. 5) each nutrient has a single annual cycle and peak. In comparison to the whole period, Silicate levels are lower in 2011, phosphate levels are typical, nitrite levels are lower in 2011-2012 but nitrate are normal.

Comparing abundance patterns of *A. anophagefferens* and *P. delicatissima*, examples of pico- and nanoeukaryotes (both photosynthetic) ,respectively, with that of all pico- and nano-sized phytoplankton (Fig. 4) shows picoeukaryotes dominate numerically by 100-1000 times. The seasonal pattern may be altered as no data was available for August and November 2011 and January, April and August 2012 and March-June 2013. However, elevated levels of both pico- and nanoeukaryotes occur in summer-Autumn of 2011 which is similar to genetically measured *A. anophagefferens* and *P. delicatissima* (Fig 3, Fig. 2B respectively). Nanoeukaryotes appear to recover their numbers at their peak in 2013 but picoeukaryotes show lower overall peak abundances in 2012 and 2013 compared to 2011. Only microscopically detected *P. delicatissima* and diatoms (Fig. 2B, 4A) show similar abundance trends with nanoeukaryoties. The genetically detected *A. anophagefferens* and *P. delicatissima*, remained at low levels in 2012 and 2013. It is clear that 2012 appears to be a poor year for both picoeukaryotes measured by FC (Fig. 4C), and for *P. delicatissima* measured microscopically (Fig. 2A). Of interest is that the pattern of microscopically measured *Pseudo-nitzschia delicatissima* follows the opposite pattern of total diatoms, which grew relatively more 2012.

**3.5 Genetic taxa trends**

After quality filtering and clustering, the total number of reads from 42 combined samples was 49033, ranging from 69-7500, with a mean of 1167.5 and median of 692 reads per sample (see Table 4). Read and OTU distributions were unequal across samples and the first sequencing run showed poorer OTU counts compared to the second. Measures of alpha diversity in Table 4 confirmed this and suggest the planktonic community was incompletely sampled. Calculated chao1 rarefaction curves for each sampling tow failed to reach plateaus (Fig. 6). Due to the bias it is likely rare taxa are not represented in sequencing run 1 and subsequently any community analysis (beta diversity) would be likely be biased.

Using UCLUST approach, Taxa at hierachy level 3 (class level or equivalent) from all major eukaryotic lineages were observed in the Febraury 2011- February 2012 samples, though most groups were relatively rare (Figure 7). Level 3 was the most complete in terms of genetic taxonomic assignment (also shown in Table S2), however UCLUST taxa assignments were made to level 5 (family/genus level), shown in Tables S3-35. The dominant taxonomic groups over all samples was uncultured eukaryotes at 34% followed by Metazoa at 26% then alveolates at 25% then a significant minority consisting of Stramenopiles at 6% and chloroplastida of 4% (see Table S2). Rare taxa contributed just 5% of total reads, but represented by 19/24 taxa while a majority of the dataset (95%) corresponded to reads shared among five taxa. The summer season had less uncultured eukaryotes and were dominated by Alveolata (samples WS17-22). The proportions of Chloroplastida were absent or minimal in summer and relatively higher in winter, Spring

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and Autumn of 2011. At taxonomic level L4, the Alveolates in samples WS17-22 were dinoflagellates. Most of the Metazoa were arthropods (see Table S3). Haptophytes mostly appeared from June onwards

Diversity assessments using the RDP classified dataset were similar to UCLUST (see Fig. 8 and Table S6); lower taxa were divided into levels that did not always correspond to taxonomic hierachies- a class or genus could be in the same level. A second caveat are some outdated taxonomic classifications. A third (299) of the 922 Eukaryotic OTUs were unidentifiable and a further 147 were unidentifiable beyong Kingdom level. The majority of other OTUs belonged to SAR supergroup or Opisthokonta. Only a third of the total number of OTUs (262) could be identified at level 3 (ranging from phylum to class) and here the majority of taxa belonged to crustacean arthropods (7%), Mamiellophyceae (9%) five alveolate groups namely, Ciliophora, Protalveolata, Dinoflagellata and Cecozoa, totalling 53% and Stramenopiles mainly Diatomea and Bicosoecida at 9%. Sixteen OTUs could be identified to genus or equivalent level for those without official genus names. Of interest, both Aureococcus and Acanthamoeba identified by UCLUST were confirmed by qPCR and sequencing respectively.

**3.6 Phylogenetic analysis of partial 18S reads of WS1-43**

Confirmation of UCLUST and RDP results using maximum likelihood method of phylogeny on selected taxa were able to confirm taxa to genera level, although to low confidence considering the short size of the partial 18S reads and the limits of the markers resolution (Fig 9). Only shallow phylogenetic positions were considered to confirm the presence of a taxa as deeper level taxa clusters were unreliable with low bootstrap support. Within the MAST group (Fig. 9A), one other OTU, sister to Biocosoecida could not be placed and the divergent nature of these sister sequences mean that OTUs in the MAST groups are often unclassified. The Haptophytes confirmed the presence of Phaeocystis and an unidentified Prymnesiophyte sister group to Chrysochromulina. Within the dinoflagellates (Fig. 9C), Prorocentrum taxa was split into different clades and one OTU grouped with one clade containing the benthic *Prorocentrum lima*, although with low support. Few OTUs could be identified to genus level with the exception of *Karlodinium*, *Torodinium* and *Protoperidinium*. Three sequences were sister to *Toridinium* and *Karlodinium* and likely to belong to Gymnodiniales. Four other sequences were sister to a *Gymnodinium* species, whilst four others were more deep branching of unknown origin. Phaeocystsis was the only haptophyte that could be identified. Three other reads were sister to two Chrysochromulina species, and could be another of the same species or a related genus.

**3.7 Detection of Amoeba and Legionella from WaMS samples**

Phylogenetic analysis of Amoeba sequences is shown in Fig. 10A. Three Acanthamoebae species were found in WaMS samples plus *Hartmanella vermiformis*, (Awaiting Genbank accession number) that was 100% identical to *H. vermiformis* public sequences using BLASTn search tool [32]. Four of these belonged to *Acanthomoeba castellanii* and identical

to sequences belonging to T4 genotype (ATCC30011), however one of these (WEC_Amoeb9) was more variant and heterozygous at four sites so may be a related species. Acanthameobae species were co-identified for group I, however group II and III co-occurred with unknown environmental Acanthamoebae species and *A. castellanii* co-occurred with *L. massiliensis* and an unknown Legionella species in WEC 35 that also harboured *H.vermiformis*.

The gammaproteobacteria, *Legionella* spp., which is an obligate parasite of amoeba in the environment, was also found in t0en out of ninety samples, all but one from 2011. All but two of the samples also contained Acanthamoeba or Hartmanella, indicating a different host in those two samples. Four environmental groups from WaMS samples could be identified and two could not be resolved beyond the genus level in Fig. 10B. Two environmental Legionalla signatures grouped with Legionella group I, that contained known pathogenic strains including *L. pneumophila and L. longbeachae*. WEC Leg 9 grouped with environmental samples and two *L. pneumophila* strains and four environmental samples contained isolates similar to *L. massiliensis*- an environmental sample from a cooling tower, sister to *L. birminghamensis* [47]. The Legionella present in WEC Leg 7 and 9 could not be identified even into groups, indicating a more diverse population in this environment.

### 3.7 **WaMS 18S genetic reads versus Microscopy results**

WCO L4, CPR WaMS genetic and CPR morphological dataset had significant different standard taxa measured (see Table 5). Table S9 shows a complete comparison of taxa present in 2011-2012 from WCO L4 with genetically detected taxa from WaMS. Due to disparity only photosynthetic cateories were compared between datasets (Fig. 10). Of the coccolithophorids observed in WCO L4 six were found in CPR category too and a reasonable comparative match. The best alignment in terms of taxa measured and observed were in the Diatoms and Dinoflagellates categories. The Flagellate and Other category of WCO L4 had fewer taxa and were least in common with CPR and WaMS genetic taxa, the latter being mostly heterotrophic flagellates (see Table S5, 59% were heterotrophic or mixotrophic excluding Metazoa). Ciliophora were measured by WCO L4 than the CPR dataset not surprisingly as they are soft-bodies organisms that would be destroyed on the CPR silk. As expected the CPR and WCO L4 morphological datasets shared the most number of photosynthetic taxa observed (31 out of a combined 100). Only 20 families within photosynthetic category could be identified and between 8-12 were shared with morphological observations. The type of taxa not observed by CPR or WCO L4 morphology included Cercozoa, SAR group, Labyrinthulomycetes, Fungi, Kathablepharidae, Picozoa and Excavata. A surprisingly large number of metazoa were identified considering the small volume of samples including Echninodermata, Cnidaria, Mollusca, Urochordata and Maxillopoda. Within the most comparable photosynthetic category smaller species under 10 microns were not represented in the morphological datasets such as Pegalophyceae (Aureococcus), Chrysochromulina and Imantionia of

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Prymnesiophyceae, Geminigera and Teleaulax of Cryptophyceae and Mamiellophyceae of Chloroplastida. Dinoflagellelates had the most shared taxa (5 families) and 4 genera.

## 4. Discussion

This study has revealed that genetic detection of plankton enhances microscopy-based surveys of marine plankton. Taxa generated from the partial 18S reads were different and were smaller and/or heterotrophic protists, although many multicellular metazoans were present. Even with small volumes range of organisms multicellular metazoan to the smallest chlorophytes (e.g mamiellophyceae) were picked up, although clearly not all diversity was captured. The species found were typical for the Western Channel [48]. All supergroups in this study were also found in the Eastern (English) Channel in other 18S amplicon sequencing surveys [49]. Of interest, that study compared 454 pyrosequencing method used here with illumina methods and found significant differences in taxa such as Radiolaria which reveals the need for standardisation and normalisaton across different genetic approaches for taxonomic surveys. Smaller chlorophytes have been reported on the French far coastal waters of the Western channel [50] and were found here. Dinoflagellates have expanded 18S copy numbers and Maxillopodia within Metazoans have elevated 18S copy numbers, thus these could be over-represented. Several parasites (Gregarines, Syndiniales and Picozoa) or potentially harmful organisms such as the amoeba Hartmannella were found, and in the latter case, confirmed separately through sequencing. Interestingly, most taxa found in January to May 2011 of WaMS in our earlier study [19] were present year round.

### WaMS Sequencing and Microscopy

A key benefit of genetic survey was the recovery of a wide variety of hetertrophs which are key to nutrient cycling but are understudied. Eleven members of Uncultured and incompletely characterised MArine STramenopiles group or MAST that represent a substantial portion of the abundance and diversity of the heterotrophic eukaryotic plankton community. These are globally abundant bacteriovores, symbionts and parasites [51, 52]. There are about 18 MAST clades are shown to be influenced by temperature, nitrite and the abundance of the cyanobaceteria, Synechococcus. Several of these clades initially identified are no longer supported phylogenetically including MAST-5 found in this study [51].This brings home the need for increased joint morphological and genetic examination of small marine plankton taxa. MAST-1, MAST-2 are named Pseudofungi and sister to hyperchytriales. MAST-3 and MAST-12 are Opalozoa within Phyla Bigyra [51]. MAST 4,6,11 are an early branching Stramenopile clade [53]. With the exception of MAST-9, all MAST groups recovered here were shown to be planktonic from associated metadata of sequence reads associated with these groups and prefer oxic environements. This would be consistent with the Western Channel surface water environment. MAST-9 has now been subdivided into 4 clades with greater sequence abundance and diversity in warmer waters, whilst subclades of MAST-1 appear to prefer cooler coastal waters [52]. prefer anoxic

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environments and split between sediment and plankton. Its possible windy environments might resuspend sediments (Massana et al. 2014). Continuous monitoring of heterotrophic organisms could be used to develop much-needed indicators of Pelagic habitats in official assessments of marine health that are unassessed due to lack of data [54].

It would be expected that surveys recorded using the same method would be more similar and CPR and WCO L4 datasets showed more overlap with each other than with WaMS, which has smaller sampling volume and a different detection method. However although the WaMS was deployed on the CPR, the taxa found were more similar to those found in WCO L4. The 240 micron mesh-size of the CPR must capture different The degree of resolution of taxa from the partial 18S reads varied from different taxa with most taxa only been able to be resolved down to the family or order level. This is typical of many metabarcoding or amplicon sequencing studies due to the small size and conserved natures of the 18S rRNA marker used, however with the common use of PACBIO or Nanopore technologies, resolution to species level are likely to be common-place [55]. At the moment there are too many differences between different taxonomic surveys based on microscopy and genetic results for them to be harmonised. However studies have shown that despite different relative abundances of taxa between genetic methods and microscopic or semi-microscopic methods like CARD-FISH, similar ecological drivers could be identified from different sets [56]

One clear benefit of this genetic study is to ability to further assess taxa that would not be uncovered without the original genetic survey. Legionella bacteria are obligate symbionts of Amoeba, including Acanthamoeba, Hartmanella [57] in marine waters. There are more than 50 known species of Legionella bacteria, of which 24 species have been reported to be associated with human pathogenesis however 90% of cases are caused by five serogroups of *P. pneumophila* (reviewed by [58]. Six different *Legionella* and three amoeboid taxa were found in open water environments of the Western Channel. Three samples contained *A. castellanii* genotype T4, reported to be the most frequent cause of pathogenisis to humans [59]. All but one occurrence of these pathogens was in 2011 and most appeared in Autumn and Winter seasons. Amoeba are normally found in freshwater biofilms and the Western Channel has a riverine influence, however all species were found throughout the channel. Gast et al. [60] reported amoeba in marine sediments, nearly have of which harboured *Legionella*, 4% of which were *Legionella pneumophila*. However reports found *Acanthamoeba* in marine invertebrates [61] and in multiple environments (soil, marine, freshwater, animal) and medical studies show human sera with antibodies raised against Acanthamoeba organisms indicating common exposure. In this scenario, the survival of amoeba is likely to be due to either associations with metazoan (internally or on surface biofilms) or they may be in an inactive, cyst form. The symbiosis is a factor in Legionella's invasive properties [62]. Clearly further work is required to determine the life cycle of both organisms in the marine environment and their threat, if any, to humans and

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marine animals. *Legionella* must be residing in other amoeboid hosts as five samples did not contain *Acanthamoeba* or *Hartmanella*.

**qPCR of harmful algae**

This study validated two out of four qPCR assays that identified the presence and abundance two species, one of which cannot be identified through microscopyHowever, challenges to this method were encountered. The inter-run variability and low reaction efficiency meant that *P. fraudulenta* assay was not reliable enough to compare seasonal abundance. Other aspects that hampered consistent detection of species were a) low volume of water collected that resulted in low cell abundance often below the dynamic range of standards used b) inconsistent performance of WaMS due to machine error. So far no automated water sampling system on a moving system has been achieved and most are static. The CPR survey tows at 20-30 knots and the speed of the boat is extremely challenging sampling environment and these problems need to be overcome.

Whilst both *P. delicatissima* and *A. anophagefferens* showed different patterns of abundance, both showed elevated levels in 2011. . Both *P. delicatsisima* and *A. anophagefferens* internal controls appeared to have worked and the reaction efficiencies did not relate to detection levels e.g. P. delicatissima reaction efficiency was lower for the PD 2011 run but the detection level was greater. Thus technical difficulties in runs for 2012 and 2013 samples are less likely reason for lowered detection of these species in 2012-2013. It is notable that lower levels of nitrogen containing nutrients in 2011 when exceptionally high levels of *A. anophagefferens* was observed. *A. anophagefferens* has been reported to prefer low light, low nutrient conditions. [63] and has the ability to take up a variety of nitrogen sources under nutrient deplete conditions [64]. The detection of this species is important as potential harmful bloom causing algae but also as an indicator species.

The microscopy results for *P. delicatissima* from WCO L4 station differed from those measured by qPCR, the former showing a summer and autumn peak in 2011 and 2013. This was similar to total nanoeukaryotes measured by flow cytometry There are several confounding factors that may contribute to this, including lack of correspondence sampling dates, missing WaMS data and differences in sampling methods. Light microscopic measurements of *P.delicatissima* is insufficient to distinguish between six similar species belonging to *P. delicatissima* complex and *P. pseudodelicatissima* complex [65, 66] so, it is possible a species complex was detected in 2013 but not in WaMS sample. The genetic detection of *P. delicatissima* without microscopically detected *P.delicatissima* complex has been reported earlier [67]. However, 2012 appeared to be an unfavour year for the genetically measured *P. delicatissima* although the data in this study did not provide any clear reason. However a study of *Pseudo-nitzschia* spp. measured on the French coast of the Western Channel (Husson et al. 2016), revealed timing, abundance toxity of Pseudo-nitzschia spp. was highly influenced by local conditions, although temperature, salinity and light all positively influenced *Pseudo-nitzshchia* growth which may influence the

timing of this genus across locally distinct systems. The synchronous timing of genetically-detect *P. delicatissima* did correspond to that observed by the Husson et al (2016) study. This study highlighted the difficulty in linking *Pseudo-nitzschia* at a genus level to toxic events. The genetic assays validated here by Andree et al. (2011) will likely improve these associations and ulitmately models to understand links between environment and toxin production. Indeed, a predictive model based on molecuar data for *Alexandrium minutum* has recently been achieved ( Valbi et al. 2019).

**Flow cytometry and microscopy**

Picoeukaryote and nanoeukaryote data from flow cytometry data were remarkably similar in timing of peak abundance, although there were differences 2013 with a double peak and july and September for the former over one single peak August 2013 for the latter. This indicates similar broad ecological drivers of these communities. Of note larger phytoplankton community showed different profile in 2013 indicting different drivers. It is tempting to speculate that lowered nitrite levels in 2013 or silicate levels in 2011 could be involved, but there is not enough data to confirm this. Of interest, Aureococcus, a 2 micron sized phytoplankton species would be amongst the picoeukaryotes. However, its specific profile did not follow the aggregated picoplankton trend for the summer 2011 peak, although may be captured in the autumn peak. This species may be atypical of the group. A longer time-series is required to investigate these possibilities. The flow cytometry data is aggregated so has limited value in tracking individual species and their relationships with drivers and trophic connections but can be informative in understanding broad-scale affects of physico-chemical drivers based on their size. Additionally using qPCR or HTS to characterise the peak abundances of aggregate size-based cells from flow cytometry is an effective way to track species succession and understand common drivers.

## 5. Conclusions

Genetically-measured taxa composition measures different communities and complements microscopic observations. The species-level detection of morphologically indistinct taxa often show different profiles to aggregate community that provided insights into their habitat preferences and promise as indicators.

**Supplementary Materials:** The following are available online at www.mdpi.com/xxx/s1, Figure S1: title, Table S1: title, Video S1: title.

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**Data Availability Statement:** All raw microscopic is publicly available. CPR microscopic phytoplankton data is available at DOI https://doi.org/10.17031/1808. WCO L4 phytoplankton data is available at DOI https://10.5285/c9386b5c-b459-782f-e053-6c86abc0d129. Sequence read data of WS1-43 is has been deposited in EBI with accession number ERP105780. Sanger sequencing of organisms are all deposited in Genbank (see materials and methods).

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## Appendix A

Appendix 1: Quantitative PCR methods and validation

## Appendix 2

Quantitative PCR raw data

## Supplementary files

1. Supplementary Table S1 WaMS sample information
2. Supplementary Table S2: Taxa summary table of reads assigned by UCLUST from WaMS samples from Febraury 2011-February 2012 at taxonomic hierarchy L3
3. Supplementary Table S3: Taxa summary table of reads from WaMS samples from Febraury 2011-February 2012 at taxonomic hierarchy L4
4. Supplementary Table S4:Taxa summary table of reads from WaMS samples from Febraury 2011-February 2012 at taxonomic hierarchy L5
5. Supplementary Table S5: Taxa summary table of reads from WaMS samples from Febraury 2011-February 2012 at taxonomic hierarchy L6
6. Supplementary Table S6: RDP classified Taxa levels and their associated counts from partial 18S reads of samples WS1-42 from WaMS samples
7. Supplementary Table S7: Summary methods used in phylogenetic analysis of sequences produced from WaMS samples
8. Table S8: Calculated Pseudo-nitzschia delicatissima cell numbers derived from qPCR Ct values for samples with detectable Ct values
9. Table S9: Calculated Aureococcus anophagefferens cell numbers derived from qPCR Ct values for samples with detectable Ct values
10. Table S10: WCO L4 Phytoplankton microscopic taxa set from 2011-2012 and each taxa's nearest match to WaMS genetically-generated taxa. Grey type shows species absent from entire L4 microscopic dataset
11. Table S11: CPR Phytoplankton taxa measured and observed (in grey font) from CPR area D3, Western Channel dervied from 2011-2012 dataset at https://doi.org/10.17031/1808 and additional taxa
12. Table S12: Flow cytometry data from gluteraldehyde-preserved samples (prefixed with B) from WaMS from 2011-2013

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**Figures**

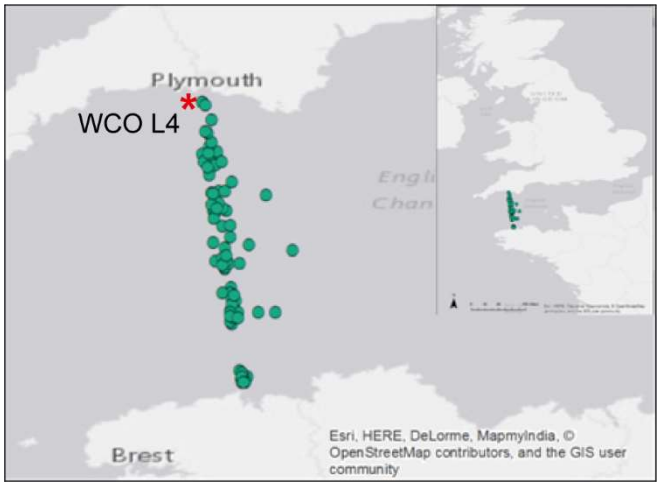


**Figure 1**: WaMS sampling positions in the Western Channel from 2011-2013. Asterix shows the approximate location of WCO station L4.

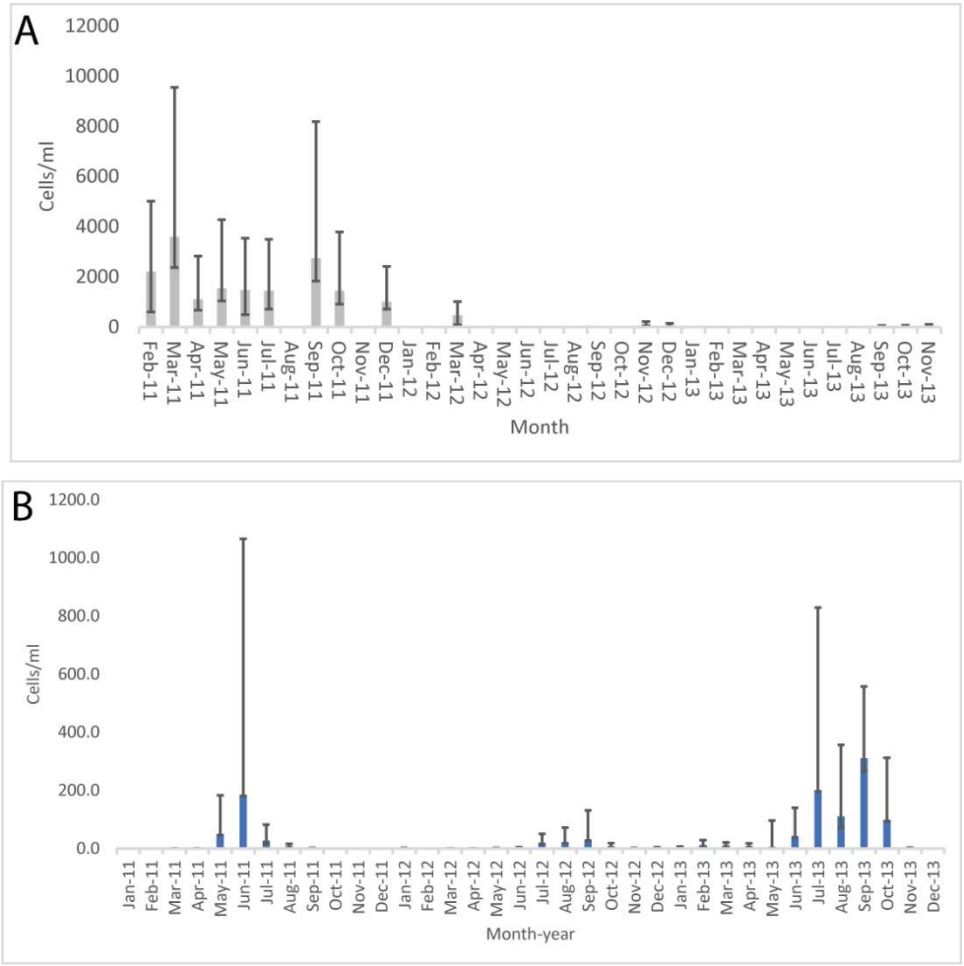


**Figure 2** Comparison of *P.delicatissima* cell counts (A) measured by qPCR averaged over all stations versus light microscopy measured at Western English Channel station L4 (B)

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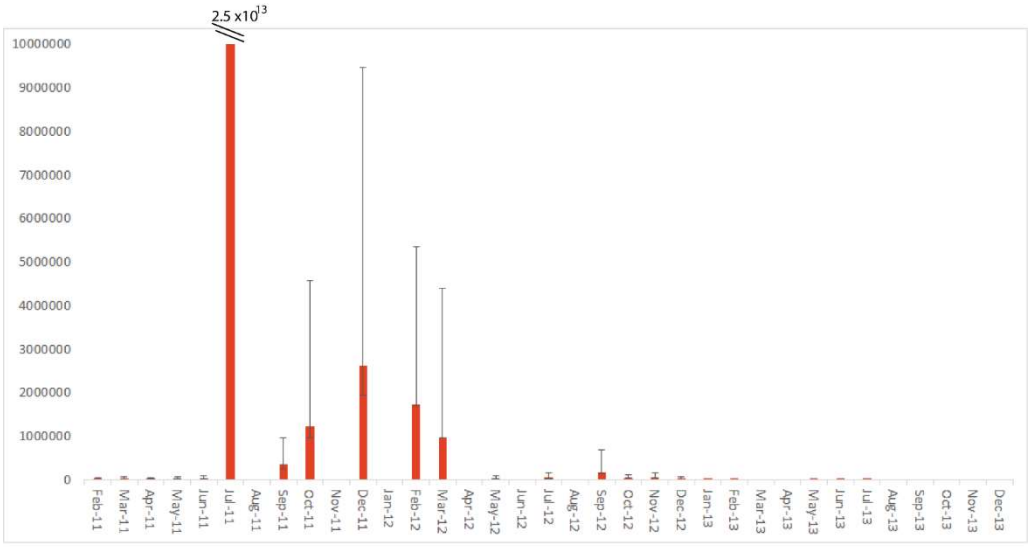


**Figure 3:** Seasonal cell abundances of *Aureococcus anophagefferens* measured by qPCR. Error bars represent maximum and minimum abundances. The y-axis on the chart represent abundance in cells/ml.

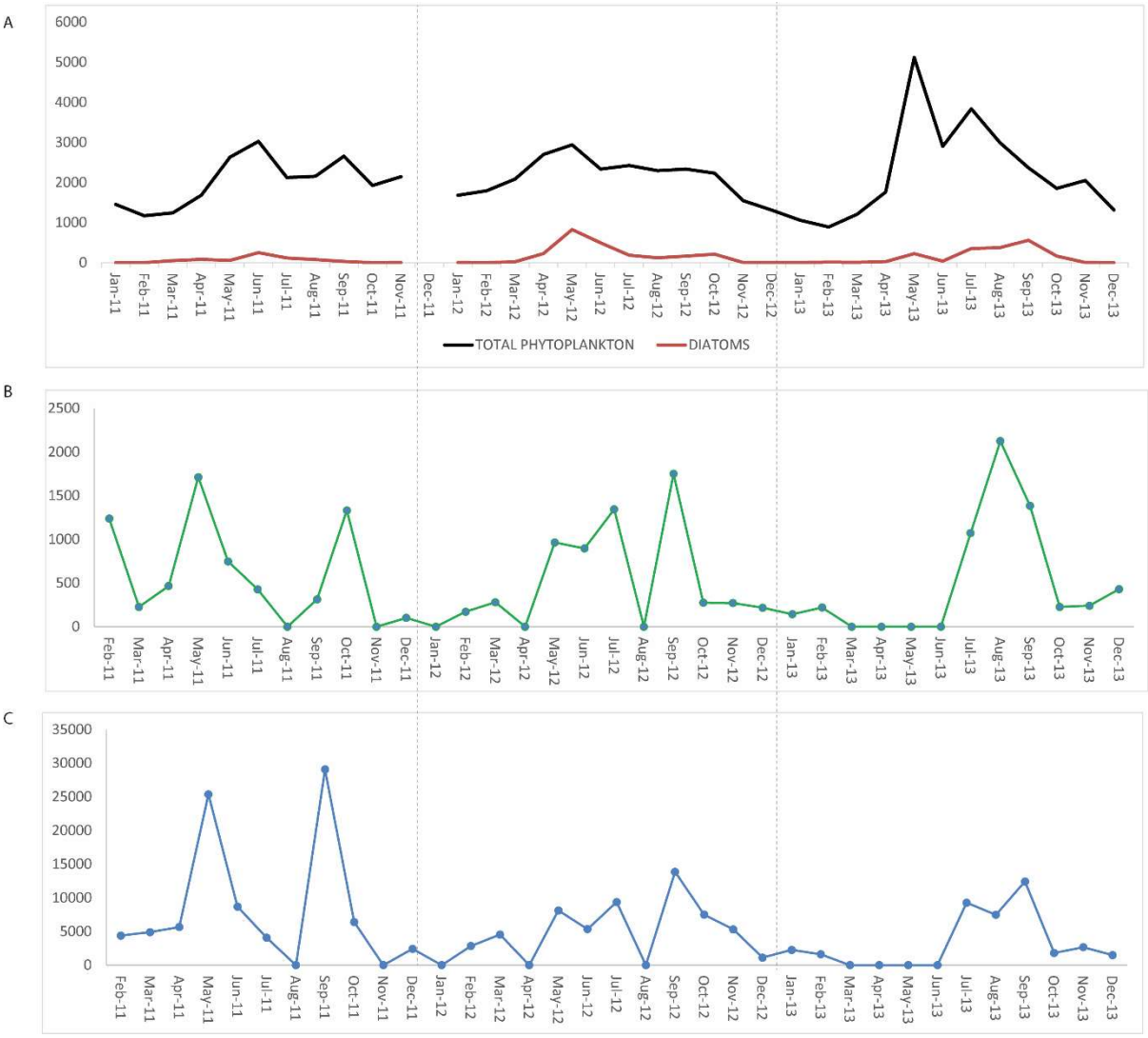

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**Figure 4:** Monthly averaged seasonal cell abundance of Phytoplankton microscopic cell counts (cells/ml) from WCO L4 versus flow cytometric measurements of aggregated photosynthetic Nanoeukaryotes that are 10 microns or less (B) and Picoeukaryotes that are 3 microns or less (C) from the WaMS 2011-2013 (cells/ml)

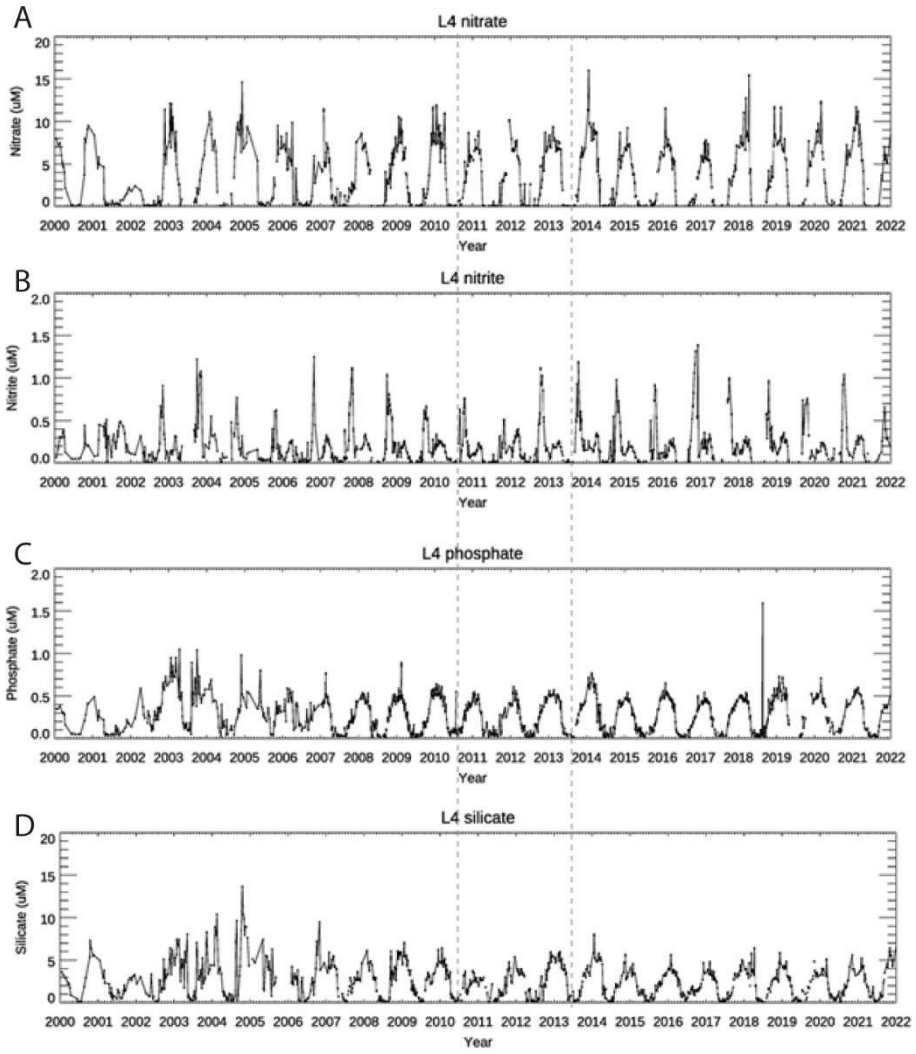


**Figure 5**: Weekly measurements of Nitrate (A), Nitrite (B), Phosphate (C) and Silica concentrations from WCO L4 station from 2000-2022. Dotted lines show 2011-2013 corresponding to this study period.

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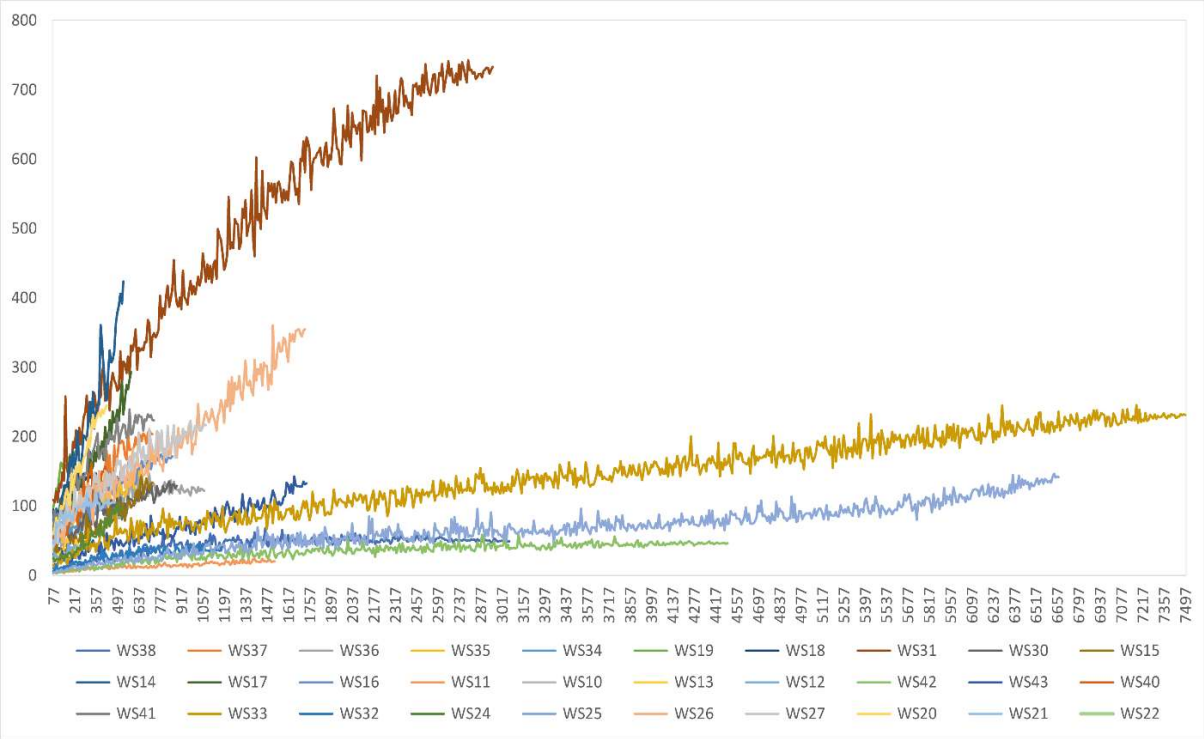


**Figure 6**: Chao1 indices of alpha diversity of sequence reads WS1-WS42 corresponding to WaMS samples from 2011-2012. Sequence reads are shown on vertical axis and Chao1 diversity values on the horizontal axis.

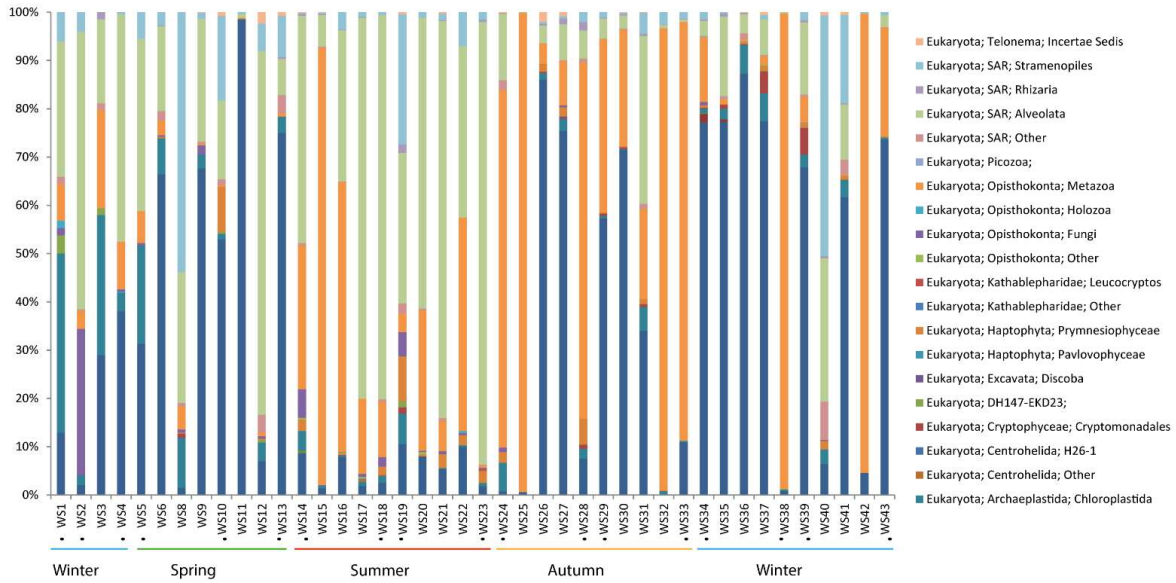


**Figure 7:** Taxa summary chart of WaMS samples from 2011-2012 partial 18S rDNA sequencing reads at level L3. Dots next to each sample indicate coastal samples. Coloured lines indicates season the sample was taken

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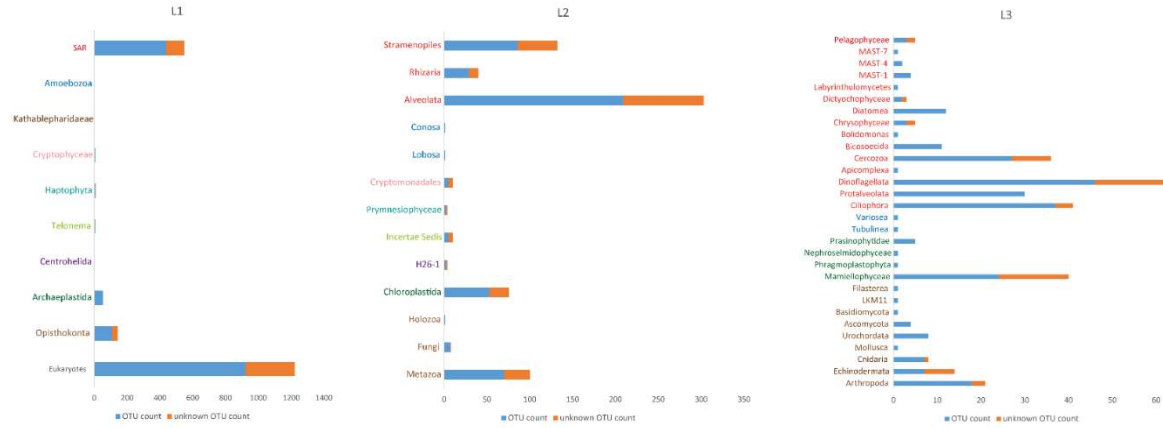
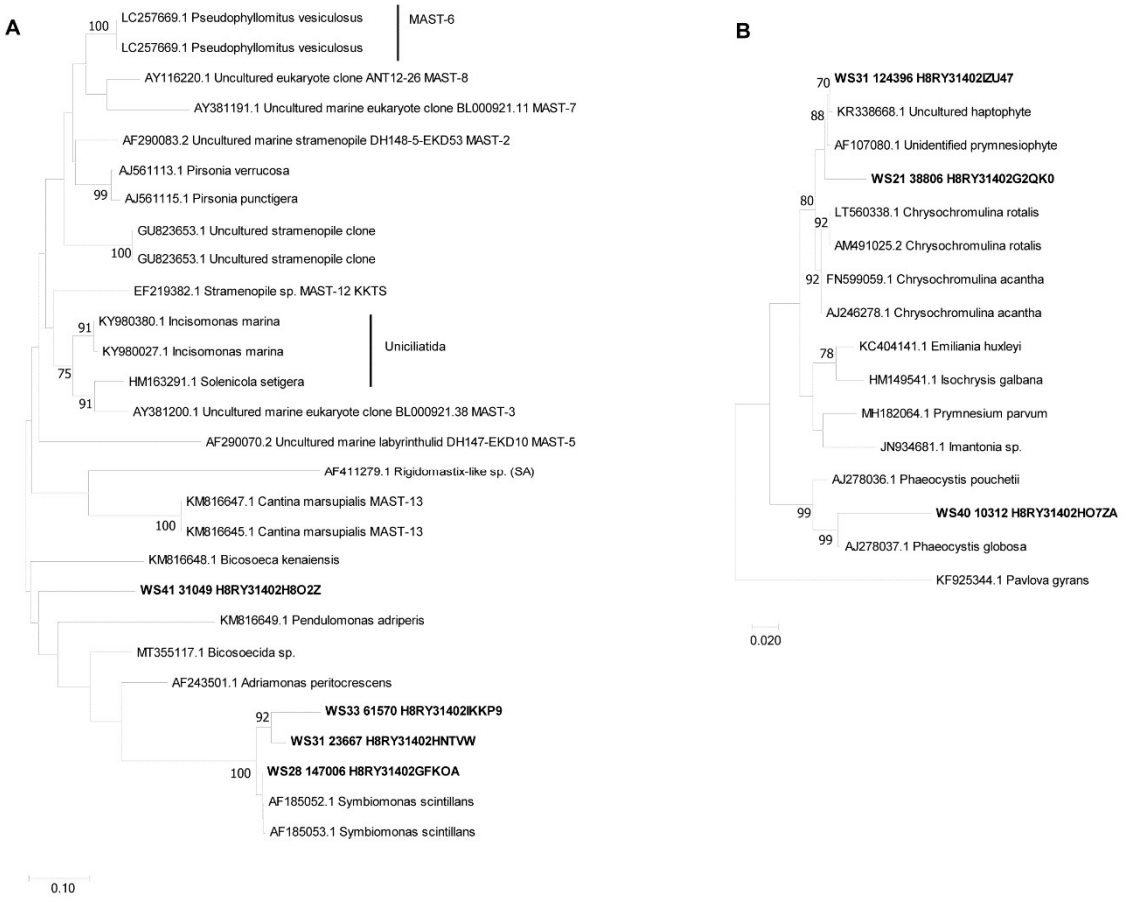



**Figure 8**: RDP classified OTU with an 80% or more confidence score at three levels L1 (Kingdom), L2 (Phyla), L3 (Phyla to class level). Blue bars are number of OTUs within taxa categories and orange bars show OTUs that have not been further classified. Horizontal axis shows number of OTUs. Type colours of taxa relate to the kingdom such as red for taxa belonging to SAR, brown for Opisthokonta, Green for Archaeplastida and blue for Amoebozoa.



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C

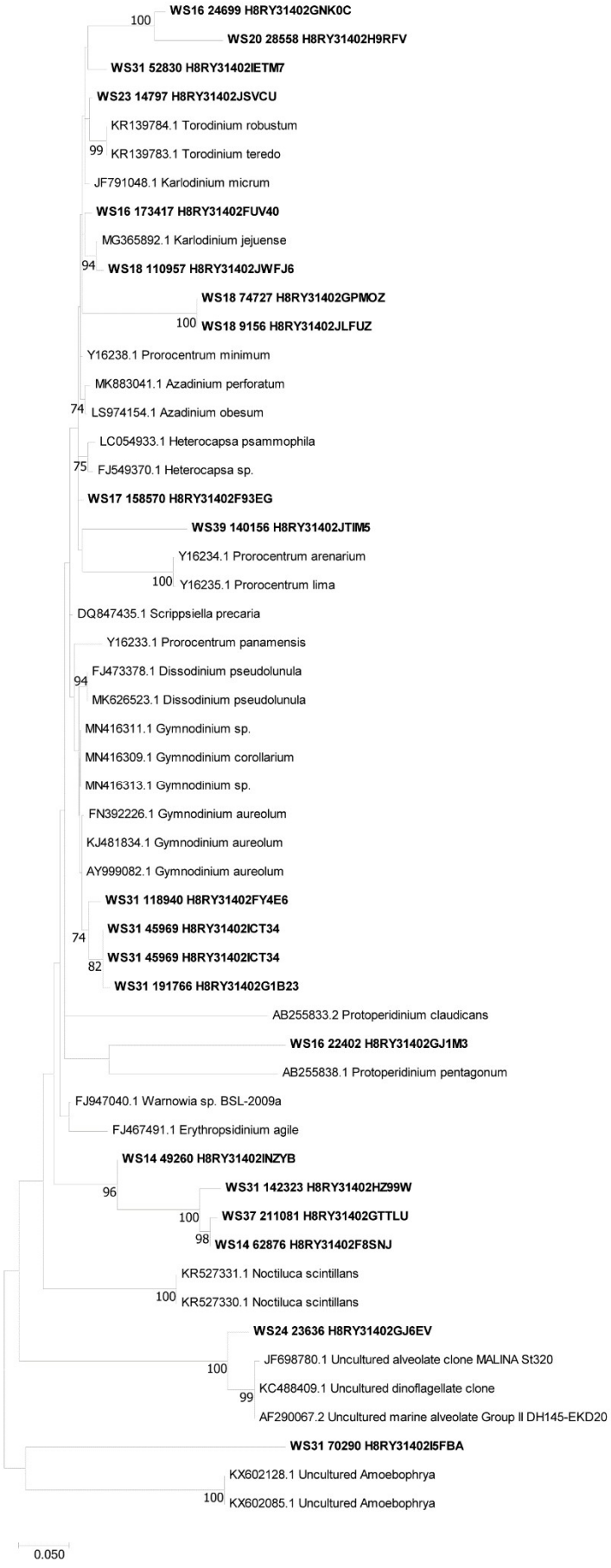

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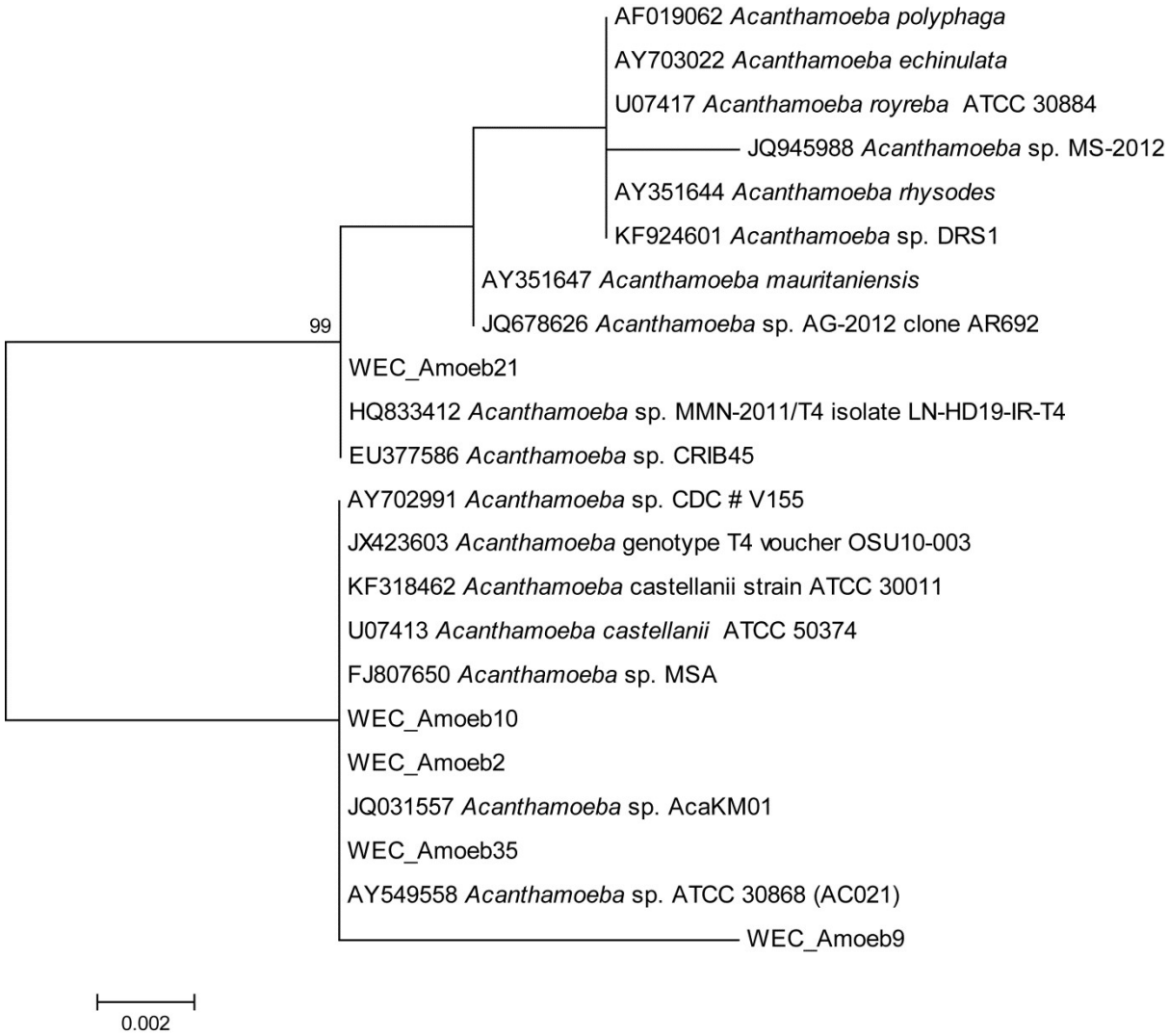
**Figure 9.** Maximum Likelihood phylogeny of OTUs belonging to MAST group (A), haptophytes (B) and dinoflagellates (C). Bootstrap support of 75 are shown by branches. Bold type indicates OTUs from this study.



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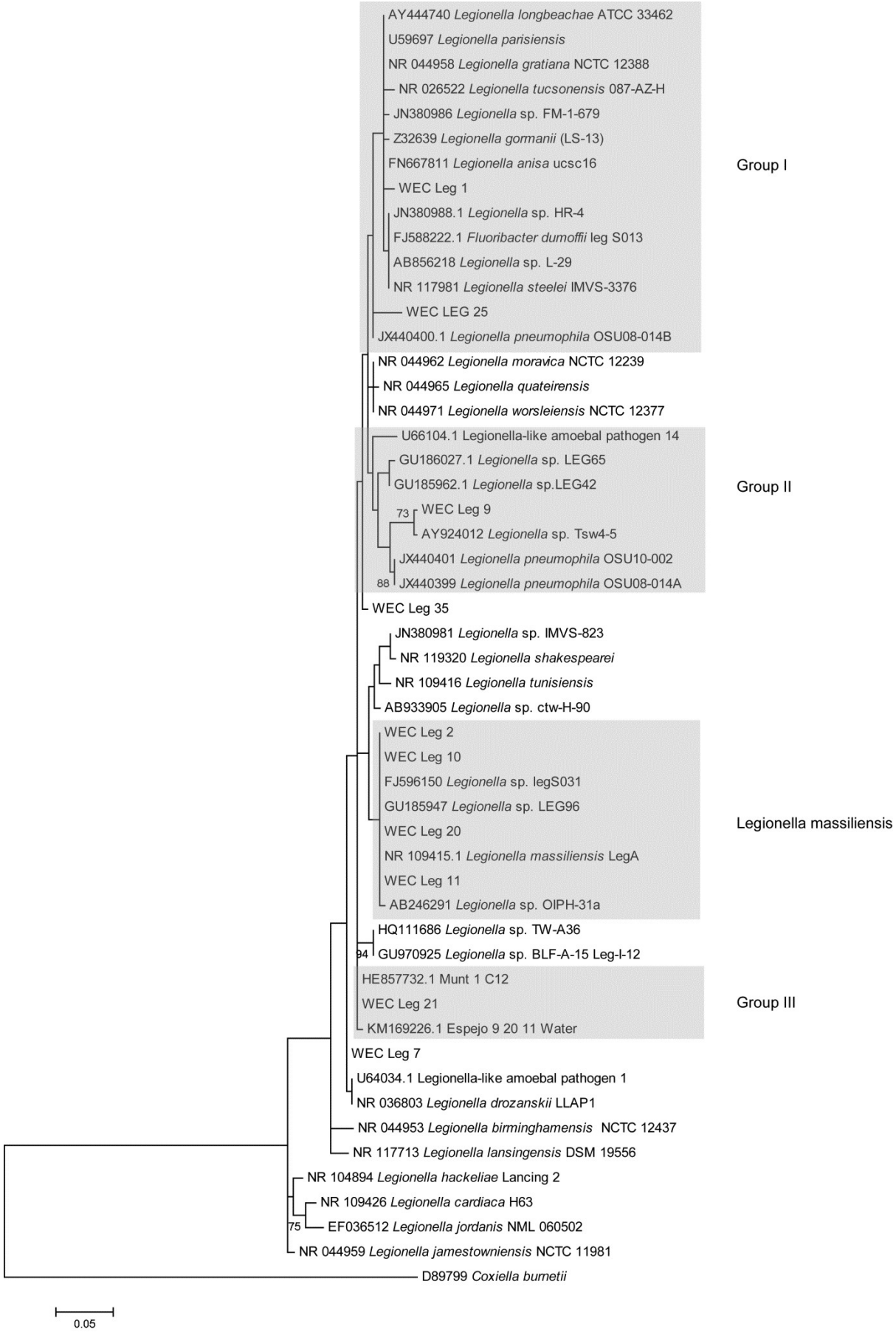

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**Figure 10**. Maximum likelihood phylogenetic analysis of Amoeba (A) and Legionella (B) found in 10 WaMS samples from 2011-2012. Bootstrap values about 70 are indicated by clades. Environmental sequences identified in this studies are prefixed with WEC.

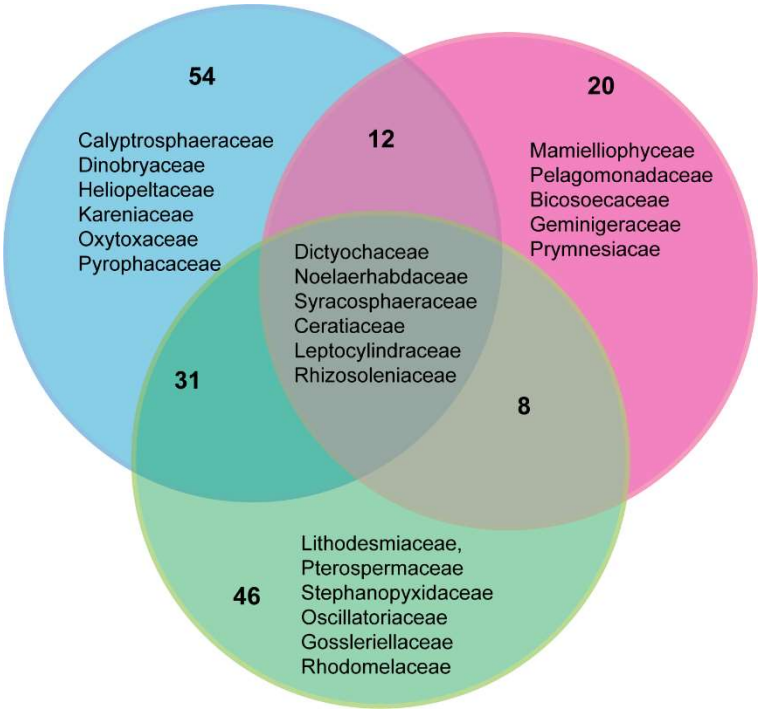


**Figure 11**: Venn-diagram showing the overlap in taxa observed in the English Channel in 2011-2012 by WaMS (pink), CPR microscopy (Green) and L4 microscopy (Blue). Examples of taxa observed in each sector are also listed but not exhaustive.

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**Tables**

**Table 1:** Summary of Samples taken in the English Channel from 2011-2013 using the WaMS deployed within the CPR sampling platform and tests carried out on them. All CPR tow samples will have microscopic plankton semi-quantitative abundance. Empty positions indicate no samples were available to analyse. Positions E1-E5 denote sample positions taken progressively from Roscoff, France to Plymouth, UK. Blue type, sample positions not collected so inferred from previous deployments. . Pn: Position, Y: Year, M: month, Lat: Latitude, Lon: Longitude, In situ T: In situ temperature, FC: Flow cytometry. AA: *A. anophagefferens*, PD: *P.delicatissima*, PF *P.fraudulenta*, PM: *P.multiseries*

| | | | | | | | | | QPCR test | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **CPR tow** | **Pn** | **Y** | **M** | **Lat** | **Lon** | ***In situ* T.** | **FC** | **Sequence sample name** | ***AA*** | ***PD*** | ***PF*** | ***PM*** |
| 344PR | E1 | 2011 | 2 | 48.03 | -3.83 | 9.56 | Yes | WS1 | Yes | Yes | Yes | Yes |
| | | 2011 | 2 | | | | | | | | | |
| | | 2011 | 2 | | | | | | | | | |
| | | 2011 | 2 | | | | | | | | | |
| | E5 | 2011 | 2 | 49.94 | -4.12 | 9.31 | Yes | WS2 | Yes | Yes | Yes | Yes |
| 345PR | | 2011 | 3 | | | | | | | | | |
| | E2 | 2011 | 3 | 49.28 | -4.02 | 9.75 | Yes | WS3 | Yes | Yes | Yes | Yes |
| | | 2011 | 3 | | | | | | | | | |
| | | 2011 | 3 | | | | | | | | | |
| | E5 | 2011 | 3 | 49.78 | -4.12 | 9.78 | Yes | WS4 | Yes | Yes | Yes | Yes |
| 346PR | E1 | 2011 | 4 | 48.80 | -3.96 | 10.5 | Yes | WS5 | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 4 | 49.08 | -4.01 | 10.25 | Yes | WS6 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 4 | 49.37 | -4.04 | 10.08 | Yes | WS7 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 4 | 49.67 | -4.11 | 10.17 | Yes | WS8 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 4 | 49.97 | -4.17 | 10.18 | Yes | WS9 | Yes | Yes | Yes | Yes |
| 347PR | E1 | 2011 | 5 | 48.82 | -3.93 | N/A | Yes | N/A | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 5 | 49.11 | -3.98 | 11.95 | Yes | WS10 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 5 | 49.40 | -3.97 | 12.4 | Yes | WS11 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 5 | 49.68 | -4.03 | 12.56 | Yes | WS12 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 5 | 49.95 | -4.09 | 12.38 | Yes | WS13 | Yes | Yes | Yes | Yes |
| 348PR | E1 | 2011 | 6 | 48.78 | -3.96 | 13.7 | yes | WS14 | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 6 | 49.10 | -4.02 | 12.9 | yes | WS15 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 6 | 49.42 | -4.10 | 13.9 | yes | WS16 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 6 | 49.68 | -4.10 | 13.9 | yes | WS17 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 6 | 49.96 | -4.13 | 13.9 | yes | WS18 | Yes | Yes | Yes | Yes |
| 349PR | E1 | 2011 | 7 | 48.83 | -3.96 | 14.5 | yes | WS19 | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 7 | 49.16 | -4.02 | 14.4 | yes | WS20 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 7 | 49.41 | -4.04 | 15.3 | yes | WS21 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 7 | 49.70 | -4.09 | 15.7 | yes | WS22 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 7 | 49.97 | -4.13 | 15.7 | yes | WS23 | Yes | Yes | Yes | Yes |

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 350PR | | 2011 | 8 | | | | | | | | | |
| | | 2011 | 8 | | | | | | | | | |
| | | 2011 | 8 | | | | | | | | | |
| | | 2011 | 8 | | | | | | | | | |
| | | 2011 | 8 | | | | | | | | | |
| 351PR | E1 | 2011 | 9 | 48.81 | -3.93 | N/A | yes | WS24 | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 9 | 49.15 | -3.78 | N/A | yes | WS25 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 9 | 49.48 | -3.69 | N/A | yes | WS26 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 9 | 49.77 | -3.83 | N/A | yes | WS27 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 9 | 50.01 | -3.97 | N/A | yes | WS28 | Yes | Yes | Yes | Yes |
| 352PR | E1 | 2011 | 10 | 48.81 | -3.95 | 14.7 | yes | WS29 | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 10 | 49.13 | -4.00 | 14.9 | yes | WS30 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 10 | 49.39 | -4.05 | 15.0 | yes | WS31 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 10 | 49.79 | -4.08 | 14.9 | yes | WS32 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 10 | 49.93 | -4.10 | 14.7 | yes | WS33 | Yes | Yes | Yes | Yes |
| 353PR | | 2011 | 11 | | | | | | | | | |
| | | 2011 | 11 | | | | | | | | | |
| | | 2011 | 11 | | | | | | | | | |
| | | 2011 | 11 | | | | | | | | | |
| | | 2011 | 11 | | | | | | | | | |
| 354PR | E1 | 2011 | 12 | 48.79 | -3.96 | 13.0 | yes | WS34 | Yes | Yes | Yes | Yes |
| | E2 | 2011 | 12 | 49.15 | -4.01 | 12.7 | yes | WS35 | Yes | Yes | Yes | Yes |
| | E3 | 2011 | 12 | 49.41 | -4.03 | 12.5 | yes | WS36 | Yes | Yes | Yes | Yes |
| | E4 | 2011 | 12 | 49.70 | -4.06 | 12.3 | yes | WS37 | Yes | Yes | Yes | Yes |
| | E5 | 2011 | 12 | 50.00 | -4.09 | 12.0 | yes | WS38 | Yes | Yes | Yes | Yes |
| 355PR | E1 | 2012 | 2 | 48.31 | -3.95 | 10.3 | yes | WS39 | Yes | Yes | Yes | Yes |
| | E2 | 2012 | 2 | 49.12 | -4 | 10.6 | yes | WS40 | Yes | Yes | Yes | Yes |
| | E3 | 2012 | 2 | 49.44 | -4.06 | 10.3 | yes | WS41 | Yes | Yes | Yes | Yes |
| | E4 | 2012 | 2 | 49.72 | -4.12 | 10.2 | yes | WS42 | Yes | Yes | Yes | Yes |
| | E5 | 2012 | 2 | 49.95 | -4.16 | 9.7 | yes | WS43 | Yes | Yes | Yes | Yes |
| 356PR | E1 | 2012 | 3 | 48.82 | -3.96 | 10.40 | yes | | Yes | Yes | | |
| | E2 | 2012 | 3 | 49.15 | -3.98 | 10.70 | yes | | Yes | Yes | | |
| | E3 | 2012 | 3 | 49.44 | -4.06 | 10.10 | yes | | Yes | Yes | | |
| | E4 | 2012 | 3 | 49.72 | -4.12 | 10.30 | yes | | Yes | Yes | | |
| | E5 | 2012 | 3 | 49.95 | -4.16 | 10.10 | yes | | Yes | Yes | | |
| 357PR | | 2012 | 4 | | | | | | | | | |
| | | 2012 | 4 | | | | | | | | | |
| | | 2012 | 4 | | | | | | | | | |
| | | 2012 | 4 | | | | | | | | | |
| | | 2012 | 4 | | | | | | | | | |
| 358PR | E1 | 2012 | 5 | 48.81 | -3.96 | 11.9 | yes | | Yes | Yes | | |
| | E2 | 2012 | 5 | 49.25 | -4.02 | 11.6 | yes | | Yes | Yes | | |

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E3 | 2012 | 5 | 49.61 | -4.06 | 11.6 | yes | | Yes | Yes | | |
| | E4 | 2012 | 5 | 49.95 | -4.07 | 12.2 | yes | | Yes | Yes | | |
| | E5 | 2012 | 5 | 50.27 | -4.17 | 11.4 | yes | | Yes | Yes | | |
| 359PR | E1 | 2012 | 6 | 48.80 | -3.96 | 13.41 | yes | | Yes | Yes | | |
| | E2 | 2012 | 6 | 49.20 | -4.02 | 13.22 | yes | | Yes | Yes | | |
| | E3 | 2012 | 6 | 49.62 | -4.13 | 13.6 | yes | | Yes | Yes | | |
| | E4 | 2012 | 6 | 49.94 | -4.15 | 13.8 | yes | | Yes | Yes | | |
| | E5 | 2012 | 6 | 50.25 | -4.16 | 14.2 | yes | | Yes | Yes | | |
| 360PR | E1 | 2012 | 7 | 48.80 | -3.96 | 14.65 | yes | | Yes | Yes | | |
| | E2 | 2012 | 7 | 49.25 | -4.06 | 14.90 | yes | | Yes | Yes | | |
| | E3 | 2012 | 7 | 49.65 | -4.11 | 15.05 | yes | | Yes | Yes | | |
| | E4 | 2012 | 7 | 49.99 | -4.14 | 14.87 | yes | | Yes | Yes | | |
| | | 2012 | 7 | | | | | | | | | |
| 361PR | E1 | 2012 | 8 | 48.80 | -3.96 | | yes | | Yes | Yes | | |
| | E2 | 2012 | 8 | 49.26 | -4.05 | | yes | | Yes | Yes | | |
| | E3 | 2012 | 8 | 49.66 | -4.09 | | yes | | Yes | Yes | | |
| | E4 | 2012 | 8 | 50.00 | -4.13 | | yes | | Yes | Yes | | |
| | E5 | 2012 | 8 | 50.32 | -4.18 | | yes | | Yes | Yes | | |
| 362PR | | 2012 | 9 | | | | | | | | | |
| | | 2012 | 9 | | | | | | | | | |
| | | 2012 | 9 | | | | | | | | | |
| | | 2012 | 9 | | | | | | | | | |
| | | 2012 | 9 | | | | | | | | | |
| 364PR | E1 | 2012 | 10 | 48.84 | -3.97 | 14.40 | yes | | Yes | Yes | | |
| | E2 | 2012 | 10 | 49.21 | -3.99 | 14.5 | yes | | Yes | Yes | | |
| | E3 | 2012 | 10 | 49.55 | -4.02 | 14.5 | yes | | Yes | Yes | | |
| | E4 | 2012 | 10 | 49.9 | -4.13 | 14.3 | yes | | Yes | Yes | | |
| | E5 | 2012 | 10 | 50.25 | -4.15 | 14.5 | yes | | Yes | Yes | | |
| Additional | E1 | 2012 | 10 | 48.84 | -3.97 | 14.40 | yes | | Yes | Yes | | |
| | E2 | 2012 | 10 | 49.21 | -3.99 | 14.5 | yes | | Yes | Yes | | |
| | E3 | 2012 | 10 | 49.55 | -4.02 | 14.5 | yes | | Yes | Yes | | |
| | E4 | 2012 | 10 | 49.9 | -4.13 | 14.3 | yes | | Yes | Yes | | |
| | E5 | 2012 | 10 | 50.25 | -4.15 | 14.5 | yes | | Yes | Yes | | |
| 365PR | E1 | 2012 | 11 | 48.83 | -3.96 | 13 | yes | | Yes | Yes | | |
| | E2 | 2012 | 11 | 49.22 | -4.01 | 13.5 | yes | | Yes | Yes | | |
| | E3 | 2012 | 11 | 49.6 | -4.05 | 13.6 | yes | | Yes | Yes | | |
| | E4 | 2012 | 11 | 49.92 | -4.09 | 13.2 | yes | | Yes | Yes | | |
| | | 2012 | 11 | | | | yes | | | | | |
| 366PR | E1 | 2012 | 12 | 48.8 | -3.96 | 11.3 | yes | | Yes | Yes | | |
| | E2 | 2012 | 12 | 49.19 | -4.01 | 12 | yes | | Yes | Yes | | |
| | E3 | 2012 | 12 | 49.52 | -4.11 | 11.8 | yes | | Yes | Yes | | |
| | E4 | 2012 | 12 | 49.88 | -4.13 | 11.6 | yes | | Yes | Yes | | |

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E5 | 2012 | 12 | | | | yes | | | | | |
| 367PR | E1 | 2013 | 1 | 48.78 | -3.96 | | yes | | Yes | Yes | | |
| | E2 | 2013 | 1 | 49.15 | -4.02 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 1 | 49.49 | -4.05 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 1 | 49.84 | -4.01 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 1 | 50.17 | -4.12 | | yes | | Yes | Yes | | |
| 368PR | E1 | 2013 | 2 | 48.8 | -3.96 | | yes | | Yes | Yes | | |
| | E2 | 2013 | 2 | 49.13 | -4.02 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 2 | 49.4 | -4.06 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 2 | 49.7 | -4.09 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 2 | 50.05 | -4.12 | | yes | | Yes | Yes | | |
| 369PR | | 2013 | 3 | 48.78 | -3.94 | | | | | | | |
| | | 2013 | 3 | 49.15 | -3.87 | | | | | | | |
| | | 2013 | 3 | 49.51 | -3.92 | | | | | | | |
| | | 2013 | 3 | 49.79 | -4.05 | | | | | | | |
| | | 2013 | 3 | 50.1 | -4.14 | | | | | | | |
| 370PR | | 2013 | 4 | 48.78 | -3.94 | | | | | | | |
| | | 2013 | 4 | 49.15 | -3.87 | | | | | | | |
| | | 2013 | 4 | 49.51 | -3.92 | | | | | | | |
| | | 2013 | 4 | 49.79 | -4.05 | | | | | | | |
| | | 2013 | 4 | 50.1 | -4.14 | | | | | | | |
| 371PR | | 2013 | 5 | 48.78 | -3.94 | | yes | | | | | |
| | | 2013 | 5 | 49.15 | -3.87 | | yes | | | | | |
| | | 2013 | 5 | 49.51 | -3.92 | | yes | | | | | |
| | E4 | 2013 | 5 | 49.79 | -4.05 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 5 | 50.1 | -4.14 | | yes | | Yes | Yes | | |
| 372PR | E1 | 2013 | 6 | 48.78 | -3.94 | | yes | | Yes | Yes | | |
| | E2 | 2013 | 6 | 49.15 | -3.87 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 6 | 49.51 | -3.92 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 6 | 49.79 | -4.05 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 6 | 50.1 | -4.14 | | yes | | Yes | Yes | | |
| 374PR | E1 | 2013 | 7 | 48.78 | -3.94 | | yes | | Yes | Yes | | |
| | E2 | 2013 | 7 | 49.15 | -3.87 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 7 | 49.51 | -3.92 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 7 | 49.79 | -4.05 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 7 | 50.1 | -4.14 | | yes | | Yes | Yes | | |
| 375PR | E1 | 2013 | 8 | 48.78 | -3.94 | | yes | | Yes | Yes | | |
| | E2 | 2013 | 8 | 49.15 | -3.87 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 8 | 49.51 | -3.92 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 8 | 49.79 | -4.05 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 8 | 50.1 | -4.14 | | yes | | Yes | Yes | | |
| 376PR | E1 | 2013 | 9 | 48.78 | -3.94 | | yes | | Yes | Yes | | |

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E2 | 2013 | 9 | 49.15 | -3.87 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 9 | 49.51 | -3.92 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 9 | 49.79 | -4.05 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 9 | 50.1 | -4.14 | | yes | | Yes | Yes | | |
| 378PR | E1 | 2013 | 11 | 48.78 | -3.95 | | yes | | Yes | Yes | | |
| | E2 | 2013 | 11 | 49.12 | -3.98 | | yes | | Yes | Yes | | |
| | E3 | 2013 | 11 | 49.45 | -4.04 | | yes | | Yes | Yes | | |
| | E4 | 2013 | 11 | 49.78 | -4.11 | | yes | | Yes | Yes | | |
| | E5 | 2013 | 11 | 50.11 | -4.15 | | yes | | Yes | Yes | | |

**Table 2: qPCR assays developed, species, primers, years tested**

| Assay | Reference | Marker | Primers | Size of product/bp | Standard range copies/µl |
|---|---|---|---|---|---|
| Pseudo-nitzschia fraudulenta | Andre et al. (2011): PN5.8SF-HRM, QPfrauR-HRM | ITS1 | PN5.8SF-HRM 5′ CAGCGGTGGATGTCTAGGTTC-3' QPfrauR-HRM 5′ CCGCTGCTAGAGCGGTCAGAG 3′ | 225 | 3.18 x$10^8$ -3.17 x $10^6$ |
| Pseudo-nitzschia multiseries | PMulsF (this study), PN5.8SR-HRM (Andre et al. 2011) | ITS2 | PMulsF-HRM 5′ CTAGACTACTGTAGTCAAACTTAACCGGCAAC 3′ PN5.8SR-HRM 5′ GAACCTAGACATCCACCGCTG 3′ | 201 | 5.75 x $10^8$-5.75 x $10^4$ |
| Pseudo-nitzschia delicatissima | QPdelRa2F (this study), PN5.8SR-HRM (Andre et al. 2011) | ITS | QPdelRa2F GTGCAATACTTTGTTGGGTTTCG PN5.8SR-HRM 5′ GAACCTAGACATCCACCGCTG 3′ | 182 | 2.5x $10^2$ to 2.5 x $10^7$ |

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| | | | | | |
|---|---|---|---|---|---|
| Aureococcus anophageffe rens | Aa1685f, Popels et al. 2003, Euk B (Medlin et al. 1988) | 18S | Aa1685f ACCTCCGGAC TGGGGTT, EukB | | $10^2$ -$10^6$ |

Table 3: Reaction efficiency of qPCR assays in this study PD (*P. delicatissima*), AA (Aureoccocus anophagefferens) for assay run 2011,2012 and 2013. E=efficiency, M=slope, B=offset or y-intercept, R, $R^2$ values indicate fit to equation

| | **PD 2011** | **PD 2012** | **PD 2013** | **AA 2011** | **AA 2012** | **AA 2013 pt1** | **AA 2013 pt2** |
|---|---|---|---|---|---|---|---|
| R | 0.999 | 0.999 | 0.996 | 0.994 | 0.994 | 0.998 | 0.984 |
| $R^2$ | 0.985 | 0.997 | 0.992 | 0.987 | 0.9888 | 0.995 | 0.969 |
| M | -3.935 | -2.900 | -3.266 | -3.502 | -3.858 | -3.979 | -3.223 |
| B | 36.361 | 28.489 | 34.76 | 43.483 | 43.900 | 38.268 | 29.398 |
| E | 0.796 | 1.212 | 1.023 | 0.9302 | 0.8163 | 0.784 | 1.04 |

**Table 4**: OTU table count of reads from WaMS sample between 2011-2012. Y: year, M: month, SR: sequence run (run 1 was carried out first and reported in Stern et al. 2015), #: number, PDM: Phlogenetic distance whole tree mean, C1M: Chao I mean, SM: Shannon mean, +/-M: above/below mean

| **Y** | **M** | **SR** | **#/sample** | **# OTU** | **+/- M** | **Above/below median** | **PDM** | **C1M** | **SM** |
|---|---|---|---|---|---|---|---|---|---|
| 2011 | 2 | 1 | WS1 | 132 | -1035.452 | -560 | 4.867548 | 36.0 | 24.100 |
| 2011 | 2 | 1 | WS2 | 346 | -821.452 | -346 | 1.6509 | 22.0 | 9.300 |
| 2011 | 3 | 1 | WS3 | 69 | -1098.452 | -623 | 4.633776 | 93.1 | 24.500 |
| 2011 | 3 | 1 | WS4 | 263 | -904.452 | -429 | 1.586147 | 9.5 | 7.200 |
| 2011 | 4 | 1 | WS5 | 182 | -985.452 | -510 | 2.120118 | 39.8 | 18.600 |
| 2011 | 4 | 1 | WS6 | 655 | -512.452 | -37 | 3.218702 | 29.3 | 14.900 |
| 2011 | 4 | 1 | WS8 | 930 | -237.452 | 238 | 1.08946 | 15.7 | 9.500 |
| 2011 | 4 | 1 | WS9 | 924 | -243.452 | 232 | 0.78551 | 9.9 | 6.400 |
| 2011 | 5 | 1 | WS10 | 257 | -910.452 | -435 | 0.68059 | 22.0 | 12.400 |
| 2011 | 5 | 1 | WS11 | 1535 | 367.548 | 843 | 0.154481 | 2.7 | 2.500 |
| 2011 | 5 | 1 | WS12 | 386 | -781.452 | -306 | 1.175051 | 23.2 | 14.100 |
| 2011 | 5 | 1 | WS13 | 268 | -899.452 | -424 | 2.10681 | 27.6 | 15.100 |
| 2011 | 6 | 2 | WS14 | 544 | -623.452 | -148 | 1.46735 | 62.5 | 23.900 |
| 2011 | 6 | 2 | WS15 | 729 | -438.452 | 37 | 1.561149 | 23.6 | 13.600 |

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| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 2011 | 6 | 2 | WS16 | 861 | -306.452 | 169 | 2.271352 | 56.7 | 25.300 |
| 2011 | 6 | 2 | WS17 | 592 | -575.452 | -100 | 2.49747 | 43.4 | 20.900 |
| 2011 | 6 | 2 | WS18 | 358 | -809.452 | -334 | 3.344375 | 74.0 | 25.200 |
| 2011 | 7 | 2 | WS19 | 237 | -930.452 | -455 | 3.779641 | 105.0 | 35.700 |
| 2011 | 7 | 2 | WS20 | 427 | -740.452 | -265 | 1.589128 | 32.9 | 27.300 |
| 2011 | 7 | 2 | WS21 | 484 | -683.452 | -208 | 1.889013 | 48.0 | 17.100 |
| 2011 | 7 | 2 | WS22 | 242 | -925.452 | -450 | 2.368124 | 57.5 | 18.400 |
| 2011 | 7 | 2 | WS23 | 445 | -722.452 | -247 | 0.71281 | 38.0 | 15.000 |
| 2011 | 9 | 2 | WS24 | 540 | -627.452 | -152 | 0.650258 | 22.1 | 12.500 |
| 2011 | 9 | 2 | WS25 | 6674 | 5506.548 | 5982 | 0.319075 | 4.5 | 2.900 |
| 2011 | 9 | 2 | WS26 | 1734 | 566.548 | 1042 | 1.622526 | 29.2 | 11.400 |
| 2011 | 9 | 2 | WS27 | 1080 | -87.452 | 388 | 1.786829 | 32.9 | 14.200 |
| 2011 | 9 | 2 | WS28 | 240 | -927.452 | -452 | 3.088336 | 79.0 | 24.000 |
| 2011 | 10 | 2 | WS29 | 928 | -239.452 | 236 | 2.057396 | 39.3 | 16.000 |
| 2011 | 10 | 2 | WS30 | 893 | -274.452 | 201 | 0.256501 | 36.7 | 12.100 |
| 2011 | 10 | 2 | WS31 | 2966 | 1798.548 | 2274 | 2.601002 | 89.1 | 31.000 |
| 2011 | 10 | 2 | WS32 | 1175 | 7.548 | 483 | 0.316267 | 7.1 | 4.300 |
| 2011 | 10 | 2 | WS33 | 7500 | 6332.548 | 6808 | 0.431583 | 12.9 | 6.500 |
| 2011 | 12 | 2 | WS34 | 451 | -716.452 | -241 | 0.772125 | 26.0 | 11.700 |
| 2011 | 12 | 2 | WS35 | 766 | -401.452 | 74 | 1.965868 | 51.0 | 13.100 |
| 2011 | 12 | 2 | WS36 | 1072 | -95.452 | 380 | 1.2185 | 20.4 | 9.800 |
| 2011 | 12 | 2 | WS37 | 736 | -431.452 | 44 | 2.471535 | 25.9 | 13.400 |
| 2011 | 12 | 2 | WS38 | 3072 | 1904.548 | 2380 | 0.046794 | 4.0 | 3.200 |
| 2012 | 2 | 2 | WS39 | 902 | -265.452 | 210 | 2.747683 | 83.8 | 18.600 |
| 2012 | 2 | 2 | WS40 | 455 | -712.452 | -237 | 0.916246 | 23.0 | 13.500 |
| 2012 | 2 | 2 | WS41 | 738 | -429.452 | 46 | 1.699598 | 66.8 | 19.200 |
| 2012 | 2 | 2 | WS42 | 4505 | 3337.548 | 3813 | 0 | 4.4 | 3.500 |
| 2012 | 2 | 2 | WS43 | 1740 | 572.548 | 1048 | 0.67496 | 10.8 | 7.200 |
| | | | **Total** | **49033** | | | | | |

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