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Evaluation of the MIDTAL microarray chip for monitoring toxic microalgae in the Orkney Islands, U.K.

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Abstract Harmful or nuisance algal blooms can cause economic damage to fisheries and tourism. Additionally, toxins produced by harmful algae and ingested via contaminated shellfish can be potentially fatal to humans. The seas around the Orkney Islands, UK currently hold a number of toxic algal species which cause shellfishery closures in most years. Extensive and costly monitoring programs are carried out to detect harmful microalgae before they reach action levels. However, the ability to distinguish between toxic and non-toxic strains of some algae is not possible using these methods. The microarrays for the detection of toxic algae (MIDTAL) microarray contains rRNA probes for toxic algal species/strains which have been adapted and optimized for microarray use. In order to investigate the use of the chip for monitoring in the Orkney Islands, samples were collected between 2009 and 2011 from Brings Deep, Scapa Flow, Orkney Islands, UK; RNA was extracted and hybridized with generation 2 and 3.1 of the chip. The data were then compared to cell counts performed under light microscopy and in the case of *Alexandrium tamarense* to qPCR data targeting the saxitoxin gene and the LSU-rRNA gene. A good agreement between cell numbers and microarray signal was found for *A. tamarense*, *Pseudo-nitzschia* sp., *Dinophysis* sp. ($r<0.5$, for all) in addition to this there the chip successfully detected a large bloom of *Karenia mikimotoi* ($r<0.70$) in August and September 2011. Overall, there was good improvement in probe signal between generation 2 and generation 3.1 of the chip with much less variability and more consistent results and better correlation between the probes. The chip performed well for *A. tamarense* group I signal to cell numbers in calibrations ($r>0.9$). However, in field samples, this correlation was slightly lower suggesting interactions between all species in the sample may affect signal. Overall, the chip showed it could identify the presence of target species in field samples although some work is needed to improve the quantitative nature of the chip before it would be suitable for monitoring in the Orkney Islands.

Keywords MIDTAL · Microarray · Harmful algae · qPCR · Monitoring · Orkney islands · *Alexandrium tamarense* · *Karenia mikimotoi* · *Dinophysis*

Introduction

In UK waters, there are several microalgal species responsible for the production of biotoxins. Filter feeding molluscs such as oysters and mussels can accumulate these toxins within their flesh; this poses a risk to human health if they are consumed. Shellfish in the UK are routinely monitored for toxins, and the shellfishery may be subject to closure due to the detection of high concentrations in the shellfish flesh of toxins responsible for three shellfish poisoning syndromes: paralytic shellfish poisoning (PSP), diarrhoeic shellfish poisoning (DSP) and amnesic shellfish poisoning (ASP). In addition to this, the presence of certain microalgal species above trigger limits in the water column (notably, the *Alexandrium* sp., *Dinophysis* sp. and *Pseudo-nitzschia* sp.) drives the requirement for increased testing and analysis of toxins within shellfish flesh (AFBI 2006a, b; Stubbs et al. 2008; CEFAS 2011).

Scapa Flow in the Orkney Islands is a typical area where monitoring for toxic algae and their toxins is required. The waters of Scapa Flow contain a number of toxic algal

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species and the area has a number of commercial shell fisheries. Any improvement to monitoring methodologies could save both time and money in this area (Hoagland et al. 2002; Hoagland and Scatas 2006). Within the waters of Orkney, using conventional techniques to distinguish between toxic and non-toxic strains of some microalgae is difficult particularly in the case of group I and group III strains of *Alexandrium tamarense* which is not possible using light microscopy (Higman et al. 2001; Leaw et al. 2005; Lilly et al. 2007). The same applies for several species of the *Pseudo-nitzschia* genus, which has a variety of non-toxic and toxic species and strains that are very similar in appearance that can only be separated by high-resolution scanning electron microscopy or molecular techniques. During the summer months in the Orkney Islands, these *Pseudo-nitzschia* species may be prevalent (Hall and Frame 2010; Davidson and Bresnan 2009) and in the Orkney Islands shellfishery closures are required in most years either due to *Pseudo-nitzschia* or *Alexandrium* toxins being detected (Tett and Edwards 2002; Fraser et al. 2006).

Cases of PSP in Orkney and the surrounding waters have generally been linked inferentially to *A. tamarense* since this species was generally believed to be the main toxic *Alexandrium* present in Scottish coastal waters (Wyatt and Saborido-Rey 1993; Collins et al. 2009; Touzet et al. 2010). However, both the PSP toxin producers *Alexandrium minutum* and *Alexandrium ostenfeldii* are also widespread in Orkney waters where increased PSP toxicity of mussels has coincided with increasing *Alexandrium* abundance (Brown et al. 2001; Töbe et al. 2001). This increases the need to be able to distinguish these species quickly and accurately.

Large microalgal blooms can also cause damage to fish farm stocks (Glibert et al. 2001). In this regard, in the UK, *Karenia mikimotoi* is of concern. A variety of haemolytic compounds have been found to occur during *K. mikimotoi* blooms, causing extensive damage to gill epithelia in finfish and death in filter feeding benthic fauna (Yamasaki et al. 2004). Also, toxin extracts from *K. mikimotoi* cultures have been shown to have a negative impact on the growth and development of mammalian cells (Chen et al. 2011), suggesting that their ingestion might negatively impact humans if ingested in large numbers. Currently there is no threshold limit for *K. mikimotoi* numbers in the UK as it is not certain if the toxins are toxic to humans, certainly it is a species that needs further study (Brand et al. 2012). In September of 1980 and 1981, *K. mikimotoi* blooms were observed at fronts between inshore mixed waters and offshore stratified waters along the coast of Scotland (Gowen 1987), which killed large numbers of fish (Jones et al. 1982; Davidson et al. 2009a, b). Davidson et al. (2009a, b) reported blooms of *K. mikimotoi* in the Orkney Islands in 1999 as well as the Orkney and Shetland Islands in 2003. In 2006, a large bloom was observed in northern Scotland, which was transported along the west coast to the east. This bloom killed large numbers of benthic invertebrates and some fish (Davidson et al. 2009a, b). *K.mikimotoi* bloom dynamics in the Orkney Islands are not well characterised, and being able detect potential blooms before they reach problematic numbers would aid management of coastal resources.

In common with many countries, the methodology used in the UK to monitor microalgal cells in the water column employs light microscopy, which is time consuming and costly. Policy states that the threshold level for shellfishery closure is the mere presence of *Alexandrium* in the water column (AFBI 2006a, b; CEFAS 2011). Being able to distinguish between toxic and non-toxic strains would therefore save money each year, avoiding unnecessary shellfishery closure and toxin testing. Molecular techniques can provide a tool for preliminary detection of harmful microalgae before toxins pass a safety threshold level, as well distinguishing between toxic and non-toxic strains (Ebenezer et al. 2012). A variety of methods based on the sequencing of nucleic acids have been developed over the past decade (Karlson et al. 2010) which have considerably improved our ability to accurately identify organisms to the species or strain level.

Quantitative PCR (qPCR) has been used to quantify microalgae to genus and species/strain level for *Alexandrium* (Galluzzi et al. 2004, 2010) and *Pseudo-nitzschia* (Fitzpatrick et al. 2010; Andree et al. 2011) and monitoring using this methodology has been undertaken in some countries (Gilmartin and Silke 2009). Although less time consuming than cell counts and eliminating the difficulties of morphological identification, it only targets single species or at most approximately four species (Kamikawa et al. 2006) in one sample. Therefore, in areas such as the Orkney Islands where there is a potential for a large number of different harmful algal bloom species, qPCR is not really suited to full-scale monitoring programmes. Additionally, the combined cost in time and consumables of doing these multi-assays is not suited to a regular monitoring programme.

Microarrays are widely used in molecular biology for the processing of bulk samples for the detection of large numbers of specific target RNA or DNA sequences. Phylochips differ from most microarrays in that the probes target a range of different species. Phylochips have previously shown to be an accurate, effective and reproducible technique to monitor microalgae (Metfies and Medlin 2004, 2008; Metfies et al. 2007; Gescher et al. 2008, 2010). The European Union (EU) project microarrays for the detection of toxic algae (MIDTAL) automated detection of harmful microalgae through the use of rRNA probes, targeting both the 18S and 28S rRNA genes in such a phylochip format.

The MIDTAL microarray chip was developed using existing probes adapted to a microarray format on a glass slide. Since its first development, the chip has gone through several versions (or generations), with probes being changed

in both length and choice of sequence, layout of probes being changed to reduce interference and optimisation of probes between each version. Generation 1 was the prototype chip; multiple probes (18-bp long) for 40 species were added to the chip. Generation 2 of the chip optimised probe lengths, lengthening probes from 18 to 25 bp to improve specificity, and probe sequences were refined to minimise cross reactivity. Additional probes were added to the chip (for ten species in total) and probes that did not work well were removed or replaced. Between generation 2 and 3, probe sequences were further refined, and several probes were removed due either to the species not being known toxin producers or multiple cross reactivity for those probes. Generation 3.1 extended the attachment of the probes to the microarray slide to improve accessibility by target molecules and developed the microarray methodology to improve stringency and signal strength. The final generation (3.3) includes some of the 163 probes (see Supplementary Table 1) at various taxonomic hierarchies covering all the major harmful algal species of current interest in the EU. Preliminary results of generation 2 of the chip have already shown a good general agreement between microarray signal and cell counts in the Orkney Islands (Taylor et al. 2013). The aim of this study was to compare seasonal data from generations 2 and 3.1 of the MIDTAL microarray chip against current UK cell counting methodologies and assess the effectiveness of the chip for monitoring microalgae in the Orkney Islands. In order to achieve this, data from the microarray chip were compared to cell counts, independent DNA qPCR methodologies for *A. tamarense*. In addition to this, improvements between generation 2 and 3.1 microarray chips were assessed. Microarray data and cell counts for *A. tamarense* were linked to toxins by comparing positive results in microarray chip to positive results for PSP in the MIDTAL toxin array. The potential for the chip to show quantitative data was also investigated. Each partner in the EU project had a target species to produce calibration curves for on the chip with cultures of the target species, data are presented here for *A. tamarense*. Calibration curves were determined for RNA extracted from *A. tamarense* group I and *A. tamarense* group III run on generation 2 and generation 3.1 of the chip and matched the cell numbers to the microarray signal.

Methodology

Calculating average RNA per cell for *A. tamarense*

Three different isolates of *A. tamarense* group I UoW 717, UoW 718, UoW 719 from the Orkney Islands and *A. tamarense* group III UoW 700, UoW 702, VG0927 from Weymouth harbour were cultured in triplicate in f2 media (Guillard and Ryther 1962) under a variety of conditions. These were varying light intensities: 26 μmol photons per square metre per second, 160 μmol photons square metre per second, 430 μmol photons square metre per second; varying nutrient conditions; (normal f/2, f/2- N, f/2-P); varying temperature (12 °C, 16 °C, 19 °C) and finally salinities (28, 33, and 38 ppt). Samples were taken daily for cell counts (1.2 mL preserved in Lugol's solution) and for RNA extraction (10 mL). Cell counts were performed using a Sedgewick Rafter counting chamber. From this dataset, an average RNA concentration per cell for *A. tamarense* group I and group III was calculated. This was used to relate the RNA amount added to the chip directly to cell numbers.

Differing amounts of RNA extracted (see below) from *A. tamarense* group I and group III were run on the generation 2 version of the MIDTAL microarray chip (1, 5, 25 and 100 ng) and from that a normalised microarray signal for each RNA amount was calculated. In addition to this, calibration curves were checked with the generation 3 version of the chip by hybridising 25 and 100 ng of *A. tamarense* group III and group I RNA on the chip. However, in these calibrations, 10 ng of *Dunaliella tertiolecta* RNA was also added to the chip in order to normalise to *Dunaliella*. This allows for normalisation to the *Dunaliella* probes, which act as internal controls for extraction efficiency and are more suited to samples where extraction efficiency may be low.

Field site and sampling

Monthly seawater samples were taken from Scapa Flow, Orkney (58°54'23.46" N, 3°13'27.72" W) from October 2009 to October 2011 (Fig. 1). Water samples were taken using an integrated tube sampler of 3-m depth. A well-mixed measured volume (1 L) was first pre-filtered through a mesh of 80 μm to remove large particles and then vacuum-filtered through nitrocellulose filters (Whatman, UK) with a pore size of 3 μm. Triplicate filters were then immediately submerged into 1 mL of Tri-Reagent (Ambion, UK) within a cryovial tube and the material stored at −20 °C before being shipped in a dry shipper to the University of Westminster where on arrival they were stored at −80 °C. Six additional nitrocellulose filters were also taken for DNA extraction and also toxin analysis and stored in the same way as the filters for RNA extraction. In addition, 200 mL of seawater was taken for microscope cell counts and preserved with acidic Lugol's iodine (Engell-Sørensen et al. 2009).

Cell counting in field samples

Cell counts were carried out using the Utermöhl (1931) method as used by monitoring agencies in the UK (AFBI 2006a, b; CEFAS 2011), to compare existing sampling

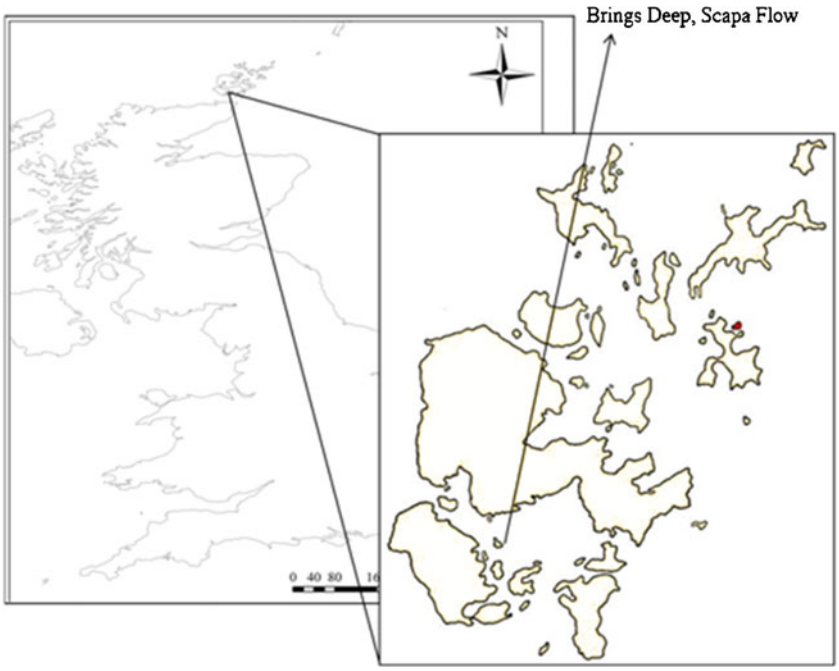

Fig. 1 Map of the Orkneys Islands, UK showing the Brings Deep sampling station (58° 54'23.46" N, 3°13'27.72" W)

methods for the Orkney Islands with the microarray method. Acidic Lugol's preserved seawater collected at the time of sampling was sedimented in Utermöhl settling chambers (20 mL). The chamber was viewed under an inverted microscope and species/genus or class identification and counts performed for the whole area for species of low abundance, or average of ten fields of view taken for species of high abundance (greater than 50 cells per field of view). Counts were performed in duplicate. In the case of *A. tamarense*, counts were assumed to be either group I or group III as identification further than this under light microscopy is not possible. *Pseudo-nitzschia* cells were counted based on size class width >5 or <5 μm. Current trigger levels to stimulate extensive toxin testing in shell fisheries or further water sampling are for *Alexandrium* sp.; its presence in counts, depending on the counting methodology this equates to 50 cells L^{-1}, for 20-mL counts, and 20 cells per litre for 50-mL counts, for *Pseudo-nitzschia* sp. its 50,000 cells per litre and *Dinophysis* sp. 100 cells per litre (AFBI 2006b).

RNA extraction of cultures and field samples

Before extraction from field samples, an aliquot of *Dunaliella tertiolecta* (5×10^5 cells) was added to each of the three filters submerged in tri-reagent as an internal control for the RNA extraction process. Glass beads (0.5 g, 100–300 μm) were also added to each of the tubes. The tubes were then transferred to a mini-bead beater (BioSpec, USA) the samples were then bead-beated (60 s at 4,800 oscillations per minute) in the Tri-reagent (Ambion, UK) to lyse the cells. For extraction from cultures, culture (10 mL) was centrifuged (2,500×*g*, 10 min) and then the supernatant was removed, the resultant pellet was resuspended in Tri-reagent (1 mL) and bead-beated as above.

After bead beating samples were heated at 60 °C for 10 min. Then 1-bromo-3-chloro-propane (0.1 mL) was added, and the mixture was added to phase lock columns (5Prime, USA) and centrifuged (12,000×*g*, 15 min) to separate organic and aqueous phases. The aqueous phase was removed, and the RNA was precipitated in 2-propanol (Sigma, UK) (−20 °C), followed by a wash with 75 % ethanol. After drying, the pellet was suspended in DEPC-treated water (50 μL, Ambion, UK) and was stored at −80 °C.

Microarray hybridisation

Protocols were carried out as detailed in Kegel et al. (2012). The RNA was labelled using a Platinum Bright 647 Infrared Nucleic Acid kit (Kreatech, USA) and fragmented in a salt buffer (Gescher et al. 2010). The epoxysilane-coated microarray chips were first pre-activated. Generation 2 chips were incubated at 60 °C for 1 h in a pre-hybridisation buffer (BSA 1 mg/mL in SST buffer) and then washed with distilled water and dried by centrifugation 3 min, 900 rpm. Generation 3.1 chips were incubated in a blocking buffer (patent pending) for 20 min at 50 °C, these were then incubated in ddH_20 for 10 min at 50 °C and washed again at room temperature for 15 min and dried by centrifugation, 3 min, 900 rpm. Hybridisation was 60 °C for generation 2 slides and 65 °C for generation 3.1 for 1 h. Un-hybridised RNA was removed from the chip surface using three washing steps with increasing buffer stringency. The slide was first washed in a low stringency buffer (2× SSC, 10 mM

EDTA, 0.05 % SDS), then a second, more stringent buffer (0.5× SSC.10 mM EDTA) was applied. Both of these washes were performed at room temperature. Finally a third most stringent wash (0.2× SSC/10 mM EDTA) was performed at 45 °C for generation 2 and 50 °C for generation 3.1 to minimise background noise and unspecific binding to the probes. The chip was scanned (GenePix 4000B, Axon, Inc.) with a resolution of 5 μm and an excitation wavelength of 635 nm. The scanned images were then analysed with GenePix analyser software (Axon, Inc., USA). The total fluorescence signal intensity from each probe was calculated by measuring the pixel intensity in the defined area for that probe minus the background fluorescence, which is calculated as an average fluorescence around the spots on the ray. Each microarray slide contained two arrays with samples being run in duplicate on separate slides. Each array contained eight spots for each probe. Therefore, for each probe, a mean value was calculated for the 16 spots specific for that probe over the two arrays. Data were normalised to an internal positive control. The internal control was 16 probes on the chip specific for a TATA box. This internal control was added to the hybridisation mixture prior to the hybridisation. The control probes target was a PCR amplicon produced by PCR amplification from a DNA extract of *Saccharomyces cerevisiae* using the primers TBP-F (5′ TTTTCAGATCTAACCTGCACCC 3′) and TBP-R-CY5 (5′ ATGGCCGATGAGGAACGTTTAA 3′). The mean signal intensities for each other class, genus, species or strain probe were divided by the mean value of the 16 control probes in order to normalise between samples for hybridisation efficiency.

The cut-off for probes deemed to be a positive hit for was a normalised signal >0.2, and in samples hybridised using version 3.1, it was a signal to noise ratio >2 due to less internal positive control (10 ng) being added to the generation 3.1 hybridisations than in generation 2 (50 ng). Year 1 (2010) samples were carried out using the second generation chip and year 2 (2011) samples with the third generation chip.

DNA extraction

The three filters for DNA extraction were transferred to tubes containing 0.5 g of glass beads. The DNA was extracted using an Invisorb DNA plant extraction kit (Invisorb, UK), where 1 mL of lysis buffer from the kit was added to the beads and filter and tubes were bead-beated (60 s at 4,800 oscillations per min). The remaining protocol was carried out in accordance with the manufacturer's instructions, and the DNA was re-suspended in 100 μL Invisorb elution buffer. The DNA concentrations were determined by Nanodrop (Thermo, UK).

Quantification of *A. tamarense* and the saxitoxin gene by qPCR

qPCR targeted both the LSU region and the saxitoxin gene for quantification of *A. tamarense* group I as an independent method of validating toxin concentration data as well. The LSU region of the rRNA gene shows significant differences between groups I and III, and the primers designed by Erdner et al. (2010) amplify only group I. qPCR was carried out in the spring and summer month samples, where *A. tamarense* had been identified in cell counts or the microarray thus providing an independent method of validating the microarray data. All qPCR reactions were carried out on a Qiagen Rotor Gene 3000 using Qiagen SYBR green QPCR kit (Qiagen, UK). All qPCR assays were performed in a final volume of 25 μL consisting of 12.5 μL Rotor Gene–SYBR Green Master Mix (Qiagen, UK) 1 μL of template DNA (~50 ng), 1 μL of each primer (0.5 μM final conc.) and 9.5 μL DEPC-treated water (Ambion, UK) all assays were performed in triplicate. Melt curve analysis was performed at the end of each cycle to confirm amplification specificity.

The primers designed to amplify the saxitoxin gene targeted a region of the genome specific for the saxitoxin *sxtA4* gene were the forward primer sxtA4F 5′-CTGAGCAAGGCGTTCAATTC-3′ and reverse primer sxtA4R 5′-TACAGATMGGCCCTGTGARC-3′, resulting in a 125-bp product (Murray et al. 2011). An initial denaturisation step was performed at 95 °C for 10 s, and then 35 replicates of 95 °C for 15 s and 60 °C for 30 s. Standards were prepared by PCR amplification using DNA extracted from an isolate of *A. tamarense* group I. The reaction contained a final volume of 50 μL consisting of 12.5 μL MYTAQ (Bioline) 1 μL of template DNA (~50 ng), 2 μL of each primer (listed above) (0.5 μM final concentration) and 19 μL DEPC-treated water (Ambion, UK). The product was then subjected to the following conditions in a thermocycler: an initial denaturation of 3 min at 95 °C then 30 cycles of 95 °C for 15 s and 60 °C for 30 s, 72 °C for 30 s and then a final extension step of 72 °C 3 min. The product size ~125 bp was confirmed using gel electrophoresis and was then purified using a Qiagen PCR Purification Kit (Qiagen, UK) and quantified using the NanoDrop (Thermo Scientific, UK); the copy number was calculated and dilutions of this product 10^7–10^4 were used as standards in qPCR.

For *A. tamarense* NA group I, the forward primer AlexLSUf2 (5′-GGCATTGGAATGCAAAGTGG GTGG-3′) and the reverse primer AF1 (5′-GCAAGTG CAACACTCCCACCAAGCAA-3′) (Erdner et al. 2010) were used to amplify a ~160-bp fragment of the 28S rRNA gene. A denaturation step of 95 °C for 3 min was carried out followed by 35 cycles of 95 °C for 10 s and 55 °C 30 s. Standards were produced by PCR amplification of a cloned

plasmid containing an insert of the region of the 28S rRNA gene from a group I *A. tamarense* UoW717 isolate from the Orkney Islands; the concentration of this PCR product was measured using a NanoDrop (Thermo Scientific, UK).

Toxin analysis

Toxin analysis was carried out at the Queen's University Belfast using methods for their toxin array and was confirmed using ELISA (protocols outlined in McNamee et al. 2012).

Results

Calibration of probes specific for *A. tamarense*

For the generation 2 chip, the calibration curves for *A. tamarense* group I were based on the two group I-specific probes (ATNA_D01_25, ATNA_D02_25) which have a different sequence and target different regions of the group I genome, and although *A. tamarense* group III does not have specific probes on the chip, the calibration was based on the single *A. tamarense* complex probe, although this probe will light in the presence of all members of the *A. tamarense* complex in normal samples. Both these curves were linear $R^2<0.97$ (Fig. 2).

The two probes with the highest signal for group I were the probe specific for the group I (ATNA_D02_25) and the *Alexandrium* genus (AlexG_D01_25) with the first group I probe (ATNA_D01_25) showing a greatly reduced signal (greater than 0.2 but less than 1) when compared to the second probe. Group III did not show a positive signal for group I specific probes showing no cross-reactivity (Fig. 2c). The probes with the highest signal for group III were the *A. tamarense* complex probe (AtamaS01_25_dT) and *Alexandrium* genus probe. RNA equivalent to 35 cells did produce a very weak signal (Fig. 2a) for the group I strain, but it was not deemed to be positive. The RNA equivalent to 240 cells was deemed to give a positive signal. The group III strain of *Alexandrium* failed to light up probes specific for *A. tamarense* NA group I (Fig. 2c).

Calibrations performed with the 3.1 generation of the chip showed similar results (Fig. 3). However, with normalisation to the *Dunaliella* probe, this clearly made the signal values higher by a factor of ~10. The probe signals for the group I-specific probe (ATNA_D02_25) were comparable between generations of the chip for probes normalised to POSITIVE_25 which was the internal control with TATA box-specific groups and showed similar signals ~5 for 100 ng of RNA. Signals for the *Alexandrium* genus probe and *A. tamarense* complex were much lower on the generation 3 chip than for the generation 2, although for both signals, was still deemed to be positive. Overall for the generation 3.1 chip, the group I-specific probes seemed to show higher affinity for the target RNA, whereas the genus and complex probes showed lower affinity for the target RNA.

Microarray performance against traditional methods of monitoring and qPCR

Between generation 2 and 3.1, there was an improvement in the reproducibility within the chip. Standard errors for each probe set were lower in the data analysed with the 3.1 generation of the chip than in the generation 2 chip. The background noise on the chip was improved overall between generation 2 and 3.1 with much better signal to noise ratios for the probes. However, modifications to the protocol and chip layout produced lower normalised signal values due to differences in the POSITIVE_25 signal, despite adding less positive control to the hybridisation signals, which were overall higher for the POSITIVE_25. Despite overall lower normalised signals, there was increased probe specificity and lower cross reactivity— this was reflected in the higher correlation values between cell counts.

The microscope cell counts for 2010 showed that *Pseudo-nitzschia* cell numbers were highest in the summer of July 2010 and in the spring of April, with *Pseudo-nitzschia* cells absent from February and November (Fig. 4a). *Pseudo-nitzschia* cell counts and generation 2 microarray data for genus probes showed a good agreement. However, the first *Pseudo-nitzschia* genus probe (PsnGS01_25) showed a relatively weak correlation ($r=0.42$; $p\leq0.05$) with array data, whereas the second genus probe (PsnGS02_25) showed a strong positive correlation ($r=0.71$; $p\leq0.05$). In 2011, the highest cell numbers of *Pseudo-nitzschia* were reached on the 20th of June, August and September (Fig. 4b). The genus probe PsnGS01_25 showed no significant correlation with cell numbers. Whereas the genus probe PsnGS02_25 showed a strong positive correlation ($r=0.68$; $p\leq0.001$).

K.mikimotoi in both years showed increased abundance in March, whereas for the rest of the winter and spring, it was absent. However, in both years, there were increases in abundance in August and September (Fig. 5a, b). Microarray data for 2010 for *Karenia* were conflicting with higher signals for most months showing positives in most months for both *Karenia* species probes and genus probes. The microarray data for generation 2 did not show good agreement for *Karenia*, the genus probe (Kb), and was poorly correlated ($r=0.43$; $p\leq0.05$). The *K. mikimotoi* species probe (L*Kare0308A_25) also showed a weak positive correlation ($r=0.43$; $p\leq0.05$); although they showed increased signal when *K. mikimotoi* was present, it also showed a positive signal for the other summer months as

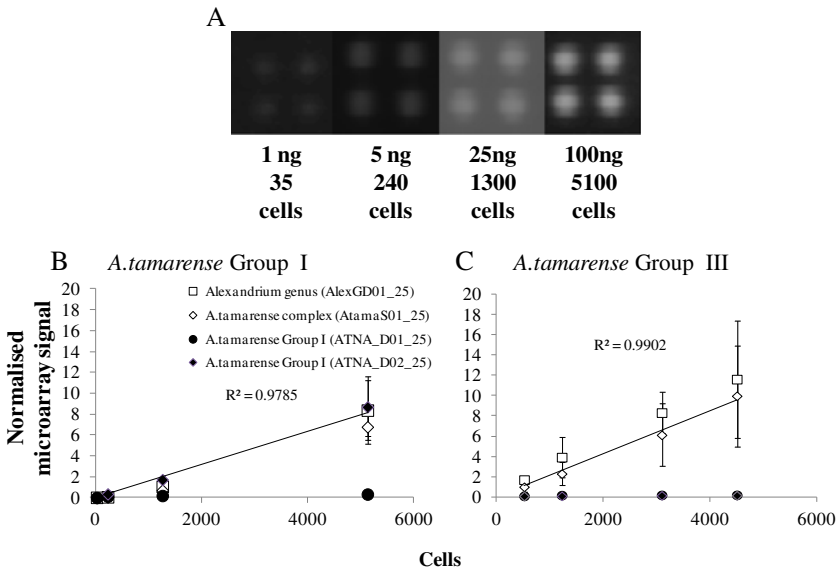

Fig. 2 **a** Microarray probes specific for *A. tamarense* group I (ATNA_D02_25) showing four replicated spots of each RNA concentration added to the chip. **b** Calibration curves for *A. tamarense* group I showing an *Alexandrium* genus probe (AlexGD01_25), *A. tamarense* complex probe (AtamaS01_25) and group I ribotype specific probes (ATNA_D01_25, ATNA_D02_25). **c** Calibration curves for *A. tamarense* NA group III *Alexandrium* genus probe (AlexGD01_25), *A. tamarense* complex probe (AtamaS01_25) and group I ribotype-specific probes (ATNA_D01_25, ATNA_D02_25)

well (Fig. 5a). In September 2011, there was bloom of *K.mikimotoi*, >100,000 cells. This was preceded in August 2011 by increased numbers of *K.mikimotoi* >10,000. When the microarray data are back calculated using the calibration curves for *Karenia*, it showed around 3,000 cells. This was confirmed by using real-time nucleic acid sequence-based amplification with internal control RNA (IC-NASBA) (Ulrich et al. 2010), which also calculated around 3,000 cells. Overall, the microarray data for the generation 3.1 version of the chip were better correlated with the cell counts (Fig. 5b) for the *Karenia* genus probe KareGD01_25_dT (r=0.70; $p \leq 0.005$), the *K. mikimotoi* species probe L*Kare0308A25_dT (r=0.85; $p \leq 0.005$) and the *K. mikimotoi* species probe (KbreD05_25_dT) (r=0.74; $p \leq 0.05$).

In 2011, there was a much better agreement between the *Dinophysis* probes and the *Dinophysis* cell numbers. The *Dinophysis* family probe (DphyF02_25_) showed a strong positive correlation (r=0.79; $p \leq 0.05$), the genus probe (DphyGS03_25) a weak correlation (r=0.39; $p \leq 0.05$) and but the genus probe (DphyGS02_25) again showed a strong

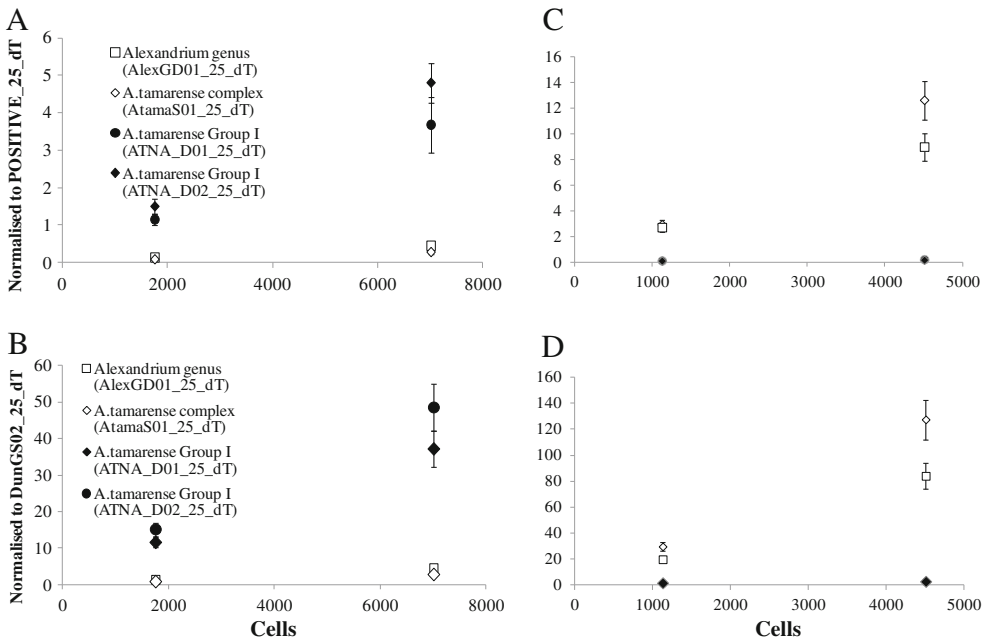

Fig. 3 Calibration curves for *A. tamarense* group I showing an *Alexandrium* genus probe (AlexGD01_25_dT), *A. tamarense* complex probe (AtamaS01_25) and group I ribotype-specific probes (ATNA_D01_25_dT, ATNA_D02_25_dT) showing normalisation to **a** POSITIVE_25_dT and **b** the *Dunaliella*-specific probe DunGS02_dT; and calibration curves for *A. tamarense* NA group III *Alexandrium* genus probe (AlexGD01_25_dT), *A. tamarense* complex probe (AtamaS01_25) and group I ribotype-specific probes (ATNA_D01_25_dT, ATNA_D02_25_dT) showing normalisation to **c** POSITIVE_25_dT and **d** the *Dunaliella*-specific probe DunGS02_dT

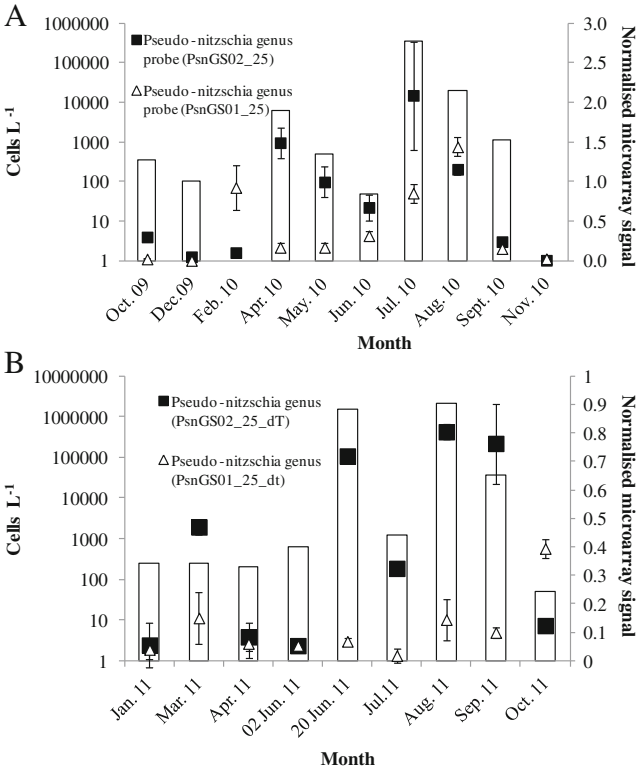

Fig. 4 Cells per litre of *Pseudo-nitzschia* (*open bars*) (primary *y* axis) and mean normalised microarray signal of probes PSNGS01_25, PSNGS02_25 specific for *Pseudo-nitzschia* genus ($n \leq 16$) to POSITIVE_25 (secondary *y* axis) (*shaded squares*) hybridised with RNA extracted from a litre of seawater. Showing samples collected in late from Brings Deep, Scapa Flow, Orkney Islands, UK in **a** 2010 and analysed with generation 2 of the microarray chip and **b** 2011 and analysed with generation 3 of the chip

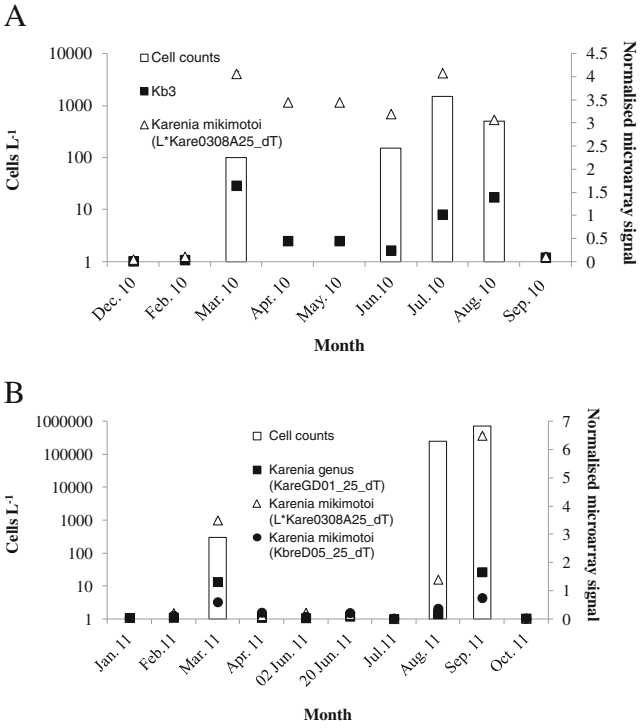

Fig. 5 **a** Cell counts (cells per litre) (*open bars*) of *K. mikimotoi* and microarray data for the *Karenia* genus-specific probe Kb3 (*shaded squares*) and the species-specific prove (L*Kare0308A25_dT) (*open triangles*) in months throughout 2010. **b** Cell counts (cells per litre) (*open bars*) of *K. mikimotoi* and microarray data for the *Karenia* genus-specific probe (KareGD01_25_dT) (*shaded squares*) and the species-specific probes (L*Kare0308A25_dT) (*open triangles*) and (KbreD05_25_dT) in months throughout 2011. Data for the probes are normalised to POSITIVE_25 and are mean values (n=16) ±SE

positive correlation (r=0.80; $p \leq 0.05$) (Fig. 6d). Correlations for species level proves for *Dinophysis acuta* (DacutaS01_25, Dacuta D02_25) all showed good correlations (r=0.77, $p \leq 0.05$; r=0.77, $p \leq 0.05$) and for the probes for *Dinophysis acuminata* (D.acumiD02_25, D.acumiS01_25) showed better correlations than the generation 2 data set (r=0.63, $p \leq 0.05$; r=0.85, $p \leq 0.05$). *Dinophysis* numbers were present in fewer months in 2011 with peak cell numbers in April and August.

Alexandrium species had a presence in the majority of months in 2010 with a high of 350 cells per litre in May (Fig. 7a). The *Alexandrium* genus probe (AlexGD01_25) showed a strong positive correlation with cell counts (r=0.83; p=0.05), the *A. tamarense* complex probe (AtamaS01_25) showed a weak positive correlation (r=≤0.31; $p \leq 0.05$) (Fig. 7a). Correlations for *A. tamarense* group I specific showed both weak correlation (ATNA_D01_25) (r=0.50; $p \leq 0.05$) and a strong positive correlation (ATNA_D02_25) (r=0.87; $p \leq 0.05$) (Fig. 7b).

In 2011, there was a much better overall agreement between probes and *A. tamarense* in the cell counts. *A. tamarense* group I was less prevalent in months throughout 2011 with a notable absence from counts in the winter months but also in March and July. *A. tamarense* numbers were highest in August with a peak of 700 cells per litre. When comparing the cell counts and the whole data set for the generation 3.1 chip for 2011, the *Alexandrium* genus probe (AlexGD01_25) showed a strong positive correlation with cell counts (r=0.66; $p \leq 0.05$) and the *A. tamarense* complex probe (AtamaS01_25) showed a strong positive correlation (r=0.70; $p \leq 0.05$). Correlations for *A. tamarense* group I-specific probes showed both a weak correlation (ATNA_D01_25) (r=0.50; $p \leq 0.05$) and a strong positive correlation (ATNA_D02_25) (r=0.84; $p \leq 0.05$). The qPCR assay confirmed the presence of *A. tamarense* group I in the months with high abundance (Fig. 5d, h), and across the whole dataset, there was an agreement with the presence of Saxitoxin and *A. tamarense* (Table 1) in the cell counts and microarray data, this also tied in with the qPCR data as well. As would be expected, the saxitoxin gene copy numbers were highly correlated with *A. tamarense* gene copy number 2010 (r=0.98; $p \leq 0.0001$) 2011 (r=0.99; $p \leq 0.0001$). Between the qPCR data and the microarray data (ATNA_S02_25) for 2010, there was a

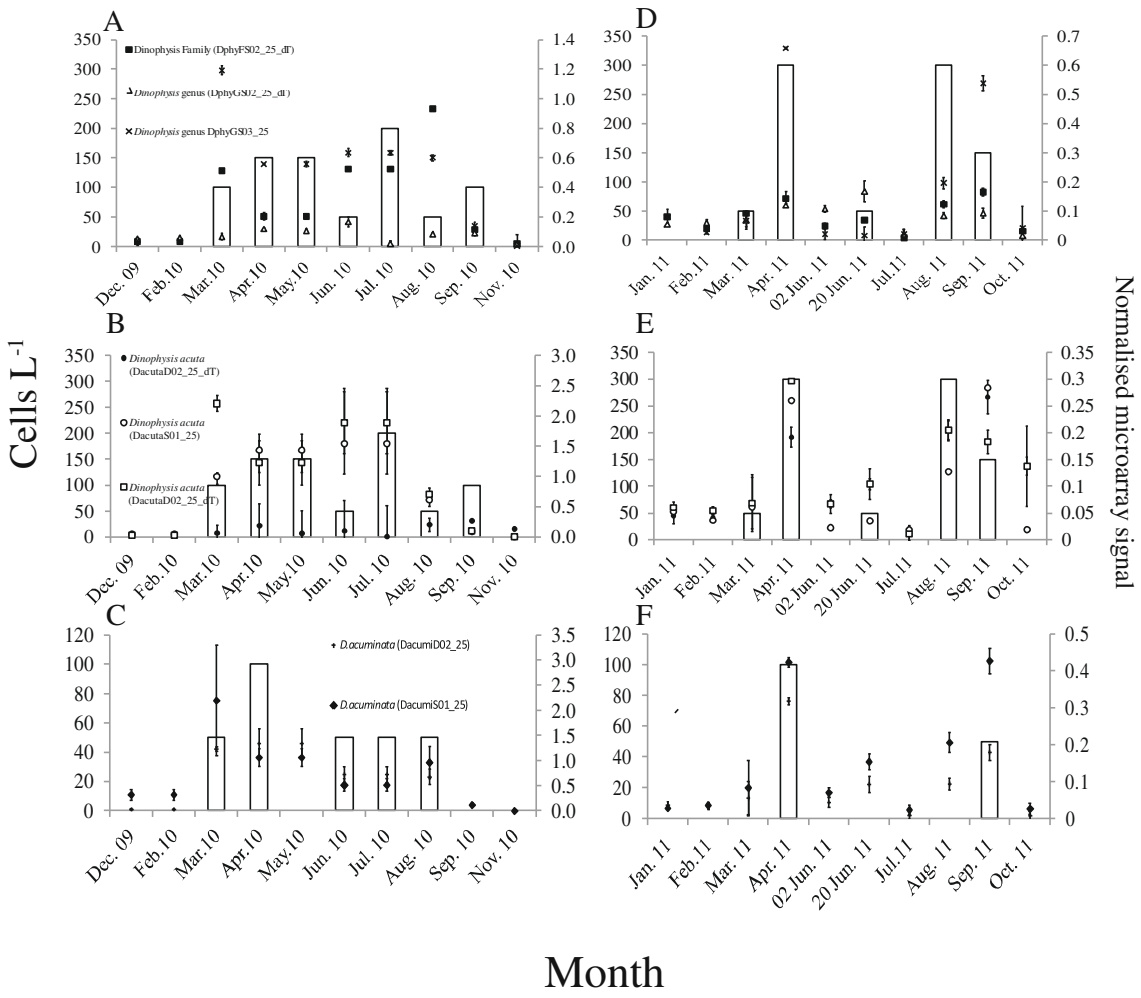

Fig. 6 Monitoring data for *Dinophysis* through 2010 and 2011 in seawater samples from Brings Deep, Scapa flow, samples represent 1 L of seawater from the top 3 m of surface water graphs represent **a** microarray data throughout 2010 showing data probes for the *Dinophysis* family (DphyFS02_25) and the *Dinophysis* genus (DphyGS02_25) and cell counts of *Dinophysis* sp. **b** Microarray data throughout 2010 showing data probes for *D. acuta* (DacutaD02_25, DacutaS02_25 and DacutaS01_25) and cell counts of *D. acuta*. **c** Microarray data throughout 2010 showing data probes for *D. acuminata* (DacumiD02_25, DacumiS01_25) and cell counts of *D. acuminata*. **d** Microarray data throughout 2011 showing data probes for the *Dinophysis* family (DphyFS02_25) and the *Dinophysis* genus (DphyGS02_25) and cell counts of the *Dinophysis* sp. **e** Microarray data throughout 2011 showing data probes for *D. acuta* (DacutaD02_25, DacutaS02_25 and DacutaS01_25) and cell counts of *D. acuta*. **f** Microarray data throughout 2011 showing data probes for *D. acuminata* (DacumiD02_25, DacumiS01_25) and cell counts of *D. acuminata*. Data for the probes are normalised to POSITIVE_25 and are mean values (n=16)±SE

weak positive correlation between the microarray and gene copy number for *A. tamarense* (r=0.46; $p \leq 0.05$) and no significant correlation with saxitoxin with much higher levels being picked up in qPCR data than were seen microarray and cell count data, for 2011 the correlation was still weak but slightly stronger (r=0.47; $p \leq 0.05$) and a weak correlation between the array data and saxitoxin gene copy number (r=0.46; $p \leq 0.05$).

Comparisons between data from the toxin array and the microarray data, in general, showed a good agreement with the microarray showing a positive signal for *A. tamarense* (Table 1) when there was a positive signal for PSP toxins apart from the 20th June 2011. The ELISA, a more sensitive technique, showed a positive result for PSP in all months this finding was also confirmed by the QPCR results. However, neither the microarray or the cell counts detected *A. tamarense* in all months throughout the whole year.

Discussion

The role of microarrays for use in monitoring has previously been assessed (Metfies and Medlin 2004, 2008; Metfies et al. 2007; Gescher et al. 2008, 2010), whereas these studies

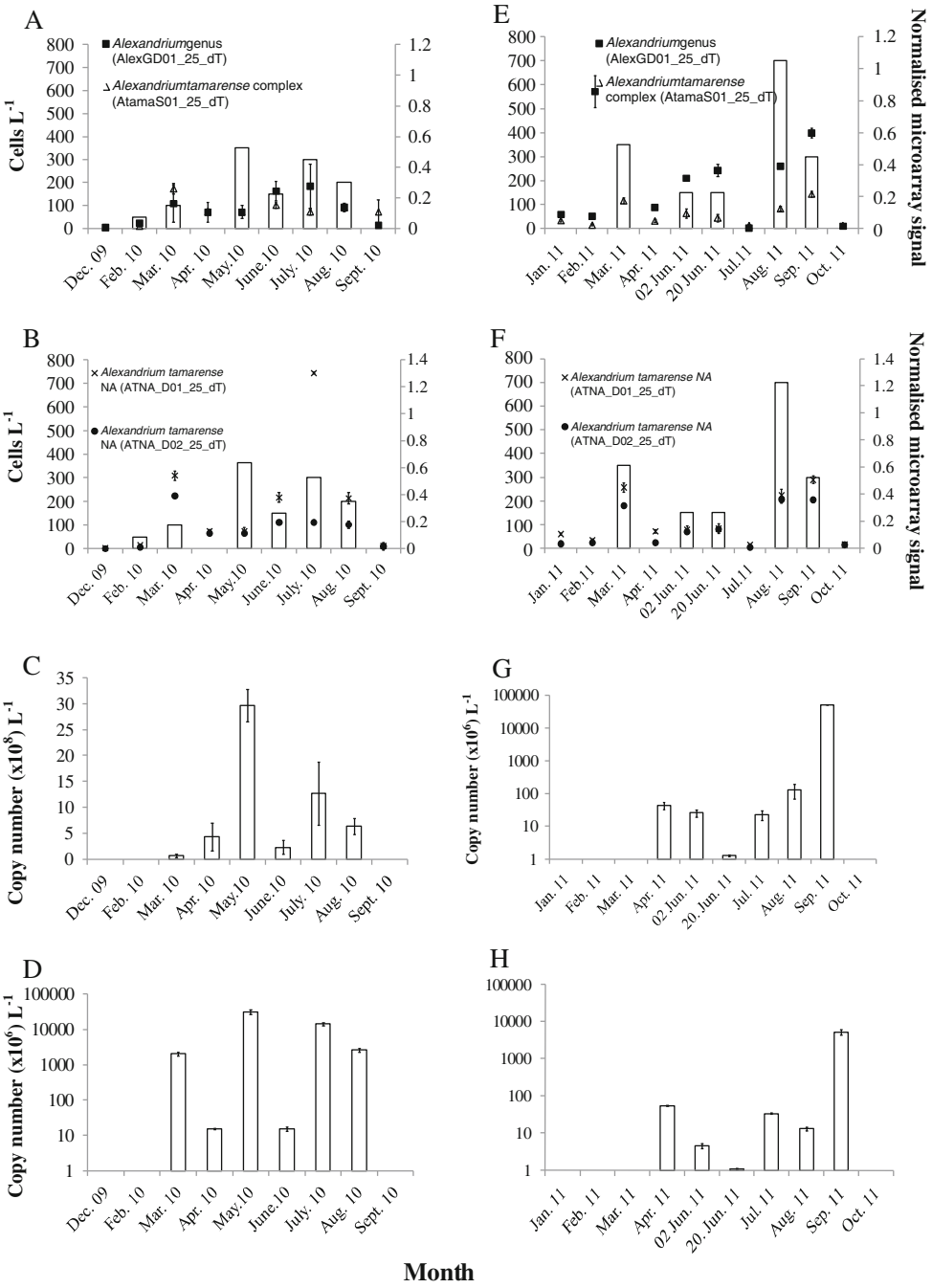

Fig. 7 Monitoring data for *A. tamarense* through 2010 and 2011 in seawater samples from Brings Deep, Scapa flow, samples represent 1 L of seawater from the top 3 m of surface water graphs represent **a** microarray data throughout 2010 showing data probes for the *Alexandrium* genus and the *A. tamarense* complex and cell counts of *Alexandrium* sp. **b** Microarray data throughout 2010 showing data probes for *A. tamarense* group I and cell counts of *A. tamarense*. **c** Copy number per litre of the saxitoxin gene stx4 in months through 2010. **d** *A. tamarense* group I 23 S rRNA gene copies per litre throughout 2010. **e** Microarray data throughout 2011 showing data probes for the *Alexandrium* genus and the *A. tamarense* complex and cell counts of *Alexandrium* sp. (cells per litre). **f** Microarray data throughout 2010 showing data probes for *A. tamarense* group I and cell counts of *A. tamarense*. **g** Copy number of the saxitoxin gene stx4 gene copies per litre throughout 2011. **h** *A. tamarense* group I 23S rRNA gene copies per litre throughout 2011. Data for the probes are normalised to POSITIVE_25 and are mean values (n=16) ±SE

have looked to target-specific groups of toxic algae the MIDTAL chip is the first to use a multi-class, genus and species approach. This is of particular use in Orkney Islands, UK were species such as *Alexandrium* sp., *Pseudo-nitzschia* sp. and *Dinophysis* sp. regularly co-occur (Davidson and Bresnan 2009; Hinder et al. 2011) and costly monitoring programs are undertaken to routinely check for these algae and their toxins to ensure they do not enter the food chain. In Europe, the method currently specified by European Food Safety legislation for official control testing for PSP and DSP are mouse bioassays (EFSA 2009) based on the protocol of Yasumoto et al. (1978). However, in the UK, this has recently been phased out in favour of liquid chromatography methods and GC-MS (CEFAS 2012).

Species such as *K. mikimotoi* currently have no threshold trigger level, and with its toxins poorly characterised, there is a need for more information on this species. It can be a routinely encountered species and its dynamics mean that blooms can appear relatively quickly (Brand et al. 2012).

Being able to distinguish between non-toxic and toxic strains or with greater sensitivity would mean that unnecessary toxin monitoring is not undertaken, therefore, saving

Table 1 Presence (+) or absence (−) of PSP toxins multi-SPR toxin array (McNamee et al. 2012) and independent ELISA techniques and copy number of the *A. tamarense* group I copy number ($\times 10^6$ copies per litre and of the saxitoxin gene $\times 10^6$ copies per litre and species present in cell counts and those with a positive signal on the array

Date	PSP toxins (STX)		*Alexandrium tamarense* gene copy number ($\times 10^6$)	stxA gene copy number ($\times 10^6$)	*Alexandrium* cell counts	*Alexandrium* on array
	Multi-SPR	ELISA				
03 March 2010	−	+	2.03	0.72	−	−
09 April 2010	+	+	0.02	4.35	+	+
13 May 2010	+	+	31.73	29.61	+	+
26 June 2010	−	+	0.02	2.34	−	−
22 July 2010	−	+	14.23	12.74	+	+
25 August 2010	−	+	2.63	6.37	+	+
27 April 2011	+	+	53.23	43.30	+	+
02 June 2011	−	+	4.51	25.90	−	+
20 June 2011	+	+	1.09	1.29	+	−
15 July 2011	−	+	33.43	22.69	−	−
10 August 2011	+	+	12.90	133.03	+	+
09 September 2011	+	+	5,146.57	51,433.33	+	+

money, and in the case of areas that may be closed immediately when threshold limits are met preventing unnecessary closure of shell fisheries. In other European areas, the chip could be useful in replacing the mouse bioassay.

Certainly, the chip operates in the range required for detection of *Alexandrium* sp. and can detect cells at the current limit of detection (presence in the counts), and it is likely this would be the limit of detection in natural samples. Interestingly, it was the group I strain which was the most dominant strain in all months rather than the non-toxic strain. The array data would suggest that in 2011 group III was also present in some months due to strong positive hits for the *Alexandrium* genus and *A. tamarense* complex probes. However, this shows that the chip is effective at distinction between *A. tamarense* group I and group III and also showing its co-occurrence in Orkney waters which has been shown previously (Collins et al. 2009; Brown et al. 2010; Touzet et al. 2010). Further work is needed to make the chip truly quantitative.

The chip shows a good suitability for monitoring *Pseudo-nitzschia* due to reasonable correlations between the signal strength and cell numbers. The genus level probes may be suitable markers for these species collectively and perhaps combined with some of the species probes they can improve the effectiveness of monitoring. *Dinophysis* was successfully detected at levels equivalent to those required for the current monitoring programme ~100 cells per litre for generation 3.1 of the chip. Further ID for *Dinophysis* is not normally required as all species in the UK are known to produce Okadoic acid and DTX toxins (Smayda 2006). However, the species probes will be useful in determining cell numbers of specific *Dinophysis* species. Due to the fact that settling chambers usually used in UK monitoring programs are either 20 or 50 mL in volume (in this study we chose a standard size of 20 mL), counting triplicate either 20 or 50 mL can only represent a maximum sampling pool of 150 mL. Therefore, the limit of detection is a minimum of 50 cells per litre for 50-mL chambers and at such low numbers the probability of counting one cell in a random 50 mL may be quite small. Of course some monitoring programs in other countries may use larger volumes but currently for the, Orkney Islands this is not the case. The microarray data presented here represents direct analysis of 1 litre. The equivalent volume analysed is dependent on the RNA content of the 1 L extracted and is normalised for when array signal is calculated.

This study further supports the evidence that microarrays have the potential to be a technique for harmful algal species detection (Metfries and Medlin 2004, 2008). The study has shown that there is a real potential for the monitoring of toxic algae using this technology and the MIDTAL chip. It has shown that there is effective detection of *Alexandrium* sp., *Pseudo-nitzschia* sp. and *Dinophysis* sp. at action levels, which are currently required in the Orkney Islands. In addition to this, it has shown its effectiveness for monitoring a lesser known problem species—*K. mikimotoi*. Although comparisons to the qPCR data suggest that more work may need to be done to make this truly quantitative, the positive correlations between the array data and qPCR and also between the cell counts and array data do show that this is achievable and it could potentially be suitable for monitoring harmful and nuisance species in the Orkney Islands.

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