AN EVALUATION OF THE APPLICABILITY OF MICROARRAYS FOR MONITORING TOXIC ALGAE IN IRISH COASTAL WATERS

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Appendices I

ABBREVIATIONS

AFLP-PCR	amplified fragment length polymorphism PCR
ASP	amnesic shellfish poisons
BCP	1-Bromo-3-chloro-propane
CCCM	Canadian Centre for the Culture of Microorganisms
CCMP	National Center for Culture of Marine Phytoplankton
CFP	ciguatera fish poisoning
CMSTAC	Center for Marine Science Toxic Algal Collection
CTP	cyanobacteria toxin poisoning
dcGTX	decarbamoyl gonyautoxins
dcNEO	decarbamoyl neosaxitoxin
dcSTX	decarbamoyl saxitoxin
ddH_20	double distilled water
DGGE	denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DSP	diarrhetic shellfish poisons
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	environmental protection agency
EtOH	ethanol
FISH	Fluorescent *in situ* Hybridization
FP	framework programme
GPR	GenePix results
GTX	gonyautoxins
HABs	harmful algal blooms
HCL	hydrochloride
H_2O	water
HPLC	high performance liquid chromatography
ITS	internal-transcribed spacer
LM	light microscopy

LSU	large subunit
MIDTAL	MIcroarrays for the Detection of Toxic Algae
multi SPR	multiplex Surface Plasmon Resonance
NaCl	sodium chloride
NEO	neosaxitoxin
NGS	next generation sequencing
NH_4Ac	Ammonium acetate
NMP	National Biotoxin Monitoring Programme
NSP	neurotoxic shellfish poisons
PCC	Pasteur Culture Collection of Cyanobacteria
Poly (dT) tail	poly (deoxythymidylic) tail
PSP	paralytic shellfish poisoning
qRT-PCR	Quantitative real-time Polymerase Chain Reaction
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAG	Sammlung von Algenkulturen der Universität Göttingen
SDS	sodium dodecyl sulphate
SEM	Scanning Electron Microscope
S/N ratio	signal-to-noise ratio
SSC	saline-sodium citrate
SSCP	single stranded conformation polymorphisms
SSU	small subunit
STX	saxitoxin
TBP	TATA-box binding protein
TEM	Transmission Electron Microscope
TGGE	temperature gradient gel electrophoresis
TRIS	tris(hydroxymethyl)aminomethane
UIO	University of Oslo
ULS	universal linkage system
$ZnCl_2$	zinc chloride

I dedicate this PhD Thesis

to my brother

Scott

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ABSTRACT

Monitoring of toxic phytoplankton is traditionally carried out by light and electron microscopy. However, the use of molecular methods for identification and quantification are now becoming more prevalent. Existing oligonucleotide probes used in whole-cell Fluorescent *in situ* Hybridisation (FISH) for *Prymnesium* species from higher group probes to species level probes were adapted and tested on to the novel MIDTAL (MIcroarrays for the Detection of Toxic ALgae) microarray format. Testing of probe specificity, cross reactivity issues, adaptations and optimisation of protocols are all reported upon during subsequent generations of the MIDTAL microarray. The applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events was tested in numerous locations around the southern and western coastline of Ireland between 2009 and 2011. Ribosomal RNA was extracted from filtered field samples, labelled with a fluorescent dye and hybridised to oligonucleotide 18S and 28S rDNA probes spotted onto a glass slide. The fluorescent signal intensity of the hybridisation to >140 probes on the chip was analysed and compared with light microscopy counts from field samples.

A correlation between RNA content and cell number with microarray signal intensities was one of the main focuses of this project whereby numbers of toxic algal cells could be inferred from the microarray signal. *Prymnesium parvum, P.* (= *Chrysochomulina*) *polylepis*, cf. *Chattonella* sp. and *Karlodinium veneficum* cells were grown under different stress conditions (light, temperature, salinity and nutrient), to see if RNA content per cell varied between treatments and over time. The study showed that total rRNA does not always positively correlate with growth rate, with no significant change in rRNA content over time when exposed to the majority of environmental stresses applied and that total rRNA content significantly correlated with increasing cell numbers.

Calibration curves for *Prymnesium* spp. with increasing labelled RNA amounts of 1 ng, 5 ng, 25 ng and 100 ng were performed on both the 2nd and 3rd generation microarrays. These calibrations revealed that the detection limit for both *P. parvum* and *P. polylepis* required an RNA amount of ≥5 ng which equates to 8,800 and 3,800 cells respectively. The detection limits of cf. *Chattonella* and *K. veneficum* species are also reported to be as

low as 1 ng, which is equivalent to <200 *cf. Chattonella* cells and ~250 *K. veneficum* cells.

During a sampling survey carried out in 2011, there was an extensive bloom of *Alexandrium minutum* in the North Channel of Cork Harbour and *Prorocentrum micans* in Bell Harbour of Galway Bay. Both *A. minutum* and *P. micans* species probes were successfully detected by the MIDTAL microarray and correlated significantly with light microscopy counts. Additionally, the detection of PSP toxins by the multi SPR biosensor, ELISA and HPLC methods during the *A. minutum* bloom further validated the presence of this species. The microarray can not only detect what species are co-occurring together in one analysis but can also track the progression of HAB events, giving us an in depth insight into the phytoplankton ecology with the potential to be used as an early warning tool.

The main aim of this thesis was to demonstrate the potential use of the MIDTAL microarray and multi SPR biosensor to support national monitoring agencies. Data presented in this thesis support the use of the MIDTAL microarray, which is now commercially available through Microbia Environnement (France), to provide a high-throughput method for fast, accurate detection and quantification of the harmful phytoplankton community and their toxins in natural water samples.

CHAPTER I

INTRODUCTION

1. INTRODUCTION

1.1. Phytoplankton

In general the term phytoplankton refers to free living photosynthetic microorganisms, which can adapt and live partly or continuously suspended and free of adhesion to substrate, encompassing a broad range of ecological niches across the world's seas, lakes ponds and rivers (Reynolds 2006). They are highly diverse and are represented by cyanobacteria, diatoms, dinoflagellates, green algae and coccolithophores (Lindsey et al. 2010). Dinoflagellates possess flagella which allow them to change positions at various speeds and their outer cell wall is composed of cellulose, while haptophytes outer cell wall is made of calcium carbonate which forms a variety of scales. On the other hand, diatoms are non-motile due to the lack of flagella and their cell wall is composed of silica. There are approximately 5,000 species of marine phytoplankton. About 300 of these can proliferate into enormous concentrations and about 80 have the ability to produce toxins (Sournia et al. 1991; Hallegraeff et al. 1993). They are the primary producers and consumers of carbon in our oceans and through the process of photosynthesis contribute to 40-50% of the oxygen in the atmosphere (Hallegraeff et al. 1993). They are responsible for most of the transfer of carbon dioxide (10 gigatonnes) from the atmosphere to the deep ocean every year (Fig. I-1). Phytoplankton thrive in upwelling zones along coastlines and continental shelves which are controlled by strong winds that drive deep ocean currents rich in nutrients to the surface (Lindsey et al. 2010).

Figure has been removed due to copyright restrictions

Fig. I-1. Carbon cycle. The transfer of carbon dioxide from the atmosphere to the deep ocean (Image from Lindsey et al. 2010).

Most algal blooms occur naturally in the environment and provide an abundance of food to a wide variety of organisms which include zooplankton and filter feeding bivalve

shellfish (oysters, mussels, scallops and clams). Some of these blooms can be harmful and are known as harmful algal blooms (HABs). They can manifest themselves in many ways causing damage to the ecosystem, reducing water quality, discolouring the seawater and affecting recreational areas due to their sheer high biomass. HABs can also be toxic even in low cell abundances which can lead to illness and death in fish, seabirds, marine mammals and humans, typically through the transfer of their toxins through the food web (Anderson 2009; GEOHAB 2001). The toxins produced by these HABs can accumulate in shellfish flesh and if consumed by humans can subsequently lead to serious gastrointestinal and neurological syndromes. The main groups of HAB toxins as delineated by syndromes are paralytic shellfish poisons (PSP), neurotoxic shellfish poisons (NSP), amnesic shellfish poisons (ASP), diarrhetic shellfish poisons (DSP), azaspiracid shellfish poisoning (AZP), ciguatera fish poisoning (CFP), and cyanobacteria toxin poisoning (CTP) (Sellner et al. 2003). It has not yet been fully established if these toxins have a harmful effect upon the shellfish themselves (Gainey 1988). A small number of around 80 HAB species can produce harmful toxins, 90% of which are dinoflagellates (Sournia et al. 1991; Smayda 1997). Of these, dinoflagellates are primarily responsible for the recent increase in HAB outbreaks reported worldwide (Anderson, 1989; 2012; Hallegraeff 1993; Smayda 1990; Sellner et al. 2003). These HAB events can have a negative economic effect on aquaculture industries on a global scale, but it is hard to estimate the exact losses occurred during such events (Anderson 2009). The impact of localised HABs can be more readily assessed. For example the dinoflagellate *Karenia mikimotoi* commonly produces red tides which can reach cell densities of over several million cells per litre and along with the presence of toxins can cause high marine life mortality which has been observed in Irish and Scottish waters (Silke et al. 2005; Davidson et al. 2009).

HAB events have also been associated with the potentially toxic diatom *Pseudo-nitzschia* with specific species such as *P. pseudodelicatissima* and *P. multistriata* reported to produce the toxin domoic acid (DA) detected in shellfish in the Atlantic and Mediterranean coastal areas (Amzil et al. 2001). However, the majority of *Pseudo-nitzschia* species present in the phytoplankton assemblage are harmless making it difficult through light microscopy (LM) alone to discern between toxic and non-toxic species.

1.2. Irish Phytoplankton and Biotoxin Monitoring Programme

The National Phytoplankton and Biotoxin monitoring programme in Ireland is underpinned by a series of European Union directives obliging coastal states to monitor shellfish production areas for the presence of toxin producing algae and their marine biotoxins in shellfish. This is carried out using biological and chemical methods in accordance with Commission Regulations (EC) No. 2010/477, No. 1664/2006, No. 853/2004 and No. 2074/2005 (Gilmartin and Silke 2009). The Irish Phytoplankton and Biotoxin Monitoring Programme is the responsibility of Irish Food Safety Authority and is carried out by the Marine Institute (MI) in Oranmore Galway. The MI use the accredited Utermohl methods and Light Microscopy for the identification and enumeration of harmful phytoplankton species of around 1,400 samples per year, sent in from numerous production areas located around the Irish coastline. They are also involved in the development of quantitative real-time PCR molecular techniques (Kavanagh et al. 2010). If there are rapid increases in the toxin producing phytoplankton observed by LM, then this will indicate the need for additional sampling and testing for potential harmful toxins in shellfish.

The Marine Biotoxin unit analyse *Mytilus edulis* (edible mussel), *Crassostrea gigas* (pacific oyster), *Ostrea edulis* (European flat oyster), *Ensis siliqua* (sword razor) and *Tapes philippinarium* (saltwater clam) tissue samples through biological and chemical means. These include bioassays, immunoassays, Liquid Chromatography Mass Spectrometry LC-MS and High Pressure Liquid Chromatography (HPLC) for the detection of AZP, DSP, ASP and PSP (Gilmartin and Silke 2009). If the detection of these toxins is over the regulatory limit, a ban on harvesting of all or selected bivalve species from that production area will be immediately implemented (Food Safety Authority of Ireland, COP on Biotoxins, version 3; 2013).

1.3. Monitoring of Toxins

The monitoring and prediction of toxic blooms is necessary to ensure the safety of seafood product consumers and the sustainability of the aquaculture industry. The monitoring of marine biotoxins is carried out by the use of biological, biochemical and physico-chemical methods. The earliest methods of detection were mouse bioassays, which were simple to perform and interpret. However these do not provide information on the range or variety of toxins present. This led to the development of sensitive

detection methods such as HPLC coupled with MS, which can routinely analyse PSP toxins (Oshima et al. 1989; Sullivan 1990). Time and skill is needed to routinely operate HPLC methods. Only recently have PSP toxin detection methods become commercially available, improving the quality of data obtained (Jellett et al. 2002). These commercially available Enzyme Linked Immunosorbent Assay (ELISA) methods come in the form of test kits, which permit the screening of PSP toxins in <20 minutes (Silva et al. 2001). However, it is important to note that HPLC methods are still superior to ELISA methods for detecting and quantifying marine biotoxin derivatives (Van Egmond et al. 1994).

In today's world we have become accustomed to the issuing of weekly, monthly or even annually forecasting of our weather systems around the globe. So why not transfer these technologies to prediction and forecasting of HABs. In the marine environment there is a combination of both physical and biological forces at play which need to be considered (Anderson et al. 2012). Satellite remote sensing combined with meteorological and environment data has been used to follow the development of *Karenia mikimotoi* blooms off the coast of Scotland (Davidson et al. 2009). Raine and co-authors (2010) has used empirical models to predict the occurrence of *Dinophysis acuminata* blooms in Bantry Bay using weekly wind-driven water exchange results to predict possible future HAB events. Studies in *Alexandrium fundyense* cyst concentrations in the Gulf of Maine showed a significant positive correlation to cyst abundance to the predictive size of *A. fundyense* blooms in subsequent years (Anderson et al. 2013). However, a similar predictive model carried out in the North Channel of Cork Harbour over a similar time scale showed the cyst densities are independent of the intensity of *Alexandrium minutum* blooms from year to year and showed insights into the reoccurring persistence of *Alexandrium* blooms (Cosgrove et al. 2014). This also indicates that not one model can be implemented to various different regions but they are quite specific as the biology and physical nature of the local areas are quite unique.

1.4. Phytoplankton Analysis

1.4.1. Light microscopy

Microscope-based cell identification methods are used to detect and enumerate specific species found in the water column to gain a better understanding of harmful algal blooms. Traditionally species classifications use compound light microscopy, inverted

microscopy or epifluorescence microscopy techniques by looking at morphological traits through increasing magnifications depending on the size class of the microalgae (Anderson and Throndsen 2003). For example, the armoured dinoflagellate *Alexandrium* contain thecal plate patterns, which are morphologically different to each species and discrimination between toxic and non-toxic *A. tamarense* by morphological analysis alone is impossible (Taylor et al. 2003). Electron Microscopy is an additional method which can further assist with specific species identification.

1.4.2. Electron microscopy

The identification of certain phytoplankton species may require the high resolution of electron microscopy for using either scanning electron (SEM) and/or transmission electron (TEM) microscopy. These methods differ in that SEM is based on the scattering of electrons which bounce off an object and are viewed on a screen which makes images three-dimensional. TEM is based on transmitted electrons which pass through the sample which are viewed as two-dimensional on a screen but at much higher magnifications.

Taxonomic and morphological identification is important in that certain species within a morphologically similar group produce potent toxins whilst others do not. This is particularly true for the genus *Pseudo-nitzschia* (Cusack et al. 2004). *Pseudo-nitzschia* species are identified by measuring width and length of valves to distinguish fine structures of girdle band patterns and poroids, density of striae and fibulae which all vary depending on the species (Cusack et al. 2004; Garcés et al. 2008). Also, the identification of species from the *Chrysochromulina*, *Prymnesium* and *Phaeocystis* genera is difficult due to their delicate nature, and requires a number of fixative techniques to avoid distortion of cellular features. Osmium tetroxide vapour is used commonly in laboratory situations for TEM grid preparation; however this cannot always be used during field work studies due to its high toxicity. A combination of Lugol's solution (acidic) and glutaraldehyde is a more refined method for the preservation of haptophytes in the field. This preserves the delicate haptonema, flagella and scales in most cells which is paramount for the identification and quantification under TEM (Fig. I-2; Jensen 1998).

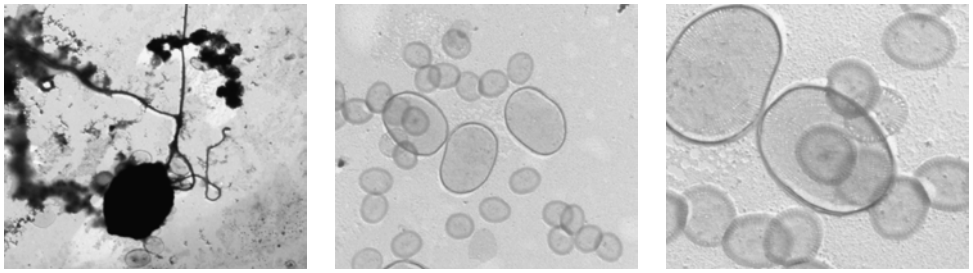

Fig. I-2. TEM images of *Prymnesium* (*Chrysochromulia*) *polylepis* fixed in a combination of Lugol solution (acidic) and glutaraldehyde (1% Lugols + 0.25% GA). (TEM shc) Left image of whole cell (x3.000); middle and right image of scales (x6.600-x12.000). Images taken by G.R. McCoy at Oslo University, Blindern, Oslo, Norway.

Monitoring programmes which analyse a high throughput of samples require methods of quantification which are rapid, without the need for slow and laborious tasks of trying to identify cells to species level using electron microscopy (Thomsen et al. 1994; Hajdu et al. 1996).

1.4.3. Molecular methods

In the study of phycology, species are not only recognised by their morphology and biology but are also increasingly identified with the use of molecular methods (John and Maggs 1997; Garcés et al. 2008). These approaches have been widely used in bacterial studies. However a growing number of methods have been developed for eukaryotes (Amann et al. 1995). Molecular technologies can greatly increase our knowledge of microalgae without the need for light microscopy identification (Ebenezer et al. 2012). Molecular techniques can advantageously discriminate between algal groups and the comparative analysis of their molecular sequences can be visualized as phylogenetic trees (Simon et al. 1997). Advances in molecular biology have shown that organisms that were assigned as different species due to morphological differences may now be considered as the same species due to their DNA signatures, an example of which are two forms of *P. parvum*, f. *parvum* and f. *patelliferum*, which are now known to be two life-cycle stages of the same species (Edvardsen and Medlin 1998; Larsen and Medlin 1997). Recently, morphological and ribosomal DNA sequence data have revealed that *C. polylepis* is more closely related to *Prymnesium* spp. than to other *Chrysochromulina* spp., and it has since been taxonomically reclassified into the genus *Prymnesium* as *Prymnesium polylepis* (Edvardsen et al. 2011). Also, the discriminate between morphologically similar but

genetically dissimilar strains, such as toxic and non-toxic forms of the same species or groups of monophyletic clades can be discerned by molecular techniques (Lilly et al. 2005; 2007).

The study of genome analysis and sequencing can shed light on the evolutionary history of algae. The construction of clone libraries using polymerase chain reaction (PCR) amplicons is one of the most common approaches to investigate both prokaryote and eukaryote diversity in the marine environment (Moon-van der Staay et al 2001; Díez et al. 2001; Giovannoni et al. 1990). A number of DNA-fingerprinting methods can be used for comparative diversity analysis when working with PCR products from a large number of samples; they include denaturing or temperature gradient gel electrophoresis (DGGE or TGGE; Muyzer et al. 1998) and single stranded conformation polymorphisms (SSCP; Schwieger and Tebbe 1998). Both DGGE and TGGE use a single large primer with a high number of GC content known as GC clamps which prevents strands from separating during electrophoresis on a gradient gel and comparisons of PCR fragments are limited to the amount of slots you can fit on the gel. SSCP however does not require GC Clamp primers or gradient gels and separates fragments by polyacrylamide gel electrophoresis which is considered a more simple and straightforward method then DGGE and TGGE (Schwieger and Tebbe 1998; Medkin and Kooistra 2010).

Higher resolution can be achieved with restriction fragment length polymorphism (RFLP), random amplification of polymorphic DNA (RAPD) and amplified fragment length polymorphism PCR (AFLP-PCR). When distinctions are needed to be made below the species level, microsatellite markers can also resolve intra-specific diversity as they use highly variable molecular markers which use internal-transcribed spacer (ITS) sequences (Medlin and Kooistra 2010; Ebenezer et al. 2012).

Entire genomes can now be sequenced using a new technology called Next Generation Sequencing (NGS) which can generate extensive transciptome data of prokaryotes and eukaryotes in a fast and cost effective way (Logares et al. 2013; Zhan et al. 2013; Kim et al. 2014). There are a number of NGS platforms such as 454 pyrosequencing, illumina, SOLiD, HiSeq and MiSeq, PacBio RS all of which use multiple PCR amplification strategies which has been applied in diversity studies of microbial eukaryotes (Kim et al. 2014). This approach generates sequence data by matching a single nucleotide to its

complementary base pair from a single strand of DNA fragment and detects which base was added at each step by the emission of light from the chemiluminescent enzymatic reaction of the DNA polymerase activity (Medlin and Kooistra 2010). The use of NGS technologies have rapidly increased in recent years and have provided many novel insights into taxonomy, phylogeny and evolutionary history of photosynthetic eukaryotes which can overcome traditional PCR biases related to amplification and primer mismatch, providing a more realistic estimation of community richness (Logares et al. 2013; Kim et al 2014). NGS also has a great potential for future work in both discrimination and quantification of microalgae worldwide (Ebenezer et al. 2012).

Techniques using molecular probes can selectively adhere to molecules specifically associated with a particular species even in complex phytoplankton communities (Scholin and Anderson, 1998). Some molecular probes include lectins, antibodies and DNA. Lectins are non-enzymatic secretary proteins, which bind to specific sugars including glycoproteins, polysaccharides and chitin on the cell surface (Brown and Hunt, 1978; Scholin and Anderson, 1998). Antibody probes bind to antigen molecules, such as peptides, glycoproteins, carbohydrates and toxins (Vrielling and Anderson, 1996; Peperzak et al. 2000). DNA probes are directed against sequences of small subunits (18s or SSU), and large subunits (28s or LSU) (Parson et al. 1999; Scholin et al. 1998). These probes can be applied to whole-cell or cell-homogenate methods for the detection and quantification of the microalgal assemblage and have been adapted as HAB monitoring tools.

1.4.4. Fluorescent *in Situ* Hybridisation

Whole-cell approaches demand that the target species remains intact throughout the assay procedure; an example is Fluorescent *in-situ* Hybridisation analysis (FISH) that is used to quantify and indicate target taxa in environmental samples by means of fluorescence microscopy or flow cytometry (Fig. I-3; Groben and Medlin 2005; Lange et al. 1996; Scholin et al. 1998; Tang et al. 2012; Touzet et al. 2007; 2010). The procedure of whole cell format is easy to operate, and intact cells can be identified by rough morphological features but advantageously nonspecific signals can be discerned from specifically labelled cells more easily (Miller and Scholin 1998). The uses of two different fluorescent dyes (FITC and Cy.3) attached to the specific oligonucleotide probes offers a faster means of species identification, especially in relation to toxic and nontoxic strains in a single hybridisation (Lilly et al. 2005; 2007; Touzet et al. 2010). However, this is also a drawback as they are limited to a few targets at a time.

Fig. I-3. Whole-cell hybridisation. Filtered cells are labelled with rRNA-targeted probes and visualized with fluorescence microscopy or flow cytometry. (Image from Bates et al. 1999).

1.4.5. Sandwich Hybridisation Assays

Sandwich hybridisation assays are DNA-based methods for rapid detection of targets which consists of two hybridisation reactions (Ayers et al. 2005). It includes two oligonucleotide probes that target ribosomal RNA (rRNA), one which binds to target nucleic acids to a binding site which is immobilises on a solid surface by the capture probe and a second probe which is species specific for the target called the signal probe and binds near the previous capture site (Fig. I-4; Diercks et al. 2008a; 2008b; Metfies et al. 2005; Scholin and Anderson 1998). These assays have proved useful on-board ships for obtaining near real-time mapping of *Alexandrium* species distributions (Anderson et al. 2005).

Fig. I-4. Sandwich hybridisation. Homogenised cells underwent two separate hybridisation reactions: capture of target RNA sequences and binding of an enzyme-tagged signal probe to a sequence near the capture site. The RNA is thus sandwiched between two probes. (Image from Bates et al. 1999).

Further advances have led to the development of DNA-biosensors for electrochemical detection of phytoplankton and their toxins on a sensor chips via a sandwich-hybridisation (Metfies et al. 2005a; Vilariño et al. 2009). The principle of which is the capture probe is immobilised on to the surface of a working electrode, the second target specific probe is attached with an antibody which in turn is coupled with horseradish peroxidase which catalyses the reduction of hydrogen peroxide to water. (Metfies et al. 2005a) The electron transfer during the reaction is measured as and electric current which is only possible if there is a link between the two probes, thereby indicating the present of the target nucleic acids of a particular species of interest (Medlin and Kooistra 2010). These systems have low detection limits and can be potentially developed into miniature easy to handle portable devices for the detection of seafood toxicity screening (Campàs et al. 2007).

1.4.6. Quantitative real-time Polymerase Chain Reaction

Quantitative real-time PCR (qRT-PCR) can provide accurate and reproducible quantification of gene copy formation during the exponential phase of a reaction (Galluzzi et al., 2004; 2010; Touzet et al., 2009; Erdner et al., 2010). In principle it uses standard PCR techniques but has a key distinction in that specific primers amplify target genes in real time and their formation is monitored after each cycle by measuring fluorescence (Galluzzi et al. 2004; 2010). The florescence is measured by either using a non-specific fluorescent dye such as SYBR Green that binds to double stranded DNA as it forms or sequence specific oligonucleotide probes such as the fluorescent reporter TaqMan (Ebenezer et al. 2012). The latter approach is considered more accurate as the SYBR Green dye method will bind to all PCR products including primer dimers which can prevent accurate quantification of intended targets (Ebenezer et al 2012). The number of PCR cycles (Ct) determines the amount of target in a sample, if there is more target in a sample the fluorescence readings for that target will significantly increase and when it hits the baseline threshold, the abundances of that target species can be determined (Galluzzi et al. 2010)

This allows for sensitive and rapid analysis for the detection and quantification of several target microalgal species at a time in environmental samples. This technology is cost effective and is currently being developed and used as a monitoring tool for HAB species (Galluzzi et al. 2004; 2010; Touzet et al. 2009; Kavanagh et al. 2010).

1.4.7. Microarrays

Microarrays are the state of the art technology in molecular biology for the processing of bulk samples for the detection of target RNA/DNA sequences. They are generally comprised of a glass surface with multiple DNA probes spotted in a defined position on its surface, which can hybridise to labelled RNA/DNA targets (Ki and Han 2006). Recently developed DNA-microarray-technology allows the simultaneous analysis of up to 250,000 probes at a time (Lockhart and Winzeler 2000). DNA-microarray technology has considerable potential to be used as a method to analyse samples from complex environments, because it has the ability to analyse samples quickly without a cultivation step (Medlin and Kooistra 2010).

The first DNA-microchip to study microbial diversity was a chip to analyse samples that contained nitrifying bacteria, which are difficult to study by cultivation (Guschin et al. 1997). In that publication a hierarchical set of oligonucleotide probes targeting the 16S ribosomal rRNA was created to analyse the bacterial samples on the DNA-chip. The use of microarrays as quantitative tools has predominately been based on gene expression and mixed bacterial communities in food, soil and water to identify and estimate abundances (Schena et al. 1995; 1996; Brodie et al. 2006; Kong et al. 2009; Lee et al. 2008; 2010; Call et al. 2003; Kostić et al. 2010).

An increasing number of molecular probes targeting the 18S or 28S rRNA gene sequence are now available for many species of toxic algae (Groben et al. 2004; Lange et al. 1996; Simon et al. 2000). However, the adaption of existing FISH probes to the microarray format was not as straight forward because the sequence length, secondary structures and proteins can block accessibility of labelled targets to the corresponding binding sites of the 18S rRNA gene molecule (Fig. I-5; Groben et al. 2004; Lane et al. 2004; Liu et al. 2007; Metfies and Medlin 2008).

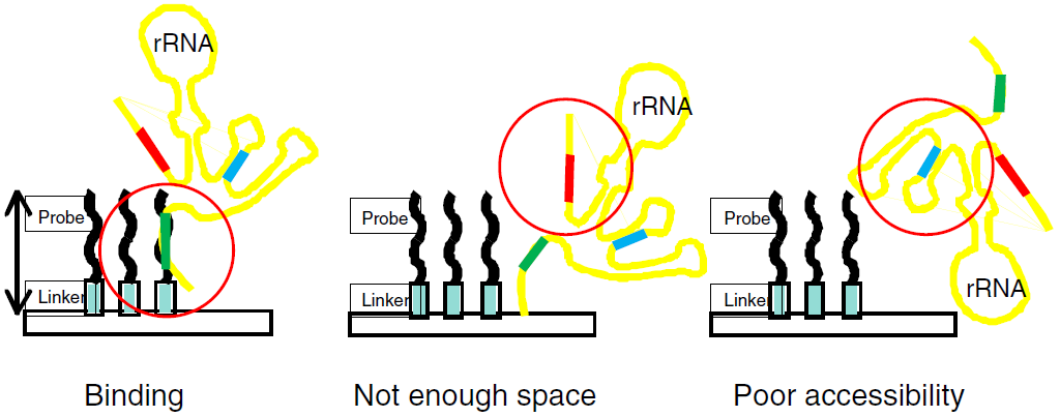

Fig. I-5. Scheme showing the basic problems in trying to achieve a good hybridisation between target and probe. (Image from Medlin 2013).

To overcome these problems, some studies working with 16S rRNA reduced the amplicon length, which improved probe accessibility (Lane et al. 2004; Liu et al. 2007). However the secondary structures in 16S rRNA are generally found in the first half of the molecule whereas they are mostly found in the second half of the molecule in 18S rRNA genes which would mean increasing the amplicon length for binding success (Metfies and Medlin 2008). The addition of a fluorescent label to the RNA extracted seawater

sample (target) prior to hybridisation provides the ability to measure the amount of target in the sample using a microarray scanner (Metfies and Medlin 2008).

Another drawback when examining with these approaches is that a number of false positives and non-specific binding may occur with environmental samples when and unknown composition of species are taken for RNA extraction and analysis on a universal microarray (Gescher et al. 2008). Some techniques PCR amplified the targets in a sample prior to hybridisation, while using shorter species specific rDNA regions (ITS1-5.8S-ITS2) which can increase specificity and sensitivity (Galluzzi et al. 2011).

Cross reactivity issues have also been dealt with in past studies by incorporating a hierarchical RNA-based approach for harmful marine phytoplankton species identification, using oligonucleotide probes that specifically target the 18S-28S rDNA domains on the microarray format (Groben et al. 2004; Groben and Medlin 2005; Gescher et al. 2008; Metfies et al. 2005b; 2007; 2010; Metfies and Medlin 2004; 2008). Thus, for a given species to be present the higher group probes such a genus, family, clade or class level must also be highlighted, thereby increase the specificity of the microarray and reducing the number of false positives (Metfies and Medlin 2008).

1.5. Introduction to MIDTAL

The MIDTAL (MICROARRAYS FOR THE DETECTION OF TOXIC ALGAE) project is funded under the European Union (EU) Seventh Framework Programme Theme 6 Environment (including climate change). It was a collaborative project encompassing 11 partners across Europe and the US (Table I-1).

Table I-1. List of partners involved in the MIDTAL Project.

Beneficiary Number *	Beneficiary name	Short name	Country
1	Marine Biological Association of the UK	MBA	UK
2	Stazione Zoologica 'A. Dohrn' di Napoli	SZN	IT
3	University of Kalmar	UniKal	SE
4	Instituto Español de oceanografía Vigo	IEO	SP
5	Ryan Institute, National University of Ireland	NUIG	IR
6	University of Oslo	UniOslo	NO
7	University of Westminster	UniWes	UK
8	Insitut for Van dog Miljo Forening	DHI	DK
9	Instituto Technoloxico para o control do Medio Marino	INTECMAR	SP
10	University of Rhode Island	URI	USA
11	Queen's University Belfast	QUB	UK

The aims and objectives in the MIDTAL project were:

1) To test and optimise existing rRNA probes for toxic species and antibodies for toxins for their application to a microarray

2) To design and test the specificity of any new probe needed

3) To construct a universal microarray from the probes tested and optimised by all of the partners for the detection of harmful algae and their toxins

4) To provide national monitoring agencies with a rapid molecular tool for monitor toxic algae and to validate or replace traditional methods used in toxic algae monitoring programmes

5) To integrate European efforts to monitor coastal waters for toxic algal species

The purpose of MIDTAL is to support the common fisheries policy and to aid national monitoring agencies by providing new rapid tools for the identification of toxic algae and their toxins so that they can comply with EC directive 91/1491/CEE and potentially reduce the need for the mouse bioassay.

1.5.1. MIDTAL microarray

The MIDTAL microarray is essentially a glass microscope slide with oligonucleotide probes that specifically target the 18S-28S rDNA domains from hierarchical groups down to the species level. Existing probes designed for FISH were adapted to the microarray format along with newly designed probes (Ludwig et al. 2004). Each probe was spotted in replicate in a planned layout on the chip surface, which also contain several controls (Fig. I-6). MIDTAL has developing what is the first commercially available universal microarray (phylochip), capable of rapidly detecting the presence of specific harmful algal species and their toxins before they develop into harmful blooms (HABs). Essentially the methodological plan development for MIDTAL in order to support monitoring can be summarised as follows:

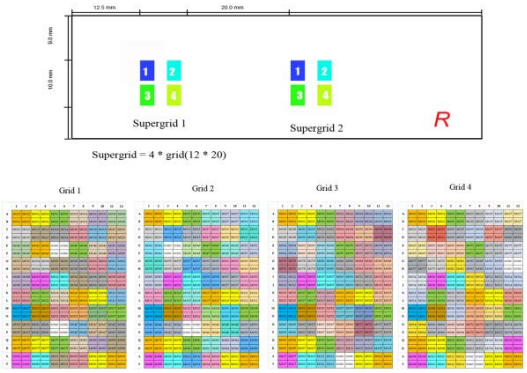

Fig. I-6. Layout and design of hierarchical probe set for a range of phytoplankton spotted on the first version of MIDTAL microarray slide (Image source from MIDTAL leaflet).

1) Take a water sample, 2) extract total RNA, 3) extract total toxins, 4) apply RNA to microarray and determine the fluorescent signal of the hybridised probe to its target RNA, 5) apply toxins to microarray and determine the fluorescent signal of the hybridised probe to its target antibody, 6) extrapolate using novel algorithms to cell counts per litre using calibration curves and 7) compare manual phytoplankton counts with signals from microarray to validate monitoring effectiveness (Fig. I-7).

The main objective of MIDTAL is to be able to use the microarray as a quantitative tool for reliable analysis of field samples, to achieve this the correlation of cell counts and RNA concentrations per cell with microarray signal intensities is essential (Gescher et al. 2008).

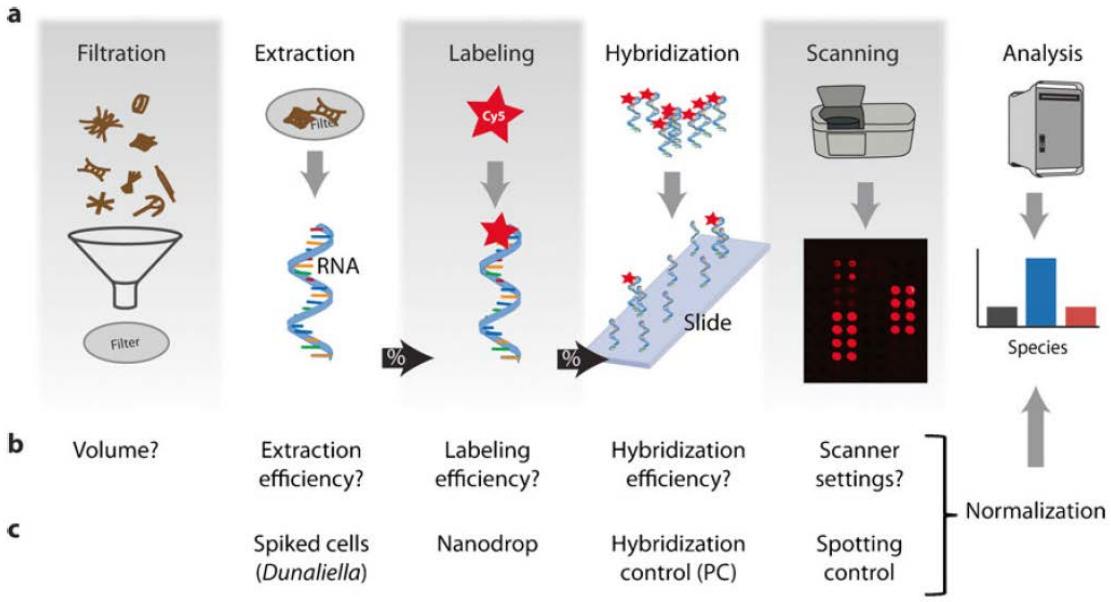

Fig. I-7. (a) Important steps that were involved in the development of the MIDTAL microarray protocol, (b) factors to consider at each stage of development and (c) to assess the best controls to use for normalisation of results. Image from Dittami and Edvardsen (2013).

1.5.2. Adaptation of toxin antibodies to microarray: MIDTAL

The adaptation of toxin antibodies to the microarray was carried out by partner 11 using a multiplex optical Surface Plasmon Resonance (SPR) biosensor assay capable of detecting a combination of ASP, NSP, DSP, PSP toxins in algal samples. The key steps in being able to develop such an assay for SPR analysis are the availability of a multiplex SPR biosensor and the design of a multiplex biosensor chip surface. Within the scope of FP6 project BioCop a prototype 16-plex SPR instrument has been developed for the detection of protein biomarkers. Multiplexing was achieved by the development of multi-lane (x 4) SPR and/or by the development of multi-spots (x 4 per lane) for different bio-recognitions within a single SPR lane (Campbell et al. 2011). Within the MIDTAL project this same technology is being applied for the development of the multiplex

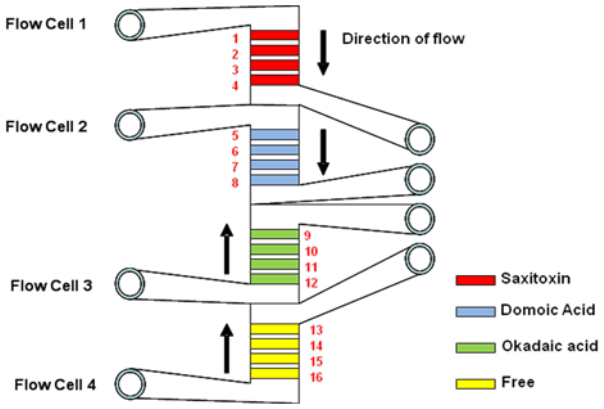

Fig. I-8. The chip surface chemistry of the prototype multi SPR biosensor chip (Image from Campbell et al. 2011).

analysis of marine biotoxin in seawater samples. The prototype multi SPR instrument will use saxitioxin, domoic acid and okadaic acid polyclonal antibodies (Fig. I-8).

1.5.3. Field sampling locations

One of the key tasks in the MIDTAL project was to demonstrate the applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events.

Monitoring sites used in national biotoxin and toxic phytoplankton monitoring programmes were selected in several countries across Europe for this purpose. The sampling sites chosen were Arcachon Bay in France, Gulf of Naples in Italy, Skagerrak West Coast in Sweden, Ria de Pontevedra (Ponta vedra) in Spain, West and South Coast of Ireland, Northern Skaderrak and Outer Oslofjord in Norway, Orkney Islands in Scotland and Galicia Rias in Spain. At each sampling site a variety of samples were taken which included Lugol's Iodine preserved discrete sample for microscopy, unpreserved non-concentrated sample for cell isolations, filtered seawater samples for RNA, DNA and toxin extraction.

1.5.4. Sampling locations in Ireland

Several sites were chosen from around the Irish coastline, the most frequently sampled of these sites was Killary Harbour, North Channel in Cork Harbour and Bell Harbour in Galway Bay (Fig. I-9). The ecological status of the coastal water quality for these three main sampling sites are also shown, assessed by the Environmental Protection Agency (EPA) of Ireland (Fig. I-9A, B and C). Both Killary Harbour and Galway Bay are unpolluted and have a thrieving shellfish industry, which was estimated to be worth approximately €60 million annually to the Irish economy (Browne et al. 2007). However, the potentially eutrophic North Channel of Cork Harbour is a location were PSP producing *Alexandrium minutum* blooms occur regularly during the summer months (Touzet et al. 2007). PSP events have resulted in the closure of shellfish harvesting in Cork Harbour in most years since 1996 (Ní Rathaille et al. 2008; 2009). The North channel is not only effected by HAB events but there has been a ban on the harvesting of oysters since 2002 due to viral contamination (ENVIRON report 2012; http://www.environ.ie). Monitoring analysis of shellfish flesh undertaken by the Marine

Institute also indicates faecal contamination in this shellfish area resulting in the bivalve mollusc production areas of North Channel West and North Channel East being classified as 'Class B' for the purposes of EC Regulation 854/2004.

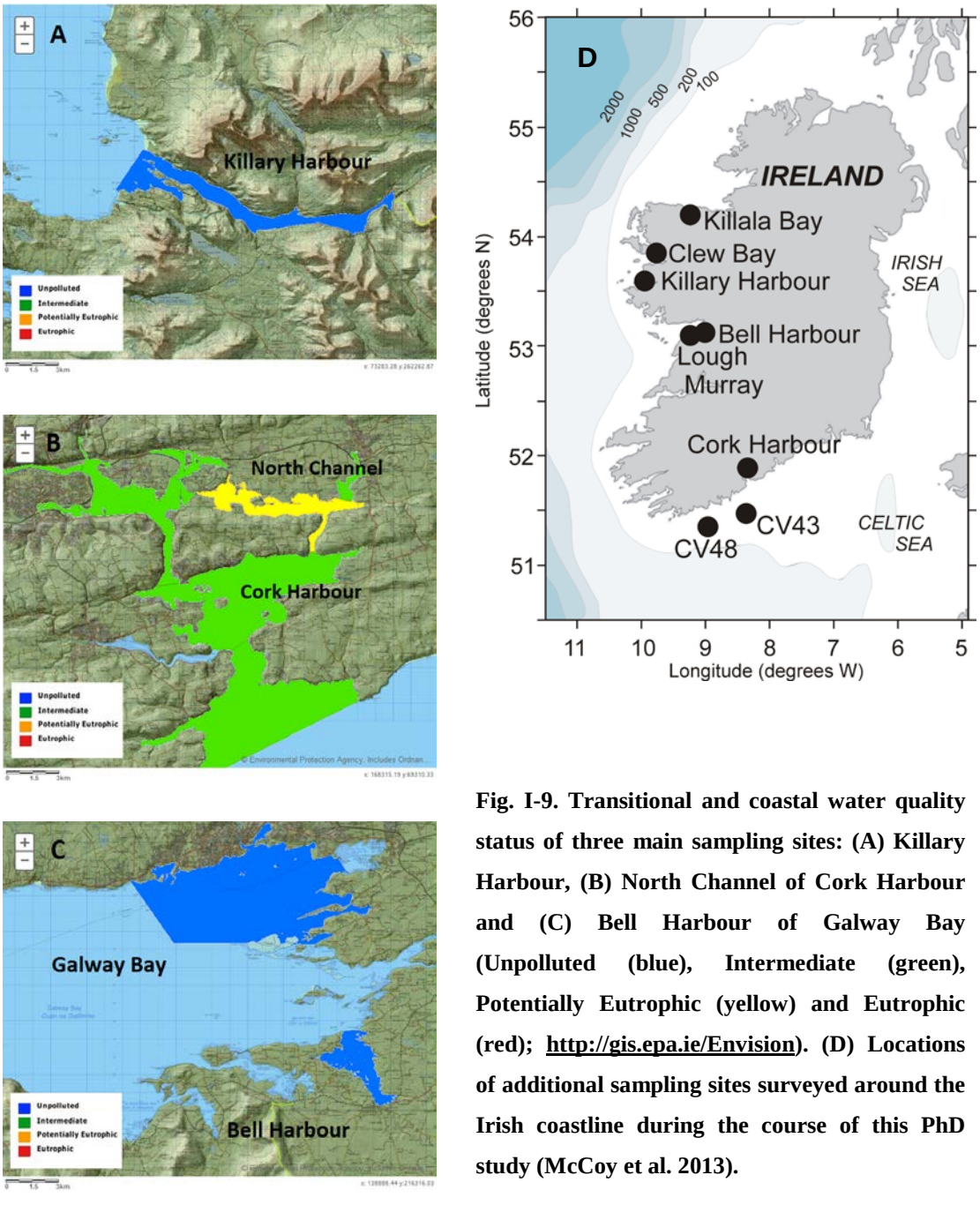

Fig. I-9. Transitional and coastal water quality status of three main sampling sites: (A) Killary Harbour, (B) North Channel of Cork Harbour and (C) Bell Harbour of Galway Bay (Unpolluted (blue), Intermediate (green), Potentially Eutrophic (yellow) and Eutrophic (red); http://gis.epa.ie/Envision). (D) Locations of additional sampling sites surveyed around the Irish coastline during the course of this PhD study (McCoy et al. 2013).

Microarrays used in regulatory monitoring could be used to track the abundances of species and strains, not only would they also be able to differentiate between toxic and non-toxic species, they may also be able to determine possible introductions of species from other regions through resting cysts in ballast waters (Lilly et al 2002; Gescher et al. 2008). There are a number of fundamental hypotheses to be addressed before national monitoring agenises are confident in using microarray technology and specifically using the MIDTAL microarray.

- Can the MIDTAL microarray reliably detect HAB species using pure cultures and environmental samples?
- Can we use microarrays to further investigate phytoplankton biodiversity and the quantitative changes in the biodiversity with time?
- In terms of quantification, how representative are the limits of detection inferred by the microarray with current national harmful phytoplankton monitoring programmes?

The aim of the work embodied in this thesis is an attempt to address these questions. As one of the partners involved in the MIDTAL project, NUIG was assigned the testing of existing *Prymnesium* spp. FISH probes and adapting them to the microarray format. Chapter 2 outlines the evolutionary process of the development of the MIDTAL microarray. This chapter focuses on the specificity of *Prymnesium* spp. probes, re-designing, optimisation, and improvements made from the initial 1st generation microarrays to the testing of Killary Harbour field samples on the latest 3rd generation (version 3.3) microarray. Chapter 3 investigates the variation of RNA content in *Prymnesium parvum*, *Prymnesium polylepis*, cf, *Chattonella* sp. and *Karlodinium veneficum* cells when grown under different environmental stress conditions (McCoy et al. 2014a). The following chapter (Chapter 4) investigates the applicability of microarrays to monitoring HABs across a broad range of ecological niches with some of the first field samples collected in this study along the Irish coastline and analysed with the 2nd generation microarray (McCoy et al. 2013). The following two chapters (5 and 6), assesses the effectiveness of the 3rd generation microarray during large *Alexandrium* and *Prorocentrum* bloom events with field samples taken from the Cork Harbour and Bell Harbour sampling sites (McCoy et al. 2014b).

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CHAPTER II

EVOLUTION OF THE MIDTAL MICROARRAY: THE ADAPTION AND TESTING OF OLIGONUCLEOTIDE 18S AND 28S RDNA PROBES AND EVALUATION OF SUBSEQUENT MICROARRAY GENERATIONS WITH *PRYMNESIUM* SPP. CULTURES AND FIELD SAMPLES

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ABSTRACT

The toxic microalgal species *Prymnesium parvum* and *Prymnesium polylepis* are responsible for numerous fish kills causing economic stress on the finfish and shellfish industries and, through the consumption of contaminated shellfish, can potentially impact on human health. Monitoring of toxic phytoplankton is traditionally carried out by light microscopy. However, molecular methods of identification and quantification are becoming more common place. This study documents the optimisation of the novel MIDTAL (MIcroarrays for the Detection of Toxic ALgae) microarray from its initial stages to the final commercial version now available from Microbial Environnement (France). Existing oligonucleotide probes used in whole-cell Fluorescent *in situ* Hybridisation (FISH) for *Prymnesium* species from higher group probes to species level probes were re-evaluated, adapted and tested on the 1st generation microarray. The combination and interaction of numerous other probes specific for a whole range of phytoplankton taxa also spotted on the chip surface caused high cross reactivity, resulting in false positive results on the microarray. The probe sequences were extended for the subsequent 2nd generation microarray. Adaptions of the hybridisation protocol and incubation temperatures significantly reduced false positive readings from the 1st to the 2nd generation chip, thereby increasing the specificity of the MIDTAL microarray. Further adaptions to the subsequent 3rd generation microarray protocols with the addition of a Poly T amino linker to the 5' end of each probe further enhanced the microarray performance but also highlighted the importance of optimising RNA labelling efficiency when testing with natural seawater samples from Killary Harbour, Ireland.

Keywords: HAB, molecular probes, MIDTAL, microarray, RNA, *Prymnesium*.

INTRODUCTION

Globally harmful algal blooms (HABs) cause major environmental problems as they can cause high economic losses to the shellfish industry and, through the consumption of contaminated shellfish, can potentially impact on human health (Anderson et al. 2012; Hallegraeff 1993; Smayda 1990). Haptophytes are frequently associated with HABs in marine and brackish waters. One such family is the Prymnesiophyceae, which contains the ichthyotoxic toxin producing species *Prymnesium parvum* and *Prymnesium* (=*Chrysochromulina*) *polylepis* that have been responsible for numerous fish kills (Moestrup 1994; Edvardsen and Paasche 1998; Edvardsen et al. 2011). The monitoring of toxic phytoplankton is necessary to predict the possible re-occurrence of toxic blooms. Early warning systems, operated by traditional light microscopy techniques, identify and quantify species by their morphological traits. This can be time consuming and discrimination between small morphologically similar species is almost impossible without the aid of electron microscopy (Larsen et al. 1993; Hajdu et al. 1996).

Molecular probes can selectively adhere to molecules specifically associated with a particular species, even in complex phytoplankton communities (Scholin and Anderson, 1998). Oligonucleotide probes are directed against sequences of small (18S or SSU) and large subunits (28S or LSU) (Simon et al. 1997; Töbe et al. 2006). These probes can be applied to whole-cell or cell-homogenate based methods of detection. The whole-cell approach maintains the target species remains intact throughout the assay procedure; an example is Fluorescent *in-situ* Hybridisation (FISH) analysis that is used to quantify and identify target taxa in environmental samples (Eller et al. 2007; Simon et al. 1997, 2000; Töbe et al. 2006; Touzet et al. 2007; 2010). Cell-homogenate probe techniques on the other hand require the lysis of cells. These include methods such as Sandwich Hybridisation Assays (SHA), Polymerase Chain Reaction (PCR) based methods and microarray analyses (Diercks et al. 2008a; 2008b; Galluzzi et al. 2008; Gesher et al. 2008; Penna and Galluzzi 2013; Scholin and Anderson, 1998; Metfies and Medlin, 2005, 2008a). The use of microarrays as quantitative tools has predominately been based on gene expression and bacterial studies (Schena et al. 1995; 1996; Brodie et al. 2006; Kong et al. 2009; Lee et al. 2010).

In the MIDTAL project an RNA approach for species identification was adapted to the microarray format, using existing and re-designed oligonucleotide probes that specifically target the 18S-28S rDNA domains from hierarchical groups down to the

species level. Using microarray-technology in this way, the simultaneous analysis of 113 (1st generation), 170 (2nd generation) and 140 (3rd generation) different probes specific for a range of harmful phytoplankton and spotted as 8 replicates, together with several controls species can be detected (Kegel et al. 2013a; 2013b; Medlin 2013).

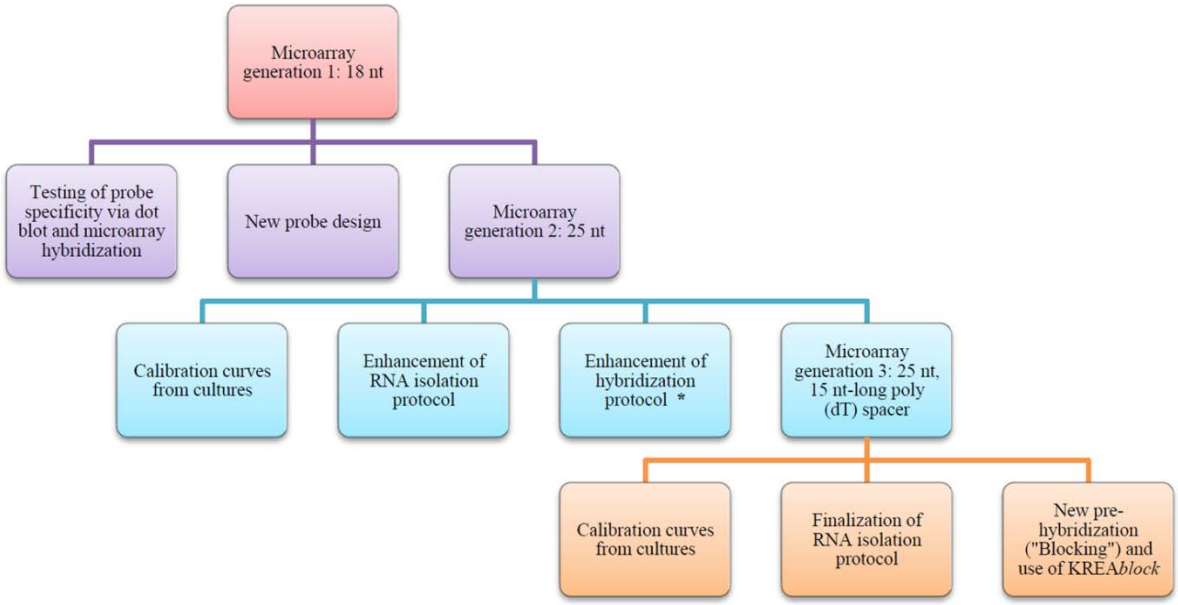

Fig. II-1. Evolutionary development of the MIDTAL microarray. The schematic shows the subsequent microarray generations with different probes, optimisation steps of protocols for RNA, hybridisations and washing steps (* Higher temperature final wash step of 50 °C). This schematic was taken from Kegel et al. (2013b).

The aim of this study was to re-evaluate existing oligonucleotide rRNA *Prymnesium* spp. probes and to assess their adapted functionality when spotted on the novel MIDTAL microarray platform. Probe specificity, cross reactivity testing and protocol optimisation were some of the key steps involved in developing the latest 3.3 version MIDTAL microarray (Fig. II-1; Kegel et al. 2013a; 2013b; McCoy et al. 2012; 2013; 2014a; 2014b; Medlin 2013), which is now commercially available from Microbia Environnement (France).

MATERIALS AND METHODS

Obtaining cultures

Cultures for probe testing were retrieved from culture collections which contained strains from different geographical regions (Table II-1). The aim of this particular work was to test probes targeting *Prymnesium parvum* and *Prymnesium* (=*Chrysochromulina*) *polylepis* species (Edvardsen et al. 2011). Stock cultures were grown in L1 medium or ½ IMR prepared using 0.22 µm filter-sterilised seawater (salinity 32 PSU) (Guillard and Ryther, 1962). Algal cultures were maintained at 15 ± 1ºC following a light-dark cycle of 14:10h with a photon flux density of 150 µE m^{-2} s^{-1}.

Table II-1. Algal cultures used during this study.

Culture Collection	Strain Code	Species name
Provasoli-Guillard	CCMP 1757	*Prymnesium polylepis*
University of Oslo	UIO038	*Prymnesium polylepis*
University of Oslo	UIO037	*Prymnesium polylepis*
University of Oslo	UIO036	*Prymnesium polylepis*
University of Oslo	UIO054 (=RHpat89)	*Prymnesium parvum*
Provasoli-Guillard	CCMP 709	*Prymnesium parvum*
SAG	SAG 127.79	*Prymnesium parvum*

CCMP (Provasoli–Guillard National Center for Culture of Marine Phytoplankton), SAG Sammlung von Algenkulturen der Universität Göttingen, UIO (University of Oslo, Department of Biology, Oslo, Norway).

FISH probes

Eight taxa-specific fluorescently labelled oligonucleotide probes were selected for the detection of *Prymnesium* species in culture and spiked field samples using whole-cell Fluorescent *in situ* Hybridisation (FISH) analysis, along with two eukaryote probes (Table II-2). Probes were purchased from MWG Biotech (Germany) with the 5'end labelled with a fluorescent CY.3 dye.

Table II-2. A list of a hierarchical probe set of higher group Eukaryote and *Prymnesiophyta* probes, *Prymnesium* genus, clade and species level probes for *P. parvum* and *P. polylepis* with the original source sequences used for FISH and spotted on the 1st generation microarray. The subsequent probes spotted on the 2nd generation were extended by 25 nucleotides and subsequent 3.2 and 3.3 versions of the 3rd generation microarray sequences included a 15 nucleotide long poly (dT) spacer attached to the 5'end, these adaptations are patent pending and the sequences are therefore not provided (Kegel et al. 2013a; 2013b).

1st generation	Specific for…	Gene	Position (E.coli)	Sequence (5' – 3')	Source	Probes on the 2nd generation	Probes on the 3.2 version microarray	Probes on the 3.3 version microarray
Higher group probes								
EUK328	Eukaryotes	18S		ACCTGGTTGATCCTGCCAG	Moon-Van 2001	EUK328	-	-
						Euk_328_25	Euk_328_25_dT	Euk_328_25_dT
EUK1209	Eukaryotes	18S	36	GGGCATCACAGACCTG	Lim et al. 1993	EUK1209	-	-
						Euk_1209_25	Euk_1209_25_dT	Euk_1209_25_dT
PRYM01	Prymnesiophyta	18S	24	ACATCCCTGGCAAATGCT	Lange et al. 1996	Prym01_25	Prym01_25_dT	Prym01_25_dT
PRYM02	Prymnesiophyta	18S	22-24	GGAATACGAGTGCCCCTGAC	Simon et al. 2000	Prym02_25	Prym02_25_dT	-
PRYM03	Prymnesiophyceae	18S	16	GTCAGGATTCGGGCAATT	Eller et al. 2007	Prym03_25	Prym03_25_dT	Prym03_25_dT
Genus level probes								
PRYMGL01A	Prymnesium	18S	41-42	TGCTCGCCAACGAGGTGT	Eller et al. 2007	PrymGS01_25	Prymparv01_25_dT*****	-
PRYMGL02B	Prymnesium	18S	40	AAGAAGTGCTCGCCAACG	Eller et al. 2007	PrymGS02_25	-	-
Clade level probes								
Clade01	Prymnesium	18S	26-27	GAACTTCCGCCGATCCCTAGT	Simon et al. 1997	Clade01	Clade01old_25_dT	Clade01old_25_dT
	Prymnesium Clade					-	Clade01new25_dT	Clade01new25_dT
Species level probes								
Cpoly01	Prymnesium	18S	26-28	GACTATAGTTTCCCATAAGGT	Simon et al. 1997	Cpoly01	-	-
						CpolyS01_25	CpolyS01_25_dT	CpolyS01_25_dT
PRYM694	Prymnesium parvum	28S	D1/D2	CAGCCGACGCCGAGCGCG	Töbe et al. 2006	PRYM694	PparvD01_25_dT	PparvD01_25_dT
						-		Prymparv01_25_dT*****

***** Prymparv01_25_dT is now considered a species level probe for *Prymnesium parvum.*

FISH analysis

Sample preparation

The re-evaluation of existing FISH probes was assessed with *P. parvum* and *P. polylepis* cultures using a custom made manifold according to the method described by Miller and Scholin (1998) with the following modifications. Approximately 2 ml of *P. parvum* (CCMP1757, SAG 127.79 and UIO054) and *P. polylepis* (CCMP709, UIO036, UIO037 and UIO038) pure cultures were transfer to new 2 ml tubes and the cells fixed with formalin (0.5% final concentration). The tubes were inverted three times and left to stand at room temperature for 15 minutes. The filtration unit with vacuum pump was set up with adapted 15 ml tube hybridisation chambers each containing a 13 mm diameter polycarbonate membrane filter (Millipore 1.2 µm pore size). The fixed cells (25-50 µl) were homogenised gently and transfer to each hybridisation chamber. The sample was then filtered through using a very low vacuum as possible, while maintaining a constant flow to prevent breakage of the delicate cells. The fixed cells on each filter were treated with 2 ml of ice cold methanol (100%) making sure the whole filter is covered with the solution and let stand at room temperature for 1 hour in the dark. The solution was removed by vacuum filtration prior to hybridisation. This procedure was also repeated for the FISH probe cross reactivity testing with *P. parvum* and *P. polylepis* cultures spiked with 250-500 µl of field sample (CH5605-Q-0m, CH5604-P-3m and CK5733-0m) obtained from the North Channel of Cork Harbour, Ireland. Collected during summer 2007 at a time when phytoplankton and diversity in the samples was substantial.

FISH hybridisation

A volume of 1 - 2 ml of 0% hybridisation buffer (5X SET and 0.1% IGEPAL) was added to each hybridisation chamber and left stand at room temperature for 2 minutes and filtered through to remove any excess methanol. In 1 ml Eppendorf tubes, 500 µl of hybridisation buffer (5X SET, 0.1% IGEPAL, 0% to 25% (v/v) formamide (FA)) was mixed with 1.5 µl of probe working stock (200 µg. μl^{-1}; kept at -20 °C) by vortexing. The probe-containing hybridisation buffer was added directly onto the whole membrane and the hybridisation chamber lids were sealed tight. The manifold was placed in a hybridisation oven (Binder BD 53) in the dark, at a fixed temperature for 60 minutes. A series of incubation temperatures (45 - 55°C) were tested to determine the optimal hybridisation temperature for each probe. After hybridisation, cells were washed once with 500 µl 0.2X SET buffer preheated to the set hybridisation temperature to remove

any excess unbound probes. Under vacuum the membrane filters were removed from the 15 ml hybridisation chambers and placed onto a microscope slide shiny side up. Before mounting a coverslips, 10 -12 µl of a 20% glycerol solution containing DAPI (1 $\mu g.ml^{-1}$) and calcofluor (100 $\mu g.ml^{-1}$) were added to the membranes, trying not to form any air bubbles.

Epi-fluorescent microscopy

Slides were observed using an inverted microscope (Olympus CKX-41) fitted with a U-RFL-T epi-fluorescence attachment, 100 W Mercury lamp and an Olympus DP70 digital camera. The following filter combinations were used to expose the fluorescent signals: calcofluor and DAPI (355DF25 excitation filter, 400DRLP dichroic mirror and 420 long pass barrier filter), and CY.3 (525AF45 excitation filter, 560DRLP dichroic mirror and 595AF60 band pass barrier filter). Observations were performed at between ×200 and ×400 magnification and the entire membrane surfaces were scanned for the presence of positive fluorescent signals. Each positive signal was recorded for *Prymnesium species* through the examination of the characteristic cellular features such as shape and size of *Prymnesium* cells. Other phytoplankton taxa and detritus material were confirmed from using the calcofluor/DAPI UV filter.

Field sampling

Killary Harbour is Ireland's only natural fjord located (53° N 27' W, 09° 48' W) in West Connemara, Co. Galway, Ireland (Fig. II-2). Six seawater samples were collected between May and September 2010 from either one of three stations in the inner (GY-KH-KI), middle (GY-KH-KM) and outer (GY-KH-KO) part of the estuary. These sites were chosen as they are routinely monitored by the Marine Institute (MI) as part of the Irish National Biotoxin Monitoring Programme (NMP) (Fig. II-2). At each sampling site unacidified Lugol's Iodine preserved samples (Throndsen 1978) collected from discrete depths were stored in 50 ml cell culture bottles and kept in the dark. Cell count determination was performed using an inverted microscope (Olympus CKX-41) following the procedure described in McDermott and Raine (2010). Water samples were pre-filtered through a 150 µm mesh. A total volume of 250-650 ml was filtered for subsequent RNA extraction through a 1 µm pore-size nitrocellulose filter (25 mm diam.), which was immediately immersed in 1 ml of TRI Reagent (Ambion) contained in 2 ml screw cap tubes. The samples were kept at 4 °C and within 6 hours of sampling stored at

-80°C on return to the laboratory. Water samples (1.0-2.0 l) for toxin analysis were filtered through glass fibre (Whatman GF/F, 47 mm diam.) filters. These were stored separately at -20 °C in 2 ml screw cap tubes and mailed to Queens University, Belfast, for extraction and analysis for PSP toxins (STX), Okadaic Acid (OA) + *Dinophysis* toxin (DTXs) and Domoic Acid (DA) using two different analytical platforms. The prototype multi SPR biosensor (GE Healthcare, Uppsala, Sweden) (Campbell et al., 2011) and a commercial ELISA kit (Centre d'Economie Rurale (CER), Belgium) (Dubois et al., 2010) were used for analysing toxin samples (Table II-S1; McNamee et al., 2013).

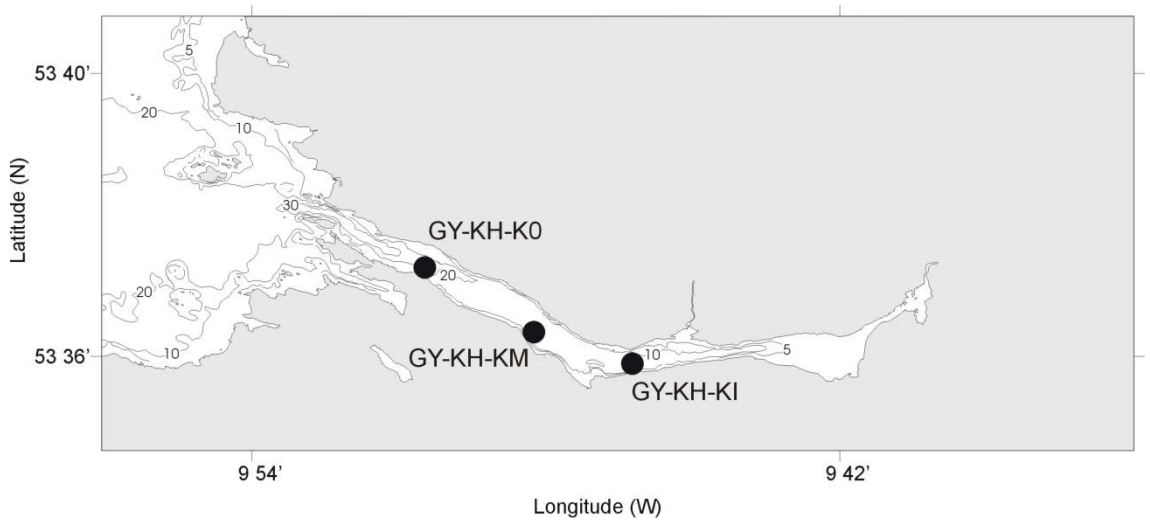

Fig. II-2. Map of Killary Harbour fjord, Co. Galway, Ireland showing the three sampling locations of inner: GY-KH-KI, middle: GY-KH-KM and outer: GY-KH-KO.

Microarray analysis

RNA extraction

For algal culture processing, approximately 5 to 15 ml of *P. parvum* and *P. polylepis* cell suspensions were placed in 15 ml polypropylene tubes and were centrifuged at 6,000 rcf for 10 minutes and the supernatant was then removed to leave around 2 ml of sample. Tubes were centrifuged a second time at 6,000 rcf for 5 minutes and the remaining supernatant completely removed using a micropipette and a vacuum pump, without disturbing the pellet. TRI-Reagent (1 ml) was immediately added to each pellet, homogenised and transferred to 2 ml screw cap tubes containing 0.1 ml of 212-300 µm acid washed glass beads (Sigma-Aldrich). Triplicate field samples were supplemented with an aliquot of TRI Reagent (25 µl) containing 125,000 cells of *Dunaliella tertiolecta* (UIO226 strain) to each filtered sample, acting as an internal control. An additional

triplicate field samples from each sampling site was also taken which was not spiked with *D. tertiolecta*. A control of 1 ml TRI-Reagent containing only 125,000 cells of *D. tertiolecta* was extracted in parallel along with the spiked and un-spiked field samples to validate the RNA extraction efficiency. The procedure was modified from the methods outlined in Kegel et al. (2013b) and McCoy et al. (2013). Frozen algal cultures and field samples were thawed and heated in a thermomixer at 60 °C for 10 minutes, at maximum shaking speed. The cells were also further disrupted twice during the heat treatment in a Hybaid RiboLyser at maximum speed for 20 seconds.

There followed a sequential extraction using 1-Bromo-3-chloro-propane (BCP:Sigma) and isopropanol (Sigma). An aliquot (100 µl) of BCP was added to the sample, the mixture was vortexed for 15 seconds and transferred to pre-spinned 2 ml heavy phase lock tubes (5-PRIME; 12,000g for 30 sec), which were then shaken (by hand) for 15 seconds and allowed to settle for 5 minutes at room temperature. The tubes were centrifuged at 4 °C for 15 minutes at 12,000g and the supernatant layer transferred to a clean 1.5 ml RNase-free tube. An equal volume of isopropanol was added (500-700 µl) and the tube was vortexed for 15 seconds. The tube was then kept at -20 °C for 1 hour, centrifuged again for 15 minutes at 4 °C, and the supernatant carefully removed using a micropipette. The RNA pellet was washed with 1 ml of 75% ethanol, centrifuged again and the supernatant was completely removed. After the final centrifugation step, the pellet was air dried for 3-5 min and suspended in 50–100 µl RNase free water by repeated flicking and vortexing. Nucleic acid concentrations in the sample were measured with a NanoDrop 1000 Spectrophotometer and the extract was stored at -80 °C. Prior to RNA labelling the eluted RNA underwent an ammonium acetate (NH_4Ac) precipitation step which was added to improve RNA quality and subsequent labelling efficiency for the V3.3 generation microarray hybridisations with field samples (Kegel et al 2013b; Lewis et al. 2012).

RNA labelling and fragmentation

The RNA (1 µg) was labelled with a CY5-ULS dye using a Platinum Bright 647 Infrared Nucleic Acid labelling kit (KREATECH Biotechnology) according to the manufacturer's instructions, with the modification of removing un-labelled RNA with Qiagen RNeasy Mini Kit (58 °C, 62 °C and 65 °C) or GE illustra MicroSpin G-50 columns (65 °C NWS) or KREApure columns (V3.3 and V3.3 microarray hybridisations). The concentration of

labelled dye was measured by NanoDrop (Microarray) and the degree of labelling (DoL %) was subsequently calculated. Fragmentation of the labelled RNA was carried out by adding 1/10 fragmentation buffer (100 mM ZnCl2 in 100 mM Tris-HCl pH 7), incubation in a thermomixer for 15 minutes at 70 °C, and the reaction was then stopped by adding 1/10 stop buffer (0.5 M EDTA pH 8) and placing samples on ice (Lewis et al. 2012; Kegel et al. 2013a; 2013b).

Internal control (TBP-Cy5) preparation

DNA from Bread Yeast powder (*Saccharomyces cerevisiae*) was extracted using Qiagen RNeasy Plant Mini Kit according to manufacturer's instructions. The PCR cycle and primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R_CY5 (5'-TTT TCA GAT CTA ACC TGC ACC C-3') were used to amplify the TATA-box binding protein (TBP) gene using the detailed procedure in the MIDTAL Manual (Lewis et al. 2012). The PCR program was as follows: initial step of 95 °C for 5 min, 40 cycle step (95 °C 1 min, 53 °C 1 min, 72 °C 2 min) and final step 72 °C for 5 min. The final PCR product was purified using the PCR MinElute Cleanup Kit (QIAGEN) and quantified with a Nanodrop and stored at -80 °C.

Probe development

Epoxysilane-coated microarray chips were pre-spotted with 113 (1st generation), 170 (2nd generation) and 140 (3rd generation) different oligonucleotide probes corresponding to a taxonomic hierarchy (kingdom, class, genus, clade and species) for a range of potentially harmful phytoplankton species. Probes were initially taken from those designed for FISH detection and were spotted and tested on the 1st generation microarray. On the 2nd generation microarray the probes were extended in length up to 18-25 base pairs and a further 15 nucleotide poly deoxythymidylic (dT) tail following the amino (NH_2) link at the 5' end was subsequently added for the 3rd generation chip. The addition of an Amino C6/MMT and Poly-T (15 nt) spacer was to lower cross reactivity between probes on the chip along with more stringent washing steps (Fig. II-1; Kegel et al. 2013b; Medlin et al. 2013). Probes for *Prymnesium* spp. hierarchy are provided in Table II-2. However, the 2nd and 3rd generation probe sequences are patent pending as the MIDTAL microarray can be obtained as a commercial kit, which is now available from Microbia Environnement (contact@microbiaenvironement.com).

Microarray hybridisation

Microarray hybridisations were carried out as described in Lewis et al. (2012) and Kegel et al. (2013a; 2013b) with the following modifications. The 1st and 2nd generation chips were pre-hybridised with 20 ml pre-hybridisation buffer (2 M NaCl; 20 mM Tris-Cl, pH 8.0; 0.01% Triton 100) for 60 minutes at a hybridisation temperature of 58 °C for the 1st generation chip and 62 °C and 65 °C for the 2nd generation chip. Slides were washed three times with deionised water (ddH_20) and dried using centrifugation in slide holders for 3 minutes at 1800 rpm.

The 3rd generation chip underwent a further blocking step by incubating the chips in blocking solution (0.02% SDS, 2 x SSC) for 20 minutes at 50 °C on a shaker ~70 rpm. The slides were washed once in 50 °C ddH_20 for 10 minutes and twice more with room temperature ddH_2O in the dark shaking ~70 rpm. A mixture of 35 µl 2x hybridisation buffer (1 mg/mL BSA, 0.2 µg/µL herring sperm DNA, 2 M NaCl, 20 mM Tris-Cl pH 8.0, 0.01% Triton 100; Lewis et al. 2012) containing the labelled *P. parvum* or *P. polylepis* RNA or labelled field sample (1 µg) and 3 µl of the TATA-box positive control (TBP-control) was prepared and made up to a final volume of 70 µl with RNase-free water. The hybridisation mixture was then incubated at 94 °C for 5 minutes to denature the target labelled nucleic acid. After denaturation, 15 µl of KREAblock (KREATECH) was added to the 3.3 version hybridisations (Kegel et al. 2013b). MicroArray mSeries LifterSlips (20 x 25 mm) (Thermo Scientific) were placed on the microarray and half (35 µl) of the hybridisation mixture was added to each array. Hybridisation was carried out at 58 °C for the 1st generation chip; 62 °C and 65 °C for the 2nd generation chip and set at 65 °C for the subsequent 3rd generation chips for 1 hour in a wet chamber comprising wet Whatman filter paper in a screw-capped 50 ml centrifuge tube (Falcon).

After 1 hour, the cover slips were removed off the array and the chip surface underwent three washing buffer steps with increasing buffer stringency in the dark while shaking (300 rpm) was applied. The first (2x SSC/10 mM EDTA/0.05% SDS) and second (0,5x SSC/10 mM EDTA) wash steps were carried out at room temperature for 10 minutes for the 3 generation chips tested. The third (0.2x SSC/10 mM EDTA) wash step was also performed at room temperature for 10 minutes for hybridisations conditions of 58 °C, 62 °C and 65 °C, however for the 65 °C new wash step (NWS) condition was performed at 50 °C for 10 minutes to minimise background noise and removal of unspecific binding of probes (Lewis et al. 2012; Kegel et al. 2013a; 2013b). The chip was scanned (Perkin

Elmer Microarray Scanner) and the fluorescence signal intensity from each probe was measured.

Scanning and analysis

The microarray chip was scanned (Perkin Elmer Microarray Scanner) with a resolution of 5 µm and excitation wavelength of 635 nm. The scanned image output (.tiff files) was then uploaded into GenePix 6.0 software programme. With the aid of uploaded gal files for 1st generation (midtal_array_ver1.3_20090428.gal), 2nd generation (midtal_ver252_20100423.gal), 3.2 version microarray (midtal_ver32_20110429.gal) and 3.3 version microarray (MIDTAL_V3.3.gal), which is a gridded map corresponding to each individual probe spotted onto the microarray chip so the fluorescent signals and background intensities can be calculated for each probe (Kegel et al. 2013a; 2013b). Results were saved as a separate GPR file, which was imported to the PhylochipAnalyzer graphical Windows programme or GPR-Analyzer version 1.27, which describes the hierarchical level of the probes from high to low signal intensities (Dittami and Edvardsen, 2013a). The signal-to-noise ratio of 2.0 was set as a cut-off for positive signals. Total signal intensities were normalised against the positive control Poly-T-CY5, POSITIVE_25_dT and DunGS02_25_dT_dT to quantify and compare results from different hybridisations.

Statistical analysis

Analysis by 1-way ANOVA followed by a Tukey multiple comparison test was carried out to determine if there were significance differences for (1) the number of false positive recordings between 1st and 2nd generation microarrays and (2) the comparison of TATA box protein amounts in hybridisations performed with the 2nd and 3rd generation microarrays. The level of significance was analysed by a paired t-test for (3) comparing the average difference between spiked and un-spiked field samples with the average RNA amounts from six *D. tertiolecta* controls and (4) assessing the variation of degrees of labelling (DoL %) concentrations between 3.2 and 3.3 version RNA extracts with or without the addition of RNA precipitate (NH_4Ac) clean up steps prior to labelling.

RESULTS

FISH analysis

Re-evaluation of existing FISH probes

The optimal hybridisation conditions were determined to be 50 °C with 20% FA for probes PRYM01, PRYM02, PRYM03 and PRYMGL01A, PRYMGL02B (Fig. II-S1a to e). Out of the three higher group and class level probes, PRYM02 probe produced the brightest fluorescing signal. The clade level probes (Clade01) optimal conditions were set at 54 °C and 20% FA and species level probes for *P. parvum* (PRYM694; 50 °C and 10% FA) and *P. polylepis* (Cpoly01; 50 °C and 15% FA) are represented as micrograph images in Fig. II-S1f. The results presented here are consistent with previous studies who were the original designers of the range of oligonucleotide probes being tested (Table II-2).

Cross reactivity testing: FISH

Cross reactivity testing was carried out on *P. parvum and P. polylepis* cultures supplemented with aliquots of North Channel in Cork Harbour field samples showed no apparent cross-reactivity issues with all the probes tested PRYM01, PRYM02, PRYM03, PRYMGL01A, PRYMGL01B, Clade01, Cpoly01 and PRYM694 (Fig. II-S2a to g).

Microarray analysis

Prymnesium spp. microarray analysis

RNA labelling

The degrees of labelling (DoL %) were between 0.3 and 1.2 % when testing *Prymnesium* cultures on the 1st, 2nd and 3rd generation microarrays (Table II-3).

Table II-3. The 1st, 2nd and 3rd generation microarray with corresponding hybridisation temperatures and wash steps, concentration of TBP-control (ng) added, total labelled *P. parvum* and *P. polylepis* RNA hybridised (ng) and the degrees of RNA labelling (DoL %).

Species tested	Microarray generation	Hybridisation temperature	TBP amount (ng)	RNA hybridised (ng)	DoL %
P. parvum	1st generation	58 °C		500	0.3
P. polylepis	1st generation	58 °C		500	0.4
P. parvum	2nd generation	62 °C		500	0.5
P. polylepis	2nd generation	62 °C		500	1.1
P. parvum	2nd generation	65 °C		500	0.5
P. polylepis	2nd generation	65 °C		500	1.1
P. parvum	2nd generation	65 °C NWS*		500	0.8
P. polylepis	2nd generation	65 °C NWS*		500	1.2
P. parvum & P. polylepis	2nd generation	65 °C NWS*	50	300	0.4
P. parvum & P. polylepis	3rd generation	65 °C NWS*	50	300	0.4
P. parvum & P. polylepis	3rd generation	65 °C NWS*	5	300	0.4

*New Wash Step (NWS)

Microarray probes

Probe signal intensities on the microarray were recorded as positive when they produced a signal-to-noise ratio ≥2. Signal intensities were normalised against the spotted control Poly-T-CY5 from higher group to species level probes when either labelled *P. parvum* or *P. polylepis* RNA was hybridised to the microarrays and the various hybridisation conditions tested (Fig. II-3b and d). Both eukaryote probes EUK1209 and EUK328 produced positive signals when hybridised with labelled *P. parvum* or *P. polylepis* RNA on the 1st generation microarray. However, the EUK1209 probe was 24 to 28 times more sensitive than the EUK328 probe, which barely reached the threshold level on the 1st generation chip and did not for all the hybridisation conditions tested on the 2nd generation chip (Fig. II-3a and c). The extended EukS_1209_25 and EukS_328_25 probes returned positive signals in all the 2nd generation hybridisation conditions, EukS_1209_25 consistently returning a higher signal compared to EukS_328_25_dT.

The Prymnesiophyta higher group probes (PRYM01 and PRYM02) and the Prymnesiophyceae class level probe (PRYM03) spotted on the 1st generation chip and their corresponding extended probes on the 2nd generation chip (PrymS01_25, PrymS02_25 and PrymS03_25) all returned positive signals, PRYM02/PrymS02_25 constantly returning higher signal out of the three. The genus level probes specific for *Prymnesium* species on the 1st generation chip (PRYMGL01A and PRYMGL02B) both returned positive microarray signals for *P. parvum* and *P. polylepis* hybridisations. However, out of the two corresponding extended 2nd generation probes (PrymGS01_25 and PrymGS02_25), only PrymGS01_25 produced a positive hybridisation signal ≥2 when labelled with *P. parvum* RNA across all the conditions tested (62 °C, 65 °C and 65 °C NWS), but did not for *P. polylepis* RNA.

The clade level probe Clade01 (Prymnesium B1 clade) is the same oligonucleotide sequence on the 1st and 2nd generation microarray and emitted a signal intensity above the threshold limit across all the generations, testing conditions and *Prymnesium* species used for the analyses. The species-specific probe for *Prymnesium parvum* (PRYM694) was spotted on the 1st and 2nd microarray generations without any new modification to its oligonucleotide sequence (Table II-2). This probe produced a positive signal for hybridisations with labelled *P. parvum* RNA for all the conditions tested on the 1st and 2nd generation chip, but also recorded signal-to-noise ratio values ≥2 for the *P. polylepis* specific probes Cpoly01 and Cpoly01_25. Species-specific probe Cpoly01 (1st and 2nd generation) and Cpoly01_25 (2nd generation) for *Prymnesium* (=*Chrysochromulina*) *polylepis* both returned positive microarray signals for both generations and testing conditions when hybridised with labelled *P. polylepis* RNA. However, there were also non-specific signals recorded for the PRYM694 probe only for the 1st generation hybridisations with labelled *P. polylepis* RNA (Fig. II-3c and d).

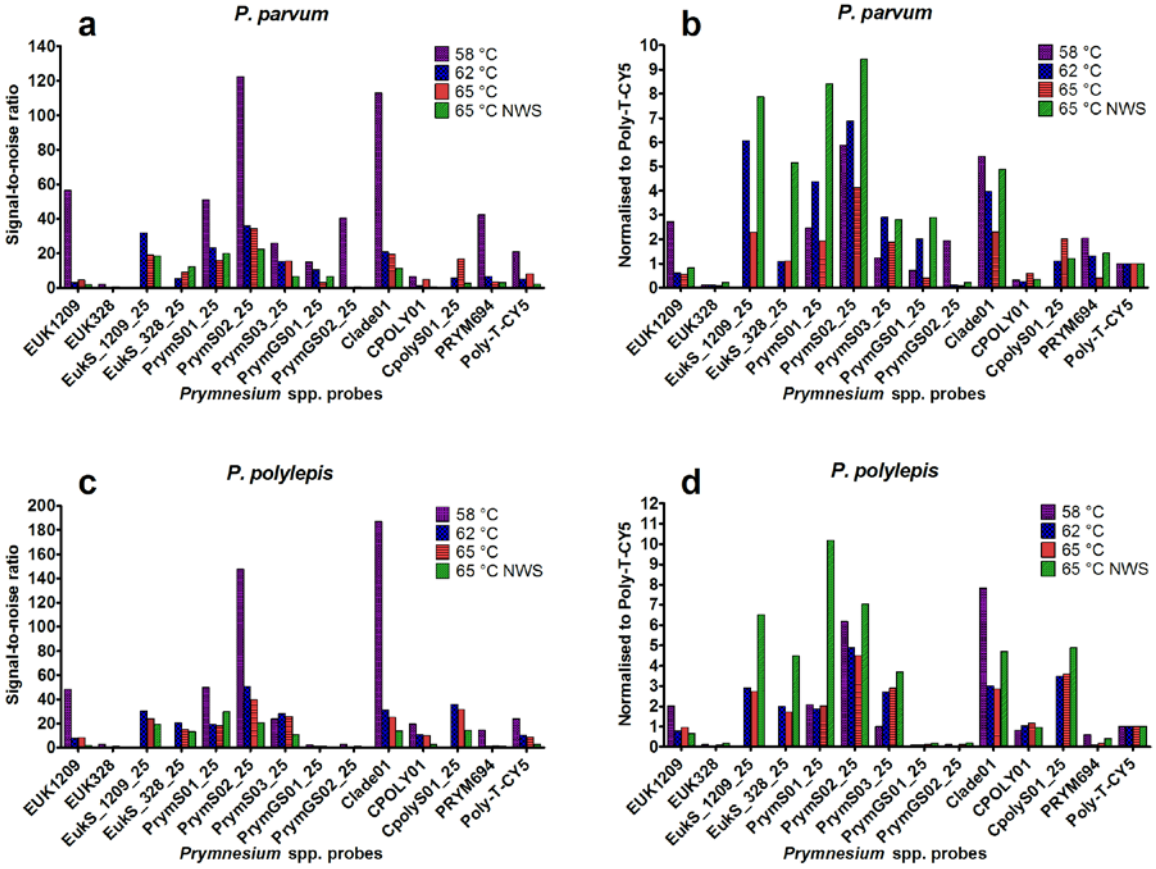

Fig. II-3. (a and c) Microarray signal-to-noise ratio values and (b and d) normalised to Poly-T-CY5 results for labelled *P. parvum* and *P. polylepis* RNA hybridised to the 1st and 2nd generation microarrays. The 1st generation microarray was hybridisation at a temperature of 58 °C and all three wash steps were carried out at room temperature for 10 minutes. The subsequent two hybridisations were carried out on the 2nd generation microarray, with hybridisation temperatures set at 62 °C and 65 °C, all three wash steps were carried out at room temperature for 10 minutes. The final hybridisation was incubated at 65 °C NWS on the 2nd generation microarray, with the first two wash steps at room temperature for 10 minutes and the final wash step incubated at 50 °C for 10 minutes. New Wash Step (NWS).

Cross reactivity testing: microarray

The 1st generation microarray designed for a defined range of HAB species produced low specificity with high cross reactivity for several species-probe combinations, with 48 % and 78 % cross reactivity recorded for hybridisations performed with pure cultures of *P. parvum* RNA and *P. polylepis* RNA respectively (Table II-4). The percentage of false positives decreased from the 1st to the 2nd generation by up to 44 % and 40 % with labelled *P. parvum* RNA hybridisations and up to 67 % and 64 % with labelled *P. polylepis* RNA hybridisations carried out at 62 °C and 65 °C respectively (Table II-4). The microarray hybridisation carried out at 65 °C with the additional third washing step incubated at 50 °C decreased the number of false positives for both labelled *P. parvum* RNA and *P. polylepis* RNA hybridisations by a further 5 % and 9 % respectively (Table II-4). With these adaptations in place, false positives were significantly ($P < 0.05$) reduced from the 1st (58 °C) to the 2nd (62 °C and 65 °C NWS) generation microarray.

TATA box protein control concentrations

An additional 15 nucleotides in the form of a Poly-T tail was added to the 5' end of the sequence for probes spotted on the 3rd generation chip. To compare the performance of the 2nd and 3rd microarrays, hybridisations with the addition of labelled TATA box protein (TBP) included in the hybridisation mix containing a combination of both labelled *P. parvum* and *P. polylepis* RNA was undertaken. Signal-to-noise ratio values observed with corresponding *Prymnesium* spp. probes from the 3rd generation microarray were higher than the 2nd generation microarray indicating improved molecule and probe interactions (Fig. II-4a). However, this was not apparent when normalised to the Poly-T-CY5 control probe (Fig. II-4b). The concentration of the TBP control was initially set at 50 ng per hybridisation; this concentration was saturating the signal intensity of the POSITIVE_25_dT probe and was therefore reduced to 5 ng (Fig. II-4). This reduction in the amount of TBP added to the hybridisation mix for the *Prymnesium* spp. hybridisations significantly ($P < 0.05$) increased the POSITIVE_25_dT normalised microarray signal values of the 3rd generation hybridisations (5 ng TBP) compared to the 2nd and 3rd generation hybridisations carried out with 50 ng of TBP (Fig. II-4c).

Table II-4. The 1st and 2nd generation of the MIDTAL microarrays tested under various hybridisation temperatures and washing conditions. Corresponding cross reactivity percentages (%) are the false positive signal-to-noise ratio values above the threshold limit set at ≥ 2 for non-specific probes when labelled *P. parvum* and *P. polylepis* RNA is hybridised to the microarray.

Microarray generation	**1st**		**2nd**		**2nd**		**2nd**	
Hybridisation temperature	58 °C		62 °C		65 °C		65 °C NWS	
Washing step	X3 (10 min, RT)		X3 (10 min, RT)		X3 (10 min, RT)		X2 (10 min, RT); X1 (10 min, 50 °C)	
No. probes on chip	113		170		170		170	
Species tested	*P. parvum*	*P. polylepis*	*P. parvum*	*P. polylepis*	*P. parvum*	*P. polylepis*	*P. parvum*	*P. polylepis*
Cross reactivity %	**48**	**78**	**4**	**11**	**8**	**14**	**3**	**5**

Room Temperature (RT); New Wash Step (NWS); minutes (min)

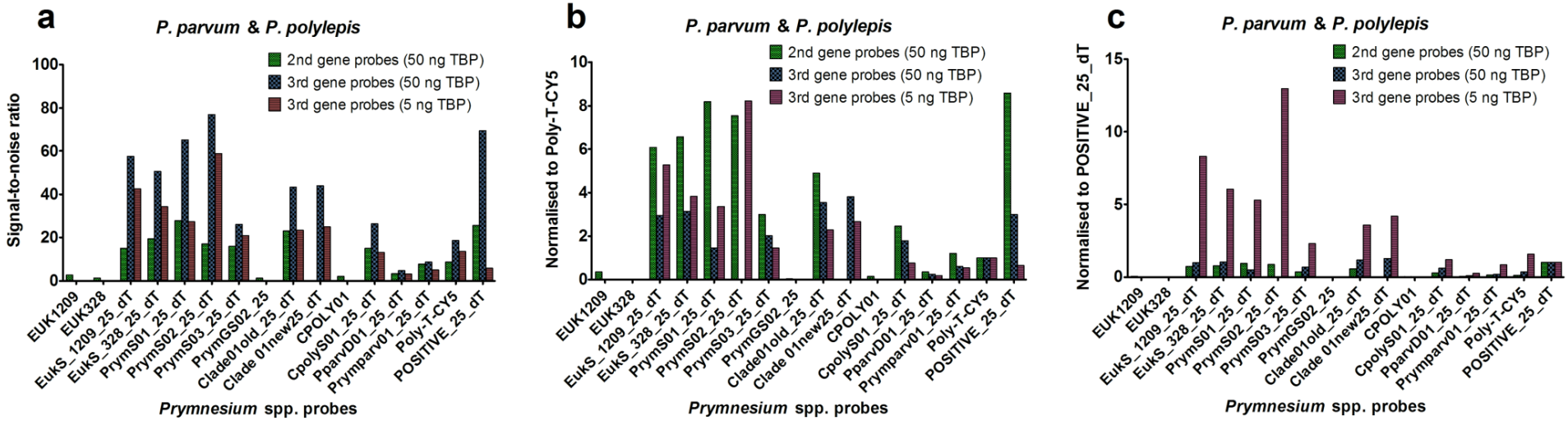

Fig. II-4. Testing the TATA box protein (TBP) positive control (POSITIVE_25_dT) concentrations with pooled labelled *P. parvum* and *P. polylepis* RNA hybridised on the 2nd generation and 3rd generation microarrays. Note: "gene" indicates generation, 50 and 5 ng TBP indicates the concentration added to hybridisation mix.

Field testing of 3.2 and 3.3 version MIDTAL microarray

RNA extraction efficiency

The comparison of RNA extracts from field samples with and without the spiking of 125,000 *Dunaliella tertiolecta* cells prior to RNA extraction is shown in Fig. II-5. The average difference between spiked and un-spiked field samples (413 ± 246) was not significantly ($P > 0.05$) different to the average RNA amount (485 ± 173) extracted from the *D. tertiolecta* controls, indicating a proficient RNA extraction efficiency (Fig. II-5).

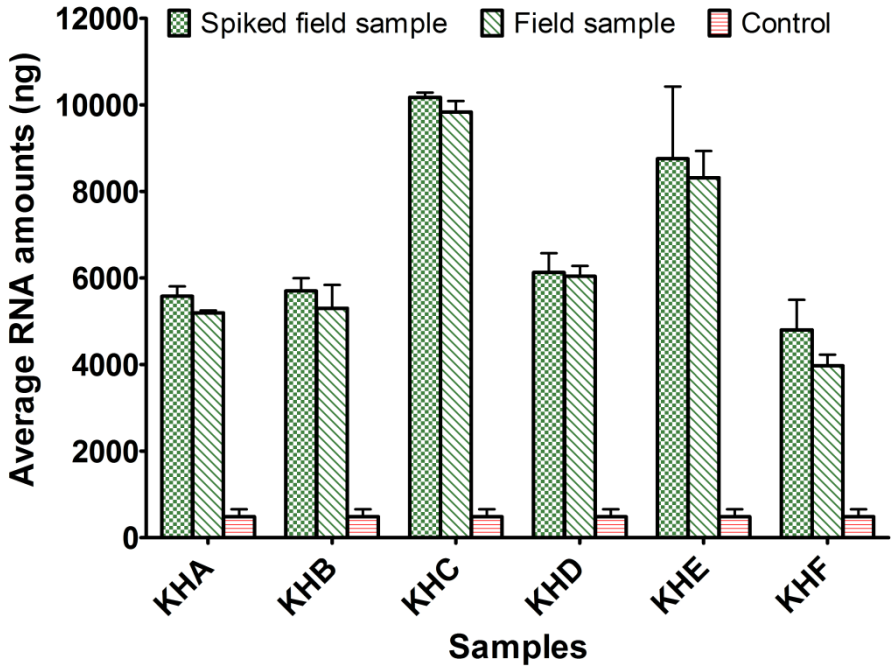

Fig. II-5. RNA extraction efficiency of Killary Harbour field samples from the 2010 sampling survey. The average RNA amount extracted from triplicate field samples spiked with or without 125,000 *D. tertiolecta* cells and the average RNA amount of six controls containing only 125,000 *D. tertiolecta* cells were extracted in parallel and compared.

RNA labelling

The degrees of labelling (DoL %) were between 0.3 and 0.7 % for hybridisations to the 3.2 version microarray with RNA extracted from the Killary Harbour samples. Prior to labelling the RNA pellet did not go through the NH_4Ac precipitate step to further clean up the RNA extract. However, when the NH_4Ac precipitate step was added to the 3.3 version protocol, the DoL % were significantly ($P < 0.05$) increased and all above the

minimum optimal DoL % requirement outlined by Kreatech Biotechnology nucleic acid labelling kit guidelines (Table II-5).

Table II-5. The 3rd generation microarray DoL % results from the 3.2 and 3.3 version hybridisations with six labelled field samples from Killary Harbour taken during the 2010 summer sampling survey.

Station name	Station code	Date	Sampling site	Vol filtered (ml)	Total RNA extracted (ng)	3.2 version labelling DoL %	3.3 version labelling DoL %
KH1601	KHA	31.05.10	middle	500	5578	0.3	2.3
KH1802	KHB	14.06.10	middle	500	5704	0.3	5.4
KH2101	KHC	05.07.10	inner	250	10172	0.3	2.6
KH2402	KHD	26.07.10	outer	650	6131	0.1	2.3
KH2703	KHE	16.08.10	outer	500	8756	0.2	1.5
KH3101	KHF	13.09.10	outer	600	4799	0.7	2.8

Comparison of 3.2 and 3.3 version microarray field results

The results of both the 3.2 and 3.3 version protocols were normalised against POSITIVE_25_dT and DunGS02_25_dT and plotted with corresponding cell counts to validate microarray results (Fig. II-6 to II-9).

Pseudo-nitzschia groups

Pseudo-nitzschia groups were detected in five out of six samples with KHE being the only sample in which their presence was not detected by LM (Fig. II-6a and b). The highest concentration of *Pseudo-nitzschia* was observed in sample KHF, reaching 70,000 cells L^{-1}, whereas in the remaining four samples the cell concentrations were all below 1,700 cells L^{-1} (Fig II-6). Only three *Pseudo-nitzschia* general probes (PgalaD01_25_dT, PmulacalD02_25_dT and PmulaD03_25_dT) were detected from the V3.2 microarray hybridisations compared to nine probes highlighting from the V3.3 microarray hybridisations, results of which are represented as normalised microarray signal against the control probes POSITIVE_25_dT and DunGS02_25_dT_dT in Fig. II-6a and 6b, respectively. Of the three V3.2 probes, two (PgalaD01_25_dT, PmulacalD02_25_dT) were highlighted from sample KHE with one V3.3 probe (PmulacalD02_25_dT) also producing a signal from this sample (Fig. II-6a and b).

Pseudo-nitzschia delicatissima groups were recorded present in LM counts from samples KHA, KHB and KHC with concentrations below 1,600 cells L^{-1} (Fig. II-6c and d). Three *P. delicatissma* group probes (Pdel4D03_25_dT Pdel3B_25_dT and Pman2D03_25_dT) out of the six V3.2 probes were highlighted in sample KHE. This was the only sample were the absence of *Pseudo-nitzschia* in LM counts was recorded.

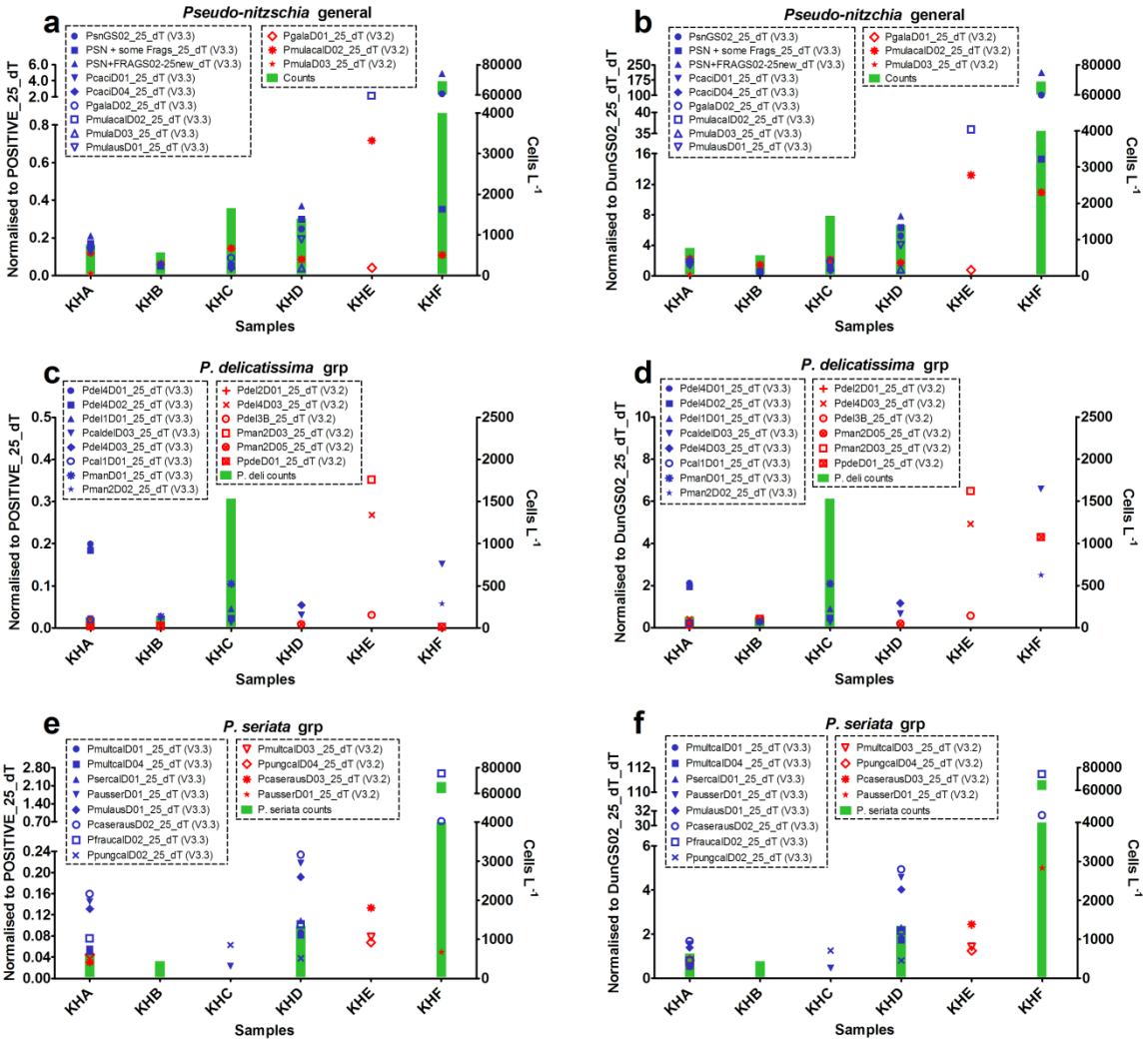

Fig. II-6. *Pseudo-nitzschia* group microarray results and cell counts from Killary Harbour 2010 sampling survey. The V3.2 (red) and V3.3 (blue) version (V) protocol signal intensity results from the 3rd generation microarray were normalised against POSITIVE_25_dT (a, c and e) and DunGS02_25_dT (b, d and f) and plotted with cell counts for *Pseudo-nitzchia* general, *P. delicatissima* and *P. seriata* groups.

LM counts indicated the presence of *Pseudo-nitzschia seriata* groups in four of the samples KHA, KHB, KHD and KHF (Fig. II-6e and f). Three out of four of *P. seriata* group probes were highlighted in sample KHE for V3.2 microarray hybridisations which indicated the presence of *P. multiseries* (PmultcalD03 _25_dT), *P. pungens* (PpungcalD04_25_dT) and *P. australis/seriata* (PcaserausD03_25_dT). Also from these hybridisations the same *P. pungens* (PpungcalD04_25_dT) and *P. australis/seriata* (PcaserausD03_25_dT) probes were highlighted in sample KHA and an additional *P. australis/seriata* probe (PausserD01_25_dT) was also recorded in sample KHF (Fig. II-6e and f). The highest diversity of *P. seriata* grp V3.3 probes was recorded from sample KHD, indicating the presence of *P. australis/seriata* (PcaserausD02_25_dT, PausserD01_25_dT and PmulausD01_25_dT), *P. multiseries* (PmultcalD01 _25_dT PmultcalD04 _25_dT) *P. seriata* (PsercalD01_25_dT) *P. fraudulenta* (PfraucalD02_25_dT) and *P. pungens* (PpungcalD02_25_dT; Fig. II-6e and f). This was also the case for the sample KHA with only two less *P. seriata* grp V3.3 probe (PmultcalD01 _25_dT and PpungcalD02_25_dT) recorded compared to sample KHD.

Dinophysis

Dinophysis acuta and *D. acuminata* were both detected and recorded in LM counts from sample KHB (130 and 190 cells L^{-1}, respectively), with *D. acuta* being also recorded in sample KHD and *D. acuminata* in sample KHC, both at low cell concentrations of 64 cells L^{-1} (Fig. II-7a and b). The only V3.2 probe to produce a microarray signal-to-noise ratio ≥2 was DacutaS01_25_dT in sample KHA. The only sample to be highlighted with the *Dinophysis* family (DphyFS02_25_dT) probe was KHE from the V3.3 microarray hybridisations, but *Dinophysis* species were not recorded in the LM counts for this sample (Fig. II-7a and b). Two genus-specific probes (DphyGD02_25_dT and DphyGS04_25_dT) were highlighted in sample KHB and a third genus specific probe (DphyGS01_25_dT) was individually highlighted in sample KHC from the V3.3 microarray hybridisations. These samples contained individually either *D. acuta* and *D. acuminata* species or both, as determined from LM counts (Fig. II-7a and b). Species level probes *D. acuta* (DacutaD02_25_dT) and *D. rotundata* (ProtuS01_25_dT) were independently highlighted in samples KHD and KHC, respectively. This is also confirmed with the presence of *D. acuta* in KHD as mentioned above, although *D. acuminata* was counted in sample KHC, the corresponding DacumiD02_25_dT probe did not produce a signal-to-noise ratio ≥2 (Fig. II-7a and b).

Prorocentrum

Prorocentrum micans was observed in three LM counts from samples KHB, KHD and KHE (Fig. II-7c and d). The *P. micans* probe PmicaD02_25_dT was positively highlighted in the samples KHC and KHD for V3.3 normalised microarray hybridisations (Fig. II-7c and d). The *Prorocentrum lima* probe PlimaFD01-25_dT recorded a positive microarray signal for samples KHA and KHB in V3.3 microarray hybridisations and in sample KHD in V3.2 normalised microarray hybridisations. However *P. lima* was not recorded in LM counts (Fig. II-7c and d).

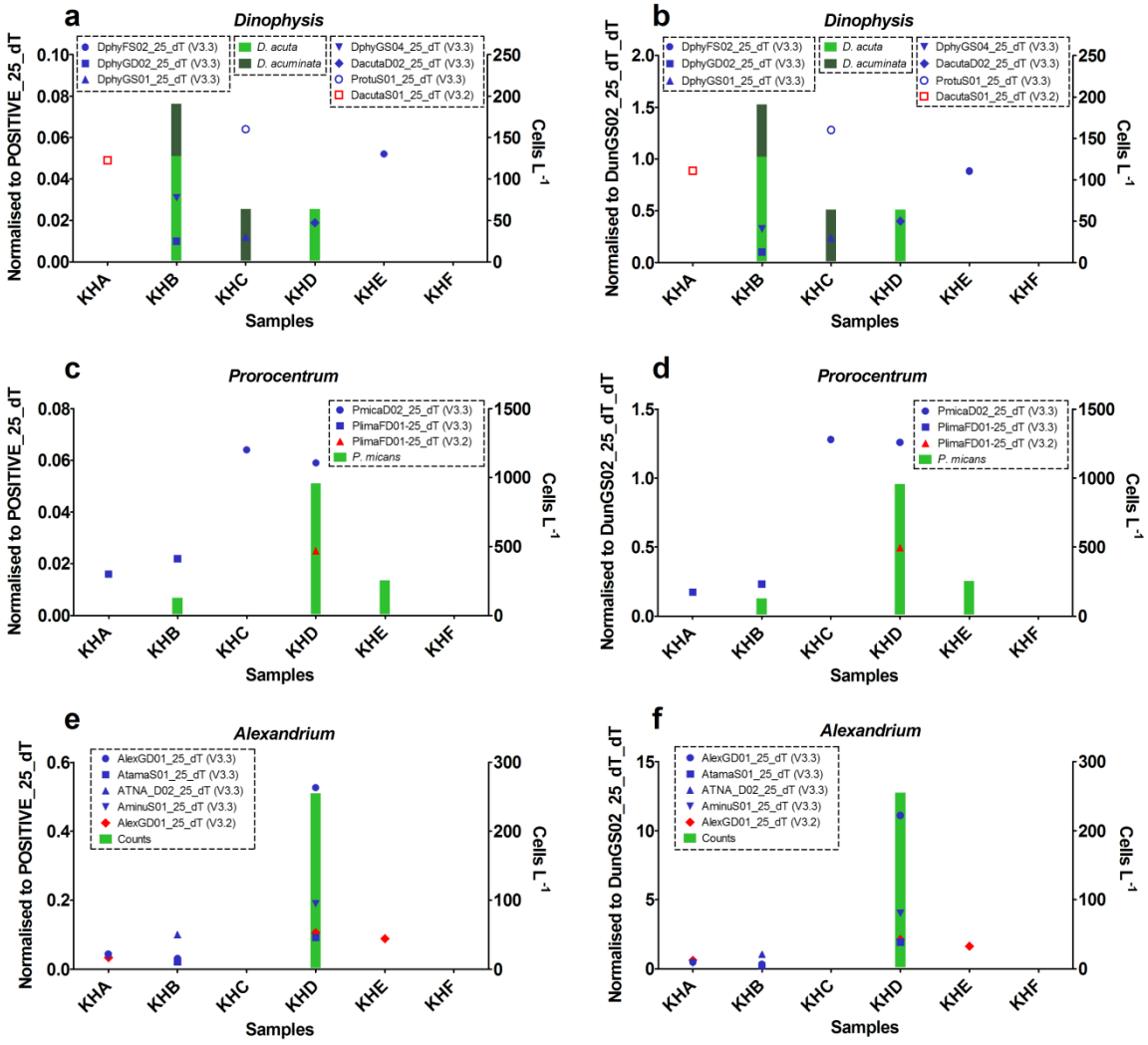

Fig. II-7. Microarray results for *Dinophysis sp.*, *Prorocentrum sp.* and *Alexandrium sp.* and corresponding cell counts from Killary Harbour 2010 sampling survey. The V3.2 (red) and V3.3 (blue) version (V) protocol signal intensity results from the 3rd generation microarray were normalised against POSITIVE_25_dT (a, c and e) and DunGS02_25_dT (b, d, and f) and plotted with LM cell counts.

Alexandrium

Alexandrium counts were compared against both genus level probes (AlexGD01_25_dT) and the species specific probes AtamaS01_25_dT (species complex probe), AminuS01_25_dT (*A. minutum*) and ATNA_D02_25_dT (*A. tamarense* NA group I ribotype; Fig. II-7e and 7f). The only sample to have the presence of *Alexandrium* genus recorded with LM counts was KHD, which equated to approximately 255 cells/L^{-1} (Fig. II-7g and h). Half the samples produced a positive microarray signal-to-noise ratio value above the threshold level with the *Alexandrium* genus (AlexGD01_25_dT) probe from both the V3.3 (KHA, KHB and KHD) and V3.2 (KHA, KHD and KHE) hybridisations (Fig. II-7e and f). The highest POSITIVE_25_dT (0.53; Fig. II-7e) and DunGS02_25_dT_dT (11.11; Fig. II-7f) normalised microarray signal was observed in station KHD. The lowest normalised signals was recorded from sample KHB (POSITIVE_25_dT 0.03 and DunGS02_25_dT_dT 0.33; respectively, Fig. II-7e and f). Microarray hybridisations using the V3.3 protocol from sample KHD were the only real positive recordings for *A. tamarense* species complex and *A. minutum* microarray probe signals, as the *Alexandrium* genus level probe produced a higher signal-to-noise ratio value. Due to the implementation of a hierarchical file, microarray signals for AminuS01_25_dT (*A. minutum*; S/N ratio 4.1) in sample KHA and AtamaS01_25_dT (*A. tamarense* species complex; S/N ratio 2.2 and ATNA_D02_25_dT (*A. tamarense* NA; S/N ratio 6.7) in sample KHB are recorded as false positives, because the AlexGD01_25_dT (*Alexandrium* genus; S/N ratio 2.0 and 2.1, respectively) probe did not produce a higher signal-to-noise ratio values (Fig. II-7e and f).

Heterosigma

LM counts were not recorded for *Heterosigma* and therefore this species could have possibly been present across the Killary Harbour 2010 sample set (Fig. II-8a and b). Two *Heterosigma* probes (LSHaka0329A25_dT and LSHaka0358A24_dT) produced a signal in V3.2 microarray hybridisations for samples KHA and KHE, respectively (Fig. II-8a and b). Five *Heterosigma* probes out of seven were highlighting from the V3.3 hybridisations, of which the two remaining probes were the only probes recording microarray signals on the V3.2 microarray mentioned above (Fig. II-8a and b).

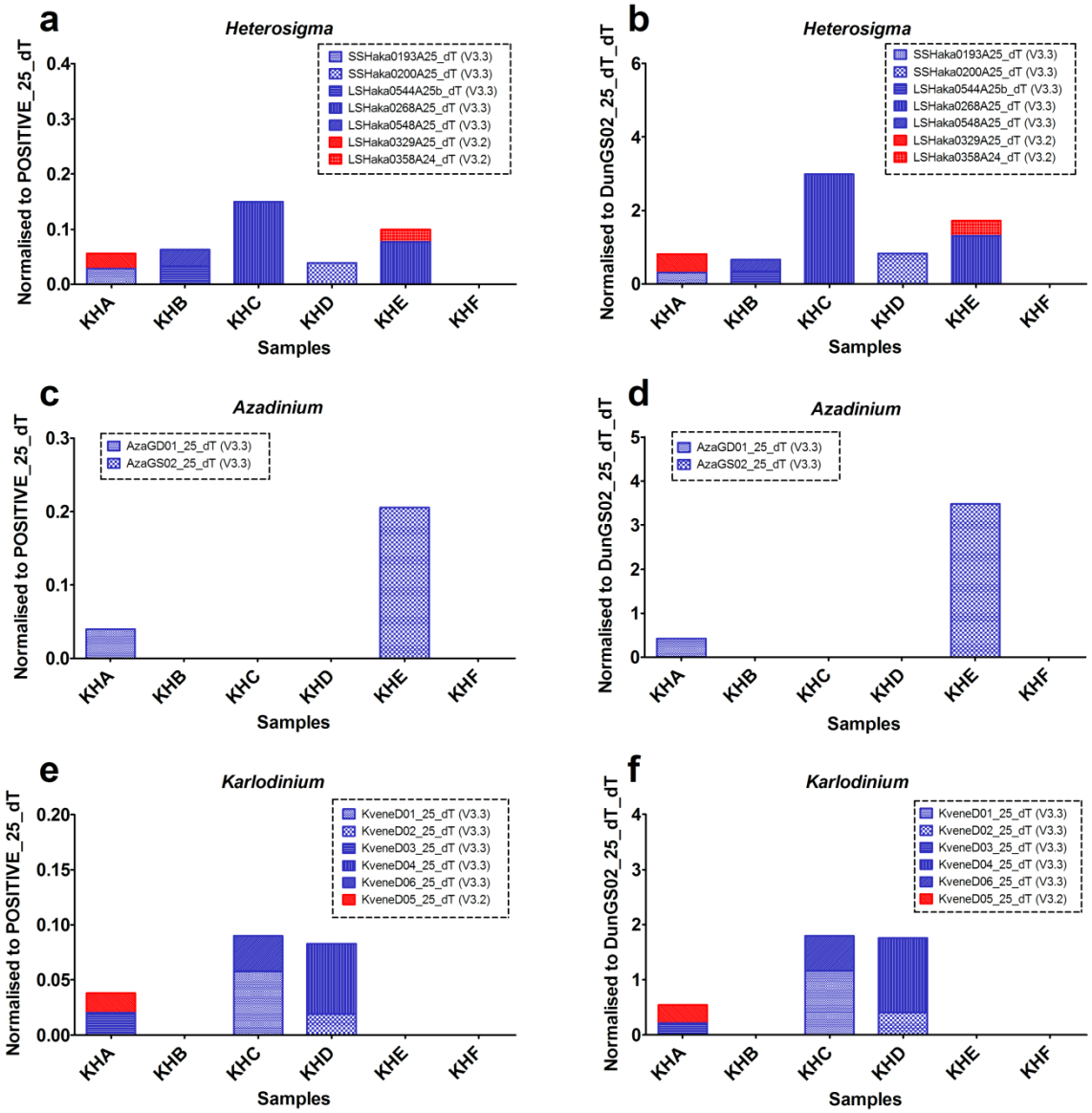

Fig. II-8. Microarray normalised signal intensity's against POSITIVE_25_dT (a, c, and e) and DunGS02_25_dT (b, d, and f) for *Heterosigma* sp., *Azadinium* sp. and *Karlodinium* sp. from Killary Harbour 2010 sampling survey. Cell counts were not recorded by LM and only the V3.2 (red) and V3.3 (blue) version (V) protocol signal intensity results from the 3rd generation microarray are plotted. Note: bars are stacked, each bar starts at the top of the one below.

Azadinium

Azadinium probes AzaGD01_25_dT and AzaGS02_25_dT were highlighted in V3.3 hybridisations from samples KHA and KHE, respectively (Fig. II-8c and 8d). This group was not detected in LM counts and would have only been recorded to genus level as *Heterocapsa* sp.

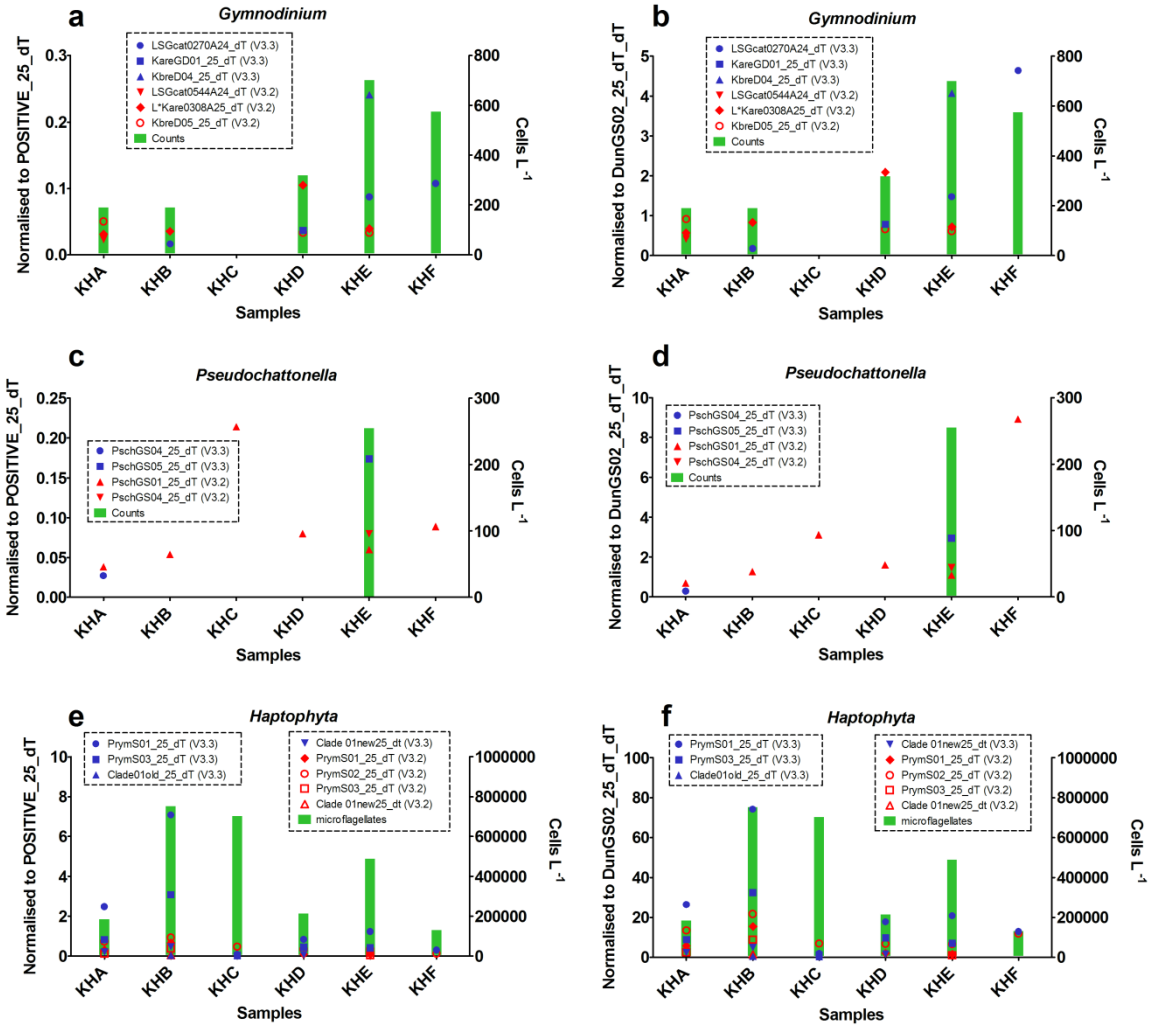

Fig. II-9. Microarray results for *Gymnodinium* sp., (a and b), *Pseudochattonella* sp. (c and d) and *Haptophyta* sp. (e and f), respectively from Killary Harbour 2010 sampling survey. The V3.2 (red) and V3.3 (blue) version (V) protocol signal intensity results from the 3rd generation microarray were normalised against POSITIVE_25_dT and DunGS02_25_dT and plotted with *Gymnodinium* sp (a and b), *Pseudochattonella* sp. (c and d) and total microflagellate LM counts (e and f).

Karlodinium

The only *Karlodinium* probe to record a microarray signal from the V3.2 hybridisations was KveneD05_25_dT (POSITIVE_25_dT 0.02 and DunGS02_25_dT_dT 0.33), however with V3.3 hybridisations this was the only probe that did not highlight across the six *Karlodinium* probes spotted on the V3.3 generation microarray (Fig. II-8e and f). *Karlodinium* was not counted for by LM.

Gymnodinium

The *Gymnodinium* genus was identified by LM and was recorded in all samples analysed except for one sample KHC taken on the 5th July 2010 (Fig. II-9a and b). The *Gymnodinium catenatum* probe LSGcat0544A24_dT from the V3.2 microarray only produced one microarray signal above the threshold limit in sample KHA. Two other probes for *Karenia* genus (L*Kare0308A25_dT) and *Karenia brevis* (KbreD05_25_dT) both recorded in samples KHA, KHD and KHE, respectively, with an additional microarray signal recorded in sample KHB for the probe L*Kare0308A25_dT on the V3.2 microarray (Fig. II-9a and b). An additional *G. catenatum* probe LSGcat0270A24_dT produced microarray signals from samples KHB, KHE and KHF from the V3.3 microarray hybridisations (Fig. II-9a and b). *Karenia* genus (KareGD01_25_dT) and *K brevis* (KbreD04_25_dT) probes also were detected by the V3.3 microarray for samples KHD and KHE, respectively (Fig. II-9a and b).

Pseudochattonella

LM counts observed the presence of *Pseudochattonella* sp. in one sample (KHE; 255 cells/Litre) taken on the 16th of August (Fig. II-9c and d). *Pseudochattonella* genus probe PschGS01_25_dT was highlighting in every sample with hybridisations on the V3.2 microarray. PschGS04_25_dT produced a microarray signal for the V3.2 KHE sample (POSITIVE_25_dT 0.08 and DunGS02_25_dT_dT 1.47), however, the corresponding probe on the V3.3 microarray was not highlighting for this sample, but did indicate the presence of *Pseudochattonella* sp. in sample KHA even though it was not observed in the LM counts (POSITIVE_25_dT 0.03 and DunGS02_25_dT_dT 0.28; Fig. II-9c and d). A positive microarray signal hybridised on V3.3 was recorded by genus probe PschGS05_25_dT (POSITIVE_25_dT 0.17 and DunGS02_25_dT_dT 2.95) for sample KHE as well (Fig. II-9c and d).

Haptophyta

Total microflagellate assemblage counts under LM comprising of the Cryptophytes, Prasinophytes, Prymnesiophytes and unknown microflagellates were compared with microarray results from the V3.2 and V3.3 hybridisations, which have been normalised to the internal control probes POSITIVE_25_dT and DunGS02_25_dT_dT (Fig. II-9e and f). All higher group probes PrymS01_25_dT, PrymS02_25_dT and PrymS03_25_dT from V3.2 microarray and PrymS01_25_dT and PrymS03_25_dT from V3.3 microarray were highlighted, however the signal intensities were higher with a greater number of S/N ratio values ≥2 detected in the V3.3 version compared to V3.2 hybridisations (Fig. II-9e and f). Clade level probes Clade01old_25_dT (samples KHB and KHC) and Clade01new_25_dT (samples KHA, KHB and KHD) on the V3.3 microarray and Clade01new_25_dT (sample KHB) on V3.2 microarray were also highlighted (Fig. II-9e and f). None of the *Prymnesium* species level probes PparvD01_25_dT, Prymparv01_25_dT and CpolyS01_25_dT were highlighted across all the samples.

DISCUSSION

FISH analysis

Re-evaluation and cross reactivity testing of FISH probes

Whole-Cell FISH probe re-evaluation was performed on all the probes assigned to NUIG. Each positive signal was recorded for *P. parvum* and *P. polylepis* and positive probe signals were confirmed using the calcofluor/DAPI UV filter set through the examination of the characteristic cellular shape, size of the *Prymnesium* speicies. Due to the small size of *P. parvum* and *P. polylepis*, the additional use of calcofluor/DAPI UV filter with FISH probes gives confirmation of the species present and also helps discriminate between fluorescent detritus material and other phytoplankton taxa found in the spiked field samples from the target *Prymnesium* sp. cells (Touzet and Raine, 2007).

Cross reactivity testing was not tested on pure culture alone as it was not necessary due to previous studies using the same *Prymnesium* probes for FISH analysis and their specificity has already been verified by dot-blot hybridisation with PCR amplified with either 18S or 28S rDNA fragments of different target and non-target microalgae (Eller et al. 2007; Töbe et al. 2006; Simon et al. 2000, 1997; Lange et al. 1996).

Specificity tests of assessing cross reactivity for every probe to be spotted on the microarray chip using FISH would be time-consuming and labour intensive. Also,

interactions of multiple probes spotted together and hybridised with labelled RNA from environmental sample could only be fully understood using the microarray format. Therefore, to determine how much potential cross reactivity there is between the higher group, class, genus and species level *Prymnesium* sp. probes from the numerous other probes spotted on the 1st and subsequent generation MIDTAL microarray chip, hybridisation with labelled *P. parvum* and *P. polylepis* RNA from pure cultures and field samples were undertaken.

Microarray analysis

Prymnesium spp. microarray analysis

RNA labelling

The recommended optimal range of RNA labelling for microarray hybridisation is between 1.0 - 3.6 % according to Kreatech Biotechnology nucleic acid labelling kit guidelines. The quality of the RNA extraction is paramount to the quality of RNA labelling DoL% (Metfies and Medlin 2005; 2008). Even though the condition of the labelled *Prymnesium* RNA were on the lower end of the optimal range of DoL %, the testing of the 1st, 2nd and 3rd generation MIDTAL chips were successfully carried out. The extraction process has been substantially improved in subsequent adaptations made to the RNA extraction protocols which are discussed below (Kegel et al. 2013b; McCoy et al., 2014a).

Microarray probes

The extended and re-designed eukaryote probes (EukS_1209_25 and EukS_328_25) on the 2nd and 3rd generation performed better than the non-extended old 1st generation probes (EUK1209 and EUK328). The Prymnesiophyta higher group probes PRYM02 and PrymS02_25 spotted on the 1st and 2nd generation, respectively, consistently returned higher signal compared to the two other higher group probes (PRYM01/PrymS01_25, and PRYM02/PrymS03_25), which is consistent with the FISH analysis reported McCoy et al. (2014b). The higher group Prym02_25_dT probe that targets the Prymnesiophyta was subsequently deleted from the latest 3.3 version microarray, as it was observed to be highlighted in the presence of non-target algae groups (Table II.B-2; Kegel et al. 2013b).

The extended genus level probe PrymGS01_25 spotted on the 2nd generation chip produced a positive signal when hybridised with labelled *P. parvum* RNA but failed to produce a positive signal ≥2 for hybridisations with labelled *P. polylepis* RNA; this

indicates that this probe is species-specific towards *P. parvum*, an observation also noted in McCoy et al. (2014a). Due to the genus level PrymGS01_25 probe being species-specific towards *P. parvum*, this probe was reclassified as a species probe and re-named Prymparv01_25_dT on the 3rd generation microarray (Table II-2).

The specificity of all the *Prymnesium* probes from higher groups to species level were greatly improved as seen in the comparison between normalised microarray signal from the 1st and 2nd generation microarrays and the significant decrease of cross reactivity among non-specific probes spotted on the chip.

Cross reactivity testing: microarray

The reason why there was extensive cross reactivity observed in the 1st generation microarray may be that the oligonucleotides routinely used for FISH hybridisation were too short, produced weak signal intensities or that the probes had inaccessible secondary structures to achieve specific hybridisation reactions in a microarray format (Chou et al. 2004; Metfies and Medlin 2008a). Lengthening the probe sequences on the 2nd generation chip by between 18 to 25 nucleotides increased the signal intensity, which has been reported previously by Chou et al. (2004). However, a number of non-specific cross-hybridisation was observed with the initial 1st generation 58 °C hybridisation temperature step (Medlin 2013). This meant that a higher melting point temperature was required and thus the hybridisation temperature was subsequently increased to 62 °C and 65 °C. The poly T spacer length linking the probe to the glass slide was also increased to address specificity issues of molecule and probe interactions respectively, along with new optimised hybridisation conditions containing fragmentation and denaturing steps to break down possible secondary structures and thereby increasing the diffusion rate of the target molecules (Kegel et al. 2013a; 2013b; Medlin 2013).

It was also observed that cross reactivity could be further reduced by integrating more stringent washing steps, thereby minimising background noise and increase specificity (Medlin 2013), which is also consistent with this study. It must be noted that that a number of false positives are likely to occur in field samples with unknown phytoplankton composition and RNA extracted and analysed on a universal microarray (Gesher et al. 2008; McCoy et al 2014a). Problems of false positive non-specific binding have been dealt with in past studies by incorporating a hierarchical probe approach (Groben et al. 2004; Gesher et al. 2008; Metfies and Medlin 2008a). By adopting this approach further cross reactivity issues observed on the 3rd generation chips were dealt

with by incorporating a customised hierarchical file, which provides the best means of eliminating false positives (Metfies et al. 2008b; Dittami and Edvardsen 2012). For the microarray analysis to indicate the presence of a species, the complete taxonomic hierarchy leading to that species must be highlighted and above the set threshold level (Kegel et al. 2013b). The conversion of microarray signals to cell abundances is generated by incorporating the slope of culture calibration curves of each species into the GPR-Analyser programme (Dittami and Edvardsen 2012; Kegel et al. 2013a 2013b; McCoy et al. 2014a).

Additionally, a known amount of *Dunaliella tertiolecta* cells are added to field samples prior to RNA extraction, which acts as an internal control for extraction efficiency (McCoy et al. 2013; 2014a). By normalising microarray signal intensities with the corresponding *D. tertiolecta* control spotted on the chip, it is possible to infer cell numbers for selected species in the field samples. However, should the signal from *Dunaliella* probe be too high, this could affect marginal yet positive signals, and possibly render them negative (McCoy et al 2014a; Medlin 2013). This was also the case with the TATA box protein added to the hybridisation mix. Therefore, the concentrations of both *Dunaliella* and TATA box protein controls were decreased for the 3rd generation microarrays. The use of the TBP control for normalisation of microarray signal-to-noise ratio values was also used in previous microarray optimising studies (Gescher et al. 2008; Metfies and Medlin 2008a). Normalisations with the Poly-T-CY5 control probe spotted on the chip surface, which can degrade over a period of time, resulting in decreasing signal intensity, thereby inferring higher normalised microarray values. This affected the comparisons between *Prymnesium* spp. probes spotted the 2nd and 3rd generation microarray when hybridised with pooled labelled *P. parvum* and *P. polylepis* RNA and normalised with Poly-T-CY5 probe, due to higher signal intensities observed in this Poly-T-CY5 probe in more recently spotted 3rd generation chips. This makes the Poly-T-CY5 probe an unreliable control to normalise against when comparing probes from new or older spotted MIDTAL microarrays.

Field testing of 3.2 and 3.3 version MIDTAL microarray

RNA extraction efficiency

RNA extraction efficiency was validated by confirming that there was no significant variation between the average difference of spiked and un-spiked field samples and the *D. tertiolecta* controls run in parallel. An optimised nucleic acid extraction method is paramount to the success of interpreting correct microarray signals into cell numbers and render the MIDTAL microarray a quantitative tool for the processing of field samples (Metfies and Medlin 2008a; McCoy et al. 2012; 2013; 2014a).

RNA labelling of field samples

The difference in NH_4Ac RNA precipitate step and addition of KREAblock to the hybridisation mix prior to microarray hybridisation to reduce background noise proved to optimise 3.3 version microarrays results (Table II-5). This returned higher percentage of V3.3 positive microarray signals corresponding to LM counts with fewer numbers of false positives compared to the V3.2 microarray signals across the range of phytoplankton taxa represented on the chip.

Comparison of 3.2 and 3.3 version microarray field results

Pseudo-nitzchia groups

The only sample not to record the presence of *Pseudo-nitzschia* groups from LM counts was KHE. However, two *Pseudo-nitzschia* spp., three *P. delicatissima* and three *P. seriata* probes from the V3.2 microarray hybridisations were highlighted for this sample. Low RNA labelling efficiency (0.2 DoL%) was recording false positive readings for the presence of *Pseudo-nitzschia* groups in sample KHE from the V3.2 microarray hybridisations, also there was high cell concentration of *Skeletonema costatum*, (1,934,000 cells L^{-1}) which may have contributed to cross reactivity issues. However, further testing would be needed to determine if this was the case. None of the V3.3 microarray hybridisations were recorded signals in sample KHE, only one *Pseudo-nitzschia* general spp. probe (PmulacalD02_25_dT), which is also one of the same corresponding V3.2 probes producing false positive readings. This was also determined by the hierarchy file which indicates that the higher group probes PsnGS02_25_dT and PSN+FRAGS02-25new_dT must be highlighted before the *Pseudo-nitzschia* spp. probe (PmulacalD02_25_dT) can be interpreted as a positive microarray signal (Barra et al. 2013). Another advantage to the V3.3 microarray is that although *P. delicatissima* grps

were not recorded in LM counts for samples KHD and KHF, along with *P. seriata* grps in sample KHC, their presence was detected to species level from the V3.3 microarray hybridisations with RNA labelling efficiencies within or above the optimal range. All species detected by the microarray have been known to occur in Irish waters (Cusack et al. 2004). Toxin analysis of the Killary Harbour 2010 seawater samples were run in parallel with two detection platforms of the multi SPR biosensor and ELISA method. The ELISA method detected the presence of Domoic Acid toxins in all six sample and the multi SPR in three samples (KHA, KHB and KHD; Table II-S1; McNamee et al 2013).

Dinophysis

The *D. acuta* probe highlighted in KHA sample was regarded as a false positive through the hierarchy file which was most likely due to the low labelling efficiency (0.3 DoL%) and the absence of *D. acuta* in the LM counts for this sample. However, it is possible that these cells could have escaped detection, because of the larger volume filtered for RNA extraction compared to just 50 ml Lugol's fixed sample volume taken for LM counts (McCoy et al 2013). This is more likely the case for KHE which was the only sample where probes detected the *Dinophysis* genus (DphyFS02_25_dT). However, LM counts did not record the presence of *Dinophysis* sp. This has also been known to occur for low cell densities of *Dinophysis* sp. which have been responsible for shellfish closure in Ireland, but have evaded LM detection (Raine et al. 2010). Interestingly, the MI detected ≤0.07 µg/g [Total Tissue,(TT)] of DSP toxin in edible mussel samples taken on the same dates from the KHD and KHE sampling sites (Table II-S2) and the multi SPR and ELISA detected the presence of OA + DTXS in all six Killary Harbour seawater samples (Table II-S1: McNamee et al. 2013). The MI toxin methods were extracted from the edible mussels (*Mytulis edulis*) tissue and the multi SPR and ELISA methods were adapted to filter seawater samples, so direct comparison cannot be made, but still are good indications for the validation of both results (McNamee et al. 2013).

Although the two genus level probes DphyGS04_25_dT and DphyGS01_25_dT were recorded in samples containing both *D. acuminata* and *D. acuta*, they were deemed false positive results because the *Dinophysis* higher group probe did not highlight this sample. The hierarchy file may need to be revised for this genus as the *Dinophysis* genus probes seem more sensitive than the higher family probe. It has also been noted in Edvardsen et al. (2013) that the estimated minimum amount of cells to record positive microarray results through calibration curves for *D. acuminata* and *D. acuta* are 345 and 950 cells,

respectively. The highest cell numbers (130 cells/L^{-1}) during the Killary Harbour sampling survey were well below these cut-off points.

Prorocentrum and Dinophyta

Prorocentrum micans (PmicaD02_25_dT) and *P. lima* (PlimaFD01-25_dT) probes both require the *Dinophyta* probe (DinoB_25_dT) to be highlighted to pass the hierarchy file and be considered a positive microarray result. The two Dinophyta probes DinoB_25_dT and DinoE12_25_dT on the V3.3 and V3.2 microarrays produced signal-to-noise ratio values above the threshold limit ≥2 for all the six samples, except for the DinoE12_25_dT probe in sample KHC and KHF on the V3.2 microarray (data not shown). This was confirmed by LM counts as all samples contained dinoflagellate species. Therefore validating the positive microarray signals for *P. micans* and *P. lima*. Toxin results from the multi SPR and ELISA both indicated the presence of OA + DTXS in all six sea water samples which is the toxin produced by the *P. lima* species (Pan et al. 1999), which gives further supports the PlimaFD01-25_dT probe signals (Table II-S1; McNamee et al. 2013). Although microarray signals were recorded in samples with and without *Prorocentrum* sp. observed in LM counts, this may be due to the volumes filtered for microarray analysis compared to those taken for LM counts as previously discussed above.

Alexandrium

Alexandrium genus (AlexGD01_25_dT) probe recorded from both the V3.3 (KHA, KHB and KHD) and V3.2 (KHA, KHD and KHE) hybridisations suggested the presence of *Alexandrium* in these samples, with only *Alexandrium* cells observed from LM counts for sample KHD. Notably, this was the only sample in which true positive microarray results were recorded for *A. tamarense* (complex) and *A. minutum*, which is further supported by the presence of PSP toxins from the ELISA results (Table II-S1: McNamee et al. 2013). The detection of *A. tamarense* complex probe has been reported to have a greater affinity towards the non-toxic *A. tamarense* group III ribotype (Taylor et al. 2013), which is the most likely species to co-occur with *A. minutum* in Killary Harbour (Touzet et al. 2009). Although, the presence of PSP was not detected by the multi SPR in any of the six Killary samples, ELISA is known to be more sensitive and where ever there was *Alexandrium* genus indicated by the V3.3 microarray there was also the presence of PSP toxins detected from the ELISA method which gives further validation to the V3.3

microarray results (McNamee et al. 2013; McCoy et al. 2014c). Toxin results from Bioassay for PSP were not under taken by the Marine Institute (MI) during these dates, presumably because of the low cell concentrations of *Alexandrium* sp. in LM counts during the survey period (Table II-S2).

Heterosigma, Azadinium, Karlodinium

The only two probes that highlighted in the V3.2 microarray were the only probes not to be represented in the V3.3 microarray. This indicates that LSHaka0329A25_dT and LSHaka0358A24_dT probe signals were probably false positives due to the low labelling of these V3.2 samples. However, the five *Heterosigma akashiwo* recorded on the V3.3 all highlighted individually on five separate samples. *Heterosigma* probe microarray analysis carried out in Blanco et al. (2013) indicated that hybridisation with labelled *H. akashiwo* RNA should highlight LSHaka0544A25b_dT, LSHaka0268A25_dT and LSHaka0548A25_dT probes together with good sensitivity. Since these probes were not all highlighted together even in one sample, the results must therefore be regarded as false positives.

Azadinium species were detected in two samples (KHA and KHE) from the V3.3 microarray hybridisations, with the KHE sample tested positive for Azaspiracids (AZP; 0.17 µg/g TT) toxins from edible mussels as analysed by the MI, which subsequently resulted in a shellfish farm closure during this period (Table II-S2). An *Azadinium* strain (SM2) of *A. spinosium* has been identified in Irish waters, which produces the toxic azaspiracid analogues AZA1 and AZA2 (Salas et al. 2011). The Azaspiracids (AZP) toxin is not yet detected for by the multi SPR biosensor (McNamee et al. 2013), but this *Azadinium* genus has only recently been identified with new toxic species continually being described (Tillmann et al. 2009; 2012; Luo et al 2013).

The *Karlodinium* species level probes (KveneD04_25_dT, KveneD03_25_dT KveneD06_25_dT) highlighted on the V3.3 microarray can detect the presence of labelled *K. veneficum* RNA cells at concentrations as low as 1 ng from pure cultures which corresponds to c. 250 *K. veneficum* cells (McCoy et al. 2014a). However, for them to be considered positive on the microarray the genus-level probe KargeD01_25_dT and species level probes need to be highlighting together, this was not the case during the Killary Harbour 2010 sampling survey.

Gymnodinium

Gymnodium catenatum and *Karenia brevis* were indicated as present by the V3.2 microarray. However, these three probes LSGcat0544A24_dT, L*Kare0308A25_dT and KbreD05_25_dT were not highlighted in the V3.3 microarray hybridisations because they were removed from this V3.3 microarray due to cross reactivity issues with other probes spotted on the microarray chip (Kegel et al. 2013b; McCoy et al. 2014a). The V3.3 microarray also indicated the presence of *G. catenatum,* and *K. brevis* but the hierarchy file dictated that these were also false positive results due to additional *G. catenatum* (GcateS01_25_dT and SSGcat0826A27_dT) and *Karenia* spp. probes (KareGD01_25_dT) not being highlighting in parallel. This result is also supported because *G. catenatum* and *K. brevis* have not been reported in Irish waters to date. KHD was the only sample which gave a true positive microarray result for the presence of *Karenia* spp. (KareGD01_25_dT) which also observed Karenia/*Gymnodinium* sp. in LM counts and is known to be associated with mortality of benthic and pelagic marine organisms in Irish coastal waters (Silke et al. 2005).

Pseudochattonella

The testing of these genus level probes by Dittamii et al. (2013b) indicated that the PschGS05_25_dT probe is the strongest genus probe out of the three in terms of microarray signal intensities when hybridised with either labelled *P. verruculosa* or *P. farcimen* RNA. This is also indicated in the hierarchical file and for the other two *Pseudochattonella* sp. genus probes (PschGS01_25_dT and PschGS04_25_dT) to be considered positive microarray signals the PschGS05_25_dT probe must produce a higher signal-to-noise ratio value. Therefore the only true microarray signal were represented in sample KHE with the PschGS05_25_dT probe recorded on the V3.3 microarray which also indicated that PschGS01_25_dT and PschGS04_25_dT probes recorded for the V3.2 microarray hybridisations were in fact false positives. The presence of *Pseudochattonella* sp. was also observed in sample KHE from the LM counts, giving further confidence in the V3.3 microarray and presence of *Pseudochattonella* sp. in this sample.

Haptophyta

Prymnesium sp. were detect to clade level for both the V3.2 and V3.3 microarrays, however *Prymnesium* sp. were detected in more samples from the V3.3 microarray compared to the V3.2 microarray, this is most likely due the improved DoL% for V3.3 hybridisations and highlights again the importance of optimal RNA labelling prior to microarray hybridisation step.

A general trend has emerged which indicates the high importance of RNA labelling efficiency prior to hybridisation which dictates a successful microarray hybridisation, with a lower amount of false positives and a higher degree of accuracy and percentage of positive microarray results which may be overlooked with a less optimal RNA labelling DOL%.

CONCLUSION

FISH analysis using species specific hierarchical fluorescent oligonucleotide probes accurately identified *Prymnesium* spp. from cultured and spiked field samples from Irish waters. This study also shows the significant improvements of the MIDTAL microarray from the initial stages of the 1st generation microarray to the latest V3.3 MIDTAL microarray. The combination of optimised protocols, toxin analysis and implementation of the hierarchy files allow for a confidence and validation of positive microarray results, further strengthening this product which is now commercially available from Microbia Environnement (France).

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SUPPORTING INFORMATION

FISH analysis

Re-evaluation of existing FISH probes

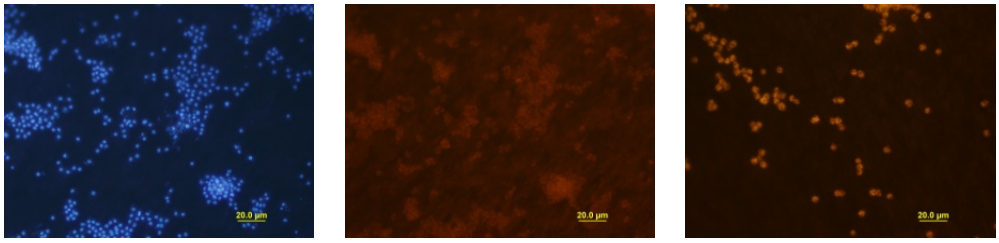

Fig. II-S1a. FISH micrograph images of *P. parvum* (strain CCMP 709) cells hybridised at 50 °C with 20% FA. (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM01 probe and (right image) highlighted cells in the presence of PRYM01 probe viewed under a CY.3 UV filter.

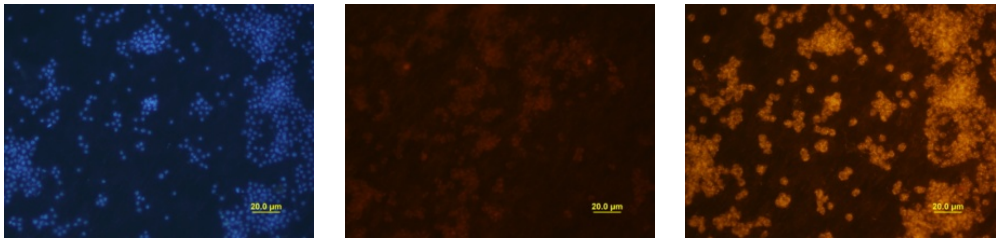

Fig. II-S1b. FISH micrograph images of *P. parvum* (strain SAG 127.79) cells hybridised at 50 °C with 20% FA. (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM02 probe and (right image) highlighted cells in the presence of PRYM02 probe viewed under a CY.3 UV filter.

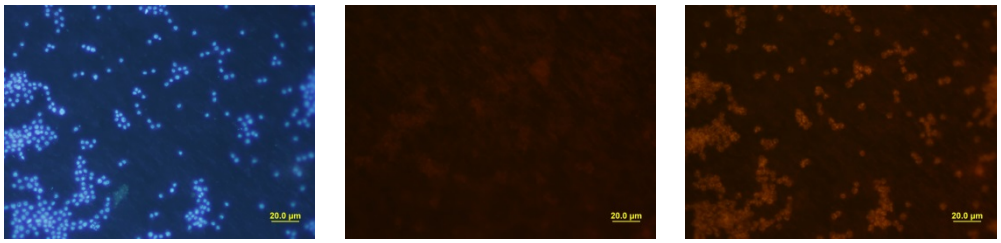

Fig. II-S1c. FISH micrograph images of *P. parvum* (strain SAG 127.79) cells hybridised at 50 °C with 20% FA. (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM03 probe and (right image) highlighted cells in the presence of PRYM03 probe viewed under a CY.3 UV filter.

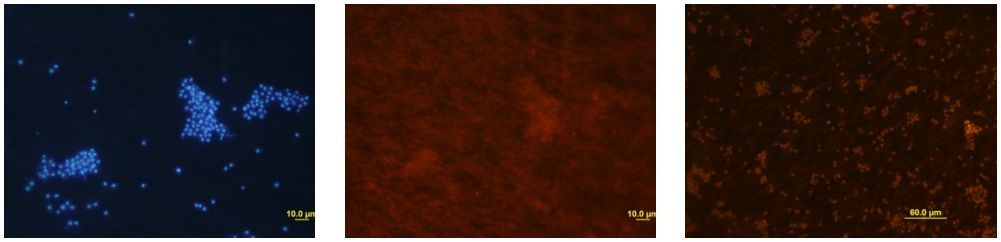

Fig. II-S1d. FISH micrograph images of *P. parvum* (strain SAG 127.79) cells hybridised at 50 °C with 20% FA. (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYMGL01A probe and (right image) highlighted cells in the presence of PRYMGL01A probe viewed under a CY.3 UV filter.

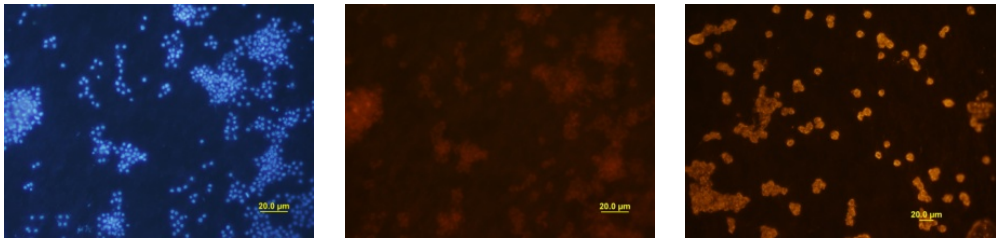

Fig. II-S1e. FISH micrograph images of *P. parvum* (strain CCMP 709) cells hybridised at 50 °C with 20% FA. (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYMGL02B probe and (right image) highlighted cells in the presence of PRYMGL02B probe viewed under a CY.3 UV filter.

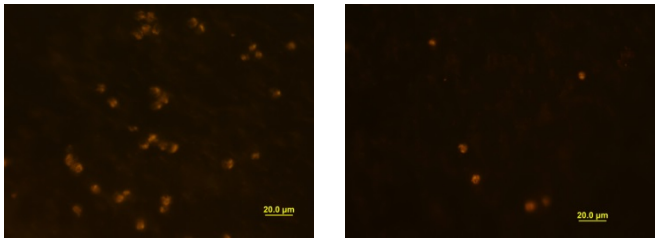

Fig. II-S1f. FISH micrograph images of *P. parvum* (strain CCMP 709) cells hybridised with Clade01 probe at 50 °C with 20% FA (left image). (middle) *P. polylepis* (strain CCMP 1757) cells hybridised with Cpoly01 probe (15% FA at 50 °C). (right, image not taken) Note that *P. parvum* was hybridised with PRYM694 probe and had an optimal hybridisation condition of 50 C at 10% FA. All images were viewed under a CY.3 UV filter.

Cross reactivity testing: FISH

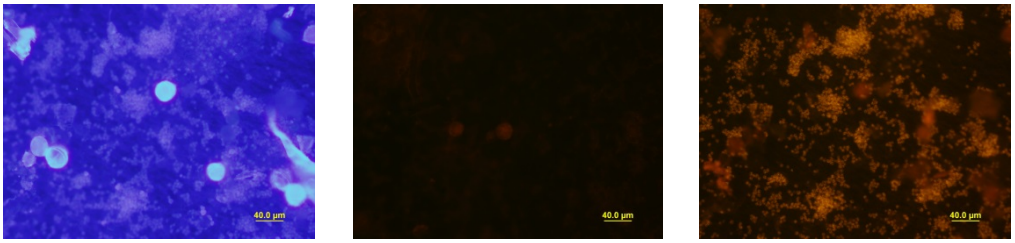

Fig. II-S2a. FISH micrograph images of *P. parvum* (strain CCMP 709) cells hybridised at 50 °C with 20% FA in a Cork Harbour field matrix (CH5605-Q-0m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM01 probe and (right image) highlighted cells in the presence of PRYM01 probe viewed under a CY.3 UV filter.

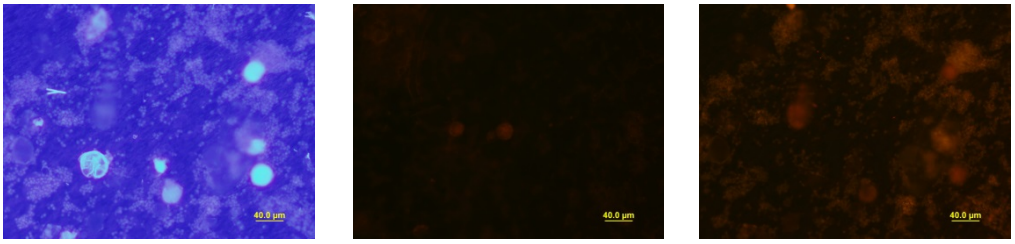

Fig. II-S2b. FISH micrograph images of *P. parvum* (strain SAG127.79) cells hybridised at 50 °C with 20% FA in a Cork Harbour field matrix (CH5605-Q-0m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM02 probe and (right image) highlighted cells in the presence of PRYM02 probe viewed under a CY.3 UV filter.

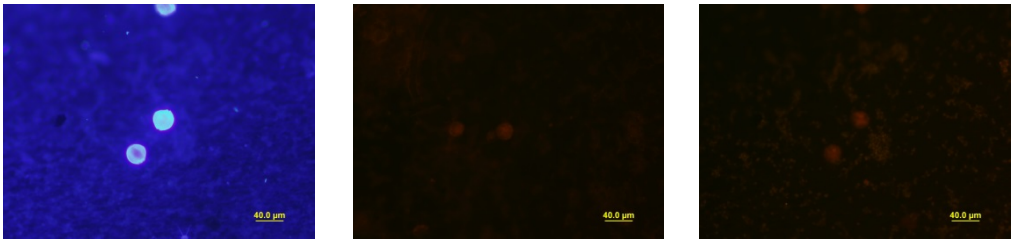

Fig. II-S2c. FISH micrograph images of *P. parvum* (strain SAG127.79) cells hybridised at 50 °C with 20% FA in a Cork Harbour field matrix (CH5605-Q-0m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM03 probe and (right image) highlighted cells in the presence of PRYM03 probe viewed under a CY.3 UV filter.

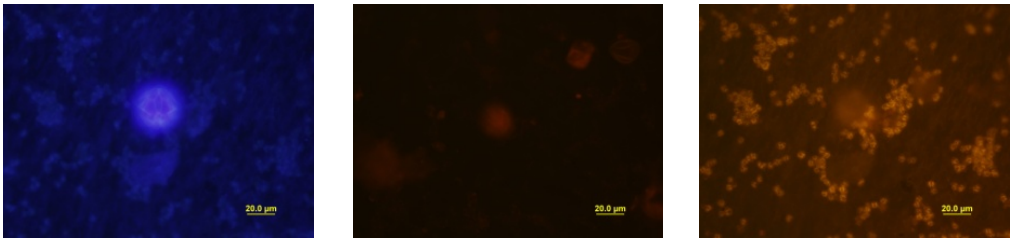

Fig. II-S2d. FISH micrograph images of *P. parvum* (strain UIO 054) cells hybridised at 50 °C with 20% FA in a Cork Harbour field matrix (CH5604-P-3m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYMGL01A probe and (right image) highlighted cells in the presence of PRYMGL01A probe viewed under a CY.3 UV filter.

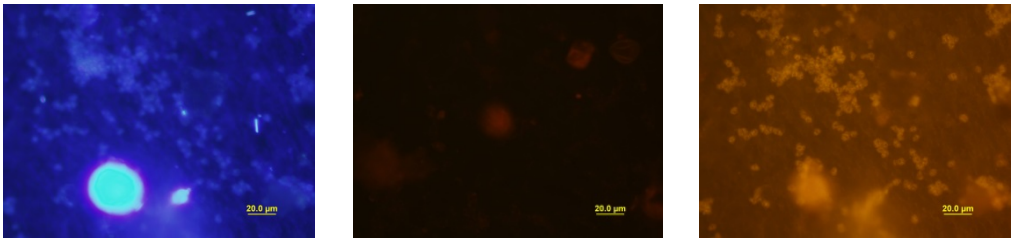

Fig. II-S2e. FISH micrograph images of *P. parvum* (strain UIO 054) cells hybridised at 50 °C with 20% FA in a Cork Harbour field matrix (CH5604-P-3m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYMGL02B probe and (right image) highlighted cells in the presence of PRYMGL02B probe viewed under a CY.3 UV filter.

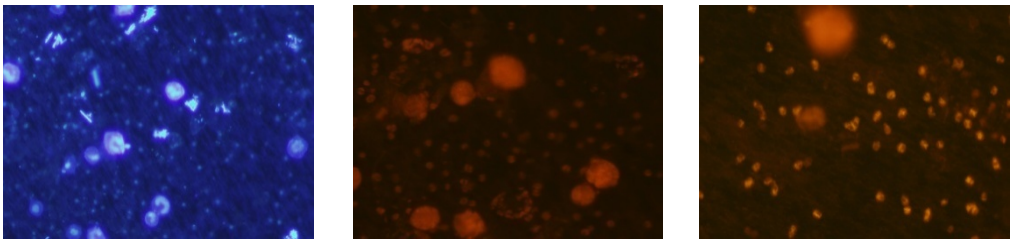

Fig. II-S2f. FISH micrograph images of *P. polylepis* (strain UIO 036) cells hybridised at 50 °C with 20% FA in a Cork Harbour field matrix (CK5733-0m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of Cpoly01 probe and (right image) highlighted cells in the presence of Cpoly01 probe viewed under a CY.3 UV filter.

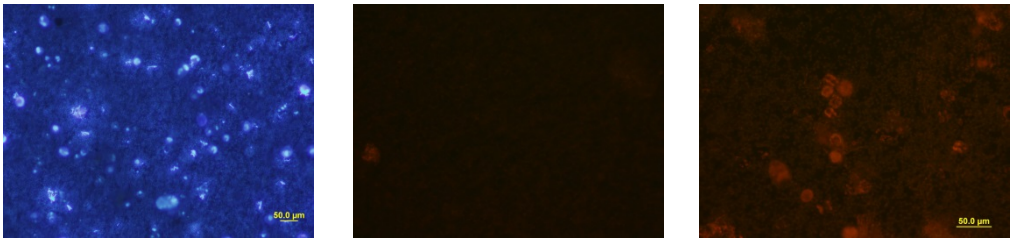

Fig. II-S2g. FISH micrograph images of *P. parvum* (strain UIO 054) cells hybridised at

50 °C with 20% FA in a Cork Harbour field matrix (CK5733-0m). (left image) cells viewed under a calcofluor/DAPI UV filter, (middle image) un-highlighted cells in the absence of PRYM694 probe and (right image) highlighted cells in the presence of PRYM694 probe viewed under a CY.3 UV filter.

Toxin results: Multi SPR and ELISA

Table II-S1. Saxitoxin (PSP), Okadaic acid (DSP) and Domoic acid (ASP) biotoxin results of filtered seawater samples from Killary Harbour during the period of the 31st May to 13th September. Samples were measured by both the prototype multiplex SPR biosensor and commercial CER ELISA and results indicated by the presence or absence of detectable levels of toxin from each method adapted from McNamee et al. (2013).

Station	Station code	Sample Date	PSP Toxins (STX)		Okadaic Acid + DTXS		Domoic Acid	
			Multi SPR	ELISA	Multi SPR	ELISA	Multi SPR	ELISA
KH1601	KHA	31-May-10	-	+	+	+	+	+
KH1802	KHB	14-Jun-10	-	+	+	+	+	+
KH2101	KHC	5-Jul-10	-	-	+	+	-	+
KH2402	KHD	26-Jul-10	-	+	+	+	+	+
KH2703	KHE	16-Aug-10	-	-	+	+	-	+
KH3101	KHF	13-Sep-10	-	+	+	+	-	+

Toxin results: Marine Institute (MI)

Table II-S2. Marine Institute toxin analysis results of the edible mussels (*Mytulis edulis*) collected on the dates during the 2010 survey of Killary Harbour. Web: www.marine.ie/habs

Station	Sample Date	Sampling Point	Species	Bioassy-DSP	Chemistry-AZP (ug/g TT)	Chemistry-DSP (ug/g TT)	Bioassy-PSP	Area-Species Status
KH1601	31/05/2010	Middle(GY-KM-KM)	*Mytilus edulis*	negative	<LOQ	<LOD		Open
KH1802	14/06/2010	Middle(GY-KM-KM)	*Mytilus edulis*	negative	0.03	<LOQ		Open
KH2101	05/07/2010	Inner(GY-KI-KI)	*Mytilus edulis*	negative	0.03	<LOQ		Open
KH2402	26/07/2010	Outer(GY-KO-KO)	*Mytilus edulis*	negative	0.07	0.07		Open
KH2703	16/08/2010	Outer(GY-KO-KO)	*Mytilus edulis*	negative	0.17	0.06		Closed
KH3101	13/09/2010	Outer(GY-KO-KO)	*Mytilus edulis*	negative	0.07	<LOQ		Open

LOD = Limit of Detection, LOQ = Limit of Quantification.

It is recommended that no harvesting takes place of species indicated as closed (*Mytilus edulis*).

CHAPTER III

AN ASSESSMENT OF RNA CONTENT IN *PRYMNESIUM PARVUM*, *PRYMNESIUM POLYLEPIS*, CF. *CHATTONELLA* SP. AND *KARLODINIUM VENEFICUM* UNDER VARYING ENVIRONMENTAL CONDITIONS FOR CALIBRATING AN RNA MICROARRAY FOR SPECIES DETECTION

Submitted to: FEMS microbiology ecology (30th September 2013)
Status: Accepted (22nd December 2013)

Reference:
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ABSTRACT

Traditional methods of identification and enumeration can be somewhat ambiguous when identifying phytoplankton that requires electron microscopic examination to verify specific morphological features. Members of the genus *Prymnesium* (division *Haptophyta*), members of the *Raphidophyceae* and naked dinoflagellates are examples of such phytoplankton whose identification can be difficult. One alternative to traditional microscopy-based methods of identification is to use molecular protocols to detect target species. Methods that measure cellular DNA and RNA content can be used to estimate the number of cells present in a sample. This study investigated the variation of RNA yields in *Prymnesium parvum*, *Prymnesium polylepis*, cf. *Chattonella* sp. and *Karlodinium veneficum* cells grown under different light, temperature, salinity, and inorganic nutrient conditions. This information was used to calibrate the signal intensity of a variety of oligonucleotide probes spotted onto the microarrays for detection of toxic algae (MIDTAL), which is being developed to aid national monitoring agencies and to provide a faster means of identifying and quantifying harmful phytoplankton in water column samples.

Keywords: Microflagellates, RNA, calibration curves, microarrays, MIDTAL.

INTRODUCTION

Phytoplankton monitoring programmes usually include the identification and enumeration of harmful species in water samples. Traditional methods, such as light microscopy, are typically used, whereby only a small sub-sample of the phytoplankton assemblage is examined (Karlson *et al.,* 2010). Traditional methods of phytoplankton identification and enumeration can be somewhat ambiguous when attempting to identify naked flagellates. Their cell body can be greatly distorted by preservative reagents, which makes their identification almost impossible; this is particularly true for the raphidophytes (Band-Schmidt *et al.,* 2012). More advanced techniques, such as transmission electron microscopy (TEM), are hence usually needed for accurate identification. Molecular methods offer a way of overcoming these problems together with reducing the time needed for analysis. Some of these molecular methods include fluorescent *in-situ* hybridisation (FISH, Scholin *et al.,* 1997; Groben & Medlin, 2005; Touzet *et al.,* 2010), sandwich hybridisation (Scholin & Anderson, 1998), quantitative PCR (Galluzzi *et al.,* 2008; Touzet *et al.,* 2009; Kavanagh *et al.,* 2010), high-throughput sequencing technologies (Logares *et al.,* 2013; Zhan *et al.,* 2013) and microarrays (Metfies & Medlin, 2005).

The most challenging aspects of molecular methods relate to quantification (Metfies & Medlin, 2008). This is an important aspect relating to the monitoring of harmful algae in particular as threshold levels apply to cell densities as well as toxin levels in certain national monitoring programmes (Karlson *et al.,* 2010). This problem also extends beyond harmful algae monitoring, where other environmental studies have used molecular techniques to identify and estimate abundances of mixed bacterial communities in food, soil and water (Call *et al.,* 2003; Loy *et al.,* 2005; Lee *et al.,* 2008; Kostić *et al.,* 2010).

In the FP7 EU funded project Microarrays for the Detection of Toxic Algae (MIDTAL, http://www.midtal.com), a molecular method based on microarray technology was employed for detecting and quantifying HAB species to help aid national monitoring agencies. Microarray signals from species level probes bound to fluorescently-labelled RNA extracts were used to estimate the number of cells present in culture and field samples. Here we investigated whether or not RNA content in microalgal cells varied when cultures were exposed over a four day period to different environmental stress conditions. The four microflagellates selected for this study were the haptophytes

Prymnesium parvum and *P. (= Chrysochomulina) polylepis*, an unidentified species closely resembling a raphidophyte, here referred to as cf. *Chattonella* sp., and the naked dinoflagellate *Karlodinium veneficum*. Results were used to calibrate the microarray signals associated for several oligonucleotide probes targeting these species spotted on the MIDTAL.

An existing hierarchical group of oligonucleotide probes specific for the haptophytes *P. parvum* and *P. polylepis*, were lengthened to 25 nucleotides and were spotted onto the microarray chip. New probes, also 25 nucleotides in length, were designed for cf. *Chattonella* sp. and *K. veneficum*. The testing of these species against various stress conditions to determine the RNA content per cell was necessary to calibrate the microarray signal so it can be used as a quantitative tool for estimating cell numbers.

MATERIALS AND METHODS

Algal strains

Prymnesium parvum and *P. polylepis* strains were purchased from CCMP (Provasoli-Guillard National Centre for Culture of Marine Phytoplankton (USA), Bigelow Laboratory for Ocean Sciences), SAG (Culture Collection of Algae (SammLung von Algenkulturen der Universität Göttingen) or kindly provided by Bente Edvardsen (Department of Biology, University of Oslo, Oslo; Supporting Information, Table III-S1). *Karlodinium veneficum* strains were purchased from CMSTAC (Center for Marine Science Toxic Algal Collection, University of North Carolina), Canadian Centre for the Culture of Microorganisms (CCCM) and The Pasteur Culture Collection of Cyanobacteria (PCC, Table III-S1). Strains and sequences of cf. *Chattonella sp.* were provided by Carmelo Tomas for probe development and testing of the microarray. Algal cultures were maintained in *f*/2 (Guillard, 1983, with silicate) or IMR½ algal media (without silicate and with 10 nM selenite) (Eppley *et al.*, 1967) at 15 ± 1 °C, under a white fluorescent light with a photon flux of 150 µE m^{-2} s^{-1} and a 14:10 light/dark cycle. The experimental setup was performed as part of an EU consortium; thus, there were slightly different aspects to the execution of the experiments because they were performed in different laboratories with different types of laboratory conditions and equipment. The experiments for *Prymnesium sp.* were conducted at the National

University of Ireland, Galway (NUIG), and cf. *Chattonella sp.* and *K. veneficum* at the Marine Biological Association (MBA) Plymouth, UK.

Cell Counts

Subsamples for cell counts were kept at 4 °C and fixed with either 5 µL of Lugol's iodine solution added to 500 µL of culture or 200 µL of glutaraldehyde 0.2% to 1 mL of the culture mix. Concentrations of cells were determined either under light microscopy using the Fuchs-Rosenthal chamber method or with a flow cytometer (Becton Dickinson FACSCalibur, BD) after adding a known amount of beads (4.8 µm, caliBRITE Beads BD Biosciences).

Experimental set-up

Stock cultures of each strain were grown in 500 mL conical flasks enriched with 300 mL of fresh medium under the previously described conditions. Every day cell counts were performed on each strain to determine when culture growth was in exponential stage. At that stage, a known amount of culture was transferred into 200 mL conical flasks containing 100 mL of fresh medium modified to the stress condition being tested. For each stress condition, the flasks at time zero (T_0) were inoculated separately with a set cell concentration, ca. 75 000 cells/mL for the three individual *P. polylepis* strains and ca. 250 000-400 000 cells/mL for the three individual *P. parvum* strains. For cf. *Chattonella sp.* and *K. veneficum* species, 20 mL of each stock culture from the three strains, which represented about 20 cells in the former and 26-30 cells in the latter, were inoculated separately into the 200 mL tissue culture flasks. The control (optimal) cultures were grown as the initial stock culture.

Light intensity, temperature stress, salinity modulation and nutrient depletion were tested, one parameter being changed with each set of cultures (Table III-S2). For the stress conditions, the strains were inoculated in flasks containing *f*/2 or IMR½ at lower (LS) and higher (HS) stress than the control conditions (Table III-S1). Nitrogen and phosphorous depletion was carried out using modified f/2 Guillard or IMR½ algal seawater medium with the absence of nitrate (N- condition) or the absence of phosphate (P- condition). Although nitrate or phosphate was not added to the medium, the presence of these compounds could have been present in trace amounts in the seawater stock and carry-over from the inoculums. An additional experiment performed at NUIG consisted of

inoculating all three strains from *Prymnesium* species together (ALL mix) across the four conditions tested and carried out along with the experiments based on the individual *Prymnesium* strains. This was done to verify whether or not the RNA content of strains grown together (ALL mix) were significantly different from individual strains grown separately under the above stress conditions because it is assumed that natural populations contain a mixture of genotypes.

Exposed cultures were incubated for 24 h (T_1), 48 h (T_2) and 72 h (T_3) after which a known volume between 10 and 15 mL was taken from each flask for RNA extraction. Samples were centrifuged and the algal pellet collected for each time point, with the exception of cf. *Chattonella sp*. and *K. veneficum* because they grew very slowly and only a single time point (T_2) was taken. For the cf. *Chattonella sp.* and *K. veneficum* cultures, the volumes collected for RNA extraction were pooled together for the three strains of each species so as to optimise extraction efficiency because of the low numbers of cells.

RNA extraction

A volume of 5-15 mL from each test culture was transferred to 15 mL tubes, which were centrifuged at 6,000 *g* for 10 min. The supernatant was then removed to leave around 2 mL of sample. Tubes were centrifuged a second time at 6,000 *g* for 5 min, and the remaining supernatant completely removed using a micropippete and a vacuum pump, without disturbing the pellet. TRI-Reagent (1 mL) was immediately added to each pellet, homogenised and transfered to 2-mL screw cap tubes containing 0.1 mL of acid washed glass beads (213-300 µm). If RNA extraction was not performed on the same day, samples were stored at -80 °C until further processing.

RNA extractions were performed as described in the MIDTAL RNA extraction protocol (Lewis *et al.,* 2012) with modifications as follows. The 2-mL screw cap tubes were incubated at 60 °C for 10 min at maximum speed on a thermoshaker and vortexing twice for 20 s during incubation. An aliquot (100 µL) of 1-bromo-3-chloro-propane (BCP; Sigma) or 200 µL chloroform was added to each tube, the mixture was vortexed for 15 s and left to settle for 5 min. The whole content of the tube was transferred to a prespun 2-mL heavy phase lock (PL) tubes (5-PRIME; 12,000 *g* for 30 s), which was then homogenised manually for 15 s and allowed to stand for a further 5 min at room temperature. The tubes were centrifuged at 4 °C for 15 min at 12,000 *g*, and the upper aqueous phase (c. 500-550 µL) from the PL tubes was transferred to a new 1.5-mL

RNase-free tube. An equal volume of isopropanol was added (500 µL) prior to vortexing for 15 s. The tube was then incubated at -20 °C for 1 h, centrifuged again for 15 min and the supernatant carefully removed without disturbing the RNA pellet using a micropipette. The RNA pellet was washed with 1 mL of 75% ethanol, centrifuged and the supernatant was completely removed. After the final centrifugation step, the pellet was air dried for 3-5 min while being kept on ice. RNA pellets from each sample were resuspended in 25-50 µL of RNase free water. An aliquot of suspended RNA sample was taken to determine its RNA concentration using a NanoDrop 1000 Spectrophotmeter. The total RNA amount (ng) was then related to the amount of cells processed in the sample, providing RNA yields (pg/cell) for all the varying environmental conditions tested. The samples were stored at -80 °C until later use.

RNA clean-up and labelling

An ammonium acetate precipitation step was added to improve RNA quality and labelling efficiency. The eluted RNA was defrosted on ice and 0.5 volume of 7.5 M ammonium acetate (NH4Ac) and 2 volumes of ethanol (EtOH absolute, stored at -20 °C) were added. RNA precipitation steps are described in detail elsewhere (Kegel *et al.*, 2013). RNA labelling and fragmentation were as described in Lewis *et al*. (2012). Prior to labelling 10 ng of *Dunaliella tertiolecta* RNA was added as an internal control to each eluted RNA sample being tested for *Prymnesium* spp., cf. *Chattonella* spp. and *Karlodinium.*

Microarray calibration

Sequences of *Prymnesium* spp., cf. *Chattonella* sp. and *K. veneficum* were analysed *in silico* using ARB (Ludwig *et al.*, 2004) to design specific probes in those instances where published FISH probes were not available. Probes for *Prymnesium* originally designed for a FISH format and for higher taxonomic levels in the *Haptophyta* division were lengthened to 25 nucleotides in length, with the exception of probes Clade01old_25_dT and PparvD01_25_dT (Table III-S3). The probe sequences for all probes designed or modified from FISH probes for the entire project for the MIDTAL microarray are patent pending as a universal microarray for the detection of toxic algae and the entire hybridisation kit including the array and all necessary reagents are commercially available from Microbia Environnement (France).

Four different amounts of CY5-labelled (cyanine-5) RNA (1 ng, 5 ng, 25 ng and 100 ng) for cf. *Chattonella* spp. and *Karlodinium*, were hybridised on the third generation MIDTAL microarray slides (SCHOTT nexterion) to create calibration curves normalised to the TATA box protein control (POSITIVE_25_dT), with the exception of *Prymnesium* spp. for which only two RNA amounts were used (25 ng and 100 ng). Calibration curves normalised to the *Dunaliella* control (DunGS02_25_dT_dT) were also generated with two different amounts of CY5-labelled RNA (25 ng and 100 ng) for *Prymnesium* spp, cf. *Chattonella* spp. and *Karlodinium*. All hybridisation mixtures contained 30 µL of 2x hybridisation buffer, 3 µL Poly-dA (1uM), 5 ng of TBP-control and were adjusted to 60 µL with nuclease-free water.

Hybridisations were carried out as in chapter 9 in Lewis *et al.*, (2012) with some modifications, which included a pre-blocking and washing step of the microarray slide by shaking for 20 min at 50 °C, denaturing of the hybridisation mixture for 10 min at 94 °C and hybridisation to the slide for 60 min at 65 °C. After hybridisation, slides were washed with washing buffers (SSC/EDTA/SDS) at room temperature, followed by a final wash at 50 °C (Lewis *et al.*, 2012; Kegel *et al.*, 2013). Finally, the slides were scanned (Perken Elmer Microarray Scanner or GENEPIX 4000B, Molecular Devices) and total signals were determined as the average of the feature-background ratio of all 8 spots for each probe using the GENEPIX 6.0 software programme. Further analysis was carried out with the GPR-ANALYZER ver. 1.24 (Dimatti & Edvardsen, 2013). Signal intensity were normalised to the internal control probes spotted on the microarray to allow comparison of signal strength between slides.

Statistical analysis

For the RNA stress experiments a two-way ANOVA was used followed by Bonferroni post test analysis to detect significant differences between each treatment at each period of time, unless stated otherwise. Linear regression analyses and Pearson's correlation tests were carried out for 1) amount of RNA and cell number and 2) microarray signal and cell number relationships. The slope of the linear regression analysis was used in the GPR-ANALYZER program to infer cell numbers from field material. A D'Agostino-Pearson test was used to determine if the data significantly differed from a Gaussian (normal) distribution ($P > 0.05$). All statistical analysis was carried out in GRAPHPAD PRISM 5.

Growth rates

Growth rate was calculated from the specific growth rate (K') equation $K' = \text{Ln}\ (N_2 / N_1) / (t_2 - t_1)$, where N_1 and N_2 correspond to cell concentration at time1 (t_1) and time2 (t_2) (Levasseur *et al.*, 1993).

RESULTS

Prymnesium parvum and *P. polylepis*

Comparison of the growth rate for all *Prymnesium* strains under the range of environmental stresses applied (light, temperature, salinity and nutrients) is shown in Fig. III-1a and b. In general, there was an increase in cell numbers even in sub-optimal conditions taken from T_0 to T_3 (72-h period). However, this was not the case for *P. polylepis*, strain UiO038, for which cell numbers from the initial inoculation fell by up to 150 fold, and growth decreased by the end of the test period in salinity and nutrient culture conditions. A similar result was observed with the *P. polylepis* culture condition containing a mixture of all the strains (UiO037, UiO038, and CCMP1757) grown together. However, cell numbers did not decrease (up to 8.3 times for salinity and 6.5 times for nutrient depletion) to the extent that occurred when UiO038 strain cultures were grown separately. This had an effect on determining RNA content per cell because the strains that had a declining growth rate showed a higher standard deviation in RNA yield (pg/cell Table III-1).

Table III-1. Average RNA content per cell (pg/cell) of individual species strains for *P. parvum, P. polylepis,* cf. *Chattonella* and *K. veneficum*.

Species	***P. parvum***			***P. polylepis***			
Strain	**UIO054**	**CCMP709**	**SAG127.79**	**UIO036**	**UIO037**	**UIO038**	**CCMP1757**
RNA content (pg/cell)	0.60	0.62	0.48	1.58	1.04	4.92	1.49
SD	0.14	0.15	0.15	0.51	0.49	4.16	0.75
Species	**cf. *Chattonella***			***K. veneficum***			
Strain	**CMSTAC300**	**CMSTAC305**	**CMSTAC307**	**CCCM734**	**PCC709**		**PCC517**
RNA content (pg/cell)	21.43	15.92	16.13	49.29	58.22		27.50
SD	11.93	6.34	6.57	65.70	28.01		21.72

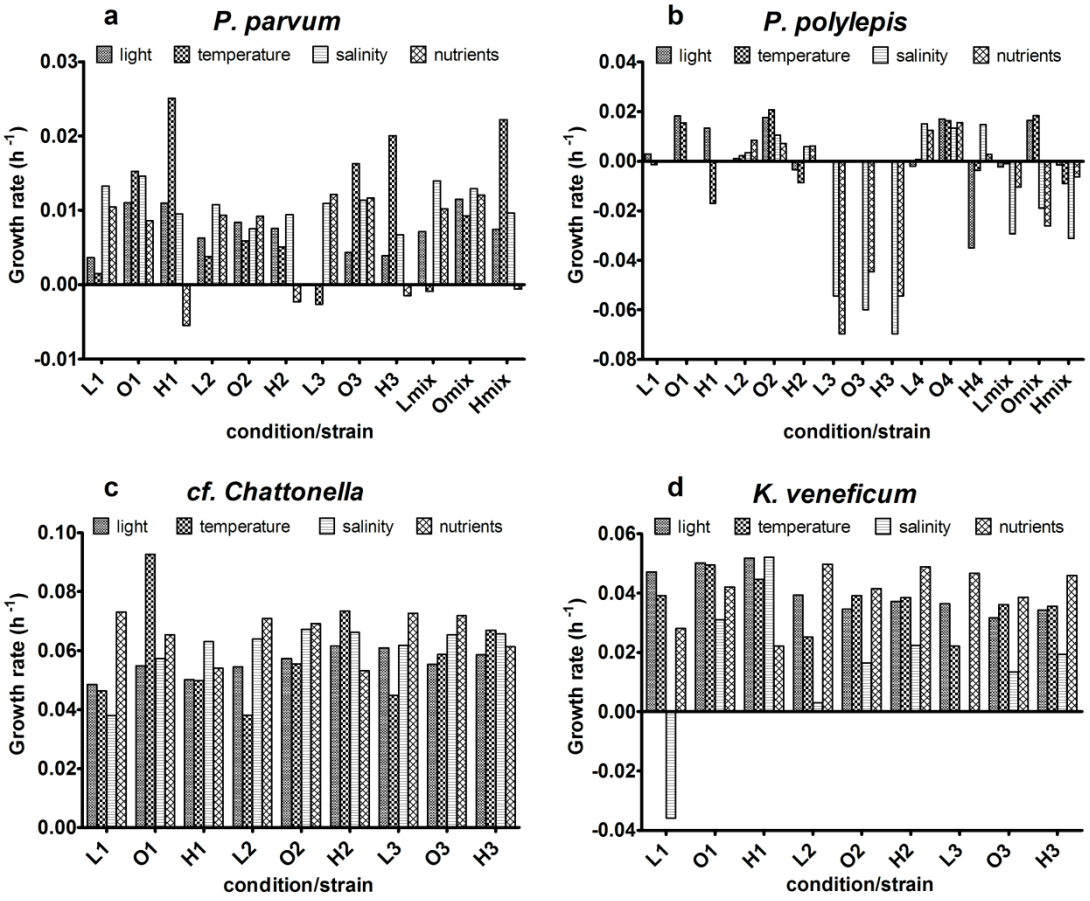

Fig. III-1. Growth rates of individual *P. parvum*, *P. polylepis*, cf. *Chattonella* and *K. veneficum* strains for low (L), optimal (O) and high (H) light, temperature, salinity and nutrient (N- and P- indicate nitrate or phosphate depletion and are represented as L (P-), O (N+P+) and H (N-)) conditions. Calculated from hour 0 (T_1= 0 h) and hour 72 (T_2 = 72 h) for *Prymnesium spp*. (a and b) and hour 48 (T_2 = 48 h) for cf. *Chattonella* and *K. veneficum* (c and d) time points. The numbers represent the different strains of *P. parvum* ["1" (UIO054), "2" (SAG127.79) "3" (CCMP709)], *P. polylepis* ["1" (UIO036), "2" (UiO037) "3" (UiO038) "4" (CCMP1757)], cf. *Chattonella* ["1" (CMSTAC300), "2" (CMSTAC305) "3" (CMSTAC307)] and *K. veneficum* ("1" (CCCM734), "2" (PCC709) "3" (PCC517)). The word "mix" represents the strains that were grown together in the same culture flasks for the *Prymnesium* species. The test conditions for each treatment follow that in Table III-S2.

Correlation between growth rate (h^{-1}) and average RNA yield (pg/cell) are shown in Fig.III-2. In general, the culture conditions had a non-significant negative correlating growth rate (h^{-1}) when compared with average RNA yields (pg/cell). However, *P. parvum* strains grown under nutrient (r = 0.7895; P = 0.0023; n = 36) treatment and *P. polylepis* strains grown under salinity (r = -0.9277; P < 0.0001; n = 36) and nutrient (r = -0.5790; P = 0.0485; n = 36) treatments had a significant correlation (Fig. III-2d, g and 2h). The only treatments to be positively correlated were *P. parvum* and *P. polylepis* strains grown under nutrient and temperature treatments, respectively (Fig. III-2d and f).

Prymnesium parvum showed no significant change in RNA yield (pg/cell) under light, temperature and salinity variation; but, there was a significant change (P = 0.006) between the treatments for nutrient stress but not for treatments over time (Fig. III-3). The average RNA content for *P. parvum* for the whole data set across three strains (UiO054, CCMP and SAG) and all conditions tested was 0.57 ± 0.16 pg/cell (n = 108; Table III-1).

Prymnesium polylepis showed no significant change in RNA yield (pg/cell) under any of the environmental conditions (Fig. III-3), with an average RNA content of 1.33 ± 0.67 pg/cell for the whole data set across the three strains (UiO036, UiO037 and CCMP) for all the conditions tested (n = 90; note excludes UiO 038 strain Fig. III-3).

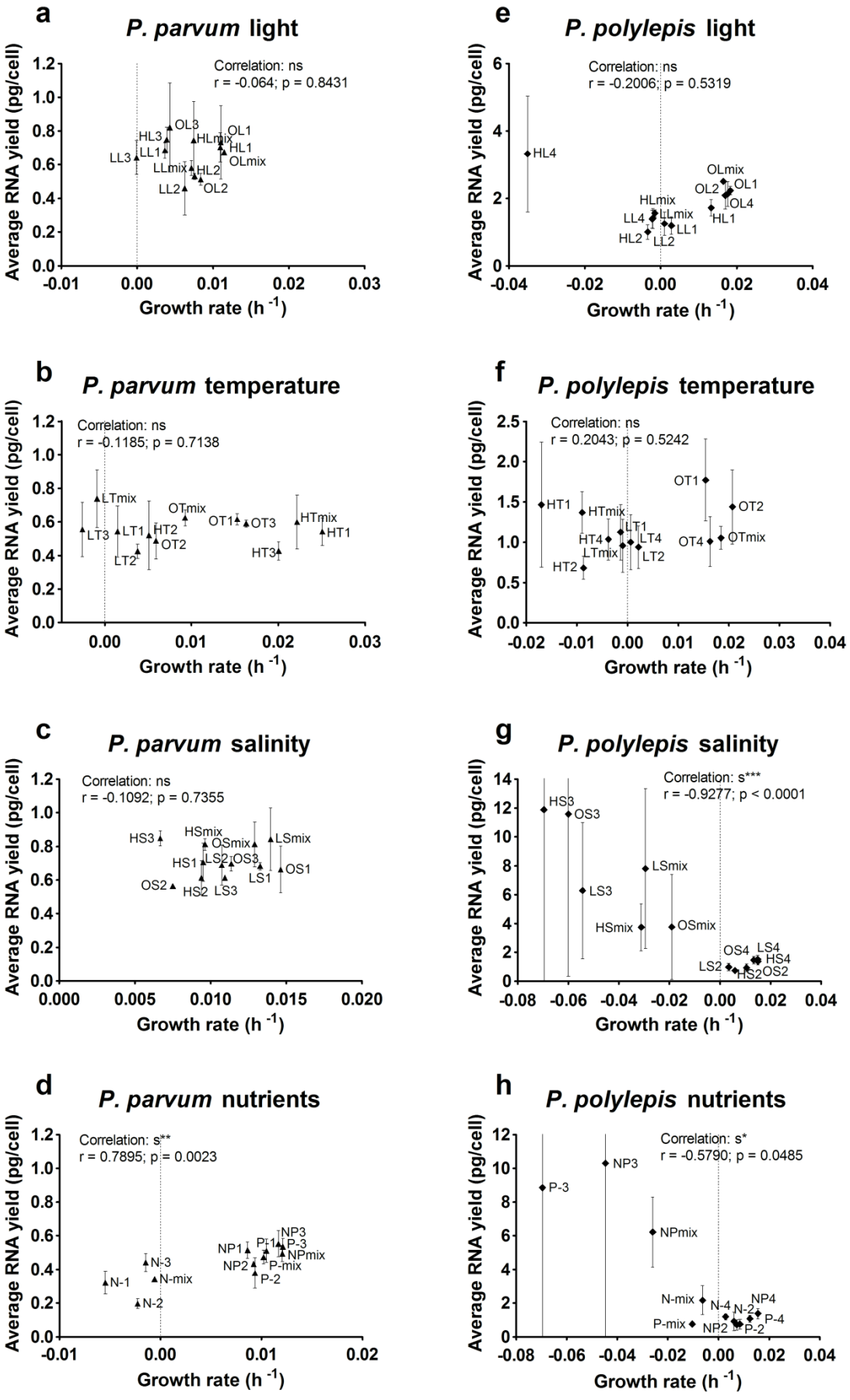
a
P. parvum light
Correlation: ns
r = -0.064; p = 0.8431
Average RNA yield (pg/cell)
Growth rate (h -1)
e
P. polylepis light
Correlation: ns
r = -0.2006; p = 0.5319
b
P. parvum temperature
Correlation: ns
r = -0.1185; p = 0.7138
f
P. polylepis temperature
Correlation: ns
r = 0.2043; p = 0.5242
c
P. parvum salinity
Correlation: ns
r = -0.1092; p = 0.7355
g
P. polylepis salinity
Correlation: s***
r = -0.9277; p < 0.0001
d
P. parvum nutrients
Correlation: s**
r = 0.7895; p = 0.0023
h
P. polylepis nutrients
Correlation: s*
r = -0.5790; p = 0.0485

Fig. III-2. Correlation between average RNA yield (pg/cell) and growth rates (h^{-1}) over different treatments and four different culture conditions (light, temperature, salinity and nutrients) for *Prymnesium parvum* (a-d) and *Prymnesium polylepis* (e-h). Each data point is indicated by a label: the first letters indicate low (L), optimal (O) and high (H) and the second letter indicates light (L), temperature (T), salinity (S) and nutrients (N- and P- indicate nitrate or phosphate depletion) experiments, the numbers represent the different strains of *P. parvum* ("1" (UIO054), "2" (SAG127.79) "3" (CCMP709)) and *P. polylepis* ("1" (UIO036), "2" (UiO037) "3" (UiO038) "4" (CCMP1757)). n.s. = not significant, *r* = Pearson correlation coefficient, *P* = significance of the correlation and * = level of significance. The test conditions for each treatment follow that in Table III-S2.

The difference in average RNA yield (pg/cell) between the three strains grown individually and ALL mix for stress conditions for *P. parvum* under light and temperature and for *P. polylepis* under light, temperature, salinity and nutrient conditions was not significant ($P > 0.05$; Figs. III-4a and b). However, there was a significant difference in the average RNA yields (pg/cell) between the temperature and salinity conditions with *P. parvum* ($P < 0.05$; Fig. III-4a). The average RNA yield (pg/cell) obtained from the ALL mix for *P. parvum* strains (*P. parvum* ALL mix = 0.64 pg/cell, s.d. = 0.18) or a combination of three *P. polylepis* ALL mix (*P. polylepis* ALL mix = 2.85 pg/cell, s.d = 2.80) from each stress condition was also determined (Fig. III-4a and b).

P. polylepis strain UiO038 used in the salinity and nutrient stress experiments was not very adaptive to changing environments and this resulted in cell numbers from initial inoculation decreasing (10-fold decrease) in numbers over the stress period of 72 h. For this reason, *P. polylepis* strain UiO 038 was not included in the salinity and nutrient results shown in Fig. III-3. Therefore, to compare average RNA yields (pg/cell) between the individual strains and the strains grown together (ALL mix), the UiO038 strain data was included (Fig. III-4b).

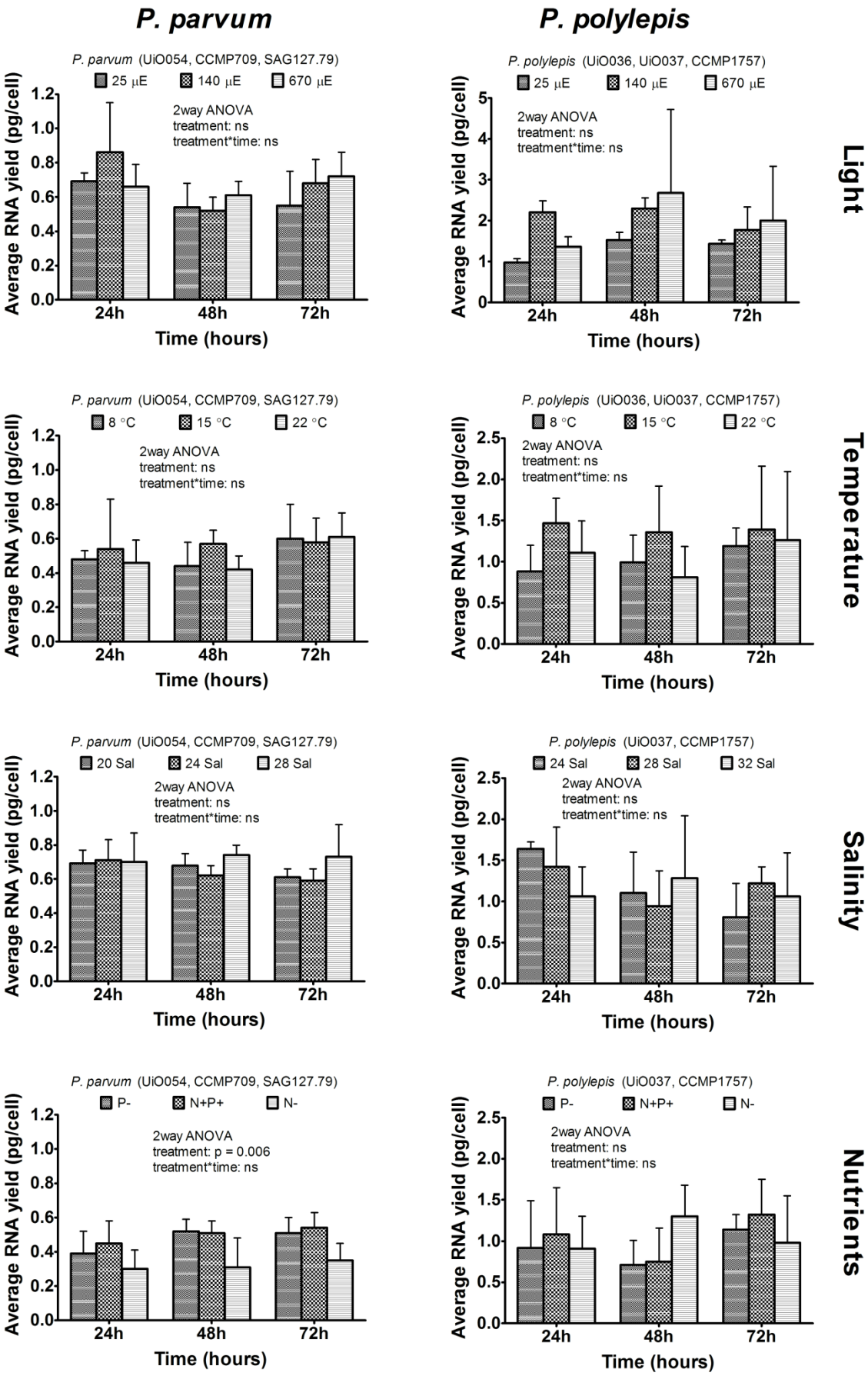
P. parvum
P. polylepis
Light
Temperature
Salinity
Nutrients
P. parvum (UiO054, CCMP709, SAG127.79)
P. polylepis (UiO036, UiO037, CCMP1757)
25 µE
140 µE
670 µE
8 °C
15 °C
22 °C
20 Sal
24 Sal
28 Sal
P. polylepis (UiO037, CCMP1757)
32 Sal
P-
N+P+
N-
2way ANOVA
treatment: ns
treatment*time: ns
treatment: p = 0.006
Average RNA yield (pg/cell)
Time (hours)
24h
48h
72h

Fig. III-3. Average RNA yield (pg/cell) for *Prymnesium parvum* and *Prymnesium polylepis* under various treatments of light, temperature, salinity and nutrient culture conditions. Note that results of salinity and nutrients conditions for *P. polylepis* do not contain UiO 038 strain data because of cell absence or death during stress period. A two-way ANOVA followed by a Bonferroni post test was used to analyse if there was any significant difference between treatments and over time. (n.s. = not significant; *P* > 0.05).

There was a positive linear relationship (*P. parvum* $R^2 = 0.76$; *P. polylepis* $R^2 = 0.74$) between cell numbers and total RNA amounts (ng) in both *Prymnesium* species (Figs. III-5a and c). The correlation was significant between cell numbers and total RNA amounts (ng) (*P. parvum* $r = 0.8717$ $P < 0.0001$, ($n = 108$); *P. polylepis* $r = 0.8578$ $P < 0.0001$ ($n = 108$)) for all the separate strains across each stress condition (Figs. III-5a and c). There was also a positive linear relationship (*P. parvum* All mix $R^2 = 0.72$; *P. polylepis* All mix $R^2 = 0.70$) between cell numbers and total RNA amounts (ng) in both *Prymnesium* ALL mix species (Figs. III-5b and d). The correlation was significant between cell numbers and total RNA amounts (ng) (*P. parvum* (All mix) $r = 0.85$ $P < 0.0001$, ($n = 35$); *P. polylepis* (All mix) $r = 0.84$ $P < 0.0001$ ($n = 35$)) for all the strains grown together across each stress condition (Figs. III-5b and d).

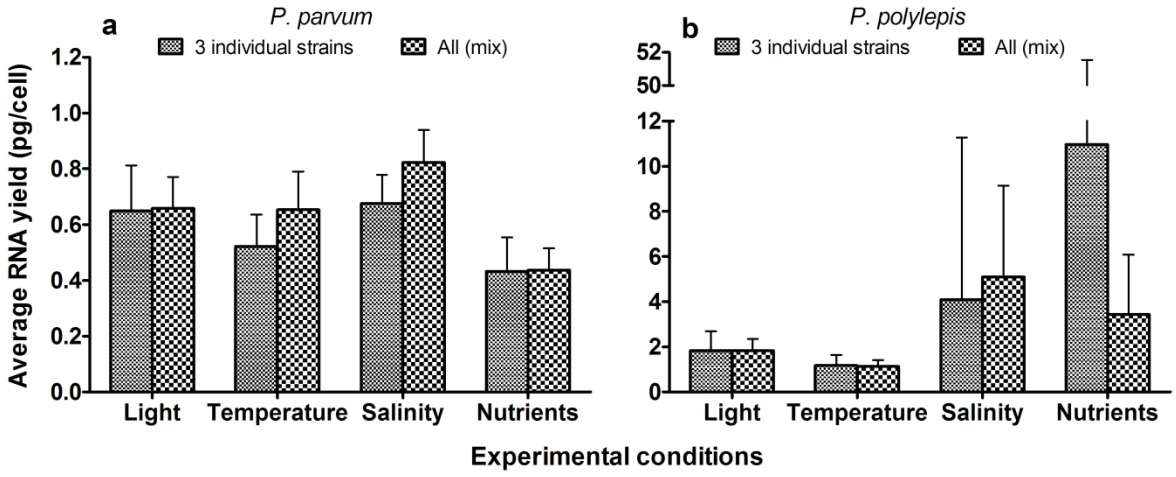

Fig. III-4. Comparisons of the average RNA amount (pg/cell) between individual strain grown separately and all (ALL mix) strains of each *Prymnesium* species grown together. (a) *P. parvum* comparison of average RNA yields for UiO 054, CCMP 709, and SAG 127.79 strains grown separately and all (ALL mix) the strains grown together over four conditions. (b) *P. polylepis* comparison of average RNA yields for UiO 036, UiO 037, and CCMP 1757 strains grown separately and all (ALL mix) the strains grown together over light and temperature conditions. For *P. polylepis* salinity and nutrient conditions it is the comparison of average RNA yields for UiO 037, UiO 038, and CCMP 1757 strains grown separately and all (ALL mix) the strains grown together.

Cf. *Chattonella* sp.

There was an increase in growth rate (h^{-1}) in all experimental culture conditions from T_0 to T_2 (48-h period) for all three strains of cf. *Chattonella* (Fig. III-1c). All culture conditions showed a negative non-significant correlation between growth rate (h^{-1}) and RNA yield (pg/cell; Fig. III-6a-d); except for salinity and nutrients which were significant (r = -0.6697; P = 0.0485; n = 9 and r = -0.6977; P = 0.0366; n = 9, respectively; Figs. III-6c and d).

Cf. *Chattonella sp.* showed no significant change ($P > 0.05$) in RNA yield (pg/cell) between strains across the treatments of light, temperature, salinity, and nutrient experiments taken at the 48 h period (Fig. III-7). The average RNA content for cf. *Chattonella* for the entire data set across three strains (CMSTAAC300, CMSTAC305 and CMSTAC307) and conditions was 17.82 ± 8.81 pg/cell (n = 36; Table III-1).

There was a positive linear relationship (R^2 = 0.58) between cell numbers and total RNA amounts (ng) for cf. *Chattonella* species (Fig. III-5e). The correlation between cell numbers and total RNA amounts (ng) (r = 0.76 $P < 0.0001$, (n = 36)) of all the strains across each stress condition was significant (Fig. III-5e).

Karlodinium veneficum

Growth rates (h^{-1}) for *Karlodinium veneficum* were estimated from only one time period at 48 h (Fig. III-1d). There was an increase in cell numbers in all experimental culture conditions from T_0 to T_2 (48-h period) except for strains CCCM734 (L1) and PCC517 (L3) at low salinity (Fig. III-1d). All tests showed a negative correlation between growth rate and RNA yields (pg/cell; Fig. III-6e-g), with salinity only being significant (r = -0.8483; P = 0.0078; n = 9; Fig. III-6g).

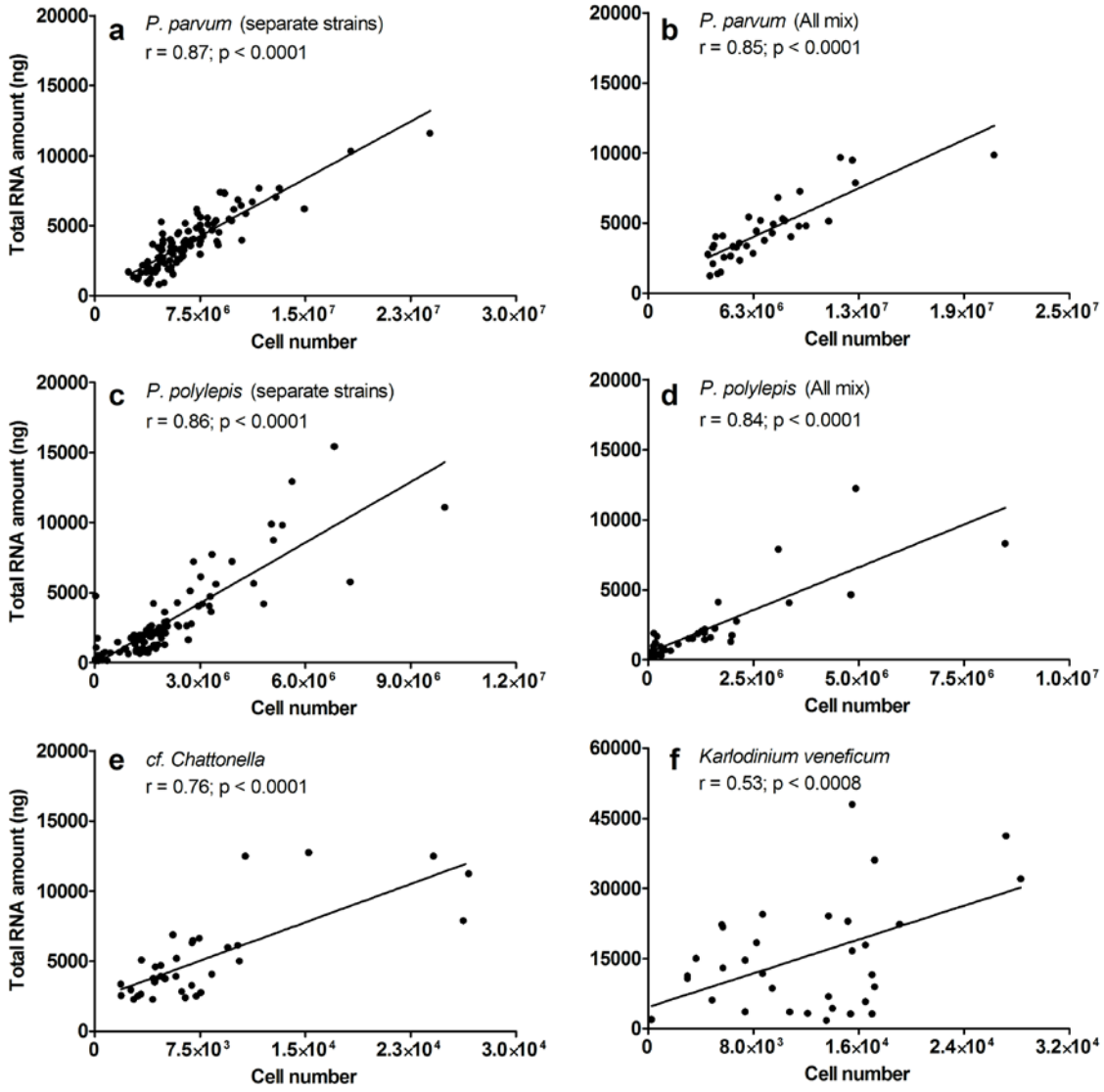

Fig. III-5. Correlation between total RNA amount (ng) and cell numbers over four different culture conditions (light, temperature, salinity and nutrient) for *P. parvum*, *P. polylepis*, cf. *Chattonella and K. veneficum*. (a) Each *P. parvum* strain (UiO 054, CCMP 709, and SAG 127.79) and (c) *P. polylepis* strain (UiO036, UiO037, UiO038 and CCMP1757) grown separately (*n* = 108) and (b) all *P. parvum* strains and (d) *P. polylepis* strains grown together (*n* = 35) from T_1 to T_3. (e) Each cf. *Chattonella* strain (CMSTAC300, CMSTAC305 and CMSTAC307) and (f) *Karlodinium veneficum* strain (CCCM734, PCC709 and PCC517) grown separately from T_2 (*n* = 36, respectively). The black line represents the linear regression, *r* = Pearson correlation coefficient, *P* = significance of the correlation (α = 0.05).

Karlodinium veneficum showed no significant change in RNA yield (pg/cell) between strains and various treatments of light, salinity and nutrient stress during the 48-h period (Fig. III-7). However there was a significant difference between treatments for temperature (P = 0.0070), but not between the strains. Due to only one period of 48 h being available, there are no replicate values to provide standard errors between strains. Therefore, to see if differences between the temperature treatments were significant, the RNA yields for the three different strains (CCCM734, PCC709 and PCC517) were averaged and analysed by a one-way ANOVA followed by a Tukey multiple comparison test. The results showed a significant difference (P = 0.0169) only between the low (10 °C) temperature treatment with either optimal (15 °C) or high (20 °C) treatments. However, due to a Gaussian distribution test indicating $P < 0.05$ for *K. veneficum* data, an additional non-parametric one-way ANOVA test called Kruskal-Wallis analysed with a Dunn's multiple comparison test was also used to verify if there was significant difference between treatments. In all cases the Kruskal-Wallis test deemed there was no significantly difference between treatments for all the stress conditions tested. The average RNA content for *K. veneficum* for the whole data set across three strains (CCCM734, PCC709 and PCC517) and conditions was 44.99 ± 43.85 pg/cell (n = 36; Table III-1).

The linear relationship (R^2 = 0.29) between cell numbers and RNA yields for *K. veneficum* and correlation [r = 0.53 $P < 0.0008$, (n = 36)] of all the strains across each stress condition was significant (Fig. III-5f).

Microarray calibration curves

Higher taxon probes specific to the hierarchy of a given species had to produce a positive signal for a species-level signal to be considered positive. Probe signal intensities on the microarray were recorded as positive when they produced a signal-to-noise ratio $\geqslant 2$. As the higher taxon probes typically gave a greater intensity than the corresponding species level probe, false positive results were thus eliminated (Table III-S3).

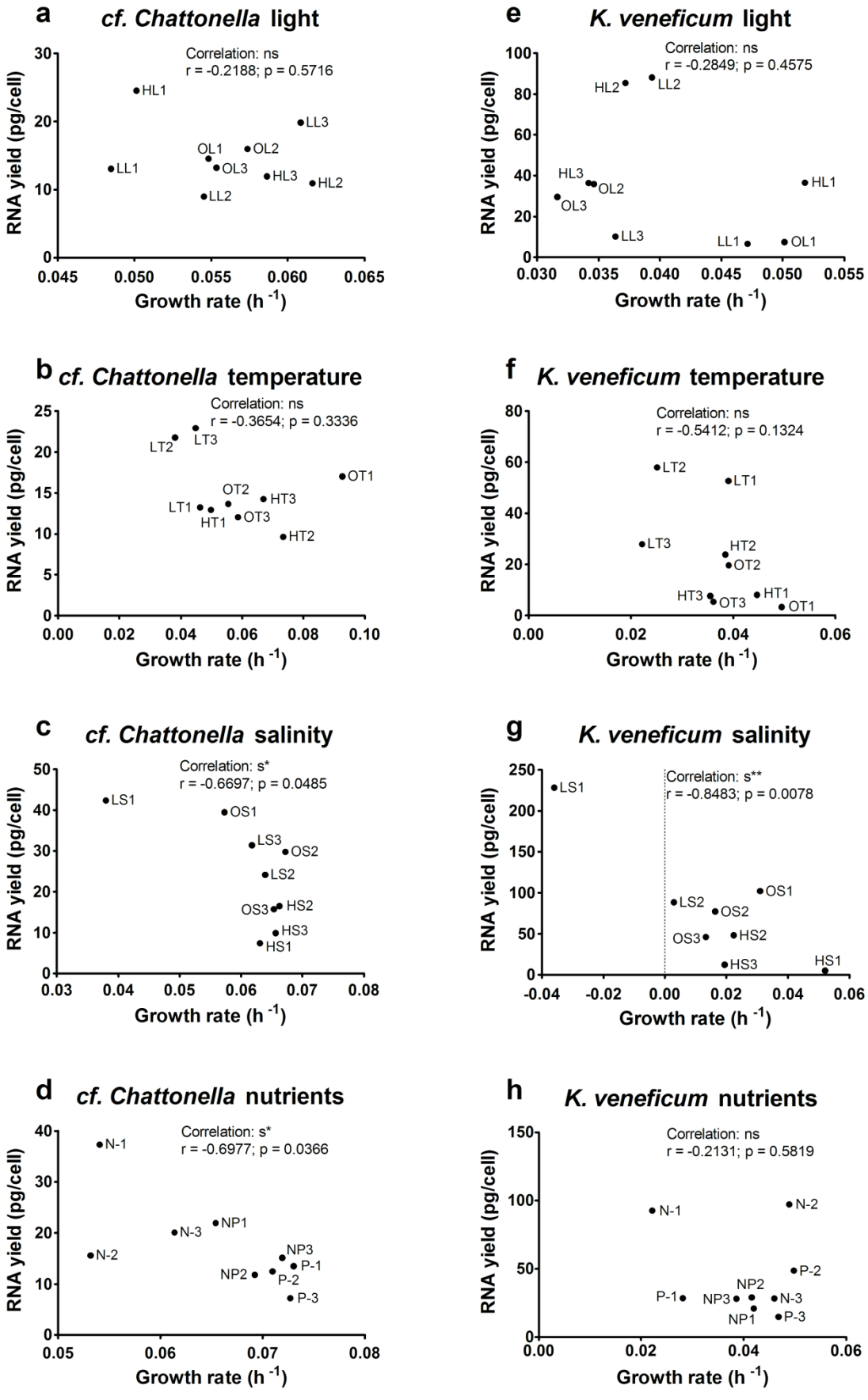
a
cf. Chattonella light
Correlation: ns
r = -0.2188; p = 0.5716
RNA yield (pg/cell)
Growth rate (h -1)
HL1
LL3
OL1
OL2
LL1
OL3
HL3
HL2
LL2
b
cf. Chattonella temperature
Correlation: ns
r = -0.3654; p = 0.3336
LT2
LT3
OT1
OT2
HT3
LT1
HT1
OT3
HT2
c
cf. Chattonella salinity
Correlation: s*
r = -0.6697; p = 0.0485
LS1
OS1
LS3
OS2
LS2
OS3
HS2
HS3
HS1
d
cf. Chattonella nutrients
Correlation: s*
r = -0.6977; p = 0.0366
N-1
N-3
NP1
N-2
NP3
P-1
NP2
P-2
P-3
e
K. veneficum light
Correlation: ns
r = -0.2849; p = 0.4575
HL2
LL2
HL3
OL2
HL1
OL3
LL3
LL1
OL1
f
K. veneficum temperature
Correlation: ns
r = -0.5412; p = 0.1324
LT2
LT1
LT3
HT2
OT2
HT3
OT3
HT1
OT1
g
K. veneficum salinity
Correlation: s**
r = -0.8483; p = 0.0078
LS1
OS1
LS2
OS2
OS3
HS2
HS3
HS1
h
K. veneficum nutrients
Correlation: ns
r = -0.2131; p = 0.5819
N-1
N-2
P-2
NP2
P-1
NP3
N-3
NP1
P-3

Fig. III-6. Correlation between RNA yield (pg/cell) and growth rates (h^{-1}) over different treatments and four different culture conditions (light, temperature, salinity and nutrients) for cf. *Chattonella* (a-d) and *Karlodinium veneficum* (e-h). Each data point is indicated by a label: the first letters indicate low (L), optimal (O) and high (H) and the second letter indicates light (L), temperature (T), salinity (S) and nutrients (N- and P- indicate nitrate or phosphate depletion) experiments, the numbers represent the different strains for cf. *Chattonella* ["1" (CMSTAC300), "2" (CMSTAC305) "3" (CMSTAC307)] and *K. veneficum* ["1" (CCCM734), "2" (PCC709) "3" (PCC517)]. n.s. = not significant, *r* = Pearson correlation coefficient, *P* = significance of the correlation and * = level of significance. The test conditions for each treatment follow that in Table III-S2.

Calibration curves in this study are represented as cell numbers of target species normalised to the microarray signals obtained from the two control probes POSITIVE_25_dT (TATA-box protein) and DunGS02_25_dT_dT (*D. tertiolecta*). The calibration curves for *P. parvum*, *P. polylepis*, cf. *Chattonella* and *K. veneficum* were generated using increasing amounts of labelled RNA (1 ng, 5 ng, 25 ng and 100 ng) for hybridisation to the third generation microarray (Fig. III-8).

Prymnesium parvum

Both Eukaryote probes EukS_1209_25_dT and EukS_328_25_dT produced a positive microarray signal when hybridised with 25 ng and 100 ng of labelled *P. parvum* RNA (Lim *et al.,* 1993; Moon-Van Der Staay *et al.,* 2001; Table III-S3). The only higher group probe for *Prymnesiophyta* (PrymS01_25_dT and PrymS02_25_dT; Lange *et al.,* 1996; Simon *et al.,* 2000; Table III-S3) to return a significant linear regression was PrymS01_25_dT normalised to POSITIVE_25_dT (R^2 = 0.98; P = 0.0400). However, both these higher group probes signal intensities were positively correlated except for PrymS02_25_dT normalised to DunGS02_25_dT_dT, which produced a negative linear regression (Table III-2). The Class level probe (PrymS03_25_dT; Eller *et al.,* 2007; Table III-S3) for *Prymnesiophyceae* normalised signal intensity with both control probes had a positive linear relationship $R^2 \geqslant 0.84$; but, only the normalised POSITIVE_25_dT curve was significant (P = 0.0276). Both the clade level probes (Clade01old_25_dT (*Prymnesium*) and Clade01new25_dT (*Prymnesium* B1 clade *sensu* Edvardsen *et al.,* 2000) normalised signal intensities were positively correlated $R^2 \geqslant 0.73$ and significant ($P \leqslant 0.0477$) except for Clade01new25_dT normalised to DunGS02_25_dT_dT (Table III-2).

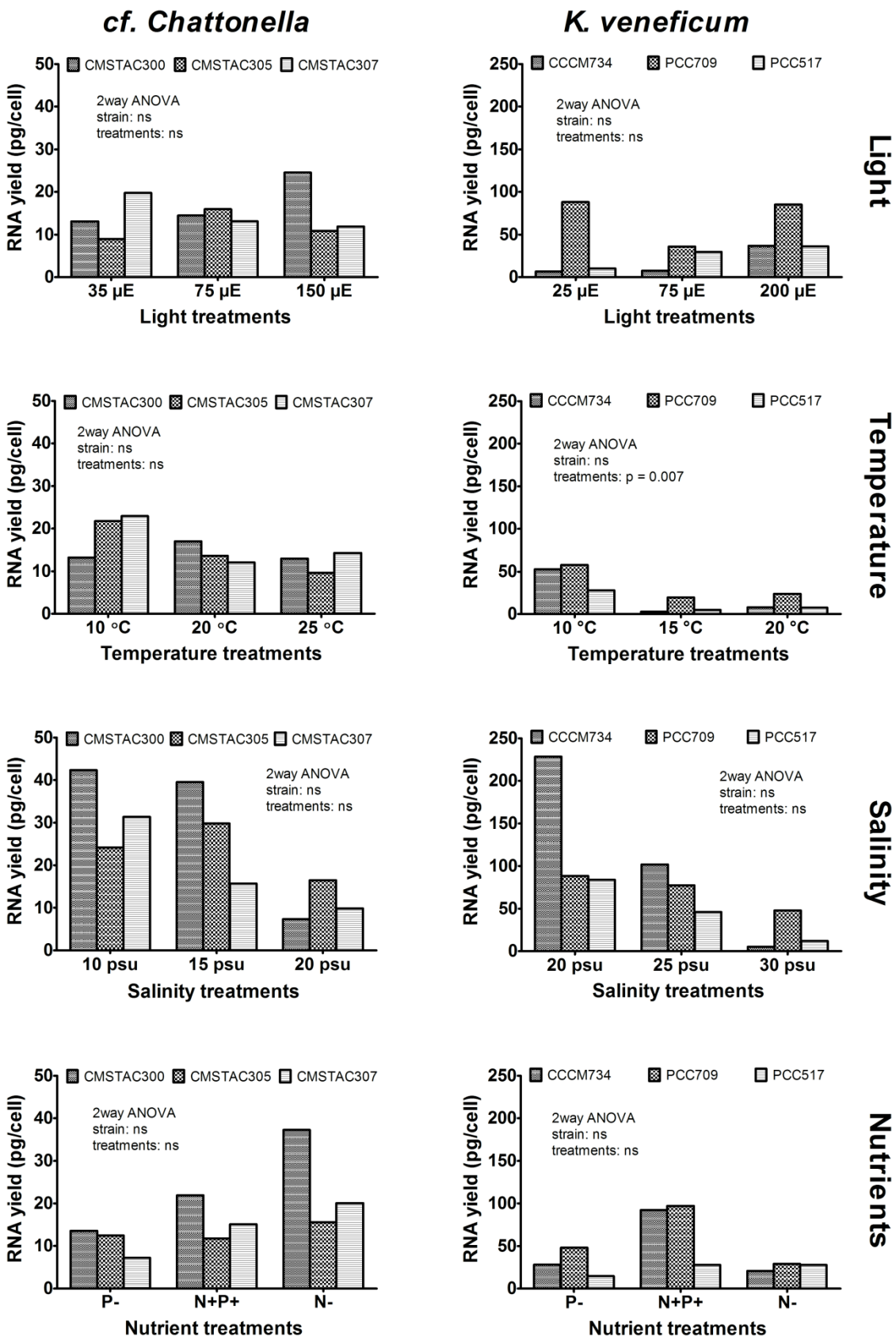
cf. Chattonella
K. veneficum
CMSTAC300
CMSTAC305
CMSTAC307
CCCM734
PCC709
PCC517
RNA yield (pg/cell)
2way ANOVA
strain: ns
treatments: ns
35 µE
75 µE
150 µE
25 µE
75 µE
200 µE
Light treatments
Light
2way ANOVA
strain: ns
treatments: p = 0.007
10 °C
15 °C
20 °C
25 °C
Temperature treatments
Temperature
10 psu
15 psu
20 psu
25 psu
30 psu
Salinity treatments
Salinity
P-
N+P+
N-
Nutrient treatments
Nutrients

Fig. III-7. RNA yield (pg/cell) for cf. *Chattonella* strains (CMSTAC300, CMSTAC305 and CMSTAC307) and *Karlodinium veneficum* strains (CCCM734, PCC709 and PCC517) under various treatments of light, temperature, salinity and nutrient culture conditions. Because replicate values are not included, it is necessary to assume that there is no interaction. In other words, this analysis assumes that the strains have the same effect (if any) at all levels of conditions using two-way ANOVA. (n.s. = not significant; *P* > 0.05).

Two species-specific probes for *P. parvum* were redesigned for the third generation chip from original sequences in the 28S (PparvD01_25_dT; Töbe *et al.*, 2007) and 18S regions (Prymparv01_25_dT; Eller *et al.*, 2007) (Table III-S3). The first probe PparvD01_25_dT signal showed a significant (R^2 = 0.99; *P* = 0.0020) linear regression when normalised to POSITIVE_25_dT, but was not significant when normalised to DunGS02_25_dT_dT (R^2 = 0.94; *P* = 0.0655; Table III-2). The second probe Prymparv01_25_dT showed a significant linear regression when signal intensities were normalised against both POSITIVE_25_dT (R^2 = 0.99; p = 0.0149) and DunGS02_25_dT_dT (R^2 = 0.98; p = 0.0401) probes (Table III-2).

Cross reactivity was observed for HeteroS01_25_dT, HeterokontComp (*Heterokonts*) and DinoB_25_dT (*Dinophytes*) probes for RNA amounts $\geqslant$ 25 ng, suggesting false-positive microarray signals. These false-positive signals increased for other probes when higher amounts of RNA were hybridised to the microarray, including probes PschGS01_25_dT (*Pseudochattonella sp.*), L*Kare0308A25_dT (*Karenia sp.*), KmGcS06_25_dT (*Karenia mikimotoi*), PcaserausD03_25_dT (Pseudo-nitzschia spp.), SSHaka0193A25_dT (*H. akashiwo*), DphyexacutaFS01_25_dT (Dinophysiaceae (*Dinophysis*+*Phalacroma*)), and KveneD06_25_dT (*Karlodinium veneficum*).

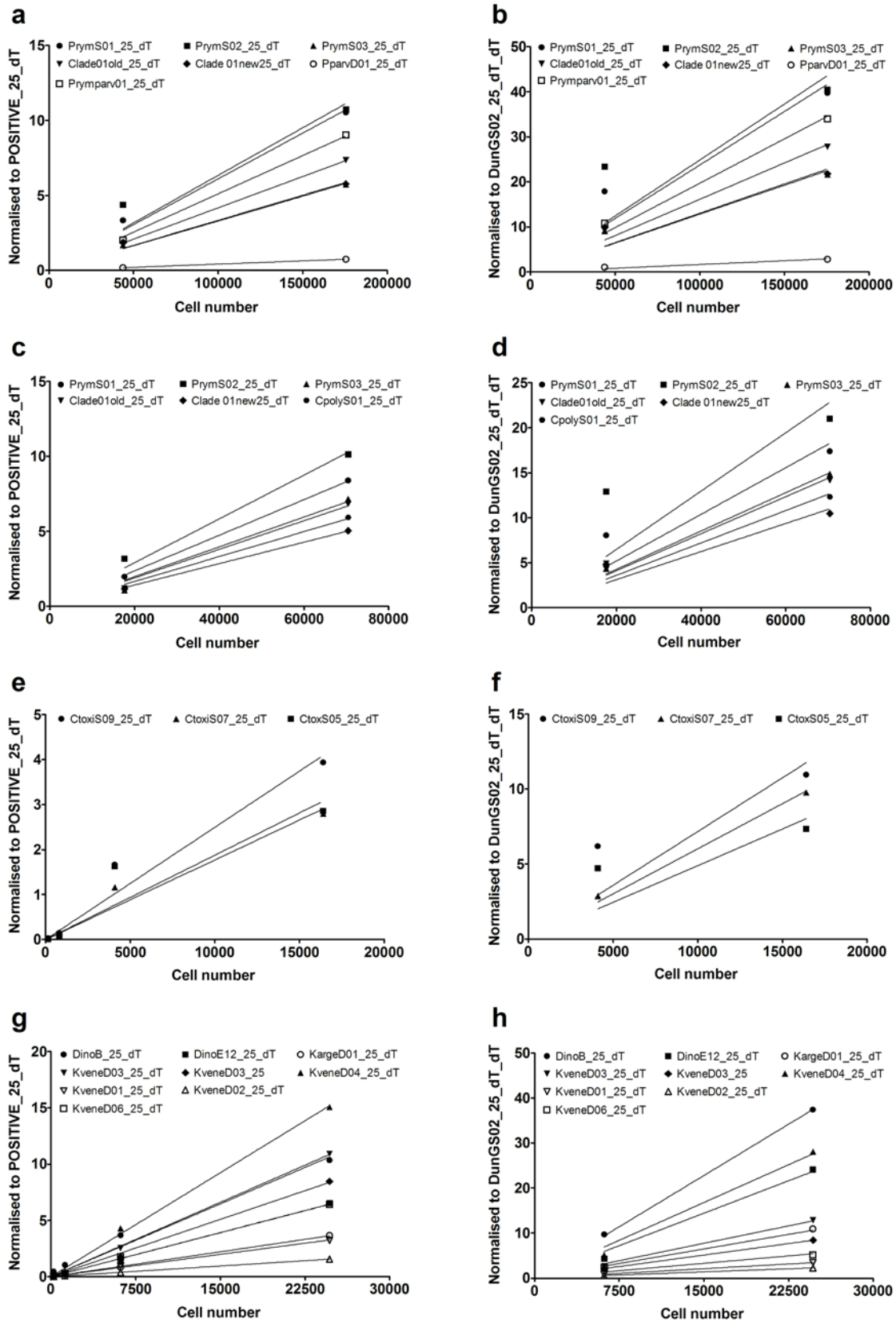

Fig. III-8. Calibrations curves between normalised microarray signals and corresponding cell numbers. (a and b) Calibration curves for *Prymnesium parvum* showing higher group probes

(PrymS01_25_dT, PrymS02_25_dT and PrymS03_25_dT), clade-level probes (Clade01old_25_dT and Clade01new25_dT) and species-level probes (PparvD01_25_dT and Prymparv01_25_dT) normalised to control probes POSITIVE_25_dT and DunGS02_25_dT_dT, respectively. (c and d) Calibration curves for *Prymnesium polylepis* showing higher group probes (PrymS01_25_dT, PrymS02_25_dT and PrymS03_25_dT); clade-level probes (Clade01old_25_dT and Clade01new25_dT) and species level probes (CpolyS01_25_dT) normalised to control probes POSITIVE_25_dT and DunGS02_25_dT_dT, respectively. (e and f) Calibration curves for cf. *Chattonella* spp. showing species-level probes (CtoxiS09_25_dT, CtoxiS05_25_dT and CtoxiS07_25_dT) normalised to control probes POSITIVE_25_dT and DunGS02_25_dT_dT, respectively. (g and h) Calibration curves for *Karlodinium veneficum* showing class-level probes (DinoB_25_dT and DinoE12_25_dT); genus-level probe (KargeD01_25_dT) and species-level probes (KveneD01_25_dT, KveneD02_25_dT, KveneD03_25_dT, KveneD03_25, KveneD04_25_dT, and KveneD06_25_dT) normalised to control probes POSITIVE_25_dT and DunGS02_25_dT_dT, respectively (Note: trendline forced intercept of zero).

Prymnesium polylepis

All probes that were meant to be highlighted when hybridised with labelled *P. polylepis* RNA successfully passed the hierarchy test. Both PrymS01_25_dT and PrymS02_25_dT probe signal intensities produced a significant linear regression when normalised to POSITIVE_25_dT ($R^2 \geqslant 0.98$; $P \leqslant 0.0373$). However, when normalised to DunGS02_25_dT_dT, the linear regression was not significant with PrymS02_25_dT (Table III-2). Class-level probe PrymS03_25_dT signal intensity was not significant ($R^2 = 0.97$; $P = 0.0615$) when normalised with POSITIVE_25_dT as opposed to DunGS02_25_dT_dT normalisation resulting in a significant linear regression ($R^2 = 0.99$; $P = 0.0251$; Table III-2). The signal intensities of the two clade-level probes Clade01old_25_dT and Clade01new25_dT had a significant linear regression when normalised to POSITIVE_25_dT ($R^2 \geqslant 0.985$; $P \leqslant 0.0451$), however, they were not significant when normalised to DunGS02_25_dT_dT (Table III-2). The only species-specific probe spotted on the third generation chip for *P. polylepis* is Cpoly01_25_dT, its corresponding signal intensity returned a significant liner regression when normalised to POSITIVE_25_dT ($R^2 > 0.98$; $P = 0.0361$); this was not significant when normalised to DunGS02_25_dT_dT (Table III-2).

Cross reactivity was observed for HeteroS01_25_dT, HeterokontComp (Heterokonts) and DinoB_25_dT (Dinophytes) for RNA amounts $\geqslant$ 25 ng. Several false-positive signals were also observed at the same RNA amount $\geqslant$ 25 ng for the probes

PschGS01_25_dT (*Pseudochattonella* sp.), L*Kare0308A25_dT (*Karenia* sp.), KbreD03_25_dT (*Karenia* sp.), PcaserausD03_25_dT (*Pseudo-nitzschia* spp.) and LSGcat0270A24_dT (*Gymnodinium catenatum*).

Cf. *Chattonella* sp.

The higher group Eukaryote probe EukS_1209_25_dT produced a positive microarray signal for all RNA amount (1 ng, 5 ng, 25 ng and 100 ng) hybridised to the third generation chip. However, EukS_328_25_dT did not produce a positive microarray signal for cf. *Chattonella* RNA amount < 25 ng, which has implications for the hierarchical groups below this probe, which would be recorded as false positives. The species-level probes for cf. *Chattonella* (CtoxS05_25_dT, CtoxiS07_25_dT and CtoxiS09_25_dT) all produced positive microarray signal-to-noise ratio values > 2 with 1 ng of labelled cf. *Chattonella* RNA being hybridised to the third generation chip, which is equivalent to < 200 cells. All three cf. *Chattonella* probe signal intensities returned significant linear regressions when normalised to POSITIVE_25_dT signals and only CtoxiS07_25_dT probe when normalised to DunGS02_25_dT_dT ($R^2 \geqslant 0.85$; $P \leqslant 0.0259$). The linear regression was not significant when CtoxS05_25_dT and CtoxiS09_25_dT probes were normalised to the signal returned by DunGS02_25_dT_dT probe (Table III-2). In terms of hierarchy, CtoxiS09_25_dT probe produced a higher signal compared to CtoxS05_25_dT and CtoxiS07_25_dT probes and thus this is reflected in the hierarchy file.

Cross reactivity was observed for KmGcS06_25_dT (*Karenia mikimotoi*) at 1 ng and by PschGS04_25_dT (*Pseudochattonella* spp.) at 5 ng. This was also the case for probes AlexGD01_25_dT (*Alexandrium* sp.) and SSGcat0826A27_dT (*Gymnodinium catenatum*) for $\leqslant$ 25 ng RNA. The number of false-positives increased for RNA amounts $\geqslant$ 100 ng for probes Clade01old_25_dT (*Prymnesium* B1 clade), PpungcalS01_25_dT (*Pseudo-nitzschia* spp.), PcalfrauD04_25_dT (*Pseudo-nitzschia* spp.), DacutaD02_25_dT (*Dinophysis* spp.), PverD01_25_dT (*Pseudochattonella verruculosa*) and ProroFBS01 (*Prorocentrum* benthic clade). All false positives are eradicated by invoking the hierarchy file.

Table III-2. The R^2 values, corresponding slope and level of significance of linear regression for normalised microarray signals against the controls POSITIVE_25_dT and DunGS02_25_dT_dT are shown below. Each probe is represented that should be highlighting when pure culture labelled RNA from *P. parvum, P. polylepis,* cf. *Chattonella* and *K. veneficum* is hybridised to the third generation microarray (Note. trendline forced intercept of zero).

Species	Probe name	Normalised probe	R^2 value	Slope	P values
P. parvum	PrymS01_25_dT	POSITIVE_25_dT	0.9814	0.000061 ± 0.000004	0.0400*
		DunGS02_25_dT_dT	0.7536	0.00024 ± 0.00004	0.1129
	PrymS02_25_dT	POSITIVE_25_dT	0.8657	0.000063 ± 0.000009	0.0910
		DunGS02_25_dT_dT	-0.122	0.00025 ± 0.00007	0.1772
	PrymS03_25_dT	POSITIVE_25_dT	0.9917	0.000033 ± 0.000001	0.0276*
		DunGS02_25_dT_dT	0.8401	0.00013 ± 0.00002	0.0969
	Clade01old_25_dT	POSITIVE_25_dT	0.9992	0.0000417 ± 0.0000006	0.0096**
		DunGS02_25_dT_dT	0.9722	0.00016 ± 0.00001	0.0477*
	Clade01new25_dT	POSITIVE_25_dT	0.9789	0.000033 ± 0.000002	0.0426*
		DunGS02_25_dT_dT	0.7345	0.00013 ± 0.00002	0.1157
	PparvD01_25_dT	POSITIVE_25_dT	0.9999	0.00000428 ± 0.00000001	0.0020**
		DunGS02_25_dT_dT	0.9435	0.000017 ± 0.000002	0.0655
	Prymparv01_25_dT	POSITIVE_25_dT	0.9888	0.000051 ± 0.000001	0.0149*
		DunGS02_25_dT_dT	0.9813	0.00020 ± 0.00001	0.0401*
P. polylepis	PrymS01_25_dT	POSITIVE_25_dT	0.9993	0.000119 ± 0.000002	0.009**
		DunGS02_25_dT_dT	0.7118	0.00026 ± 0.00005	0.1191
	PrymS02_25_dT	POSITIVE_25_dT	0.9841	0.000156 ± 0.000009	0.0373*
		DunGS02_25_dT_dT	-0.667	0.00032 ± 0.00010	0.1946
	PrymS03_25_dT	POSITIVE_25_dT	0.9735	0.000099 ± 0.000001	0.0615
		DunGS02_25_dT_dT	0.9934	0.000213 ± 0.000008	0.0251*
	Clade01old_25_dT	POSITIVE_25_dT	0.985	0.000095 ± 0.000007	0.0451*
		DunGS02_25_dT_dT	0.9597	0.00021 ± 0.00002	0.0559
	Clade01new25_dT	POSITIVE_25_dT	0.9992	0.000071 ± 0.000001	0.0095**
		DunGS02_25_dT_dT	0.7204	0.00016 ± 0.00003	0.1177
	CpolyS01_25_dT	POSITIVE_25_dT	0.9897	0.000083 ± 0.000005	0.0361*
		DunGS02_25_dT_dT	0.9209	0.00018 ± 0.00002	0.0712
cf. *Chattonella*	CtoxiS09_25_dT	POSITIVE_25_dT	0.9557	0.00025 ± 0.00002	0.0016**
		DunGS02_25_dT_dT	0.0101	0.00072 ± 0.00020	0.1716
	CtoxS05_25_dT	POSITIVE_25_dT	0.855	0.00019 ± 0.00003	0.0086**
		DunGS02_25_dT_dT	-1.2066	0.00049 ± 0.00016	0.2064
	CtoxiS07_25_dT	POSITIVE_25_dT	0.9593	0.00018 ± 0.00002	0.0014**
		DunGS02_25_dT_dT	0.993	0.00060 ± 0.00002	0.0259*
K. veneficum	DinoB_25_dT	POSITIVE_25_dT	0.9756	0.00043 ± 0.00003	< 0.0001***
		DunGS02_25_dT_dT	0.9997	0.00152 ± 0.00001	0.0054**
	DinoE12_25_dT	POSITIVE_25_dT	0.9969	0.000261 ± 0.000007	< 0.0001***
		DunGS02_25_dT_dT	0.9866	0.00096 ± 0.00006	0.042*
	KargeD01_25_dT	POSITIVE_25_dT	0.9991	0.000147 ± 0.000002	< 0.0001***
		DunGS02_25_dT_dT	0.9686	0.00043 ± 0.00005	0.0681
	KveneD01_25_dT	POSITIVE_25_dT	0.9966	0.000132 ± 0.000003	< 0.0001***

	DunGS02_25_dT_dT	0.9995	0.000139 ± 0.000002	0.0074**
KveneD02_25_dT	POSITIVE_25_dT	0.9993	0.0000630 ± 0.0000008	< 0.0001***
	DunGS02_25_dT_dT	0.9999	0.0000917 ± 0.0000004	0.0027**
KveneD03_25	POSITIVE_25_dT	0.9961	0.000339 ± 0.000001	< 0.0001***
	DunGS02_25_dT_dT	0.9999	0.000341 ± 0.000002	0.0037**
KveneD03_25_dT	POSITIVE_25_dT	0.9988	0.000440 ± 0.000007	< 0.0001***
	DunGS02_25_dT_dT	0.9941	0.00052 ± 0.00002	0.0268*
KveneD04_25_dT	POSITIVE_25_dT	0.9977	0.00062 ± 0.00001	< 0.0001***
	DunGS02_25_dT_dT	0.9872	0.00112 ± 0.00007	0.041*
KveneD05_25_dT	POSITIVE_25_dT			
	DunGS02_25_dT_dT			
KveneD06_25_dT	POSITIVE_25_dT	0.9988	0.000261 ± 0.000004	< 0.0001***
	DunGS02_25_dT_dT	0.7542	0.00022 ± 0.00004	0.1127

P values (>0.05 = not significant; 0.01 to 0.05 = significant*; 0.001 to 0.01 = very significant** and <0.001 = extremely significant***)

Karlodinium veneficum

Higher group Eukaryote probes EukS_1209_25_dT and EukS_328_25_dT produced a positive microarray signal when hybridised with low to high labelled RNA amounts of *K. veneficum*. This was also the case with the class-level probes DinoB_25_dT and DinoE12_25_dT, which returned a significant linear regressions when the signals were normalised to both probe controls POSITIVE_25_dT ($R^2 \geqslant 0.98$; $P < 0.0001$) and DunGS02_25_dT_dT ($R^2 \geqslant 0.99$; $P \leqslant 0.042$). The genus-level probe KargeD01_25_dT signal produced a significant linear regression when normalised to the signal of POSITIVE_25_dT ($R^2 > 0.99$; $P < 0.0001$). However, this was not significant when normalised to DunGS02_25_dT_dT (Table III-2). All the species specific probes (KveneD01_25_dT, KveneD02_25_dT, KveneD03_25, KveneD03_25_dT, KveneD04_25_dT and KveneD06_25_dT) had significant linear regressions ($R^2 \geqslant 0.99$; $P \leqslant 0.0268$) except KveneD06_25_dT when normalised to DunGS02_25_dT_dT ($R^2 = 0.75$; $P = 0.1127$; Table III-2). Species-specific probes KveneD04_25_dT, KveneD03_25_dT KveneD06_25_dT and KveneD03_25 produced a positive microarray signal-to-noise ratio values > 2 with labelled RNA amount of 1 ng, which corresponds to *c.* 250 *K. veneficum* cells. KveneD01_25_dT and KveneD02_25_dT probes required at least 5 ng to produce a positive microarray signal.

Cross reactivity leading to false positives was observed at the lowest levels of 1 ng with probes HeterokontCOMP, PrymS02_25_dT (Prymnesiophyta), KbreD05_25_dT (*Karenia brevis*) and PmulacalD02_25_dT (*P. multistriata* + *P. calliantha* + *P.*

australis). The number of false positives increased with higher amounts of *K. veneficum* RNA, including PrymS01_25_dT (Prymnesiophyta), AlexGD01_25_dT (*Alexandrium* sp.), L*Kare0308A25_dT (*Karenia* sp.), DphyFS02_25_dT (Dinophysiaceae (*Dinophysis*+*Phalacroma*) and DphyexacutaFS01_25_dT [Dinophysiaceae (*Dinophysis*+*Phalacroma*)] at hybridisations of 5 ng. Multiple false positives were recorded for $\geqslant$ 25 ng RNA amounts; however all false positives are eliminated by the hierarchy file.

DISCUSSION

The determination of the relationship between RNA amount, microalgal cell numbers and microarray signals was a key objective of the MIDTAL project. A reliable estimate of toxic algal cell concentrations in environmental samples could then be obtained. To achieve this, however, it was necessary to evaluate how RNA yields varied in microalgal strains subjected to different stress conditions.

Prymnesium parvum and *P. polylepis*.

Prymnesium spp., which belong to the division *Haptophyta*, have been well documented for their ability to form golden brown blooms often associated with large fish mortality and extensive economic loss (Edvarsden & Paasche, 1998). This, in turn, has prompted widespread studies into the ecology of these harmful algae and the factors driving their growth, especially that of *P. parvum*, which is one of the most toxic and well-studied species (Guo *et al.*, 1996; Edvarsden & Paasche, 1998; Landsberg, 2002; Granéli *et al.*, 2012). Another prymnesiophyte is *Chrysochromulina polylepis*, a very toxic species that caused a devastating bloom in south-western Sweden and parts of the Norwegian Sea in 1988, killing over 800 tons of farmed fish with losses estimated at €10 million (Edvarsden & Paasche, 1998). Recently, morphological and ribosomal DNA sequence data have revealed that *C. polylepis* is more closely related to *Prymnesium* spp. than to other *Chrysochromulina* spp., and it has since been reclassified into the genus *Prymnesium* as *Prymnesium polylepis*. (Edvarsden *et al.*, 2011).

For the two *Prymnesium* spp. strains tested in this study, salinity modulation caused the lowest variation in cellular RNA yield (pg/cell) over the period tested. This is not surprising because it has been reported that *P. parvum* can grow over a wide range of

salinities (Edvarsden & Paasche, 1998) whereas *P. polylepis* is not usually found in estuarine waters where salinity varies. Several nutrient studies have been carried out on *P. parvum* and *P. polylepis* strains in relation to nitrogen : phosphorus ratio modulation to assess intracellular carbon, nitrogen and phosphorus dynamics together with the potential production of haemolytic substances (Johannsson & Granéli, 1999; Granéli *et al.*, 2012). Toxic effect potential has been reported highly variable among the haptophytes under nutrient limiting conditions, which can display allopathy related inhibitions toward other phytoplankton species and other marine organisms (Johannsson & Granéli, 1999; Granéli *et al.,* 2012). Potentially impacting on the intracellular RNA amount caused by growth suppression, which may explain the highly variable *P. polylepis* RNA yields (pg/cell) for UiO037, UiO038, and CCMP1757 strains grown separately and all the strains grown together (ALL mix) for nutrient stress conditions. This also may suggest that strains within the same species may also try to outcompete one another during nutrient limitation. This possibly had an effect on finding statistical differences for *P. polylepis* between the three individual strains and the ALL mix cultures under varying salinity and nutrient conditions because of the substantial standard deviations, which made the statistical analysis less conservative.

cf. *Chattonella* spp.

An unknown microflagellate was isolated from a fish kill in Torquay Canal, Rehoboth Bay Delaware, USA (Bowers *et al.,* 2004; C.R. Tomas unpublished). It was initially identified as *Chattonella* cf. *verruculosa* based on its multiple plastids and a flagellar structure similar to that of other raphidophytes. A total of about 7 strains were isolated from this first fish kill and subsequent blooms from Delaware, Maryland and North Carolina. A phylogenetic analysis using 18S rDNA data revealed that the strains belonged to a new algal class, sister to a clade containing raphidophytes, xanthophytes and phaeophytes. A description of a new algal class has been submitted for publication (Tomas *et al.,* unpublished) and to not invalidate the old name yet, we referred to the strains used in this study as cf. *Chattonella.* To date, all strains have been tested toxic for a breve-like toxin (Bourdelais *et al.,* 2002).

Cf. *Chattonella* spp. was most affected by salinity, with lower salinity producing the highest RNA yield (pg/cell). Raphidophyte species have been known to be salinity tolerant, producing increases in the level of toxins in low salinity environments, which

are thought to have evolved from predation pressures (Strom *et al.,* 2013). This could be a possible explanation for cf. *Chattonella* increased ribosomal activity under low salinity conditions. Nutrient depletion only affected the RNA yield (pg/cell) of one strain (CMSTA 307). Reactions to light and temperature were not significantly different among the three strains. Since this is a new algal class, very little is known of the biodiversity and distribution of its species. It is hence not feasible to speculate about how representative the results obtained in this study are for the strains tested.

Karlodinium veneficum

The genus *Gymnodinium* until recently comprised a diverse assemblage of naked (unarmored) dinoflagellates. With molecular techniques and enhanced SEM techniques, several new genera such as *Karenia, Karlodinium,* and *Takayama* have been delineated from *Gymnodinium* and the corresponding species numbers have steadily increased (Daughberg *et al.,* 2000; Bergholtz *et al.,* 2009). *Karlodinium veneficum*, formerly *Karlodinium micrum*, is a common member of temperate, coastal phytoplankton assemblages, occasionally forming blooms and whose toxins, karlotoxins, cause membrane permeabilisation associated with fish kills (Van Wagoner *et al.,* 2008; Bachvaroff *et al.,* 2009).

Among the strains of *Karlodinium veneficum* tested, lower temperatures and lower salinities affected the growth rate and RNA yield (pg/cell) more than the other stress conditions. The low $R^2 = 0.29$ value obtained from the linear relationship between cell numbers and RNA yields, maybe due to the low sample set (n = 36) of only one time point of 48 hours being available and the high standard deviations of RNA content per cell obtained from the individual *K. veneficum* strains. The type locality of its synonym, *K. micrum,* is cold temperate, and *K. micrum* has been reported from a broad geographic range in cold temperate waters in both hemispheres and in river habitats presumably exhibiting estuarine conditions (Bergholtz *et al.,* 2005). It is unclear how many of these reports could represent a cryptic species because *K. micrum* is now a later synonym of *K. veneficum* based on identical morphology and near identical rDNA LSU sequences (Bergholtz *et al.,* 2005).

Microarray calibration curves

The *P. parvum* species specific probe PparvD01_25_dT originally called PRYM694 was not extended by 25 nucleotides as the name suggests, and is still the original sequence plus the addition of a 15 nucleotide poly-T tail from the second generation chip (McCoy *et al.*, 2013). This may explain the poor performance of the PparvD01_25_dT probe compared to the other *P. parvum* specific probe Prymparv01_25_dT, which was re-designed from the genus-level probe PrymGS01_25 from the second generation chip (Eller *et al.*, 2007). It was established that the PrymGS01_25 probe had more affinity towards *P. parvum* species when extended by 25 nucleotides plus poly-T tail on the third generation chip (McCoy *et al.*, 2013). The clade-level probes Clade01old_25_dT for *Prymnesium* target species contains the poly-T tail but was not extended by 25 nucleotides and is the same original sequence from Simon *et al.* (1997) and was renamed Clade01 in the second generation chip (McCoy *et al.*, 2013). However, given that the probe still produces a good signal for *Prymnesium* targets, it was included on the third generation chip.

Prymnesium spp. detection limits were determined from the second generation microarrays at levels > 5 ng of RNA, which approximately corresponds to 8800 cells of *P. parvum* and 3800 cells of *P. polylepis*. The only difference between the second and third generation microarray was that probes that showed non-specificity in the former were omitted from the latter (McCoy *et al.*, 2013). The slopes of the calibration curves generated using varying amounts of labelled RNA were then used to infer cell numbers from the microarray signal intensities via a GPR-Analyzer program (Dittami & Edvardsen, 2013).

Detection limits were as low as 1 ng RNA for cf. *Chattonella* and *K. veneficum*, respectively, when normalised to the control probe POSITIVE_25_dT. Low detection limits of 1 ng and 5 ng RNA obtained from pure cultures may not necessarily be achievable with field samples as there may be potential interferences from non-target RNA. For example, McCoy *et al.* (2014) showed that during a bloom of *Alexandrium minutum* in the North Channel of Cork Harbour (Ireland) a minimum of 3900 cells were needed to produce a positive signal for the species-specific probe AminuS01_25_dT in field samples, whereas in J.D. Taylor, J.U. Kegel, J. Lewis and L.K. Medlin (accepted), 1 ng of RNA extracted from a culture of *A. minutum* returned a suitable microarray signal intensity corresponding to 270 *A. minutum* cell equivalents.

In the environment, cells will be present in a variety of growth stages. Whereas the experiments described were all carried out during the exponential growth phase, some cells in environmental samples will be present at late exponential or stationary phase, and some may even be dead or senescent. It might be expected that RNA will be present at varying levels. The presence of cells in, for example, stationary phase will likely underestimate cell abundances in environmental samples based on RNA content per cell. In the environment, the life time of micro-algal blooms is *c.* 10 days (Parsons *et al.*, 1984; Mann & Lazier, 1996), during which most of the time will be spent in exponential growth. One must also bear in mind that the MIDTAL microarray was designed for use as part of an early warning system, that is, in a situation where cells would be actively growing. Therefore, it would be detecting cells as they are starting to bloom and thus would be in exponential growth phase and the inference of cell numbers would be more accurate then. Currently all monitoring programs are based on cell numbers and as cell numbers increase above a critical threshold, the fisheries are closed. Thus, the optimal use of the microarray is fully in keeping with current monitoring practices. We therefore consider the technique to be robust for monitoring purposes. In bacterial studies it has been observed that in times of nutrient deprivation together with other stresses, microorganisms survive by down regulating rRNA biosynthesis, ribosomal proteins and DNA replication, which is dictated by up regulating the levels of regulatory gene RpoS. This leads not only to physiological changes but also to stress resistance in the form of secondary metabolites, antibiotics and toxins and the stability of rRNA (Navarro Llorens *et al.*, 2010). Hence, it is concluded that an adequate correlation between cell counts and microarray signals will be obtained when developed with exponentially growing cells.

The differences between hybridisations with pure cultures or field samples have proved statistically significant in terms of detection limits (McCoy *et al.*, 2014). Experiments consisting of spiking field samples with known amounts of RNA extracted from pure cultures should be considered to ascertain this aspect further. The discrepancy observed with *A. minutum*-containing culture and field samples is somewhat harder to observe when dealing with microflagellates. Indeed identification of the flagellates by light microscopy alone is difficult and the cell counts obtained for preserved water samples may be underestimated. Light microscopy analysis is often inadequate to carry out specimen identification at the species level.

The cross reactivity issues observed in this study were somewhat consistent across all of the four species used during the experiments described above. This would suggest that in order to minimise false positives, all that is required is to remove the probes that are causing problems. However, it is not a simple matter because some of the cross-reacting probes are part of hierarchies used for other species and would then require the development of a new hierarchy file. The principal of the hierarchy file has provided the best means to eliminate false positives which are likely to occur when field samples with an unknown composition of species are taken for RNA extraction and analysis.

The linear regressions carried out on the data were more consistent when normalised to the positive TATA box protein control POSITIVE_25_dT than those normalised to the *Dunaliella*-specific control DunGS02_25_dT, which may lead to microarray calibration curves returning erroneous cell count estimates. One reason for this observation may be that the same standardised stock solution of TATA box protein was used by all the MIDTAL partners and hence a more consistent control for data normalisation. In addition, each partner cultivated and harvested their own *Dunaliella* sp. control strain, which could have led to inconsistencies across different laboratories. This error could be reduced if the extracted RNA of *Dunaliella* sp. spiked to each sample for normalisation was standardised and included in the MIDTAL kit produced by Microbia Environnement (France) and not made up by each individual user. However, the inclusion of *Dunaliella* cells is meant to be a control on the extraction efficiency of the sample. Also if the signal from *Dunaliella* is too high, then this will tend to affect marginal but positive signals, and render them negative. In a different series of experiments carried out with the *Dunaliella* controls, the probe was spotted in three different concentrations to minimise the possibility of observing a saturated high signal (data not shown). A deterioration of the stock solution of TATA box protein was observed over time in some occasions, which caused the corresponding signal to be absent on the microarray chip. There is no warning prior to hybridisation as to the deteriorating state of that the stock solution of TATA box protein, hence having a back-up positive control consisting of known amounts of spotted *Dunaliella* sp. on the chip still remains a necessity despite the issues mentioned above. To test the accuracy of the microarray, actual cell counts taken from natural seawater field samples were correlated with those inferred from the microarray with a good correlation (Medlin, 2013). The toxicity of field samples was also used as an

indicator of species presence and compared against the microarray signal and cell count results.

Although there are number of molecular techniques being developed that can accurately detect and quantify low abundances of harmful phytoplankton species, many of these methods only target one particular group or species present in a field sample. The main advantage of the MIDTAL microarray over other quantitative tools is its capacity to detect and quantify multiple species in a single analysis. A further advantage is that since there is no PCR step, it is not susceptible to any unknown inhibitors in the field sample. The results obtained from this study will aid with the further development and improvement of the MIDTAL microarray.

CONCLUSION

The series of experiments reported here showed a positive linear response of increasing RNA yield with increasing microalgal cell numbers. The average RNA content per cell was generally not significantly affected by the environmental stress caused by modulations in light, temperature, salinity and nutrients and over time for the species *Prymnesium spp.* cf. *Chattonella* and *K. veneficum.* The calibration curves showing the relationship between microarray signal intensities and RNA amounts for each species provide a confident indication of the presence of a given species in an environmental water sample as well as its abundance.

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SUPPORTING INFORMATION:

Table III-S1. Algal cultures used during the study.

Culture Collection	Strain Code	Species name
Provasoli-Guillard	CCMP 1757	*Prymnesium polylepis*
University of Oslo	UIO038	*Prymnesium polylepis*
University of Oslo	UIO037	*Prymnesium polylepis*
University of Oslo	UIO036	*Prymnesium polylepis*
University of Oslo	UIO054 (=RHpat89)	*Prymnesium parvum*
Provasoli-Guillard	CCMP 709	*Prymnesium parvum*
SAG	SAG 127.79	*Prymnesium parvum*
University of N. Carolina	CMXTAC300	cf. *Chattonella sp.*
University of N. Carolina	CMXTAC305	cf. *Chattonella sp.*
University of N. Carolina	CMXTAC307	cf. *Chattonella sp.*
Canadian Centre	CCCM734	*Karlodinium veneficum*
Pasteur	PCC709	*Karlodinium veneficum*
Pasteur	PCC517	*Karlodinium veneficum*

CCMP (Provasoli–Guillard National Center for Culture of Marine Phytoplankton), SAG Sammlung von Algenkulturen der Universität Göttingen, CMSTAC (Center for Marine Science Toxic Algal Collection, University of North Carolina), CCCM (Canadian Centre for the Culture of Microorganisms), PCC (The Pasteur Culture Collection of Cyanobacteria)

Table III-S2. Experimental setup for *P. parvum*, *P. polylepis*, cf. *Chattonella* and *K. veneficum* under light, temperature, salinity and nutrient experimental conditions all with their own treatment ranges.

		Light (µmol photons m^{-2} s^{-1})	**Temperature** (°C)	**Salinity**	**Nutrients**
P. parvum	**Constant**	15 °C, 14:10 l/d, 24 Sal, N+P+	150 µE, 14:10 l/d, 24 Sal, N+P+	15 °C, 150 µE,14:10 l/d, N+P+	15 °C, 150 µE, 14:10 l/d, 24 Sal
	Strains	UiO 054, CCMP 709, SAG 127.79	UiO 054, CCMP 709, SAG 127.79	UiO 054, CCMP 709, SAG 127.79	UiO 054, CCMP 709, SAG 127.79
	Cells counted	Light Microscopy	Light Microscopy	Light Microscopy	Light Microscopy
	Range tested	25, 150, 670 µE	8, 15, 22 °C	20, 24, 28 Sal	P-, N+P+, N-
P. polylepis	**Constant**	15 °C, 14:10 l/d, 28 Sal, N+P+	150 µE, 14:10 l/d, 28 Sal, N+P+	15 °C, 150 µE, 14:10 l/d, N+P+	15 °C, 150 µE, 14:10 l/d, 28 Sal
	Strains	UiO 036, UiO 037, CCMP 1757	UiO 036, UiO 037, CCMP 1757	UiO 037, UiO 038, CCMP 1757	UiO 037, UiO 038, CCMP 1757
	Cells counted	Light Microscopy	Light Microscopy	Light Microscopy	Light Microscopy
	Range tested	25, 150, 670 µE	8, 15, 22 °C	24, 28, 32 Sal	P-, N+P+, N-
cf. *Chattonella*	**Constant**	20 °C, 14:10 l/d, 15 Sal, N+P+	75 µE, 14:10 l/d, 15 Sal, N+P+	20 °C, 75 µE,14:10 l/d, N+P+	20 °C, 75 µE, 14:10 l/d, 15 Sal
	Strains	CMSTAC300, CMSTA305, CMSTA307	CMSTAC300, CMSTA305, CMSTA307	CMSTAC300, CMSTA305, CMSTA307	CMSTAC300, CMSTA305, CMSTA307
	Cells counted	Flow Cytometer	Flow Cytometer	Flow Cytometer	Flow Cytometer
	Range tested	35, 75, 150 µE	10, 20, 25 °C	10, 15, 20 Sal	P-, N+P+, N-
K. veneficum	**Constant**	15 °C, 14:10 l/d, 25 Sal, N+P+	75 µE, 14:10 l/d, 25 Sal, N+P+	15 °C, 75 µE,14:10 l/d, N+P+	15 °C, 75 µE, 14:10 l/d, 25 Sal
	Strains	CCCM734, PCC709, PCC517	CCCM734, PCC709, PCC517	CCCM734, PCC709, PCC517	CCCM734, PCC709, PCC517
	Cells counted	Flow Cytometer	Flow Cytometer	Flow Cytometer	Flow Cytometer
	Range tested	25, 75, 200 µE	10, 15, 20 °C	20, 25, 30 Sal	P-, N+P+, N-

Table III-S3. Summary of probes designed or modified from those published for FISH hybridisation and used to form the third generation of the MIDTAL microarray for the flagellate species shown in this study. Probe sequences are not provided because the microarray is commercially available.

Probe Name	Targeted Species	Gene	Tm (GC% method)	Source/Designer
Higher Group Probes				
EukS_328_25_dT	Eukaryotes	18S	79	Moon-van der Stay *et al.*, 2001
EukS_1209_25_dT	Eukaryotes	18S	79	Lim *et al.*, 1993
PrymS01_25_dT	*Prymnesiophyta*	18S	77.3	Lange *et al.*, 1996
PrymS02_25_dT	*Prymnesiophyta*	18S	80.6	Simon *et al.*, 2000
Class Level Probes				
DinoB_25_dT	*Dinophyceae* (incl. *Apicomplexa*)	18S	75.7	John *et al.*, 2003
DinoE12_25_dT	*Dinophyceae* (incl. *Apicomplexa*)	18S	77.3	Groben, John & Medlin, unpublished
PrymS03_25_dT	*Prymnesiophyceae*	18S	77.3	Eller *et al.*, 2007
Clade Level Probes				
Clade01old_25_dT*****	*Prymnesium*	18S		Simon *et al.*, 1997
Clade 01new_25_dT	*Prymnesium* B1 clade	18S		MIDTAL
Genus Level Probes				
KargeD01_25_dT	*Karlodinium*	28S	75.6	MIDTAL
Species Level Probes				
CpolyS01_25_dT	*Prymnesium polylepis*	18S	77.3	Simon *et al.*, 1997
PparvD01_25_dT*****	*Prymnesium parvum*	28S	67.4	Töbe *et al.*, 2006
Prymparv01_25_dT	*Prymnesium parvum*	18S	79	Eller *et al.*, 2007
CtoxS05_25	Cf. *Chattonella* sp.	18S	78.8	MIDTAL
CtoxiS07_25	Cf. *Chattonella* sp.	18S	80.6	MIDTAL
CtoxiS09_25	Cf. *Chattonella* sp.	18S	78.8	MIDTAL
KveneD01_25	*Karlodinium veneficum*	28S	77.3	MIDTAL
KveneD02_25	*Karlodinium veneficum*	28S	72.4	MIDTAL
KveneD03_25	*Karlodinium veneficum*	28S	74.1	MIDTAL
KveneD04_25	*Karlodinium veneficum*	28S	80.6	MIDTAL
KveneD05_25	*Karlodinium veneficum*	28S	79	MIDTAL
KveneD06_25	*Karlodinium veneficum*	28S	75.7	MIDTAL

*Probes not extended by 25 nucleotides as name suggests.

CHAPTER IV

AN EVALUATION OF THE APPLICABILITY OF MICROARRAYS FOR MONITORING TOXIC ALGAE IN IRISH COASTAL WATERS

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ABSTRACT

The applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events has been one of the key tasks in the EU seventh Framework Programme (FP7) funded MIDTAL (MIcroarrays for the Detection of Toxic ALgae) project. The technique has a strong potential for improving speed and accuracy of the identification of harmful algae and their toxins to assist monitoring programmes. Water samples were collected from a number of coastal sites around Ireland, including several that are used in the Irish National Phytoplankton and Biotoxin Monitoring Programme. Ribosomal RNA was extracted from filtered field samples, labelled with a fluorescent dye and hybridised to probes spotted in a microarray format on a glass slide. The fluorescent signal intensity of the hybridisation to >120 probes on the chip was analysed and compared with actual field counts. There was a general agreement between cell counts and microarray signal. Results are presented for field samples taken from a range of stations along the Irish coastline known for harmful algal events during the first field trial (July 2009-April 2010).

Keywords: Microarrays, Hierarchical probes, ribosomal RNA, HABs, Environmental monitoring, Cell concentrations

INTRODUCTION

Blooms of toxic or harmful microalgae, commonly referred to as harmful algal blooms (HABs), represent a significant threat to fisheries resources and human health throughout the world. These HABs manifest themselves in many ways, ranging from high phytoplankton biomass that discolours seawater and reduce water quality, to low cell density but highly toxic populations which can contaminate shellfish (GEOHAB 2001). The aquaculture industry in Ireland is a valuable resource and has been estimated to be worth approximately €60 million annually to the Irish economy (Browne et al. 2007). Monitoring programmes have become a necessity because of the potential dangers to human health and the significant economic impacts of contaminated seafood posed by harmful events. In Europe, this requirement for monitoring is established in a series of directives in which monitoring of coastal waters for the presence of potentially harmful phytoplankton is mandatory (Council Directive 91/492).

Traditionally phytoplankton monitoring has been carried out by identification and enumeration using light microscopy. It has been recognised for some time that this technique requires a high degree of skill on behalf of the operator, and is time-consuming (Penna et al. 2007; Karlsen et al. 2010). Furthermore, the morphological similarity between different species within or even across phytoplankton genera has meant that light microscopy alone is often insufficient to assess the potential toxicity of water. A variety of identification methods based on the sequencing of nucleic acids has been developed over the past decade or so that have considerably improved our ability to accurately identify organisms to species level (Karlson et al. 2010). DNA based molecular probe methods, such as Fluorescent *in-situ* Hybridisation analysis (FISH), have been developed that can identify and quantify specific species in complex phytoplankton communities (Scholin et al. 1997; Scholin and Anderson 1998; Touzet et al. 2010). Utilisation of microsatellites is another molecular technique that is now becoming a popular genotyping method to answer ecological questions (Evans et al. 2004; Masseret et al. 2009). Further advances have led to the development of DNA-biosensors for electrochemical detection of phytoplankton and their toxins (Metfies et al. 2005; Campàs et al. 2007; Vilariño et al. 2009) and real-time quantitative PCR techniques which can provide accurate and reproducible quantification of gene copies (Galluzzi et al. 2008; Touzet et al. 2009; Kavanagh et al. 2010).

Microarrays are the state of the art technology in molecular biology for the processing of bulk samples for the detection of target RNA/DNA sequences. They are essentially a glass microscope slide with specific RNA/DNA sequence probes spotted on the surface. Each spot is complementary to an extracted target (RNA or DNA) through the process of hybridisation. The addition of a fluorescent label to the extracted target prior to hybridisation provides the ability to measure the amount of target in the sample using a microarray scanner (Metfies and Medlin 2008). One of the first DNA-microchips involved in the study of microbial diversity was used to analyse nitrifying bacteria (Guschin et al. 1997). In the present study, existing rRNA probes (18S, 28S) and antibodies for algal toxins have been adapted and optimized for microarray format in order to develop a monitoring technique that strengthens our ability to monitor bulk water samples for toxic algae. The purpose was to provide a rapid test to aid national monitoring agencies by providing new rapid tools for the identification and enumeration of toxic algae and their toxins so that they can comply with EC directive 91/492/CEE. This paper presents the results from the first year of trials of field samples taken from a range of Irish coastal stations which have had a history of harmful algal events and hybridised to the 2nd generation microarray.

MATERIALS AND METHODS

Algal cultures

Prymnesium parvum N.Carter, *Prymnesium polylepis* (Manton & Parke) Edvardsen, Eikrem & Probert, and *Dunaliella tertiolecta* Butcher algal cultures were maintained in IMR½ algal medium (without silicate and with selenite) (as outlined in Eppley et al. 1967, modified by E. Paasche, UiO) at 15 ± 1°C, under a white fluorescent light with a photon flux of 100 µE m^{-2} s^{-1} and a 14:10 light/dark cycle (see Chapter II Table II-1). When required, concentrations of cells in sub-samples used for calibration were enumerated using a Fuchs-Rosenthal chamber.

Field Sampling

Water samples were taken from a number of coastal sites along the Irish coast. These sites included the North Channel of Cork Harbour, Bell Harbour and Lough Murray of Galway Bay, Killary Harbour, Clew Bay, Killala Bay and two sampling stations off the south west coast of Ireland (Fig. IV-1). At least one of the sampling stations shown in Fig, IV-1 was sampled every month. Water samples were pre-filtered through a 150 µm mesh prior to filtration through 1 µm pore-size nitrocellulose filters (25 mm diam.). The actual volume filtered depended on the turbidity of the water: 0.2-1 L was filtered up to the point when the filter started to clog. The filter was then immediately submersed in 1 ml of Tri-Reagent (Ambion, UK) within 2 ml screw-cap tubes containing an aliquot of *Dunaliella tertiolecta* (5 x 10^6 cells) added as an internal control for the RNA extraction

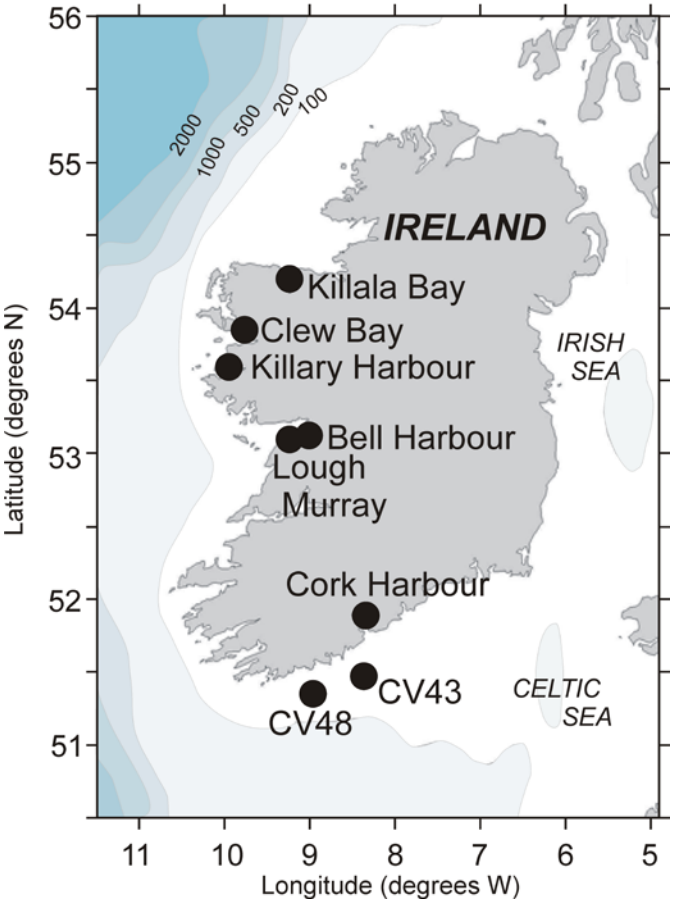

Fig. IV-1. Location of sites sampled during 2009-2010 along the Irish coastline.

process. The tubes were then stored at -80 °C. For phytoplankton cell enumeration a sample volume of 50 ml was fixed with Lugol's iodine solution (Throndsen 1978) and stored in the dark. Enumeration was carried out using an inverted microscope after settlement for 24 h in either 25 ml Utermöhl chambers or in the original 50 ml cell culture bottle, and concentrations calculated accordingly (Hasle 1978; McDermott and Raine 2010).

RNA Isolation

Field samples contained in the 2 ml tubes were thawed on ice and the cells removed from the filter through the addition of 100 µl of 212-300 µm acid washed glass beads (Sigma-Aldrich) and heating on a thermoshaker at 60 °C for 10 min, shaking at maximum speed. The samples were periodically ribolyzed for 20 s during this treatment. There followed a sequential extraction using 1-Bromo-3-chloro-propane (BCP:Sigma) and isopropanol (Sigma). An aliquot (100 µl) of BCP was added to the sample, the mixture vortexed for 15 s and transferred to pre-spinned 2 ml heavy phase lock tubes (5-PRIME; 12,000xg for 30 sec), which were then shaken (by hand) for a 15 s and then allowed stand for 5 min at room temperature. The tubes were centrifuged at 4 °C for 15 min at 12,000xg and the supernatant layer transferred to a clean 1.5 ml RNase-free tube. An equal volume of isopropanol was added (500 µl) and the tube was vortexed for 15 s. The tube was then kept at -20 °C for 1 h, centrifuged again for 15 min, and the supernatant carefully removed using a micropipette. The RNA pellet was washed with 1 ml of 75% ethanol, centrifuged and the supernatant was completely removed. After the final centrifugation step, the pellet was air dried for 3-5 min and suspended in 50–100 µl RNase free water by repeated flicking and vortexing. Nucleic acid concentrations in the sample were measured with a NanoDrop 1000 Spectrophotometer and the extract was stored at -80 °C.

RNA extraction efficiency

The efficiency of the RNA extraction method was determined by using species-specific probes for *Dunaliella tertiolecta* that had been spotted onto the microarray and acted as controls. Linearity of the extraction was investigated by extracting RNA from increasing cell numbers of *D. tertiolecta* culture carried out in triplicate; with quantification of the RNA concentration estimated using the NanoDrop 1000 Spectrophotometer. The RNA extraction of samples was also checked by preparing extractions from field samples

alongside duplicate extracts to which 5 x 10^6 cells of *D. tertiolecta* had been spiked. These were run against extracts of 5 x 10^6 cells *D. tertiolecta* alone.

RNA labelling and fragmentation

The RNA (1 µg) was labelled using a Platinum Bright 647 Infrared Nucleic Acid labelling kit (KREATECH Biotechnology) according to the manufacturer's instructions. The concentration of labelled dye was measured by NanoDrop (Microarray) and the degree of labelling (DoL) was subsequently calculated. Fragmentation of the labelled RNA was carried out by adding 1/10 fragmentation buffer (100 mM $ZnCl_2$ in 100 mM Tris-HCl pH 7), incubation in a thermoshaker for 15 min at 70 °C, and the reaction was then stopped by adding 1/10 stop buffer (0.5 M EDTA pH 8) and placing samples on ice (Lewis et al. 2012).

Internal control (TBP-Cy5) preparation

DNA from Bread Yeast powder (*Saccharomyces cerevisiae*) was extracted using Qiagen RNeasy Plant Mini Kit according to manufacturer's instructions. The PCR cycle and primers TBP-F (5'-ATG GCC GAT GAG GAA CGT TTA A-3') and TBP-R_CY5 (5'-TTT TCA GAT CTA ACC TGC ACC C-3') were used to amplify the TATA-box binding protein gene (TBP) using the detailed procedure in the MIDTAL Manual (Lewis et al. 2012). The PCR program was as follows: initial step of 95 °C for 5 min, 40 cycle step (95 °C 1 min, 53 °C 1 min, 72 °C 2 min) and final step 72 °C for 5 min. The final PCR product was purified using the PCR MinElute Cleanup Kit (QIAGEN) and quantified with a Nanodrop (Microarray) and stored at -80 °C.

Probe Development

Probes were initially taken from those designed for FISH detection tested in a first generation microarray and modified to extend their length as the 18 base pair oligonucleotides routinely used for FISH hybridisation were too short to achieve specific hybridisation reactions in a microarray format. Details are provided in Table IV-1 but the sequences are patent pending and are not shown. Where possible the original FISH probe that was modified is indicated.

Microarray Hybridisation

Details of the microarray chip development can be found in Lewis et al. (2012). Epoxysilane-coated microarray chips were pre-spotted with over 120 oligonucleotide probes for a range of potentially harmful phytoplankton species. The 2nd generation microarray chip was pre-hybridised with 20 ml pre-hybridisation buffer (2 M NaCl; 20 mM Tris-Cl, pH 8.0; 0.01% Triton 100) for 60 min at a hybridisation temperature of 65 °C. Slides were washed with deionised water and dried using centrifugation in slide holders for 3 min at 1800 rpm. A mixture of 35 µl 2x hybridisation buffer (1 mg/mL BSA, 0.2 µg/µL herring sperm DNA, 2 M NaCl, 20 mM Tris-Cl, pH 8.0, 0.01% Triton 100; Lewis et al. 2012) containing the labelled RNA (1 µg) sample and 100 ng of the TATA-box positive control (TBP-control) was prepared and made up to a final volume of 70 µl with RNase-free water. The hybridisation mixture was then incubated at 94 °C for 5 min to denature the target labelled nucleic acid. MicroArray mSeries LifterSlips (20 x 25 mm) (Thermo Scientific) were placed on the microarray and half (35 µl) of the hybridisation mixture was added to the microarray. Hybridisation was carried out at 65 °C for 1 h in a wet chamber comprising wet Whatman filter paper in a screw-capped 50 ml centrifuge tube (Falcon). After 1 h, the cover slips were removed off the array and the hybridised chip surface underwent thee washing buffer steps for 10 min with increasing stringency involving EDTA at room temperature, thereby minimising background noise (Lewis et al. 2012). The chip, pre-spotted with over 120 oligonucleotide probes corresponding to a taxonomic hierarchy (kingdom, class, genus and species) for harmful algal species, was then scanned (Genepix 4000B Axon Inc.) and the fluorescence signal intensity from each probe was measured.

Scanning and analysis

The microarray chip was scanned using a Perkin Elmer Microarray Scanner. This output (.tiff files) were then uploaded into GenePix 6.0 software programme and with the aid of an uploaded gal file (midtal_ver252_20100423.gal) which is a gridded map corresponding to each individual probe spotted onto the microarray chip, the fluorescent signals and background intensities could be calculated for each probe. The results were then saved as a separate GPR file which was imported into the PhylochipAnalyzer graphical Windows programme or GPR-analyser (gpr-analyzer ver 1.25), which allows description of the hierarchy of the probe set (Dittami and Edvardsen 2012). The signal-to-noise ratio of 2.0 was set as a cut-off for positive signals. Total signal intensities were

normalised against the positive controls (Poly-T-CY5, Positive_25 and DunGS02_25) to quantify results from different hybridisations.

Microarray results were then compared with light microscopic counts of the original water samples. This was carried out on samples taken over a period of 1 year.

RESULTS AND DISCUSSION

RNA extraction efficiency

The relationship between cell numbers of *D. tertiolecta* and the amount of RNA extracted was linear with a satisfactory coefficient of determination (R^2 = 0.9243; n = 14 Fig. IV-2). Fig. IV-3 shows a comparison between RNA extractions from field samples with and without spiking of 5 x 10^6 cells of *D. tertiolecta.* The RNA extraction efficiency was satisfactory because the average difference between spiked and un-spiked samples (mean = 9,800 ng, ± 2,600 ng) was very similar to the average RNA amount (mean = 11,000 ng, ± 1,400 ng) extracted from parallel *D. teriolecta* controls. This validated the nucleic acid extraction method, making it suitable for quantification in both laboratory studies and *in-situ* field sample analysis.

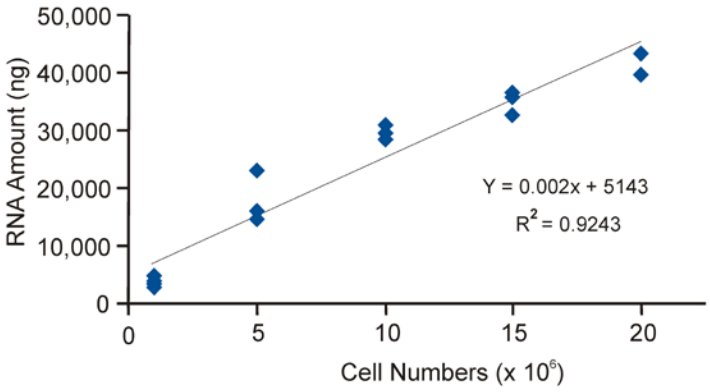

Fig. IV-2. Calibration curve of RNA amount against cell numbers using a culture of *Dunaliella tertiolecta* (UIO 226).

Chip development and Sensitivity

The first chip designed for a specified range of HAB species produced weak signals for several species-probe combinations. A second generation chip was subsequently designed in which the probes were increased in length by up to 25 base pairs with the main aim of increasing probe specificity and decreasing cross reactivity (Fig. IV-4). The

melting point temperature was subsequently altered to 65 °C, which was adopted as standard. Fig. IV-4 compares data obtained from the 1st and 2nd generation chips. The sample chosen was from Bell Harbour, where a bloom of *Prorocentrum micans* occurred in 2009. Examination under the light microscope (LM) showed that this was the dominant species (360,000 cells l^{-1}). The original *P. micans* probe spotted on the 1st generation chip (Pmica02; 37.15 s/n ratio) gave a substantial lower signal than its modified version on the 2nd generation microarray. Cross reactivity was also reduced using the 2nd generation chip with a consequent increase in specificity. There was also a reduction in signal from the class level probe for Dinoflagellates, Heterokonta, *Pseudo-nitzschia* species, *Dunaliella* genus level, Eukaryotes kingdom level probes and the spotted controls Poly-T and Poly-T-CY5.

Table IV-1. Summary of probes designed or modified from those published for FISH hybridisation and used to form the second generation of the MIDTAL microarray. Probe sequences are not provided because the microarray is patent pending.

Probe Name	Targeted Species	Gene	Tm (GC% method)	Source/Designer
Higher Group Probes				
EukS_328_25	*Eukaryotes*	18S	79	Moon-van der Stay et al., 2001
EukS_1209_25	*Eukaryotes*	18S	79	Lim et al., 1993
HeteroS01_25	*Heterokonta*	18S	77.3	Eller et al. 2007
PrymS01_25	*Prymnesiophyta*	18S	77.3	Lange et al., 1996
PrymS02_25	*Prymnesiophyta*	18S	80.6	Simon et al., 2000
PrymS03_25	*Prymnesiophyceae*	18S	77.3	Eller et al. 2007
Class Level Probes				
DinoB_25	*Dinophyceae (incl. Apicomplexa)*	18S	75.7	John et al., 2003
DinoE12_25	*Dinophyceae (incl. Apicomplexa)*	18S	77.3	Groben, John & Medlin, unpublished
ProroFD01	*Prorocentrum Clade*	28S	77.3	Groben, Lange & Medlin, unpublished
DphyFS01_25	*Dinophysiaceae (Dinophysis + Phalacroma)*	18S	77.3	Edvardsen, Groben, Brubak & Medlin, unpublished
DphyFS02_25	*Dinophysiaceae (Dinophysis + Phalacroma)*	18S	79	Edvardsen, Groben, Brubak & Medlin, unpublished
Genus Level Probes				
PrymGS01_25	*Prymnesium*	18S	79	Eller et al. 2007
PrymGS02_25	*Prymnesium*	18S	79	Eller et al. 2007
PsnGS01_25	*Pseudo-nitzschia*	18S	77.3	Eller et al. 2007
PsnGS02_25	*Pseudo-nitzschia*	18S	79	Eller et al. 2007
KareGD01_25	*Karenia*	28S	77.4	MIDTAL project
AlexGD01_25	*Alexandrium*	28S	75.7	MIDTAL project
DphyGD01	*Dinophysis in part*	28S	77.3	Guillou et al. 2002

DphyGD02	*Dinophysis*	28S	75.6	Guillou et al. 2002
PschGS01_25	*Pseudochattonella (genus)*	18S	77.3	Riisberg & Edvardsen, unpublished
PschGS02_25	*Pseudochattonella (genus)*	18S		Riisberg & Edvardsen, unpublished
PschGS03_25	*Pseudochattonella (genus)*	18S		Riisberg & Edvardsen, unpublished
PschGS04_25	*Pseudochattonella (genus)*	18S	77.3	Riisberg & Edvardsen, unpublished
PschG05_25	*Pseudochattonella (genus)*	18S	79	Riisberg & Edvardsen, unpublished
DphyGS01_25	*Dinophysis genus sensu stricto*	18S	75.9	Edvardsen, Groben, Brubak & Medlin, unpublished
DphyGS02_25	*Dinophysis genus sensu stricto*	18S	79	Edvardsen, Groben, Brubak & Medlin, unpublished
DphyGS03_25	*all Dinophysis and Phalacroma*	18S	80.6	Edvardsen, Groben, Brubak & Medlin, unpublished
DphyGS04_25	*all Dinophysis*	18S	77.3	Edvardsen, Groben, Brubak & Medlin, unpublished
PrymGS01_25	*Prymnesium*	18S		MIDTAL project
KargeD01_25	*Karlodinium genus*	28S	75.6	MIDTAL project
AzaGD01	*Azadinium Genus*	28S	75.9	MIDTAL project
AzaGD03	*Azadinium Genus*	28S	75.7	MIDTAL project
AzaGS01	*Azadinium Genus*	18S	79	MIDTAL project
AzaGS02	*Azadinium Genus*	18S	79	MIDTAL project
L*Kare0308A25	*Karenia genus*	28S	80.6	MIDTAL project
ProtuS01_25	*Phalacroma Genus*	18S	79	Edvardsen, Groben, Brubak & Medlin,
Species Level Probes				
AtamaS01_25	*Alexandrium NA,WE,TA, species complex*	18S	77.3	John et al., 2003
AminuS01_25	*Alexandrium minutum*	18S	79	Miller & Scholin, 1998
ATNA_D01_25	*Alexandrium tamarense (North America)*	28S	79	John et al., 2003
ATNA_D02_25	*Alexandrium tamarense (North*	28S	77.3	Guillou et al. 2002
ATTA _D01_25	*Alexandrium tamarense (Temperate Asian)*	28S	77.3	MIDTAL project
AostD01 _25	*Alexandrium ostenfeldii*	28S	75.7	John et al., 2003
AostS02 _25	*Alexandrium ostenfeldii*	18S	79	John et al., 2003
CpolyS01_25	*Chysochomulina polylepis*	18S	77.3	Simon et al., 1997
PparvD01_25	*Prymnesium parvum*	28S		Töbe et a. 2007
KbreD03_25	*Karenia mikimotoi and brevis*	28S		Milkulski et al. 2005
KbreD04_25	*Karenia mikimotoi and brevis*	28S	79	Milkulski et al. 2005
KmikiD01_25	*Karenia mikimotoi*	28S	79	Guillou et al. 2002
KbreD05_25	*Karenia brevis*	28S	80.6	Milkulski et al. 2005
SSKbre1448A25	*K. brevis*	18S	80.6	MIDTAL project
LSKbre0548A25	*K. brevis*	28S	82.3	MIDTAL project
KveneD01_25	*Karlodinium veneficum*	28S	77.3	MIDTAL project
KveneD02_25	*Karlodinium veneficum*	28S	72.4	MIDTAL project
KveneD03_25	*Karlodinium veneficum*	28S	74.1	MIDTAL project
KveneD04_25	*Karlodinium veneficum*	28S	80.6	MIDTAL project
KveneD05_25	*Karlodinium veneficum*	28S	79	MIDTAL project
KveneD06_25	*Karlodinium veneficum*	28	75.7	MIDTAL project
PlimaS01_25	*Prorocentrum lima*	18S	77.3	Groben, Lange & Medlin, unpublished
PlimaD01_25	*Prorocentrum lima*	28S	80.6	Groben, Lange & Medlin, unpublished
PmicaD02_25	*Prorocentrum micans*	28S	80.6	Groben, Lange & Medlin, unpublished

PminiD01_25	*Prorocentrum minimum*	28S	79	Groben, Lange & Medlin, unpublished
GcateS01_25	*Gymnodonium catenatum*	18S	76	Diercks et al 2009
DacumiD02_25	*Dinophysis acuminata+ dens+sacculus*	28S	79	Guillou et al. 2002
DacutaD02_25	*Dinophysis acuta+D.fortii*	28S	79	Guillou et al. 2002
DacumiS01_25	*Dinophysis acuminata*	18S	80.6	Edvardsen, Groben, Brubak & Medlin, unpublished
DacutaS01_25	*Dinophysis acuta*	18S	77.3	Edvardsen, Groben, Brubak & Medlin, unpublished
DnorvS01_25	*Dinophysis norvegica*	18S	77.3	Edvardsen, Groben, Brubak & Medlin, unpublished
ProtuS01_25	*Phalacroma rotundatum*	18S	79	Edvardsen, Groben, Brubak & Medlin, unpublished
PaustS01_25	*Pseudo-nitzschia australis*	18S	80.6	Diercks et al 2008
PmultS01_25	*P. multiseries*	18S	80.8	Diercks et al 2008
PpungS01_25	*P. pungens*	18S	79	Diercks et al 2008
PamerD01_25	*P. americana*	28S	79	MIDTAL project
PaustD01_25	*P.australis & P.multistriala*	28S	77.3	MIDTAL project
PdeliD02_25	*P.delicatissima*	28S	75.7	MIDTAL project
PfrauD02_25	*P.fraudulenta and P.subfraudulenta*	28S	82.3	MIDTAL project
PfrauD04_25	*P.fraudulenta*	28S	82.1	MIDTAL project
PaustD02_25	*P.australis & P.seriata*	28S	77.3	MIDTAL project
PaustD03_25	*P.australis & P.seriata*	28S	83.9	MIDTAL project
PbrasD01_25	*P.brasiliana*	28S	79	MIDTAL project
PbrasD02_25	*P.brasiliana*	28S	78.9	MIDTAL project
PbrasD03_25	*P.brasiliana*	28S	79	MIDTAL project
PcaciD01_25	*P.caciantha*	28S	74.1	MIDTAL project
PcaciD02_25	*P.caciantha*	28S	79	MIDTAL project
PcaciD04_25	*P.caciantha*	28S	75.7	MIDTAL project
Pcal1D01_25	*P.calliantha*	28S	77.3	MIDTAL project
Pcal2D01_25	*P.calliantha*	28S	77.3	MIDTAL project
Pcal2D02_25	*P.calliantha*	28S	75.7	MIDTAL project
Pcal2D03_25	*P.calliantha*	28S	77.4	MIDTAL project
Pcal2D05_25	*P.calliantha*	28S	77.4	MIDTAL project
Pdel1D01_25	*P.delicatissima*	28S	74.1	MIDTAL project
Pdel2D01_25	*P. cf. delicatissima Clade4*	28S	79	MIDTAL project
Pdel2D02_25	*P. cf. delicatissima Clade4*	28S	74.1	MIDTAL project
Pdel3D01_25	*P. arenysensis*	28S	79	MIDTAL project
Pdel1D03_25	*P.delicatissima*	28S	79	MIDTAL project
PgalaD01_25	*P.galaxiae*	28S	75.8	MIDTAL project
PgalaD02_25	*P.galaxiae*	28S	75.7	MIDTAL project
PgalaD04_25	*P.galaxiae*	28S	74.1	MIDTAL project
PhemeD2_25	*P.hemeii*	28S	77.3	MIDTAL project
PmultD01 _25	*P.multiseries*	28S	80.8	MIDTAL project
PmultD02 _25	*P.multiseries*	28S	77.3	MIDTAL project
PmultD03 _25	*P.multiseries*	28S	84.2	MIDTAL project
PmultD04 _25	*P.multiseries*	28S	80.8	MIDTAL project
PmulaD02_25	*P.multistriata*	28S	81	MIDTAL project
PmulaD03_25	*P.multistriata*	28S	77.3	MIDTAL project
PpdeD01_25	*P.pseudodelicatissima &*	28S	74.1	MIDTAL project

PpdeD02_25	*P.cuspidata* *P.pseudodelicatissima & P.cuspidata*	28S	79	MIDTAL project
PpungD02_25	*P.pungens*	28S	82.7	MIDTAL project
PpungD04_25	*P.pungens*	28S	80.8	MIDTAL project
PsubpD01_25	*P.subpacifica*	28S	77.3	MIDTAL project
PseriD01_25	*P.seriata*	28S	79	MIDTAL project
PturgD1_25	*P.turgiduloides*	28S	74.2	MIDTAL project
PturgD3_25	*P.turgiduloides*	28S	79	MIDTAL project
Pdel4D01_25	*P.cf. delicatissima Clade4*	28S	79	MIDTAL project
PvigoD01	*P. hasleana*	28S	79	MIDTAL project
PvigoD03	*P. hasleana*	28S	79	MIDTAL project
CtoxiS05	*Chloromorum toxicum*	18S	78.8	MIDTAL project
CtoxiS06	*C. toxicum*	18S	78.9	MIDTAL project
CtoxiS07	*C. toxicum*	18S	80.6	MIDTAL project
CtoxiS09	*C.toxicum*	18S	78.8	MIDTAL project
SSGcat0826A27	*Gymnodinium catenatum*	18S	77.4	MIDTAL project
LSGcat0270A24	*G. catenatum*	28S	80.8	MIDTAL project
LSGcat0544A24	*G. catenatum*	28S	82.5	MIDTAL project
SSHaka0193A25	*Heterosigma akashiwo*	18S	79	MIDTAL project
SSHaka0200A25	*H. akashiwo*	18S	77.4	MIDTAL project
LSHaka0544A25b	*H. akashiwo*	28S	82.3	Tyrrell et al. 2001
LSHaka0268A25	*H. akashiwo*	28S	82.3	Tyrrell et al. 2001
LSHaka0544A25c	*H. akashiwo*	28S	82.3	Tyrrell et al. 2001
LSHaka0548A25	*H. akashiwo*	28S	82.3	Chen et al. 2008
LSHaka0329A25	*H. akashiwo*	28S	82.3	MIDTAL project
LSHaka0358A24	*H. akashiwo*	28S	82.5	Bowers et al. 2006
PfarD01_25	*Pseudochattonella farcimen*	28S	78	MIDTAL project

The sensitivity of the extraction procedure to hybridisations of increasing amounts of labelled RNA was investigated by testing a range of probes that should be highlighted by a particular organism. Fig. IV-5a and Fig. IV-5b show calibration curve results for probes designed for prymnesiophytes that were tested using a culture of *Prymnesium parvum* and *Prymnesium polylepis*, respectively. The probes were adapted for the microarray from the original sequences published by Lange et al. (1996), Simon et al. (1997; 2000), Töbe et al. (2006) and Eller et al. (2007). The RNA extract was quantified after the labelling and clean-up steps so that approximately 1, 5, 25 and 100 ng was hybridised to the chip. The performance of a series of hierarchical probes is demonstrated in Fig. IV-5.

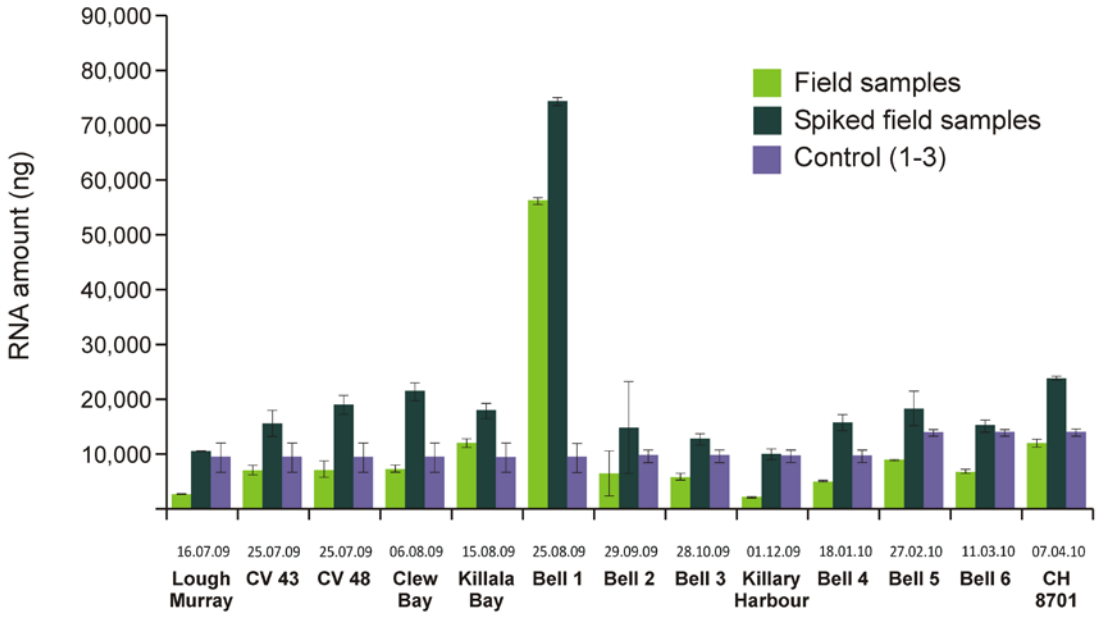

Fig. IV-3. RNA extraction efficiency of field samples. Extracted RNA amounts from triplicate filters with and without an internal spike (*D. tertiolecta)* are shown. Controls represent amounts of RNA extracted from known aliquots of three separate RNA extracts from pure *D. tertiolecta* cultures. Three controls were (1): Jul to Aug, (2): Sept to Jan and (3): Feb to April.

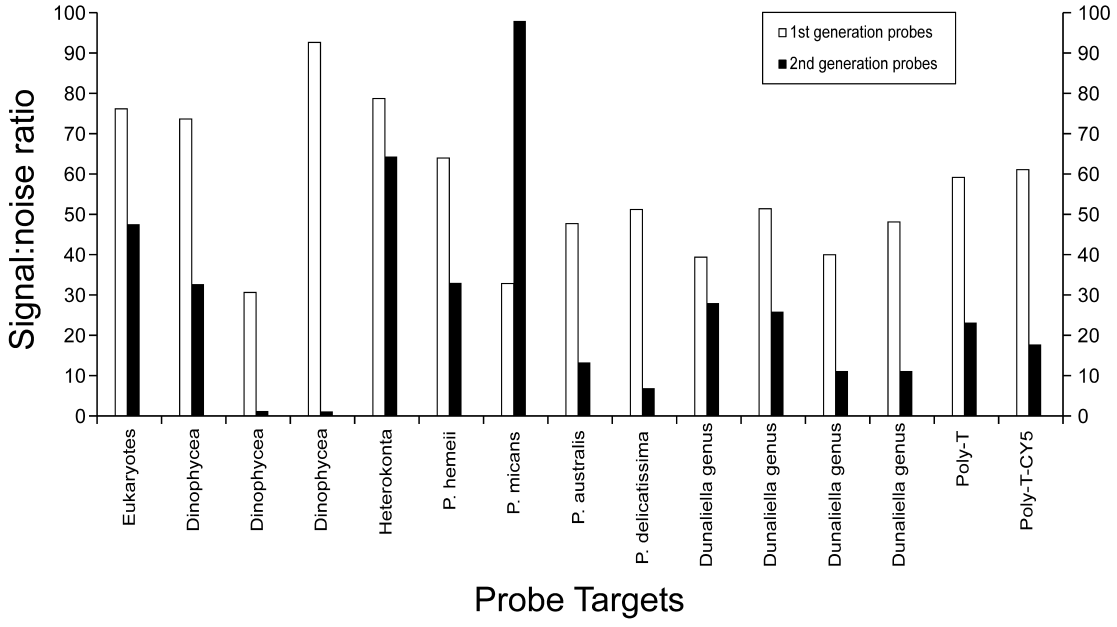

Fig. IV-4. Microarray results of 1st and 2nd generation chips both hybridised with the same Bell Harbour field extract (25/08/09) at a temperature of 58 °C and 65 °C respectively. Note the difference between signal noise ratio values in the 1st generation (open bar) compared with its extended 2nd generation probe (filled bar).

Certain probes also listed in Table IV-2, performed poorly and results from these spots were not used in the calibration or for field samples. The quantification limit was represented by a signal-to-noise ratio of 2, a value also obtained for several other probes. Thus, if the Prymnesiophytes probe (PrymS01_25; Lange et al., 1996) is applied, the microarray cannot measure RNA amounts below 5 ng, which is equivalent to 8,800 cells for *P. parvum* and 3,800 cells for *P. polylepis* species (Fig. IV-5).

Table IV-2. Characteristics of calibration curves for probes targeting *Prymnesium polylepis and Prymnesium parvum.*

Scientific Classification	Probe	*P. polylepis*		*P. parvum*	
		R^2 values	Intercept	R^2 values	Intercept
Eukaryotes	EukS_1209_25	0.91	2.02	0.95	1.66
Eukaryotes	EukS_328_25	0.48	3.74	0.96	2.26
Prymnesiophyta	PrymS01_25	0.83	2.88	0.98	2.83
Prymnesiophyta	PrymS02_25	0.88	2.63	0.97	2.44
Prymnesiophyceae	PrymS03_25	-	-	0.92	1.35
Prymnesium clade	Clade01	0.98	1.49	0.98	1.49
P. polylepis	CpolyS01_25	0.85	1.88	-	-
P. parvum	PRYM694	-	-	0.80	1.16
Probe considered unsatisfactory					
Eukaryotes	EUK1209	0.83	1.31	0.96	1.04
Eukaryotes	Euk328	0.97	1.43	0.33	1.03
Prymnesiophyceae	PrymS03_25	0.29	2.08	-	-
Prymnesium genis	PrymGS01_25	0.96	1.31	0.99	1.20
Prymnesium genis	PrymGS02_25	0.90	1.07	0.14	1.13
Prymnesium polylepis	CpolyS01_25	-	-	0.76	1.05
P. polylepis	CPOLY01	0.97	1.32	0.76	1.08
P. parvum	PRYM694	0.80	1.16	-	-

In order to derive cell numbers from RNA quantities, stress experiments (irradiance, nutrients, salinity and temperature) were performed on multiple strains of *P. parvum* and *P. polylepis* cultures (see Chapter II Table II-1). This allowed to determine the average amount of RNA per cell of *P. parvum* (mean = 0.570 ± 0.160 pg/cell) and (*P. polylepis* (mean = 1.331 ± 0.674 pg/cell) over a range of environmental conditions (data not shown).

An example of increasing intensities is shown in Fig. IV-5 with the Prymnesiophyta probe (PrymS01_25) being used with increasing amounts of *P. parvum* and *P. polylepis* RNA. This approach allowed the construction of calibration curves for each probe on the microarray chip, enabling the conversion from signal intensity to cell numbers and hence the use of the microarray for quantification purposes.

Light microscopy and microarray field results

A total of 13 samples were collected between 16.07.09 to 07.04.10 and the RNA extracted. These extracts were hybridised onto the modified (2^{nd} generation) chip. Results from a selected number of probes are shown in Fig. IV-6.

A comparison between light microscopy counts of *Pseudo-nitzschia* spp. and microarray results from samples obtained from the 16^{th} July 2009 to 7^{th} May 2010 are shown in Fig. IV-6 (a-c). A total of 7 out of 13 stations samples contained *Pseudo-nitzschia* spp., classified from light microscopy (LM) analysis either as *seriata* group (>5 µm width) or *delicatissma* group (<3 µm width). In August 2009, an assemblage of *P. seriata* like organisms were numerically dominant (113, 000 cells l^{-1}) in Killala Bay. The microarray analysis detected in these samples *P. australis* (PaustD02_25), *P. brasiliana* (PbrasD03_25), *P. fraudulenta* (PfrauD02_25), *P. multiseries* (PmultD01_25), *P. multistriata* (PmulaD03_25), *P. pungens* (PpungS01_25, PpungD02_25, PpungD04_25), *P. seriata* (PseriD01_25) and *P. sub-pacifica* (PsubpD01_25). In samples CV43, CV48 Clew Bay, Bell 1, Bell 3 and Bell 6 *P. seriata* group (range 64 to 4800 cells l^{-1}) were detected via LM analysis. Correspondingly in station CV43, *P. australis* (PaustD02_25), *P. brasiliana* (PbrasD03_25), *P. fraudulenta* (PfrauD02_25), *P. multiseries* (PmultD01_25), *P. pungens* (PpungS01_25, PpungD02_25, PpungD04_25), *P. seriata* (PseriD01_25) were highlighted by the microarray with a convincing signal-to-noise ratio above 2. Samples CV48, Clew Bay, Bell 3 returned false negative results (LM positive but microarray negative) for all the *P. seriata* group probes because the signals were below the signal-to-noise ratio threshold level of 2 (Fig. IV-6a and c).

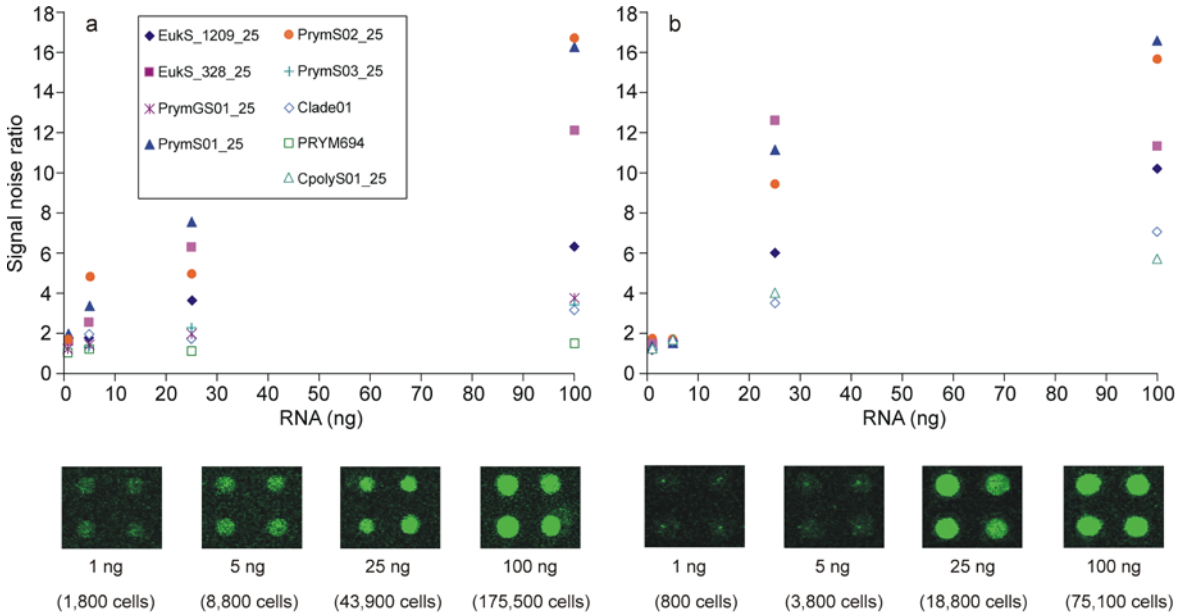

Fig. IV-5. Standardisation of the (a) *Prymnesium parvum* and (b) *Prymnesium polylepis* microarray signal. Calibration was carried out for 1ng, 5ng, 25ng and 100ng of RNA against signal-to-noise ratio values for a range of probes. Images are from use of the probe PrymS01_25 when increasing amounts of RNA are hybridised to the microarray. Increasing signal intensity represents increasing cell numbers.

P. delicatissma group were detected by LM in stations CV48, Killala Bay, Bell 1 and Bell 6 (maximum of 4000 cells l^{-1}). The probe Pdel3D01_25 which is specific for all *P. delicatissma* Clade1 *and P. multistriata*, resulted in a positive microarray signal for all of the four samples. The probe also returned a false positive result (detected by the microarray, but not present in the cell counts) for sample CV43. *P. turgiduloides* probes PturgD1_25 and PturgD3_25 were also highlighted on the microarray. Lough Murray showed false positive readings for both these probes and another false positive for PturgD1_25 in sample CV43. There were also some false negatives for both *P. turgiduloides* probes in sample CV48 but these values were very close to the threshold limit (1.90 & 1.97 s/n ratio, respectively), PturgD3 _25 also had a false negative in sample Bell 6 (Fig. IV-6b).

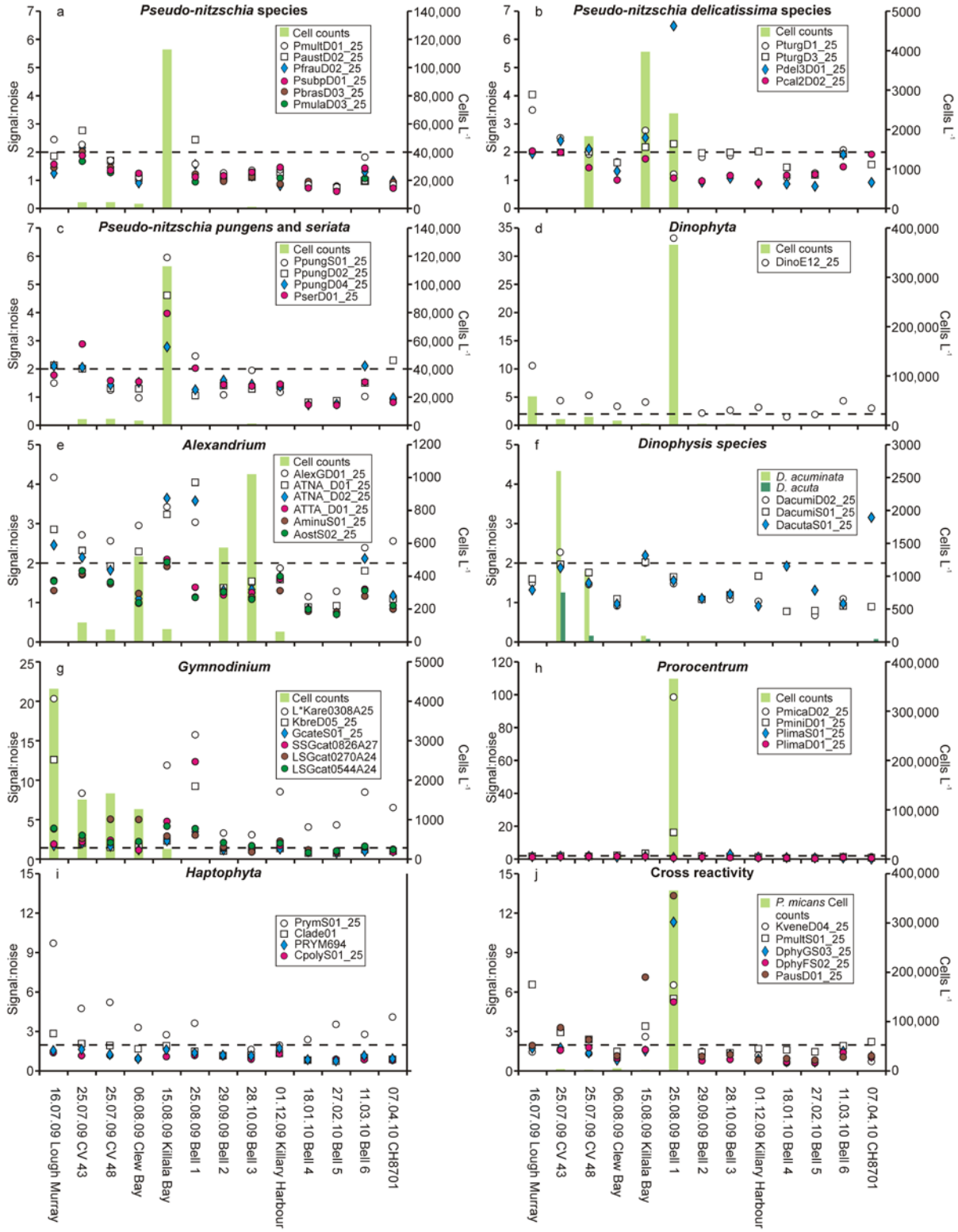
a
Pseudo-nitzschia species
Cell counts
PmultD01_25
PaustD02_25
PfrauD02_25
PsubpD01_25
PbrasD03_25
PmulaD03_25
b
Pseudo-nitzschia delicatissima species
Cell counts
PturgD1_25
PturgD3_25
Pdel3D01_25
Pcal2D02_25
c
Pseudo-nitzschia pungens and seriata
Cell counts
PpungS01_25
PpungD02_25
PpungD04_25
PserD01_25
d
Dinophyta
Cell counts
DinoE12_25
e
Alexandrium
Cell counts
AlexGD01_25
ATNA_D01_25
ATNA_D02_25
ATTA_D01_25
AminuS01_25
AostS02_25
f
Dinophysis species
D. acuminata
D. acuta
DacumiD02_25
DacumiS01_25
DacutaS01_25
g
Gymnodinium
Cell counts
L*Kare0308A25
KbreD05_25
GcateS01_25
SSGcat0826A27
LSGcat0270A24
LSGcat0544A24
h
Prorocentrum
Cell counts
PmicaD02_25
PminiD01_25
PlimaS01_25
PlimaD01_25
i
Haptophyta
PrymS01_25
Clade01
PRYM694
CpolyS01_25
j
Cross reactivity
P. micans Cell counts
KveneD04_25
PmultS01_25
DphyGS03_25
DphyFS02_25
PausD01_25
Signal:noise
Cells L-1
16.07.09 Lough Murray
25.07.09 CV 43
25.07.09 CV 48
06.08.09 Clew Bay
15.08.09 Killala Bay
25.08.09 Bell 1
29.09.09 Bell 2
28.10.09 Bell 3
01.12.09 Killary Harbour
18.01.10 Bell 4
27.02.10 Bell 5
11.03.10 Bell 6
07.04.10 CH8701

Fig. IV-6. Comparisons between light microscopy counts and 2nd generation microarray results for field samples taken during 2009-2010. Results are presented for (a-c) *Pseudo-nitzschia spp*., (d) the taxonomic class probe for the *Dinophyta*, (e) genus level and five species level probes for *Alexandrium*, (f) *Dinophysis acuta* and *D. acuminata* probes, (g) probes for various *Gymnodinium* forms (see Table IV-2 for further details), (h) *Prorocentrum spp*., (i) haptophyte probes: no light microscopy counts were available for this group, (j) examples showing cross reactivity of various probes against counts of *P. micans.*

In Fig. IV-6d, a comparison of the microarray probe signal with the total Dinophyta count in the water sample showed a good correspondence for the class level probe DinoE12_25. There was dinoflagellate species present in 11 out of 13 sampling stations. Bell 4 (18.01.10) and Bell 5 (27.02.10) were the only stations to have an absence of dinoflagellates which was reflected in the microarray result with signal-to-noise ratio values below 2 (Fig. IV-6d).

Alexandrium species were found to be present in 8 of the 13 stations sampled and cell numbers ranged from 64 to 1020 cells l^{-1} (Fig. IV-6e). The genus level probe AlexGD01_25 gave a positive result for 5 of the 8 samples containing *Alexandrium* cells (CV43, CV48, Clew Bay, Killala Bay & Bell 6). The three remaining samples (Bell 2, Bell 3 & Killary Harbour) gave false negative results; this may be because of the extraction method problems. Out of all the 13 samples, only 3 (Lough Murray, Bell 1 & CH8701) gave false positive results for this genus level probe. The microarray results for species level probes *Alexandrium tamarense* North American (NA) and Temperate Asian (TA) ribotypes, *Alexandrium minutum* and *Alexandrium ostenfeldii* are also presented in Fig. IV-6e. There were positive microarray signals for *A. tamarense* (NA) probe ATNA_D01_25 in stations CV43, Clew Bay & Killala Bay, however there were false negative results in stations CV48, Bell 2, Bell 3, Killary Harbour and Bell 6. Lough Murray and Bell 1 producing false positive results. The second *A. tamarense* (NA) probe ATNA_D02_25 showed a positive signal-to-noise ratio >2 in four of the 8 samples (CV43, Killala Bay, Killary Harbour and Bell 6). However, again Lough Murray and Bell 1 showed a false positive result for this species. The reason for these false positives may be cross reactivity with other phytoplankton genera; there was a high number of *Scrippsiella sp*. present in Lough Murry and a high number of *Prorocentrum micans* present in Bell 1, which are also grouped to the Dinophyta class. *A. tamarense* (TA) probe ATTA_D01_25 only gave a >2 signal-to-noise ratio in Killala Bay sample. All the

other samples showed a signal-to-noise ratio <2, not a surprising result as this ribotype is absent in Irish coastal waters (Lilly et al. 2002). *Alexandrium catenella* isolates that are grouped with the *A. tamarense-A. catenella-A. fundyense* species complex have been reported present in the Thau Lagoon of the French Mediterranean coast and are of the Japanese ribotype of Temperate Asian clade (Lilly et al. 2002; Masseret et al. 2009).They are also highly unlikely to be present in field samples from Ireland. *A. minutum* (AminuS01_25) and *A. ostenfeldii* (AostS02_25) probes showed a weak signal across all samples; however *A. minutum* is the most likely species present in the field samples especially in the North Channel Cork Harbour sample CH8701, which has been known for its contamination of shellfish with paralytic shellfish poisoning (PSP) toxin (Touzet et al. 2007; 2011). In general the *Alexandrium* microarray results correlated poorly with the cell counts, with numerous false positives and negatives. Further adaptations will be required to improve the *Alexandrium* probe set in the 3rd generation chip.

Dinophysis sp. cells were observed in four field samples. *D. acuta* (range 40-760 cells l^{-1}) was present but the signal-to-noise ratio did not exceed 2 in CV43 and CV48 and relationship with field samples was weak (Fig. IV-6f). Only in Killala Bay and CH8701 samples was the signal-to-noise ratio >2 with the species specific probe DacutaS01_25, even though the cell numbers were below that of CV43 and CV48. It is possible that there were larger numbers of cells on the filters than in the Lugol's samples because of the greater volume filtered for RNA extraction. *Dinophysis* have been known to escape detection with LM because of very low densities. This does not negate from their potential to cause diarrheic shellfish poisoning (DSP) events, which is the one of the main causes of closures of Irish mussel farms during late spring and early autumn (Raine et al. 2010). *Dinophysis acuminata* was present in CV43, CV48 and Killala Bay (range 40-1840 cells l^{-1}) and detected on the microarray via the two species specific probes DacumiS01_25 and DacumiD02_25. DacumiD02_25 only gave a signal-to-noise ratio above 2 in the CV43 sample, whilst DacumiS01_25 probe only gave a signal-to-noise ratio above 2 in the Killala Bay sample. Sample CV48 recorded a false negative result for both probes. Dinophysiaceae (*Dinophysis* + *Phalacroma*) family DphyFS02_25 and genus (DphyGS03_25) probes did not correlate well either and gave very low signals or may have been cross reacting with other species, such as the dominating *P. micans* bloom in Bell Harbour 1 (Fig. IV-6j).

Gymnodinium species were detected by LM in 7 of 13 stations sampled. *Karenia* genus level probe (L*Kare0308A25) gave a signal-to-noise ratio >2 for 7 samples (Lough Murray, CV43, CV48, Clew Bay, Killala Bay, Bell 1 and Bell 6); however there was also a false positive result for all the remaining 6 samples. The other *Karenia* genus level (KbreD05_25) probe gave positive microarray signal for 4 samples (Lough Murray, CV43, Killala Bay & Bell 1) containing *Gymnodinium* species. However CV48, Clew Bay and Bell 6 showed false negative results. The elevated signal in the Bell 1 sample may be caused by *Gymnodinium* cells being overlooked because of the extensive *P. micans* bloom (Fig. IV-6h). There also may be some cross reactivity with this bloom event along with *Gymnodinium catenatum* species probe (SSGcat0826A27) and a number of other genera probes shown in Fig. IV-6j. Two other *G. catenatum* species level probes LSGcat0270A24 and LSGcat0544A24 showed in general a good correlation with LM counts. There are however two exceptions with both these probes as there was a false positive result from Killary Harbour and a false negative from Bell 6 which may be because of the low numbers recorded. *Karenia mikimotoi* is one of the most frequently observed red tide causing dinoflagellates in the North Atlantic and has been known to occur all along the Irish coastline (Raine et al. 2001), being responsible for the major mortality of benthic and pelagic marine organisms which occurred in 2005 (Silke et al. 2005).

An extensive bloom of *Prorocentrum micans* (360,000 cells l^{-1}) was observed in Bell Harbour on the 25th August 2009. This was reflected in the microarray results. However, *P. micans* was also detected in six other field samples, with cell numbers ranging from 64 to 4700 cells l^{-1}. Only the Killala Bay sample gave a microarray signal above the signal-to-noise ratio value of 2. All the other five samples (CV43, CV48, Clew Bay, Bell Harbour 3 and Killary Harbour) gave a low signal and this species was not detected by the microarray (false negatives). This may have resulted from the low cell numbers or RNA extraction protocol not being stringent enough in relation to breaking up of the cells. There was a substantial difference between the *P. micans* bloom event and the other samples containing cell numbers below 5,000 cells l^{-1}. However the counts were well corroborated by the microarray signal data (Fig. IV-6h). The *P. minimum* (PminiD01_25) probe also gave a signal-to-noise ratio above 2 in two samples from Killala Bay and Bell Harbour 1. This organism may have been present in the samples but was not counted in

the LM counts. *Prorocentrum lima* (PlimaS01_25 & PlimaD01_25) was not detected in the cell counts, confirming the microarray results.

Haptophyta results were difficult to analyse because of the inability of identification to species level in preserved samples using LM. During LM analysis they are mostly recorded as unidentified microflagellates and were most likely grouped along with a number of various other microflagellate species. Therefore, comparing cell counts with the microarray can become troublesome and may be inaccurate without the aid of more skilled techniques, such as Electron Microscopy (Fig. IV-6i).

CONCLUSION

It was apparent that the Eukaryote higher group probes extended to 25 nt produced stronger signal intensities compared to their corresponding shorter 18 nt probes also spotted on the 2nd generation microarrays. The testing of the *Prymnesium* higher group, clade a species level probes with increasing amounts of labelled *P. parvum* and *P. polylepis* RNA from pure cultures allowed for the successful construction of calibration curves for determining the linear slope and cut off limits of detection for each probe and also to convert microarray signal intensities back to cell numbers. This also verifies the correct order of the *Prymnesium* species hierarchical probe set from higher group probes which produce higher signals right down to species level probes which produce weaker signals.

In general the field sample cell count results have supported the microarray data. However, there were a few false positive results detected by the microarray, possibly indicating the presence of species that were not recorded in light microscopy cell counts. This was most likely caused by the larger volume used for filtration (~0.2 to 1L) compared to the small volume taken for cell counts (50 ml), or else an inability to identify cells to species level by light microscopy alone. Unspecific binding is another issue that was particularly apparent when comparing the *P. micans* counts with false positive microarray signals from the Bell Harbour 1 (August, 2009) sample (Fig. 6j). Although these are just a few comparisons of light microscopy counts with microarray results in field samples, all of Year 1 samples were hybridised to the 2nd generation chips and in many cases had a low labelling efficiency, which can be a direct reflection of the quality of the extracted RNA, where crossover of NaCl, TRIS, EDTA or proteins may have occurred. These methodological issues will be addressed in the third generation

microarray. Adaptations to the RNA extraction protocol, such as the addition of longer bead beating to ensure successful breaking of cells, the introduction of RNA revised clean-up steps to improve labelling efficiency, and improvements to hybridisation protocol, should decrease the number of false positives, reduce cross reactivity and increase specificity and sensitivity.

The aim of the MIDTAL project is to provide a new method to support toxic algal monitoring, to contribute to human health and common fisheries policies. These first field results indicate that there remains further development work to be done but point towards the potential successful development of a 'universal' HAB microarray. Further adaption and optimisation of existing rRNA probes to a 3rd generation microarray are still ongoing.

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CHAPTER V

MONITORING A TOXIC BLOOM OF *ALEXANDRIUM MINUTUM* USING NOVEL MICROARRAY AND MULTIPLEX SURFACE PLASMON RESONANCE BIOSENSOR TECHNOLOGY

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ABSTRACT

Blooms of *Alexandrium* occur annually during the summer months in the North Channel of Cork Harbour on the south coast of Ireland. This study monitored an extensive bloom of the toxin producing *Alexandrium minutum* during the summer of 2011 with the use of the MIDTAL (Microarrays for the Detection of Toxic Algae) microarray and a prototype multiplex surface plasmon resonance (multi SPR) biosensor. Microarray signal intensities and toxin results from three testing platforms of the prototype multi SPR biosensor, commercial (CER) enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC) were compared against light microscopy counts. The main aim was to demonstrate the use of these methodologies to support national monitoring agencies by providing a faster and more accurate means of identifying and quantifying the harmful phytoplankton community and their toxins in natural water samples. Both the microarray signals and multi SPR biosensor results followed a significant trend with light microscopy results and both techniques indicated detection limits of <4,000 cells of *A. minutum* in natural seawater samples.

Keywords: *Alexandrium*; microarray; MIDTAL; biosensor; saxitoxins; RNA.

INTRODUCTION

The dinoflagellate genus *Alexandrium* encompasses approximately 30 morphologically defined species of which at least half produce potent toxins (Balech, 1995; Anderson et al., 2012). These toxins bio-accumulate through the food chain, impacting humans, fish, birds and marine mammals on a global scale (Hallegraeff 1993; Anderson et al., 2012). Saxitoxins and spirolides are the known toxins produced by this genus. Saxitoxin and its analogues are the causative agents of the human illness paralytic shellfish poisoning (PSP), a condition that can be potentially fatal following ingestion of contaminated shellfish (Huang et al., 2006; Anderson et al., 2012). It is often difficult to discriminate between armoured dinoflagellate species and it requires a high degree of skill using traditional light microscopy (LM) methods. Moreover, it is challenging to discriminate between morphologically similar but genetically dissimilar strains, such as toxic and non-toxic forms of the same species or groups of monophyletic clades (Lilly et al., 2005; 2007). A variety of molecular methods have been adapted for the detection and quantification of *Alexandrium* species with gene probes. Fluorescent *in situ* Hybridization (FISH) permits the selective detection of the genus *Alexandrium,* using oligonucleotide probes within a whole-cell format by means of fluorescence microscopy (Touzet et al., 2007; Tang et al., 2011). A sandwich hybridization assay, involving cell lysis with two hybridization reactions, has proved useful in obtaining near real-time mapping of the distribution of *Alexandrium* species when used onboard a ship (Diercks et al., 2008). Quantitative real-time PCR (qRT-PCR) can provide accurate and reproducible quantification of gene copy formation during exponential phase of the reaction (Galluzzi et al., 2004; Touzet et al., 2009; Erdner et al., 2010; Toebe et al., 2013). Further advances have led to the development of DNA-biosensors for electrochemical detection of phytoplankton and their toxins (Metfies et al., 2005).

Microarrays are the state of the art technology in molecular biology for processing bulk samples for the detection of target RNA/DNA sequences. An RNA-based approach for species identification, using oligonucleotide probes that specifically target the 18S-28S rDNA domains from hierarchical groups down to the species level have been adapted for use with microarrays (Metfies and Medlin, 2004; 2008; Galluzzi et al., 2011; Gescher et al., 2008). Using the microarray technology in this way, the simultaneous analysis of 136 different probes and 4-8 replicates including several controls specific for a range of

harmful phytoplankton species can be carried out using the recently developed MIDTAL microarray (Lewis et al., 2012; Kegel et al., 2013a).

A novel multiplex optical surface plasmon resonance (multi SPR) prototype biosensor has been applied to the analysis of marine biotoxins (Campbell et al., 2011). Using this approach, the identification of domoic acid, okadaic acid and paralytic shellfish toxins using a single multi-biosensor chip is now possible. This method has been tested in parallel with the enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography (HPLC) to verify the presence or absence of these toxins in seawater samples (McNamee et al., 2013).

The North Channel of Cork Harbour on the south coast of Ireland was chosen for a sampling survey of *Alexandrium* spp. in 2011. In this location, PSP producing *Alexandrium minutum* blooms occur regularly during the summer months, including the summer of 2011 (Touzet et al., 2007). This paper presents the results of inter-comparisons between light microscopy counts and microarray results using the MIDTAL microarray, as well as PSP toxin data derived from the multi SPR method and high performance liquid chromatography (HPLC) (Lewis et al., 2012; McNamee et al., 2013; Kegel et al., 2013a). The aim of both these newly developed technologies is the provision of new methods to support toxic algal monitoring, thus contributing to the safeguarding of human health and supporting common fisheries policies across Europe and the US (Lewis et al., 2012).

MATERIALS AND METHODS

Sampling site and field sampling

Cork Harbour located on the south coast of Ireland is separated by Great Island, north of which is an eastern arm of the harbour known as the North Channel (Fig. V-1A). The North Channel was the main area of sampling. At least two stations from locations N/O, P, and R (Fig. V-1B) were sampled every week between 25th May and 13th July 2011 (Table V-1). At each sampling site 50 mL seawater sample from discrete depths were placed in 50 mL cell culture bottles and preserved with 0.4 mL of unacidified Lugol's Iodine (Throndsen, 1978). The Lugol's preserved samples were kept in the dark until used for cell count determination, which was performed using an inverted microscope (Olympus CKX-41) following the procedure described in McDermott and Raine (2010). Water samples for RNA analysis were pre-filtered through a 150 µm mesh and then filtered through a 1 µm pore-size nitrocellulose filter (25 mm diam.). A total volume of 200-250 mL was filtered due to relatively high suspended matter found in the North Channel (Table V-1). All filters for RNA analysis were immediately immersed in 1 mL of TRI Reagent (Ambion) contained in 2 mL screw cap tubes, kept at 4 °C during sampling and within 6 hours of sampling stored at -80 °C on return to the laboratory. Water samples (0.6 – 1.0 L) for toxin analysis were filtered through glass fibre (Whatman GF/F, 47 mm diam.) filters (Table V-1). These filters were stored separately at -20 °C in 2 mL screw cap tubes.

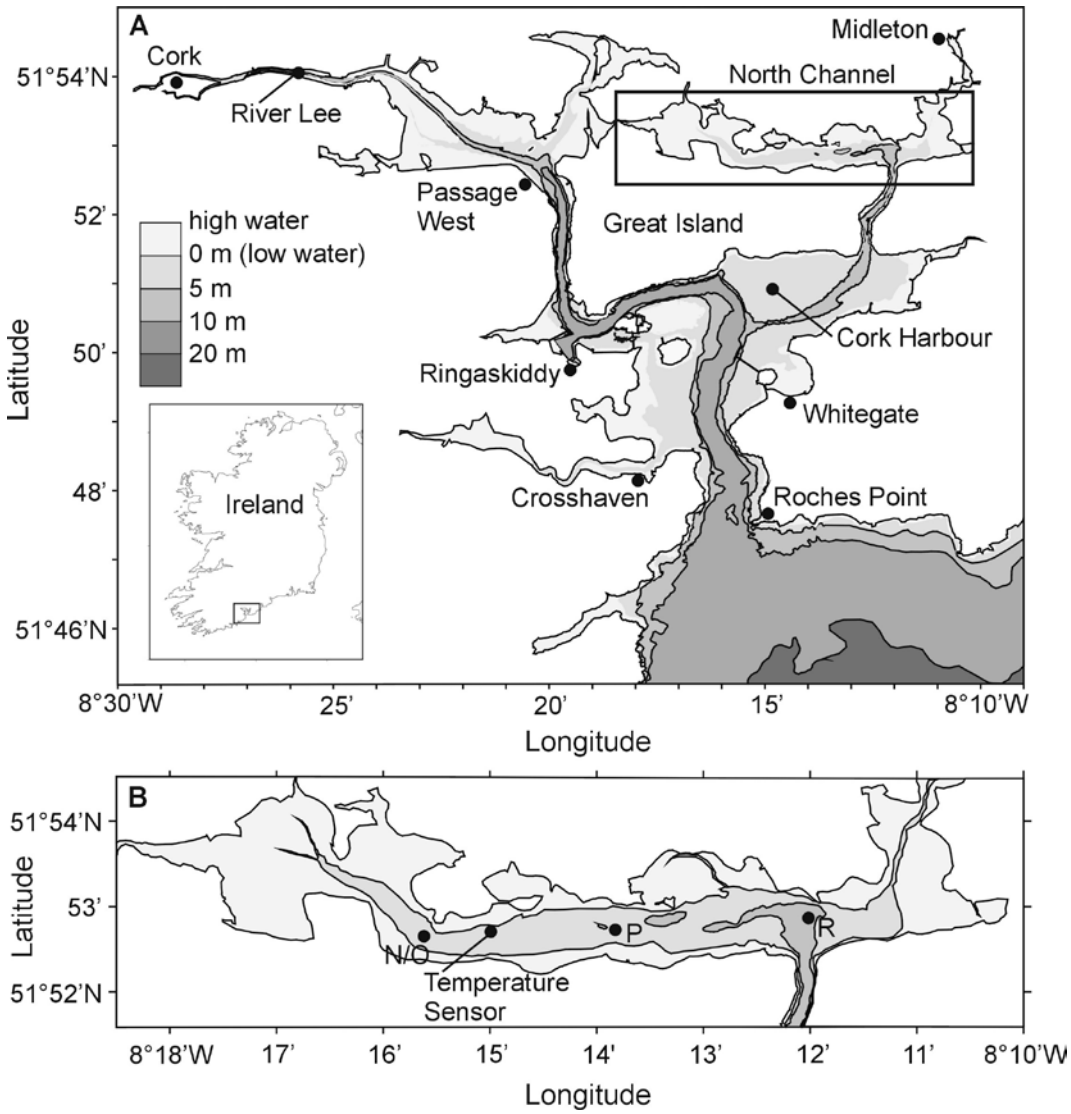

Fig. V-1. (A) Map of Cork Harbour showing the location of the North Channel. (B) Sampling station positions in the North Channel (N/O, P, and R) and the temperature sensor position which was moored at this location throughout the survey period.

Water temperature at 0.5 m off the sea bed was recorded hourly using a TidBit temperature sensor (HOBOware) moored between stations N/O and P throughout the sampling period (Fig. V-1B). Temperature and salinity profiles were also measured *in situ* using a temperature salinity probe (WTW, 197i). Tidal ranges were derived from published tide tables.

Table V-1. Field samples taken from the North Channel of Cork Harbour during the summer 2011 *Alexandrium* survey, sampling dates, location, sample, time, depth of samples taken in meters, volume filtered for RNA (millilitre) and toxin samples (litres), total RNA extracted and degrees of RNA labelling (DoL %).

Date	Location	Sample	Time (local)	Depth sampled (m)	RNA Volume filtered (mL)	Toxin Volume filtered (Litre)	Total RNA extracted (ng)	DoL %
25.05.11	N/O	S1a	11:01	1	200	1	7000	1.1
25.05.12	P	S1b	13:13	2	200	1	5200	1.1
01.06.11	N/O	S2a	10:00	1	200	0.8	4500	0.4
01.06.12	R	S2b	11:10	3.5	200	0.8	5000	2.1
08.06.11	N/O	S3a	09:50	2	200	0.6	6300	2.5
08.06.12	R	S3b	11:00	3	200	1	3200	2.2
15.06.11	N/O	S4a	10:00	2	200	0.6	8200	2.2
15.06.12	R	S4b	11:47	1	200	0.9	8800	3.0
22.06.11	R	S5a	06:00	1	250	1	5000	2.0
22.06.12	P	S5b	10:50	1	200	1	4900	1.7
29.06.11	P	S6a	11:20	0.5	200	1	11800	1.9
29.06.12	R	S6b	09:37	1	200	1	10000	2.3
06.07.11	R	S7a	11:59	1	200	0.6	10000	2.1
06.07.12	N/O	S7b	09:56	3	200	0.8	11300	2.1
13.07.11	R	S8a	09:34	1	200	0.8	6900	2.0
13.07.12	P	S8b	11:04	0	200	0.8	11900	2.5

Microarray design

Oligonucleotide probes routinely used for FISH were modified and adapted to the microarray, newly designed probes were also developed with the open software package ARB (Ludwig et al., 2004). The oligonucleotides including the positive and negative controls were synthesized (Eurofins MWG Operon or Thermo Fisher Scientific, Ulm, Germany) with a C6 aminolink at the 5' end of the molecule. The probes on the second generation chip had a length between 18 and 25 nucleotides, and a further 15 nucleotide poly deoxythymidylic (dT) tail following the amino (NH_2) link at the 5' end was subsequently added for the 3rd generation chip. The addition of an Amino C6 and Poly-T (15 nt) spacer was to lower cross reactivity between probes on the chip along with more stringent washing steps (Kegel et al., 2013a).

The MIDTAL microarray probe sequences are patent pending and the entire hybridisation kit including the array and all necessary reagents are commercially available from Microbia Environnement (France; contact@microbiaenvironnement.com). The *Alexandrium* specific probes were originally based on sequences described by Miller and Scholin (1998), Guillou et al. (2002), John et al. (2003) and Kegel et al. (2013b) (Table V-2). Duplicate arrays were spotted with 4-8 replicates of 136 different probes and as well as three negative controls (NEGATIVE1_dT, NEGATIVE2_dT, NEGATIVE3_dT), one positive control (TATA box protein), a Poly-T-Cy5 spotting control, and three internal controls (DunGS02_25, DunGS02_25_dT and DunGS05_25_dT for *Dunaliella tertiolecta*). After spotting, slides were incubated for 30 min at 37 °C and then stored at -20 °C. A list of the probes and targeted taxon made from the 18S or 28S rRNA gene to form the third generation of the MIDTAL microarray can be found in Kegel et al. (2013a).

Microarray analysis

Microarray analysis involved a multistep process of RNA extraction, labelling and hybridisation onto a microarray containing 136 probes (Kegel et al., 2013a). The methods were essentially those outlined in Kegel et al. (2013a). Prior to RNA extraction an aliquot of TRI Reagent (100 µL) containing 500,000 cells of *Dunaliella tertiolecta* (UIO226 strain) was added to each filtered sample, acting as an internal control.

Table V-2. List of *Alexandrium* genus and species specific probes designed or modified from published sources and adapted for the third generation of the MIDTAL microarray. Probe sequences are not provided as they are patent pending.

Probe name	Target	Gene	Source/Designer	Toxin type
AlexGD01_25_dT	*Alexandrium* genus	28S	Kegel et al., 2013b	Saxitoxins; Spirolides; Gonidomains
AminuS01_25_dT	*Alexandrium minutum*	18S	Miller and Scholin, 1998	Saxitoxin
AostD01_25_dT	*A. ostenfeldii*	28S	John et al., 2003	Saxitoxins; Spirolides
AostS02_25_dT	*A. ostenfeldii*	18S	John et al., 2003	Saxitoxins; Spirolides
AtamaS01_25_dT	*A. tamarense* (NA, WE, TA), species complex	18S	John et al., 2003	Saxitoxins
ATNA_D01_25_dT	*A. tamarense* (North American)	28S	John et al., 2003	Saxitoxins
ATNA_D02_25_dT	*A. tamarense* (North American)	28S	Guillou et al., 2002	Saxitoxins
ATTA_D01_25_dT	*A. tamarense* (Temperate Asian)	28S	Kegel et al., 2013b	Saxitoxins

RNA was extracted from thawed frozen filters in Tri Reagent, separated out using 1-Bromo-3-chloro-propane (BCP: Sigma), chloroform and isopropanol (Sigma) sequentially, followed by three ethanol (70% EtOH) wash steps and a clean-up step involving ammonium acetate (7.5 M NH4Ac; Applichem). The extracted RNA was labelled using a Platinum Bright 647 labelling kit (KREATECH Biotechnology) and fragmented by adding fragmentation buffer ($ZnCl_2$ in Tris-HCl pH7) followed by incubation (70 °C, 15 min). Prior to hybridisation, the microarray chips were blocked (0.2%SDS), washed, spun down and dried and stored at 4 °C until required. The hybridisation cocktail containing, 2x hybridisation buffer, labelled RNA, TBP-control, Poly-dA and RNase free water was denatured (94 °C, 5 min) and then hybridised to the microarray chip. All experimental details can be found in Kegel et al. (2013a). The only deviations from this was that the quality of the RNA was periodically checked using a NanoDrop 1000 spectrophotometer and 15 µL of KREAblock (KREATECH) was not added to the denatured hybridisation cocktail.

Scanning and Data analysis.

A Perkin Elmer Microarray Scanner was used to scan the array and the signal-to-noise ratios (S/N ratio) and total signal intensities were calculated by superimposing a GenePix array list file (midtal_ver32_20110429.gal) which acts as a gridded map that describes the size, position, layout and name of every probe and all eight replicates spotted in four different areas of the microarray chip and differentiates them from local background noise using GenePix 6.0 software (Dittami and Edvardsen, 2013). The GAL file data output was then interpreted with the GPR-Analyzer version 1.27 which describes the hierarchy of the probe set (Dittami and Edvardsen, 2013). An S/N ratio value of ≥2 was set as the cut-off threshold level for positive signals. To compare values from different hybridisations, signals were normalised using the internal controls and replicates averaged.

Toxin analysis

Toxin sample filters frozen at -20 °C contained in 2 mL screw cap tubes were shipped to Queens University, Belfast for extraction and analysis for PSP toxins using three different platforms. The prototype multi SPR biosensor (GE Healthcare, Uppsala, Sweden) (Campbell et al., 2011) and a commercial ELISA kit (Centre d'Economie

Rurale (CER), Belgium) (Dubois et al., 2010) were used for analysing toxin samples (McNamee et al., 2013).

For the separation, identification and quantification of the individual PSP toxin analogues a Supelcosil reversed phase C18 column (15 cm x 4.6 mm id, 5 µm particle size) in line with Waters alliance 2695 separation module HPLC system equipped with a Waters 2475 fluorescence detector (Waters, Ireland) was employed. Analytical standards of saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), neosaxitoxin (NEO), decarbamoyl neosaxitoxin (dcNEO), gonyautoxins (GTX1/4, GTX2/3), decarbamoyl gonyautoxins (dcGTX2/3), C1/2 and GTX5 (B1) were purchased from the National Research Council of Canada (NRCC), Halifax, Canada and prepared as 1000 ng mL^{-1} stocks separately in deionised water. Calibration standards were prepared in three separate mixes (Mix 1: STX, C1,2, GTX2,3; Mix 2: NEO, GTX1,4, dcNEO; Mix 3: dcGTX2,3, dcSTX, GTX5) in known negative extracted seawater at a concentration of 50 ng mL^{-1}. A modification of the Lawrence HPLC method using pre-chromatographic oxidation by periodate and peroxide oxidants as described in the AOAC official method (AOAC, 2005.06) was utilised (Lawrence et al., 2005). Modifications to this method were that the toxins were extracted from the seawater and not shellfish samples and as such the C18 clean-up stage was not required. A total of 16 seawater samples were prepared and extracted for toxin analysis using the same method previously described by McNamee et al. (2013) with extracts used neat (in duplicate) derivatised with periodate and peroxide oxidants prior to analysis by HPLC with fluorescence detection. However, due to the use of the GF/F filters at 47 mm diameter a higher volume (5 mL) of wash buffer was used to ensure cells and toxin were washed off the filter thus reducing the concentration of toxin in the sample.

Statistical analysis

A two-tailed Pearson's correlation of significance test was carried out for (1) normalised microarray signal and cell number relationships, (2) HPLC toxin results and cell numbers and (3) multi SPR toxin results and cell numbers. All statistical analysis was carried out in GraphPad Prism 5.

RESULTS

Alexandrium sp. cell counts and hydrographic data

Fig. V-2 shows cell densities of *Alexandrium* cells using light microscopy (LM) counts in the North Channel of Cork Harbour, recorded every week between the 25th May to the 13th July 2011. The LM count results presented are the average result of two samples taken on the same sampling date from two different stations, together with the tidal range (Fig. V-2A). A duplicate set of samples analysed by FISH showed that the bloom was >95% *A. minutum* (Cosgrove et al., 2014). The bloom gradually increased in mid-June reaching a maximum cell density of 1,700,000 cells L^{-1} recorded on the 6th July. The bloom then declined steeply the following week to a low of 1,700 cells L^{-1} on the 13th July. Salinity and temperature data recorded during the survey are presented in Fig.V-2B. Salinity fluctuated between 27.7 and 31.6 and temperature rose from 12.9 °C at the start of the survey to almost 18 °C in mid-July (Fig. V-2B).

Microarray analysis

RNA extraction and labelling.

The total RNA extracted ranged between 4,500 to 11,900 ng $sample^{-1}$ from the phytoplankton assemblage filtered, from which an aliquot of 1000 ng was used for labelling (Table V-1). The degrees of labelling (DoL) concentrations were all between 1.1% and 3.0%, except for station CH9601 taken on 1st June which was only 0.4%, and within the recommended optimal range of labelling for microarray hybridization (1.0 - 3.6%) according to Kreatech Biotechnology nucleic acid labelling kit guidelines (Table V-1).

Microarray results and cell counts.

Fig. V-3 shows a comparison between *Alexandrium sp.* cell numbers derived from LM counts with results from the 3rd generation microarray which have been normalised to the internal control probe DunGS02_25. Counts were compared against both genus level probes (AlexGD01_25_dT; Fig. VI-3A and B) and the species specific probe AminuS01_25_dT for *A. minutum* (Fig. VI-3C and D). A total of 14 samples out of 16 produced a positive microarray S/N ratio value above the threshold level for the *Alexandrium* genus (AlexGD01_25_dT) probe (Table V-3), with the highest positive normalised microarray signal observed in station S7a (28.64; 352,900 cells per volume

filtered; Fig. V-3A). The lowest normalised signal was that for sample S2b (0.51; 1,400 cells per volume filtered; Fig. V-3A). A total of 10 out of 16 samples produced a positive microarray signal for the species level probe for *A. minutum* (AminuS01_25_dT; Table V-3). The lowest normalised signal was found at station S3b (0.15; 3900 cells per volume filtered; Fig. V-3C).

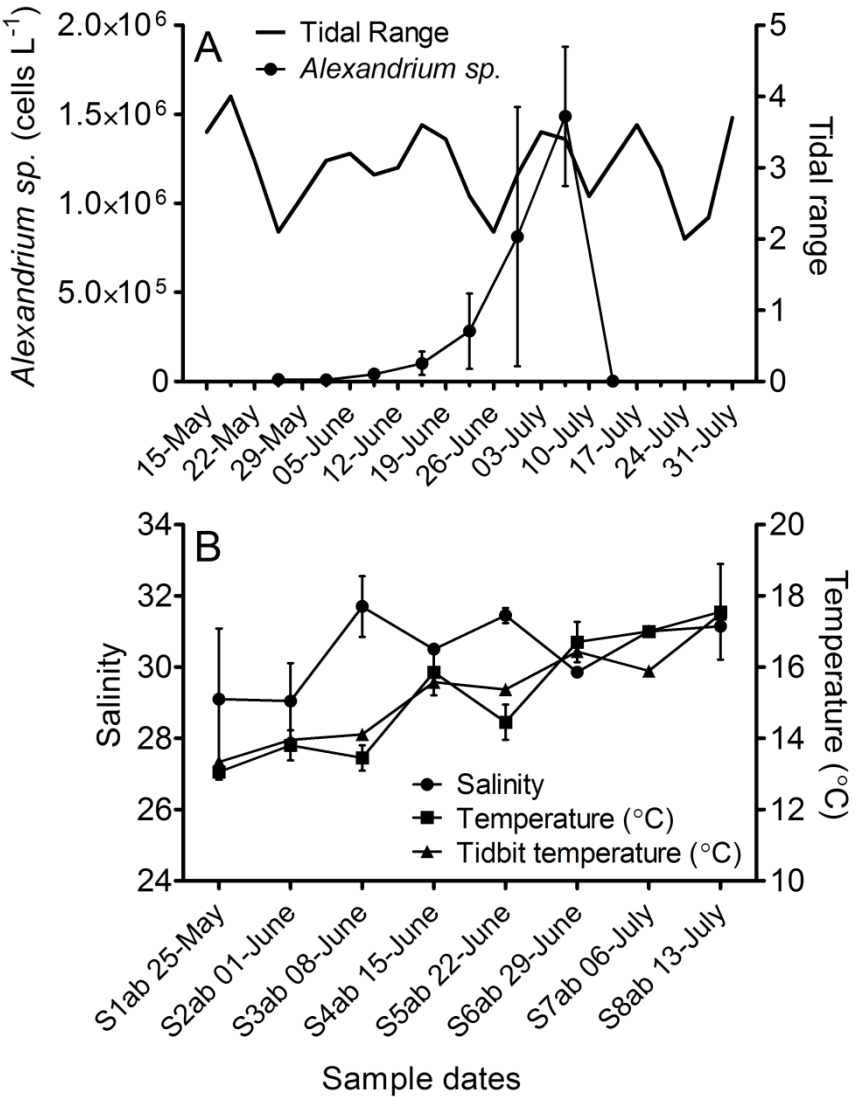

Fig. V-2. (A) *Alexandrium sp.* cell densities in the North Channel of Cork Harbour during the summer 2011 survey derived from light microscopy counts. The mean of two stations sampled in the same day is presented with tidal range. (B) Temperature and salinity reading from the *in situ* TS Probe together with the daily average temperature readings from the anchored TidBit sensor during the survey period.

Positive normalised microarray signal results for both *Alexandrium* genus (r = 0.97; P < 0.0001; n = 14) and *A. minutum* (r = 0.88; P < 0.001; n = 10) probes significantly correlated with cell counts (Fig. V-3B and D). The only *Alexandrium* species specific probe other than that for *A. minutum* (AminuS01_25_dT) which produced a positive microarray signal was that for *A. tamarense* NA (ATNA_DO2_25_dT). This was evident in sample S1a (S/N ratio 2.25) and S6b (S/N ratio 2.68), suggesting that this species was present in the *Alexandrium* bloom assemblage at these stations (Table V-3). However, the negative microarray result for both the *A. tamarense* complex probe (AtamaS01_25_dT) and the second *A. tamarense* NA probe (ATNA_D01_25_dT) spotted on the microarray indicated that these are false positive microarray signals for the NA probe (ATNA_D02_25_dT; Taylor et al., 2013a).

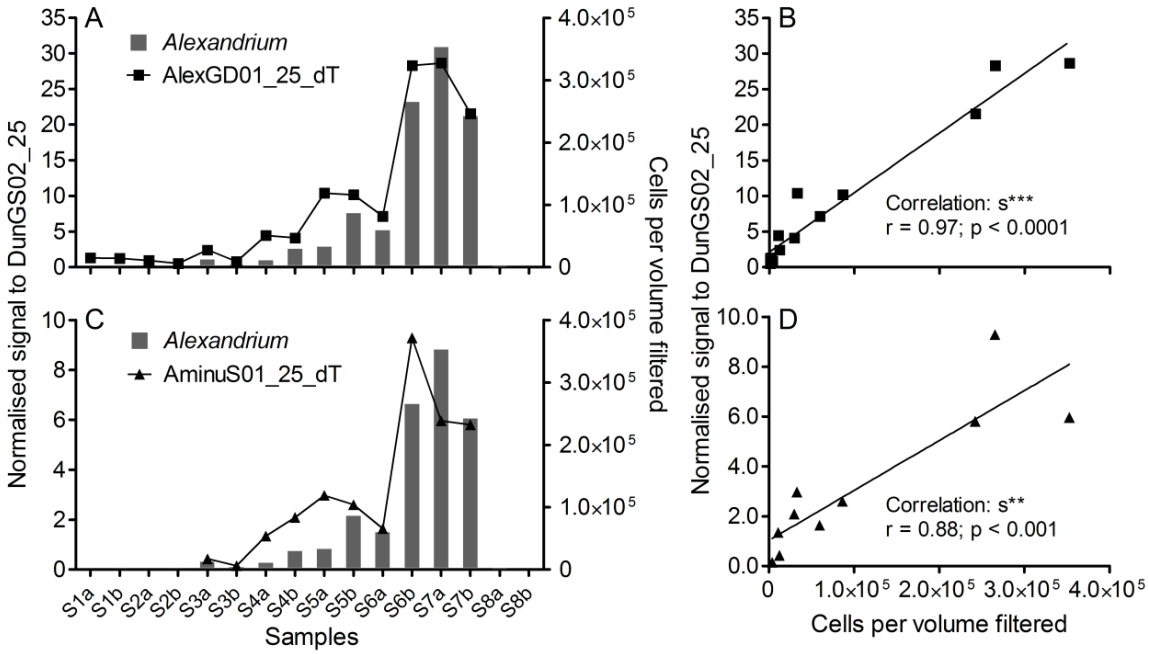

Fig. V-3. Comparison of microarray results with light microscopy counts of *Alexandrium* in the North Channel of Cork Harbour during the 2011 survey. Microarray data have been normalised to an internal control (DunGS02_25) using a known amount of *Dunaliella tertiolecta* (5 x 10^5 cells) cell numbers spiked to each filter prior to RNA extraction. Cell numbers per volume filtered derived from light microscopy counts of *Alexandrium spp.* are compared against (A, B) normalised microarray signal for *Alexandrium* genus level probe (AlexGD01_25_dT) and (C, D) *Alexandrium minutum* species specific probe (AminuS01_25_dT). Calibration curves in (B) and (D) show the corresponding normalised r = Pearson correlation coefficient, p = significance of the correlation and * = level of significance for the *Alexandrium* genus and *A. minutum,* respectively.

The absence of *A. tamarense* NA in the samples was confirmed when FISH analysis on a parallel sample set using the TamToxC probe (CY.3 label, 5'end) specific for toxic *A. tamarense* NA (group I ribotype) and Tam.A^2 probe (FITC label, 5' and 3' end) specific for non-toxic *A. tamarense* WE (group III ribotype) was carried out (Touzet et al., 2010). The results showed the absence of *A. tamarense* NA (group I ribotype), but the presence of *A. tamarense* WE (group III ribotype) in both stations with cell concentrations of 60 and 150 cells L^{-1}, respectively (data not shown; Cosgrove et al., 2014).

Toxin analysis

Toxin results and Cell Counts. HPLC analysis for paralytic shellfish toxins showed that the saxitoxin analogues GTX2, 3 were detected in all samples (Fig. V-4A; Table V-4). The highest level of GTX2, 3 in the samples (1.08 µg L^{-1}) was observed at station S6b, corresponding to 1,330,000 cells L^{-1} of *Alexandrium* cells and the lowest detectable concentration was observed at S8b (0.002 µg L^{-1}; 1,000 cells L^{-1}; Fig. V-4A). There was a significant correlation ($r = 0.87$; $P < 0.0001$; $n = 16$) between GTX2, 3 and LM counts for *Alexandrium* sp. (Fig. V-4B). The mean GTX2, 3 content per cell was 1.29 pg cell^{-1}. dcGTX2, 3 was detected in 8 out of 16 samples with levels ranging from 0.007 µg L^{-1} (S3a; 61,000 cells L^{-1}) to 0.098 µg L^{-1}; (S6b; 1,330,000 cells L^{-1}; Fig. V-4C). This dcGTX2, 3 toxin analogue also gave a significant correlation with *Alexandrium sp.* LM counts ($r = 0.83$; $P < 0.05$; $n = 8$) which allowed an estimate of 0.14 pg cell^{-1} (Fig. V-4D). dcSTX and STX were detected at only two stations, both taken on 6th July (samples S7a and S7b) when *Alexandrium* cell concentrations were at their highest (Fig. V-4C).

Table V-3. Microarray signal to noise ratios for *Alexandrium* genus (AlexGD01_25_dT) and species *A. minutum* (AminuS01_25_dT) and *A. tamarense* NA (ATNA_D02_25_dT) probes spotted on the third generation microarray. Corresponding *Alexandrium sp.* cell numbers, expressed as cells per volume filtered for RNA are also shown. Signal-to-noise ratio values ≥2 were above the threshold limits set for microarray indicating a positive microarray result, negative microarray results <2.

Table V-3. Continued

Environmental data				Signal to noise ratios		
Sample	**Date sampled**	**Volume filtered (mL)**	***Alexandrium* cells (per filter)**	**AlexGD01_25_dT**	**AminuS01_25_dT**	**ATNA_D02_25_dT**
S1a	25.05.11	200	2900	**12.68**	1.94	**2.25**
S1b	25.05.11	200	1400	**8.43**	1.18	1.78
S2a	01.06.11	200	2200	**2.87**	1.04	1.05
S2b	01.06.11	200	1400	**4.48**	1.80	1.19
S3a	08.06.11	200	12300	**5.94**	**2.06**	1.05
S3b	08.06.11	200	3900	**7.52**	**2.48**	1.16
S4a	15.06.11	200	10800	**8.22**	**4.01**	1.41
S4b	15.06.11	200	29500	**11.93**	**8.63**	1.76
S5a	22.06.11	250	26300	**24.07**	**9.20**	1.22
S5b	22.06.11	200	86500	**18.19**	**6.75**	1.15
S6a	29.06.11	200	59600	**14.21**	**5.09**	1.32
S6b	29.06.11	200	265500	**32.84**	**14.21**	**2.68**
S7a	06.07.11	200	242400	**24.88**	**8.46**	1.18
S7b	06.07.11	200	352900	**34.30**	**10.49**	1.23
S8a	13.07.11	200	500	1.25	0.88	1.26
S8b	13.07.11	200	200	1.21	1.04	1.07

Levels of these compounds were in the range 0.002 - 0.008 µg L^{-1}. Likewise, the PSP toxin analogue C1, 2 was present in samples 7a and 7b (0.010 - 0.011 µg L^{-1}), and a smaller level was detected in sample S1a (0.003 µg L^{-1}; Fig. V-4C). GTX 1,4, GTX 5 and NEO were not detected in any of the 16 samples analysed (Fig. V-4C).

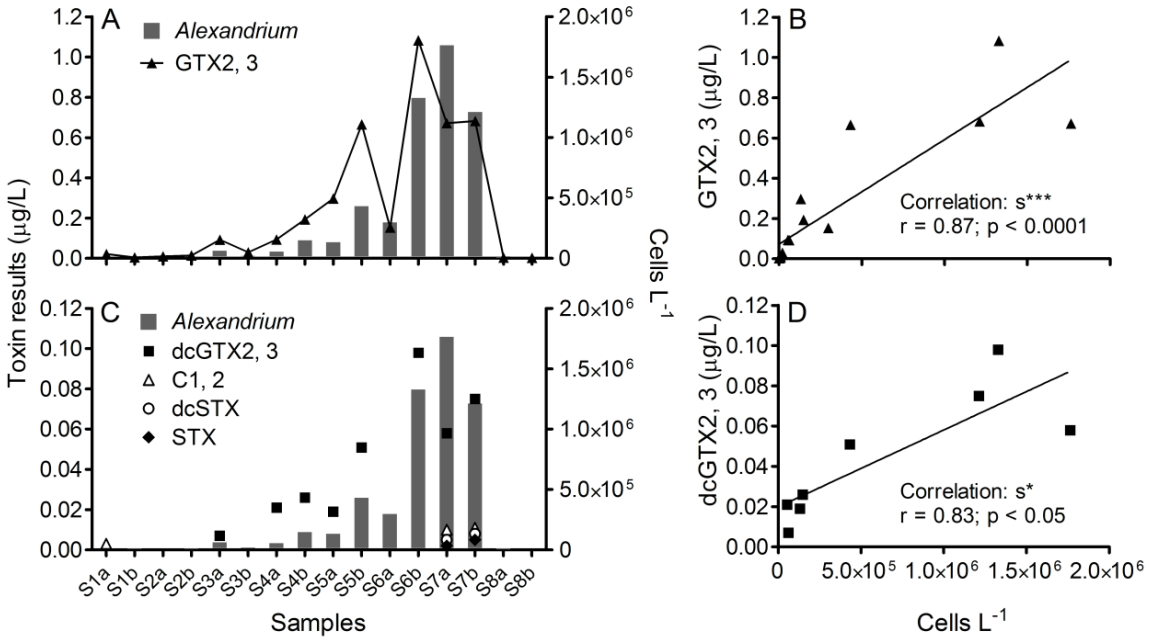

Fig. V-4. Comparison of PSP toxin results obtained from high performance liquid chromatography (HPLC) with light microscopy counts of *Alexandrium sp*. in the North Channel of Cork Harbour. HPLC results for the saxitoxin analogue are indicated for (A) GTX2, 3, (C) dcGTX2, 3, C1, 2, dcSTX and STX. Calibration curves showing corresponding r = Pearson correlation coefficient, p = significance of the correlation and * = level of significance are represented for the saxitoxin analogues (B) GTX2, 3 and (D), dcGTX2, 3.

The accurate quantification of total PSP toxin concentration when applying antibodies requires the antibodies to detect all the toxin analogues to a similar extent. Due to the variability of the structural analogues of PSP toxins that can be present the cross-reactivity of the antibody favours those most closely aligned in structure to the toxin analogue to which the antibody was raised. This is complicated further when toxicity equivalence factors are applied for the detection of the toxic potency of these toxins in seafood as STX equivalents. For both the multi SPR and ELISA the cross-reactivity profiles have been presented previously (McNamee et al., 2013).

Table V-4. Comparison of Multi SPR, ELISA and HPLC analogue results for PSP toxins represented in concentrations of ng/filter, against *Alexandrium* cell numbers for all the samples during the 2011 survey. (nd = not detected).

Environmental data				Multi SPR	ELISA	HPLC				
Sample	Date sampled	Volume filtered (L)	*Alexandrium* cells (per filter)			C1, 2	GTX2, 3	STX	dcGTX2, 3	dcSTX
S1a	25.05.11	1.0	14600	13	0.7	2.8	22.0	nd	nd	nd
S1b	25.05.11	1.0	7050	3	0.4	nd	5.3	nd	nd	nd
S2a	01.06.11	0.8	8900	7	0.5	nd	7.9	nd	nd	nd
S2b	01.06.11	0.8	5650	7	0.6	nd	11.3	nd	nd	nd
S3a	08.06.11	0.6	36900	12	> 2	nd	55.4	nd	4.4	nd
S3b	08.06.11	1.0	19700	8	> 2	nd	30.0	nd	nd	nd
S4a	15.06.11	0.6	32700	13	> 2	nd	55.5	nd	12.4	nd
S4b	15.06.11	0.9	133000	24	> 2	nd	173.9	nd	23.5	nd
S5a	22.06.11	1.0	132000	22	> 2	nd	297.3	nd	18.9	nd
S5b	22.06.11	1.0	432000	33	> 2	nd	665.4	nd	51.5	nd
S6a	29.06.11	1.0	298000	27	> 2	nd	151.5	nd	nd	nd
S6b	29.06.11	1.0	1330000	47	> 2	nd	1082.9	nd	97.6	nd
S7a	06.07.11	0.8	1410000	30	> 2	8.0	537.3	1.93	46.2	4.4
S7b	06.07.11	0.6	730000	26	> 2	6.8	409.2	3.20	44.7	5.0
S8a	13.07.11	0.8	2000	6	0.4	nd	2.7	nd	nd	nd
S8b	13.07.11	0.8	800	6	0.4	nd	1.7	nd	nd	nd

A calibration curve was used to semi-quantify the toxin concentration in these assays but due to the varying cross-reactivity profiles of the antibodies the accuracy in these methods compared to HPLC is difficult to achieve. Nonetheless, 14 samples out of 16 that presented on the dynamic range of the multi SPR curve between the IC_{20} = 8.2 ± 2.5 ng filter^{-1} and IC_{80} = 35.7 ± 6.2 ng filter^{-1} were significantly correlated with cell counts ($r = 0.64$; $P < 0.001$; $n = 14$; Table V-4). Due to the sigmoidal nature of the calibration curve, sample S1b was presented as less than the IC_{20} and below the detection limit of the multi SPR assay and the remaining sample S6b was deemed highly positive for PSP toxins and beyond the IC_{80} concentration (Table V-4).

As the ELISA assay is more sensitive compared to the multi SPR method all samples presented as positive for PSP toxins with 10 of these samples greater than the level of 2 ng filter^{-1} equivalent to the top standard on the ELISA calibration curve (Table V-4).

Detection limits of the Microarray and Multi SPR biosensor chip

Table V-3 shows the microarray S/N ratio values for the probes AlexGD01_25_dT (*Alexandrium* genus), AminuS01_25_dT (*A. minutum*) and ATNA_D02_25_dT (*A. tamarense* NA) when hybridised with labelled RNA extracted from field samples on the third generation microarray. S/N ratio values above the threshold level of 2 are compared with the corresponding *Alexandrium sp*. cell numbers on each RNA filter sampled during the survey (Table V-3). It can be seen that 1,400 *A. minutum* cells are required to generate a positive signal for the *Alexandrium* genus probe AlexGD01_25_dT and 3,900 cells to produce a positive signal for the species specific probe AminuS01_25_dT (Table V-3).

All the Cork Harbour seawater samples contained the PSP toxin producing *A. minutum* and the ELISA method could detect PSP toxins in all the samples. A comparison between the multi SPR biosensor results, ELISA and cell counts suggest that 8,900 *Alexandrium* cells are required to give a signal ≥ IC_{20} of 8.2-2.5 ng filter^{-1} and can be considered positive with confidence (Table V-4). This is based on the result for sample S2a as all other filters with numbers of cells higher than this sample gave a positive multi SPR result.

DISCUSSION

Hydrographic and physical forces are the main influences on *Alexandrium* bloom dynamics in the North Channel estuary (Ní Rathaille, 2007; Ní Rathaille et al., 2008). *Alexandrium* blooms occur during the period in June when there are weak spring tides. This takes place around the summer solstice, when water temperatures are favourable for growth (>15 °C) and follow a period of maximum *in situ* excystment (Ni Rathaille and Raine, 2011). These blooms develop as a consequence of a favourable balance between *in situ* growth rate and tidal dilution (Ní Rathaille et al., 2009; Raine, 2014). The *Alexandrium* bloom of 2011 initiated during the spring tide of ~17th June and then exponentially increased over the following neap tide supporting the current understanding of *Alexandrium* blooms in the North Channel of Cork Harbour (Ní Rathaille and Raine, 2011). The dominant *Alexandrium* species found was the toxin producing *A. minutum* (Cosgrove et al., 2014). In Cork Harbour this species co-occurs with a non-toxic *A. tamarense* WE group III ribotype and spirolide-producing *A. ostenfeldii* (Touzet et al., 2008; 2011). The variability in intensity of *A. minutum* blooms have fluctuated over the years, with more extensive blooms (~10^6 cells L^{-1}) occurring in 2004 and in the present study of 2011 (Cosgrove et al., 2014). Reasons for the rapidity of bloom decay are as yet unresolved, but are likely to be a mix of factors such as grazing, parasitism and encystment.

The efficiency of RNA extraction and labelling was within the optimal range across the sample set. Except for a lower degree of labelling in sample S2a (0.4 % DoL) which may have been due to the quality of the extracted RNA for that particular sample even after clean up and precipitation steps. This would explain why the S/N ratio value for the genus level probe (AlexGD01_25_dT) in station S2a (2.87 S/N ratio; 0.4 DoL %) was so much lower than station S2b (4.48 S/N ratio; 2.1 DoL %) and S1b (8.43 S/N ratio; 1.1 DoL %), even though the corresponding *Alexandrium* cell numbers were higher in samples S2a (2,200 cells) compared with stations S1b and S2b (1,400 cells). This also affected the detection limit of the species specific probes such as *A. minutum* (AminuS01_25_dT). It is important to note that the quality of the RNA extraction is paramount to the success of labelling and to the accuracy and sensitivity of the microarray (Metfies and Medlin, 2004; 2008; McCoy et al., 2013).

The dominant *Alexandrium* species during the 2011 bloom was *A. minutum*. The microarray detected positive signals for both *Alexandrium* genus and *A. minutum* and the

multi SPR biosensor detected the presence of PSP toxins in all of the samples taken during the bloom. Data from FISH analysis showed *A. tamarense* (WE) to be present but in very low cell densities (Cosgrove et al., 2014). The toxin producing *A. tamarense* NA (group I) has occurred in Belfast Lough, along the west and north of Scotland, but has yet to be definitively identified in water samples from the west and south coasts of Ireland, including Cork Harbour (Touzet et al., 2008; 2010). The positive microarray result for *A. tamarense* NA (group I) obtained for samples S1a and S6b were false positive signals for the ATNA_D02_25_dT probe. Taylor et al. (2013a) reported that the *Alexandrium* genus probe, as well as both probes for the *A. tamarense* NA and *A. tamarense* complex should produce positive microarray signals on the MIDTAL microarray if *A. tamarense* NA group I ribotype is present. They have shown that the *Alexandrium* genus and *A. tamarense* (NA, WE, TA) species complex probes have a greater affinity towards the non-toxic *A. tamarense* group III ribotype, which suggests that the microarray is effective at distinguishing between the two groups (Taylor et al., 2013a). The dominance of *A. minutum* during the survey period with low numbers of the non-toxic *A. tamarense* may have affected the *A. tamarense* complex probe producing a positive microarray signal. The spirolide producing *A. ostenfeldii* was not targeted in LM counts or FISH analysis. However although it is known to occur in Cork Harbour, it appears to exist in much lower numbers then either *A. minutum* or *A. tamarense* (WE) (Touzet et al., 2011). *A. ostenfeldii* probes (AostD01_25_dT and AostS01_25_dT) did not produce a positive microarray signal during the survey period.

PSP events have resulted in closure of shellfish harvesting in Cork Harbour in most years since 1996 (Ní Rathaille et al., 2008; 2009). Previous studies that have applied a combination of morphology-based species identification techniques using oligonucleotide probes, rDNA sequencing and toxin composition using HPLC with post-column derivatisation and fluorescence detection have shown that toxic and non-toxic strains of *Alexandrium* both co-occur in Irish coastal waters (Touzet et al., 2008). The proportionally higher amounts of GTX2, 3 detected with HPLC in the 2011 survey compared with other toxin analogues tested further confirms the microarray results and LM counts and FISH results which detected the dominating *Alexandrium* species as *A. minutum*. GTX2, 3 are the main toxins found in cultures of *A. minutum* isolated from Cork Harbour (Touzet et al., 2008). This is similar to isolates from France (Ledoux et al., 1993) although the toxin content of this species can vary on a global scale (see e.g. MacKenzie and Berkett, 1997). The toxin content found in this study of 1.29 ± 0.58 pg

GTX2, 3 cell^{-1} is comparable with the toxicity of this species in culture of 1.6 - 2.6 pg STX equivalent cell^{-1} (Touzet et al., 2008). A field study by Touzet et al. (2008) described the detection of low amounts of sulfocarbamate groups such as C-toxins in Cork Harbour. They suggested that these were a chemotype of *A. minutum,* and is consistent with the present study which detected C1, 2 in stations S1a, S7a and S7b.

There is a need for fast, reliable and high-throughput detection methods for marine phytoplankton and their biotoxins due to increasing concerns in relation to marine food safety. A range of analytical methods is now available for the detection of marine biotoxins in shellfish. These include HPLC, liquid chromatography with mass spectrometry, and commercial kits such as ELISA and Jellet (McNamee et al., 2013). The development of biosensor-based immunological assays such as SPR has opened the field to detection of specific low molecular weight marine biotoxins. It has demonstrated the simplicity, ease of use, speed and accuracy over other analytical methods (Campbell et al., 2007; 2011). The prototype multiplex SPR biosensor has the advantage of distinguishing between toxin families on a single chip format over single channel SPR arrays for PSP toxins, okadaic acid and domoic acid toxins (Campbell et al., 2011; McNamee et al., 2013). The PSP toxin results presented for the multi SPR biosensor chip, were also detected by both ELISA and HPLC methods, giving a further confidence of the multi SPR technique and its quantitative potential for monitoring toxic algae events such as those observed in Cork Harbour.

McNamee et al. (2013) demonstrated that the ELISA method for measuring PSP toxins is some 80-fold more sensitive than that of SPR method, a finding that has been supported in the data set presented in the current study. The lowest biosensor detection limit determined from the error margins of the IC_{20} was 5.7 ng/filter^{-1} for saxitoxin. It has already been stated that, for the sample set shown here, this limit relates to 8,900 cells. Toxin extraction from GF/F filters together with buffering resulted in a total extract volume of 5 mL. This volume would be reduced to 2 mL if polycarbonate filters had been used, as a consequence of the reduction in buffer volume to wash the filter (see McNamee et al., 2013). Thus the sensitivity of the method would be improved and the detection limit in terms of cells filter^{-1} would be reduced to 3,500 cells if a different type of filter had been used in the sample preparation step. It should be noted that samples S1b, S2a, S2b, S3b, S8a and S8b all contained low levels of GTX2/3 toxin only and the detection by SPR is borderline on the IC_{20} determined for STX. As the antibody in this assay was raised to the hydroxylated toxin neosaxitoxin it has only 6.4% cross-reactivity

to this toxin when compared to 100% for STX. For known toxin profiles of a bloom in a particular region that reoccur frequently the antibody selected for the assay should be tailored to the toxin content of the bloom for improved detection of other toxins compared to the parent saxitoxin.

The microarray results presented here were hybridised to a third generation MIDTAL chip (Kegel et al., 2013a). The microarray detection limit was quite similar to that deduced for the multi SPR biosensor at 3,900 *A. minutum* cells. Taylor and co-workers demonstrated positive microarray signals for *A. tamarense* probes in samples where cells are as low as 240 cells and *A. minutum* probes as low as 270 cells in laboratory based calibration curves (Taylor et al., 2013a; 2013b). The higher detection levels reported for this 2011 Cork Harbour study suggests that there may be interference from other organisms present in the phytoplankton assemblage and additional adjustments may be required when dealing with field samples as opposed to microarray hybridisation results from pure cultures. This could also be further reduced with the addition of KREAblock (Kreatech) to the hybridisation mixture, which reduces background noise and improves signal intensity, particularly when using field samples. This step has been added to the MIDTAL protocol subsequent to this study in Cork Harbour (Lewis et al., 2012; Kegel et al., 2013a).

A study of the data archive from the Irish biotoxin monitoring programme indicates that ~5000 cells L^{-1} is equivalent to the cell density approximately a week in advance of shellfish harvest closure resulting from PSP toxin contamination. For example, in 2005, mussel (*Mytilus edulis*) samples taken from the North Channel of Cork Harbour tested positive for PSP toxins on 7th June and again on 13th June, the date corresponding to the *A. minutum* bloom maximum. A week prior to this toxic event (30th May - 2nd June) *Alexandrium* cell counts were 1,500 - 3,000 cells L^{-1} in the North Channel (Cosgrove et al., 2014). Similarly in 2006 mussel flesh, this time from Cobh (Fig. V-1), tested positive for PSP toxins on 27th and 29th June in 2006 two weeks after *A. minutum* levels determined by FISH increased over 7,000 cells L^{-1}. In 2007, closures in Cobh started on the 27th June, after *A. minutum* cell counts of 2,000 (14th June) and 7,000 cells L^{-1} (21st June) were observed (Cosgrove et al., 2014).

The results presented here demonstrate the usefulness of combining the MIDTAL microarray and multiplex SPR biosensor for monitoring *Alexandrium minutum* and PSP toxins in water samples. The detection limits of both these methods are in keeping with the above examples of biotoxin monitoring cell count numbers equating to shellfish

closures. Their combination provides a means of identifying and quantifying the harmful phytoplankton community and their toxins that can be used in conjunction with methods currently routinely employed by monitoring agencies in compliance with European Union directives (Directive 91/492 and Commission Decision 2002/225). Their use may also assist development of early warning systems in coastal waters. Both methods are capable of detecting multiple toxins and a whole range of phytoplankton groups in one analysis. Evaluation of their use during other HAB events is underway. Probe sequences are patent pending and the MIDTAL microarray is now commercially available from Microbia Environnement.

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CHAPTER VI

MONITORING AN EXTENSIVE BLOOM OF *PROROCENTRUM MICANS* IN BELL HARBOUR OF GALWAY BAY USING A NEWLY DEVELOPED MIDTAL MICROARRAY

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ABSTRACT

An extensive bloom of *Prorocentrum micans* was recorded in Bell Harbour of Galway Bay during the late summer months on the west coast of Ireland in 2011. This study monitored the bloom from its initiation to the peak of the bloom during July 2011 with the use of the MIDTAL (Microarrays for the Detection of Toxic Algae) microarray. Information obtained from this study can be used to further assess the capabilities of the oligonucleotide 18S and 28S rRNA gene probes corresponding to a whole range of phytoplankton taxa, including the 28S gene probe specific for *P. micans*, spotted on the 3rd generation MIDTAL microarray. This technology is being developed to aid national monitoring agencies and to provide a faster means of identifying and quantifying the harmful phytoplankton community in natural water samples. Results presented show a significant correlation between total RNA extracted and the total chlorophyll present in each sample, indicating the high quality of both extractions. There was also a significant correlation of increasing cell numbers of *P. micans* with increasing microarray signal intensities with additional results for other species in good agreement with light microscopy counts, proving the use of the MIDTAL microarray as a quantification tool.

Keywords: rRNA probes, MIDTAL, Microarrays, RNA, *Prorocentrum micans*.

INTRODUCTION

The bloom forming dinoflagellate *Prorocentrum micans* Ehrenberg is a cosmopolitan species commonly found in cold temperate and tropical waters and is known to produce extensive red tides on a global scale (Fukuyo et al. 1990). However this species is considered relatively harmless as it produces no toxins that enter into the food chain and any substances it does produce seem only to inhibit diatom growth (Anderson et al. 1985; Graneli et al. 1990; Uchida 1977). However, it has been associated with shellfish kills during extensive blooms (Horstman 1981; Pinto and Silva 1956) which are likely triggered from oxygen depletion (Lassus and Berthome 1988). Although these blooms are naturally occurring, they are not particularly attractive as regards tourism, and many people would avoid swimming into a visible algal bloom with a preconception of associating it with pollution. Some toxic species, however, can cause skin and lung irritation (Hallegraff 2003).

Monitoring of Harmful Algal Blooms (HABs) by national monitoring agencies is mandatory under EU directives and is traditionally carried out using light microscopy (LM). This procedure, however, requires a high degree of skill on behalf of the operator and is also time-consuming. A number of molecular biological techniques have been developed to assist in the identification and quantification of species and their toxins as most shellfish farm closures are determined from threshold levels of cell densities and/or toxin levels in shellfish (Anderson et al. 2012a; Ebenezer et al. 2012; Karlsen et al. 2010).

Microarrays are molecular tools which allow the analysis of a large number of target probes from higher taxonomic levels right down to species level in a single analysis, reducing the amount of reagents and time required, while producing a large amount of information (Ye at al. 2001). DNA microarrays are relatively new as regards identifying marine algae (Galluzzi et al. 2011; Gescher et al. 2008). In the European Union (EU) 7th Framework Program project MIDTAL (microarrays for the detection of toxic algae; Kegel et al. 2013a; Medlin 2013) a DNA microarray has been developed for the identification and quantification of HAB organisms which are difficult to discriminate to species level under LM (Metfies and Medlin 2005, 2008; Metfies et al. 2010). The MIDTAL technology can simultaneously analyse over 136 different probes and 4-8 replicates including several controls specific for a range of harmful phytoplankton species (Lewis et al., 2012; Kegel et al., 2013b). In conjunction with this microarray

technology an antibody based biotoxin detection platform was also developed called the Multiples Surface Plasmon Resonance (multi SPR) biosensor (Campbell et al. 2011; McNamee et al. 2013). This approach can simultaneously test for domoic acid, okadaic acid and saxitoxins using a single multi-biosensor chip (McNamee et al. 2013).

Blooms of *P. micans* have occurred in Galway Bay since 1980 and 1981, particularly in the inlets along the southern coastline such as Bell Harbour, Co, Clare (Pybus 1990). Bell Harbour is an estuarine brackish water environment, which has extensive mudflats exposed at low tide (Fig. VI-1). The incoming tide is forced through Shanmuckinish straight, a narrow harbour mouth which faces directly west in line with the flow of water filling Galway Bay. However, Bell Harbour geographically takes a south easterly turn and the combination of this tight bend in the harbour with the narrow mouth allows a substantial amount of water to be retained during a tidal cycle. Low flushing rates combined with favourable environmental conditions allows blooms of *P. micans* to reach exceptionally high numbers which can cause water discolouration, causing it to turn a bright copper red (Pybus et al. 1984; Pybus 1990).

This chapter presents the results of inter-comparisons between light microscopy counts and microarray results using the MIDTAL microarray, with an emphasis on following the progression of a *P. micans* bloom in Bell Harbour of Galway Bay during the summer of 2011.

MATERIALS AND METHODS

Sampling site and field sampling

A fixed station at Bell Harbour pier was sampled every week at high tide between the 03rd May to 23rd August 2011, with one additional sample taken on 27th September (Fig. VI-1). Unacidified Lugol's Iodine preserved surface samples (Throndsen 1978) were collected in 50 ml cell culture bottles and kept in the dark until used for cell count determination, which was performed using an inverted microscope (Olympus CKX-41) following the procedure described in McDermott and Raine (2010). Water samples for RNA analysis were pre-filtered through a 150 µm mesh and then a total volume of 50-300 ml was filtered through a 1 µm pore-size nitrocellulose filter (25 mm diam.) depending on the turbidity of the water (Table VI-1). All filters for RNA analysis were immediately immersed in 1 ml of TRI Reagent (Ambion) contained in 2 ml screw cap tubes, kept at 4 °C during sampling and within 6 hours of sampling stored at -80 °C on return to the laboratory. Water samples (600– 1000 ml) for toxin analysis were filtered through glass fibre (Whatman GF/F, 47 mm diam.) filters (Table VI-1). These filters were stored separately at -20 °C in 2 ml screw cap tubes. Water temperature was recorded hourly using a TidBit temperature sensor (HOBOware) moored at a position north of Bell Harbour pier (53° 07.571' 9° 04.376'; Fig. VI-1) 0.5 m off the sea bed. This sensor was deployed from the 2nd June throughout the rest of the sampling period. Temperature and salinity profiles were also measured *in situ* using a temperature salinity probe (WTW, 197i). Tidal ranges were derived from published tide tables.

Chlorophyll a analysis

Samples for chlorophyll analysis were taken in order to estimate the phytoplankton biomass. A known volume (usually 1 L) of seawater was filtered through glass fibre (Whatman GF/C, 47 mm diam.) filters using a hand held vacuum pump. If the water turbidity and/or plankton biomass of the sample was extremely high a lower volume ranging between 100 ml to 800 ml was filtered.

Fig. VI-1. Map of the sampling location in Bell Harbour of Galway Bay. One station located at the pier situated at the most southern point of Bell Harbour was sampled from 3rd May to 27th September during the 2011 survey period. A shellfish farm off Poulnaclough Bay is also located north of the sampling site.

The filter was placed in a clean 15 ml tube and kept cool (~4 °C) and in the dark until it was brought back to the laboratory where it was immediately stored at -20 °C until further processing. For the analysis, the filters in the 15 ml tubes were immersed overnight in 10 ml of 90% acetone (90 ml acetone, 10 ml deionised water (ddH_2O)) solution in the dark and at 4 °C. The volume of the chlorophyll extract was then noted and the chlorophyll fluorescence before and after acidification with 2 drops 50% HCL was measured on a calibrated fluorometer (Turner Model 10). Chlorophyll levels were then estimated using the method outlined in Tett (1987), with the fluorescence values calibrated against spectrophotometric readings.

Microarray design

Oligonucleotide probes routinely used for fluorescent *in situ* hybridisation (FISH) were modified and adapted to the microarray (Kegel et al. 2013a; 2013b). Newly designed probes were also developed with the open software package ARB (Ludwig et al., 2004). The oligonucleotides including the positive and negative controls were synthesized (Eurofins MWG Operon or Thermo Fisher Scientific, Ulm, Germany) with a C6 aminolink at the 5' end of the molecule. The probes on the second generation chip had a

length between 18 and 25 nucleotides, and a further 15 nucleotide poly deoxythymidylic (dT) tail following the amino (NH_2) link at the 5' end was subsequently added for the 3rd generation chip. The probe sequences are patent pending (Microbia Environnement; France) and a list of the probes and targeted taxon made from the 18S or 28S rRNA gene to form the third generation of the MIDTAL microarray can be found in Kegel et al. (2013b). Duplicate arrays were spotted with 4-8 replicates of 136 different probes and as well as three negative controls (NEGATIVE1_dT, NEGATIVE2_dT, NEGATIVE3_dT), one positive control (TATA box protein), a Poly-T-Cy5 spotting control, and three internal controls (DunGS02_25, DunGS02_25_dT and DunGS05_25_dT for *Dunaliella tertiolecta*). After spotting, slides were incubated for 30 minutes at 37°C and then stored at -20°C.

Microarray analysis

Microarray analysis involved a multistep process of RNA extraction, labelling and hybridisation onto a microarray containing 136 probes (Kegel et al., 2013b). The methods were essentially those outlined in Kegel et al. (2013b). Prior to RNA extraction an aliquot of TRI Reagent (100 µl) containing 500,000 cells of *Dunaliella tertiolecta* (UIO226 strain) was added to each filtered sample, acting as an internal control. RNA was extracted from thawed frozen filters in Tri Reagent, separated out using 1-Bromo-3-chloro-propane (BCP: Sigma), chloroform and isopropanol (Sigma) sequentially, followed by three ethanol (70% EtOH) wash steps and a clean-up step involving ammonium acetate (7.5 M NH4Ac; Applichem). The extracted RNA was labelled using a Platinum Bright 647 labelling kit (KREATECH Biotechnology) and fragmented by adding fragmentation buffer ($ZnCl_2$ in Tris-HCl pH7) followed by incubation (70 °C, 15 mins). Prior to hybridisation, the microarray chips were blocked (0.2%SDS), washed, spun down and dried and stored at 4 °C until required. The hybridisation cocktail containing, 2x hybridisation buffer, labelled RNA, TBP-control, Poly-dA and RNase free water was denatured (94 °C, 5 mins) and then hybridised to the microarray chip. All experimental details can be found in Kegel et al. (2013b). The only deviations from this was that the quality of the RNA was periodically checked using a NanoDrop 1000 spectrophotometer and 15 µl of KREAblock (KREATECH) was not added to the denatured hybridisation cocktail.

Scanning and Data analysis.

A Perkin Elmer Microarray Scanner was used to scan the array and the fluorescent signal and background intensity were calculated by superimposing a gridded map of circles (midtal_ver32_20110429.gal) onto the scanned image using GenePix 6.0 software. The results were processed with the GPR-Analyzer version 1.27 which describes the hierarchy of the probe set (Dittami and Edvardsen, 2013). A signal-to-noise (S/N ratio) ratio of ≥2 was set as the low threshold for positive signals. To compare values from different hybridisations, signals were normalised using the internal controls and replicates averaged.

Statistical analysis

A two-tailed Pearson's correlation of significance test was carried out for (1) total RNA extracted (ng) with total chlorophyll (per sample) and (2) normalised microarray signal and cell number relationships. A D'Agostino-Pearson test was used to confirm that the data did not differ significantly from a normal distribution ($P > 0.05$ in all cases). All statistical analysis was carried out in GraphPad Prism 5.

RESULTS

Environmental data and Prorocentrum micans cell counts

Water temperatures generally increased till they peaked on the 26th July with an average daily temperature of 18.95 °C. On this date, the surface water temperature at the Bell Harbour pier was 23.3 °C (Fig. VI-2a). Water temperatures generally decreased after this event, with no record above 18 °C from the 27th July onwards, except on 23rd August when the surface water temperature was 18.1 °C (Fig. VI-2a). A chlorophyll maximum of 71 µg /litre coincided with a *P. micans* cell density maximum of 8.7 x 10^7 cells/litre (Fig. VI-2b).

P. micans cell densities were plotted with tidal range which is defined as the vertical difference between the high tide and the succeeding low tide (Fig. VI-2c). Relatively low cell densities (350 – 15,500 cells/litre) of *P. micans* cells were found between the dates of 23rd May to 25th June. After this date concentrations rapidly increased to 2.9 x 10^6 cells/litre on 18th July. The data is plotted with tidal range in Fig. VI-2c, where it can be seen that the *P. micans* bloom reached its maximum concentrations (8.7 x 10^7 cells/litre) during a neap tide on the 26th July. The bloom then rapidly decreased in concentration (3.7 – 4.2 x 10^5 cells/litre) between the 4th - 9th August, respectively. Prior to the following spring tide event at the end of August the *P. micans* numbers increased again to concentration of 1.2 - 2.6 x 10^6 cells/litre. No samples were taken from Bell Harbour from the 24th August until the 27th September were *P. micans* cells was still present in cell concentrations of 1.7 x 10^5 cells/litre during a spring tide cycle with the highest tidal range recorded during the survey period of between 1.9 - 5.7 m (Fig. VI-2c).

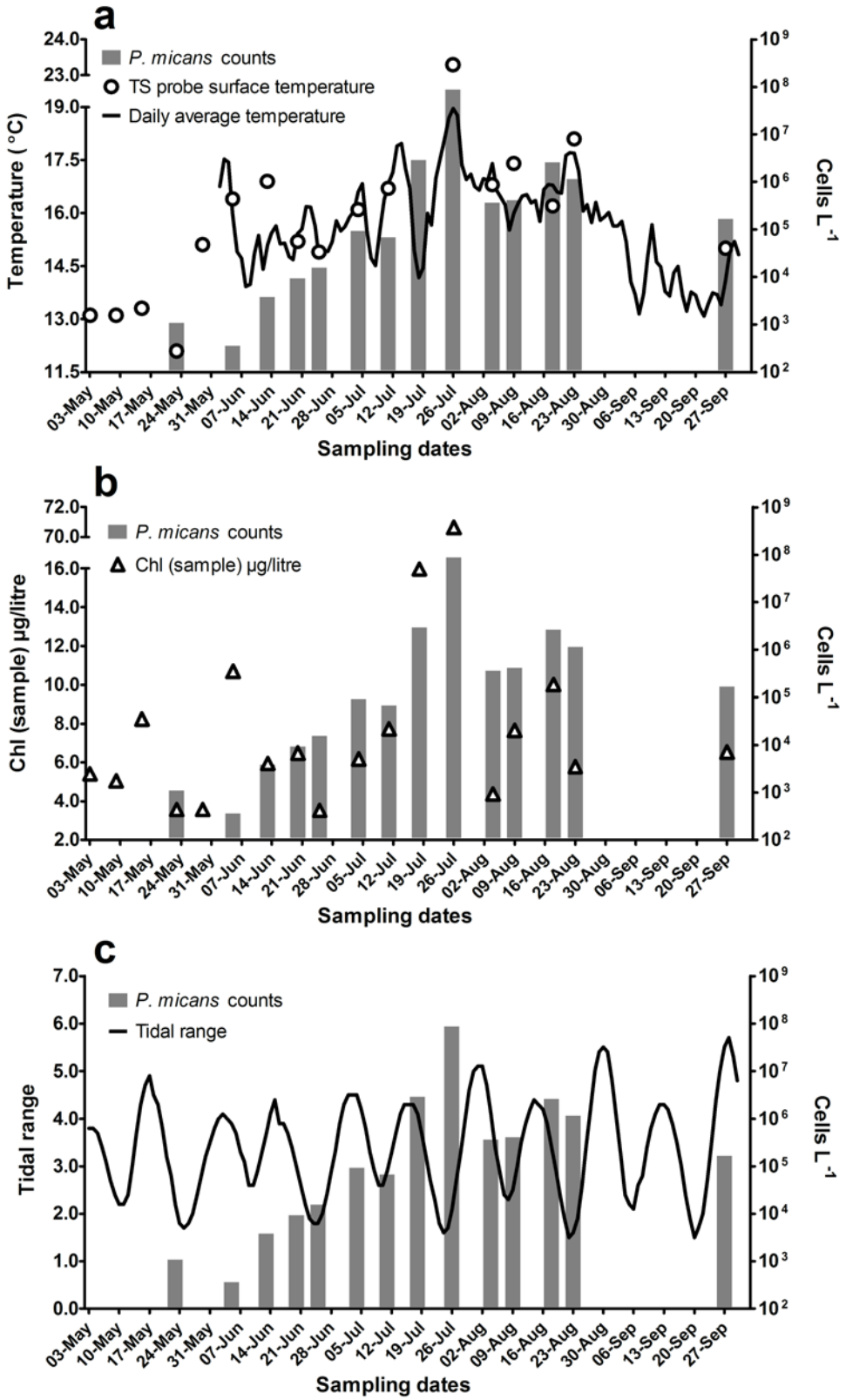

Fig. VI-2. (a) *Prorocentrum micans* cell densities in Bell Harbour of Galway Bay during the summer 2011 survey derived from light microscopy counts with temperature readings from the *in situ* TS Probe together with the daily average temperature readings from an anchored TidBit sensor

positioned east of Poulnaclough Bay deployed from 2nd June to the end of the survey period. (b) *P. micans* cell densities plotted with total chlorophyll concentrations per litre. (c) *P. micans* cell densities presented with tidal range. Note the logarithmic scale on the right y-axis.

Chlorophyll and RNA

RNA extractions were carried out on a total of 18 Bell Harbour samples, with the total RNA extracts ranging between 3,900 ng to 33,500 ng per sample prior to the NH_4Ac clean-up step (Table VI-1). Total RNA (µg/ml) extract was plotted with total chlorophyll *a* present in each sample. Both calibrations were significantly correlating ($r = 0.9074$; $P < 0.0001$; $n = 17$; Fig. VI-3).

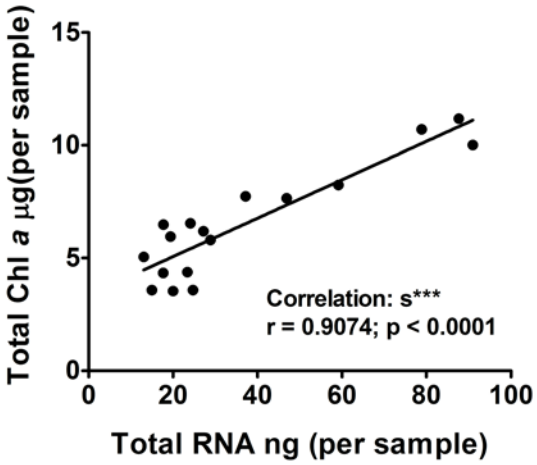

Fig. VI-3. Calibration of total Chlorphyll *a* (Chl) with total RNA extracted per sample from the Bell Harbour 2011 sampling survey. Note: Bell Hr 21 26/07/11 (BHJ) has been excluded.

Microarray analysis

RNA labelling.

A total of 10 out 18 Bell Harbour field samples were chosen for microarray analysis to show the progression of the *P. micans* bloom from early stages till its cell density maximum on the 26th July on the 3rd generation microarray. An aliquot of 1000 ng was taken from the eluted NH_4Ac clean-up RNA solution for RNA labelling (Table VI-1). The degrees of labelling (DoL%) concentrations were all between 1.9 and 4.8 DoL% for 8 of the 10 samples. Samples BHA and BHB DoL% taken on 15th May and 29th May were 0.6 and 0.8 DoL%, respectively. The recommended optimal range of labelling for microarray hybridisations according to Kreatech Biotechnology nucleic acid labelling kit guidelines should be between 1.0 - 3.6 DoL% (Table VI-1).

Table VI-1. All field samples were spiked with 100 µl of TRI Reagent containing 500,000 cells of *Dunaliella tertiolecta* prior to RNA extraction. Total RNA extracted (ng) is the concentration of the eluted RNA pellet prior to NH4Ac clean-up step. Total Chlorophyll and Total Pigment extracts from each sample are represented per Litre. The corresponding RNA labelled DoL% for each RNA extract post NH4Ac clean-up was calculated for the 10 Bell Harbour samples hybridised to the 3rd generation microarray.

Sample name	MA Station code	Date	Vol filtered (ml)	Total RNA extracted (ng)	Chl µg /litre	TP µg /litre	RNA DoL %
Bell Hr 9		03/05/11	300	5294	5.41	8.65	
Bell Hr 10		09/05/11	300	3906	5.05	7.85	
Bell Hr 11	**BHA**	**15/05/11**	**200**	**11831**	8.24	10.60	**0.6**
Bell Hr 12		23/05/11	300	4490	3.57	7.85	
Bell Hr 13	**BHB**	**29/05/11**	**300**	**7409**	3.57	5.71	**0.8**
Bell Hr 14	**BHC**	**05.06.11**	**200**	**15772**	10.70	13.56	**2.5**
Bell Hr 15	**BHD**	**13.06.11**	**350**	**6772**	5.95	7.14	**2.0**
Bell Hr 16	**BHE**	**20.06.11**	**300**	**5309**	6.48	7.06	**2.1**
Bell Hr 17	**BHF**	**25.06.11**	**300**	**6003**	3.53	5.65	**2.2**
Bell Hr 18	**BHG**	**04.07.11**	**300**	**8144**	6.18	7.42	**2.1**
Bell Hr 19	**BHH**	**11.07.11**	**250**	**9293**	7.73	9.63	**1.9**
Bell Hr 20	**BHI**	**18.07.11**	**150**	**13150**	15.98	23.21	**4.8**
Bell Hr 21	**BHJ**	**26.07.11**	**50**	**33561**	70.64	162.47	**4.7**
Bell Hr 22		04/08/11	300	7020	4.37	5.99	
Bell Hr 23		09/08/11	200	9380	7.65	9.18	
Bell Hr 24		18/08/11	200	18188	10.01	13.42	
Bell Hr 25		23/08/11	250	7214	5.79	7.29	
Bell Hr 26		27/09/11	300	7228	6.54	9.28	

Hr: Harbour; MA: Microarray; Vol: Volume; Chl: Chlorophyll; TP: Total Pigment; DoL: Degrees of Labelling.

Microarray results and cell counts

Prorocentrum

Fig. VI-4a and VI-4b shows a comparison between *P. micans* cell numbers derived from LM counts with results from the 3rd generation microarray which have been normalised to the internal control probe POSITIVE_25_dT and DunGS02_25_dT_dT. Counts were compared against both a higher group level probe for *Dinophyta* (DinoE12_25_dT) and the species specific probe PmicaD02_25_dT for *P. micans* (Fig. VI-4a and b). Two Dinophyceae (incl. Apicomplexa) probes spotted on the chip called DinoD_25_dT and DinoE12_25_dT recorded positive microarray signal-to-noise (S/N) ratio values above the threshold level of ≥2 for 10 and 9 samples, respectively, out of a total of 10 microarray hybridisations.

A total of 7 samples out of 8 which contained *P. micans* cells produced positive microarray S/N ratio value ≥2 for the *P. micans* species probe (Fig. VI-4a and b), with the highest POSITIVE_25_dT (9.71) and DunGS02_25_dT_dT (142.99) normalised microarray signals observed in sample BHJ (*P. micans* 8.7 x 10^7 cells/litre; Fig. VI-4a and b).

The lowest positive *P. micans* normalised signal was recorded in sample BHD (POSITIVE_25_dT 0.36 and DunGS02_25_dT_dT 1.69) which contained 3,800 cells/litre. Both normalised microarray signal results for *P. micans* species specific PmicaD02_25_dT probes had a positive linear regression with LM counts. However, it was only DunGS02_25_dT_dT normalised microarray results that were significantly correlating (POSITIVE_25_dT r = 0.7543; P = 0.051; n = 7 and DunGS02_25_dT_dT r = 0.9453; P = 0.0013; n = 7) with cell counts (Fig. VI-4a and b).

It was also observed that the *Dinophyta* probe DinoE12_25_dT significantly correlated with the *P. micans* probe PmicaD02_25_dT for both normalised microarray signals (POSITIVE_25_dT r = 0.9718; P = 0.0003; n = 7 and DunGS02_25_dT_dT r = 0.9975; P < 0.0001; n = 7), however the *Dinophyta* probe DinoB_25_dT only significantly correlated under DunGS02_25_dT_dT normalised microarray signals (r = 0.95; P = 0.0010; n = 7).

Alexandrium

Alexandrium sp. LM counts were compared against both the normalised microarray signals for the genus level probe (AlexGD01_25_dT) and the species probe (ATNA_D01_25_dT) specific for *A. tamarense* (North American (NA) group I ribotype) (Fig. VI-4c and d). LM counts indicated the presence of *Alexandrium sp.* in samples BHA, BHB, BHC, BHD, BHE and BHG, with the highest numbers (15,250 cells/litre) observed in sample BHB taken on the 29th May and the lowest (550 cells/litre) observed in sample BHG taken on the 4th July 2011. The *Alexandrium* genus probe (AlexGD01_25_dT) was recorded in 5 samples, four of which contained *Alexandrium* cells and one that did not (BHI). Sample BHI, also recorded a signal for the *A. tamarense* species (NA group I ribotype) probe (ATNA_D01_25_dT) (Fig. VI-4c and d).

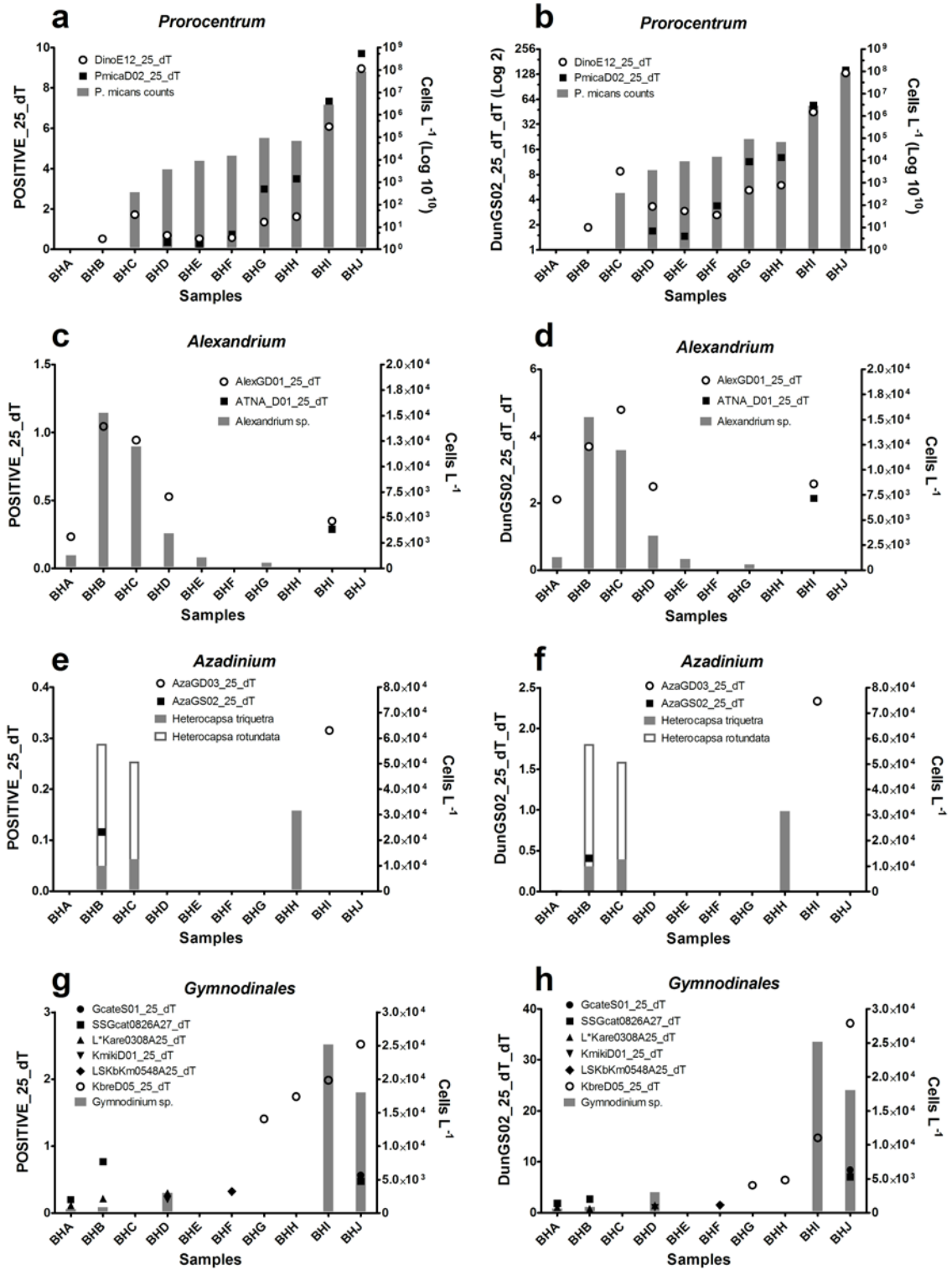

Fig. VI-4. Dinoflagellate microarray results for *Prorocentrum sp*. *Alexandrium sp*., *Azadinium sp*. and *Gymnodinales* sp. and their corresponding LM cell counts from Bell Harbour 2011 sampling survey. Signal intensity results from the 3rd generation microarray were normalised against POSITIVE_25_dT (a, c, e and g) and DunGS02_25_dT (b, d, f and h) probes and plotted with LM

cell counts. Note: (a and b) right y-axis is represented as Log 10 logarithmic scale to the power of 10; (b) left y-axis is represented as Log 2 logarithmic scale.

Azadinium

Microarray signals from two *Azadinium* probes AzaGS02_25_dT and AzaGD03_25_dT were recorded in samples BHB and BHI, respectively (Fig. VI-4e and f). It is difficult under LM to differentiate between *Heterocapsa* and *Azadinium spinosum* species, which superficially resemble each other with regards to size and shape (Hernández-Becerril et al. 2012). Therefore, under LM, each observation was recorded as either *Heterocapsa triquetra* or *Heterocapsa rotundata*. LM counts observed the presence of *H. triquetra* in samples BHA, BHB, BHC and BHH with cell concentrations not reaching above 31,500 cells/litre. *H. rotundata* was also recorded in samples BHB and BHC at higher concentration of 51,000 to 58,000 cells/litre, respectively (Fig. VI-4e and f).

Gymnodinales

Five samples were identified as containing *Gymnodinale sp.* ranging between 640 – 25,200 cells/litre (Fig VI-4g and h). Two *Gymnodinium catenatum* specific probes (GcateS01_25_dT and SSGcat0826A27_dT) were both recorded in sample BHJ which contained 18,000 cells/litre (Fig. VI-4g and h). SSGcat0826A27_dT probe was also highlighting above the threshold limit ≥2 in samples BHA and BHB which also contained *Gymnodinale sp*. cells from LM count of 600 – 900 cells/litre, respectively (Fig. VI-4g and h).

Karenia species probe (L*Kare0308A25_dT) microarray signal were recorded in samples BHA, BHB and BHD, all containing *Gymnodinale sp*. cells from LM count of ≤ 3,000 cells/litre. Species specific *Karenia mikimotoi* probe (KmikiD01_25_dT) was also recorded in sample BHD (Fig. VI-4g and h). *Karenia brevis* specific probe (KbreD05_25_dT) was recorded in four samples BHG, BHH, BHI and BHJ with *Gymnodinale sp*. cells only being noted in the last two samples from LM count (Fig. VI-4g and h). A second *K. brevis* probe (LSKbKm0548A25_dT) was singly highlighted in sample BHF, which had no *Gymnodinale sp*. cells noted in LM counts.

Pseudo-nitzschia group.

LM counts observed the presence of diatom species in 6 out of 10 samples, with the highest concentration found in sample BHG, with a dominating assemblage of *Pseudo-*

nitzschia delicatissima group (Fig. VI-5a and b). The *Pseudo-nitzschia spp.* probe (PmulacalD02_25_dT) was recorded in nine of ten samples, BHJ being the only sample not to record any microarray signal. *Pseudo-nitzschia seriata* grp. probe specific for *P. fraudulenta* (PfraucalD02_25_dT) produced a microarray S/N ratio ≥2 in samples BHB, BHC and BHH, with only sample BHB indicating the presence of diatoms in LM counts out of the three (Fig. VI-5a and b). *Pseudo-nitzschia spp*. probes for *P. multiseries* (PmultS01_25_dT), *P. calliantha* (PmultcalD04 _25_dT) and *P. caciantha* (PcaciD04_25_dT) were all highlighted in sample BHI, the only other sample that PmultS01_25_dT probe was recorded was BHB. *Pseudo-nitzschia seriata* grp. probe specific for *P. pungens* (PpungcalD04_25_dT) was highlighted in sample BHA and BHB which contained diatom cells, with sample BHA containing 2,800 cells/litre of *P. seriata* grp species (Fig. VI-5a and b).

Pseudo-nitzschia delicatissima grp probe Pdel4D03_25_dT and species specific *P. manii* probe Pman2D03_25_dT were both recorded in sample BHA, BHB and BHD. Pdel4D03_25_dT probe was also recorded in samples BHC and BHH (Fig. VI-5a and b).

Haptophyta

LM counts for haptophyta comprised of the Prasinophytes and Prymnesiophytes from the total microflagellate assemblage. These counts were compared with normalised microarray results from the 3rd generation hybridisations (Fig. VI-5c and d). All but two samples BHB and BHJ contained the presence of the Prasinophytes and Prymnesiophytes under LM counts. The higher group probes PrymS01_25_dT and PrymS02_25_dT specific for Prymnesiophyta, all had S/N ratio values ≥2 across the entire sample set analysed under microarray hybridisations (Fig. VI-5c and d). The third higher group probe PrymS03_25_dT specific for Prymnesiophyceae produced positive microarray signals between samples BHC to BHH. Clade level probes (Clade01old_25_dT and Clade 01new25_dt) for *Prymnesium sp*. were both recorded in samples BHC, BHD and BHH, respectively, the Clade 01new25_dt was also highlighted in sample BHE. Species specific probes for *P. parvum* (PparvD01_25_dT and Prymparv01_25_dT) and *P. polylepis* (CpolyS01_25_dT) were both recording positive microarray signals in samples BHC and BHH with PparvD01_25_dT also highlighted in sample BHD, all of which contained *Haptophyta* cells (Fig. VI-5c and d).

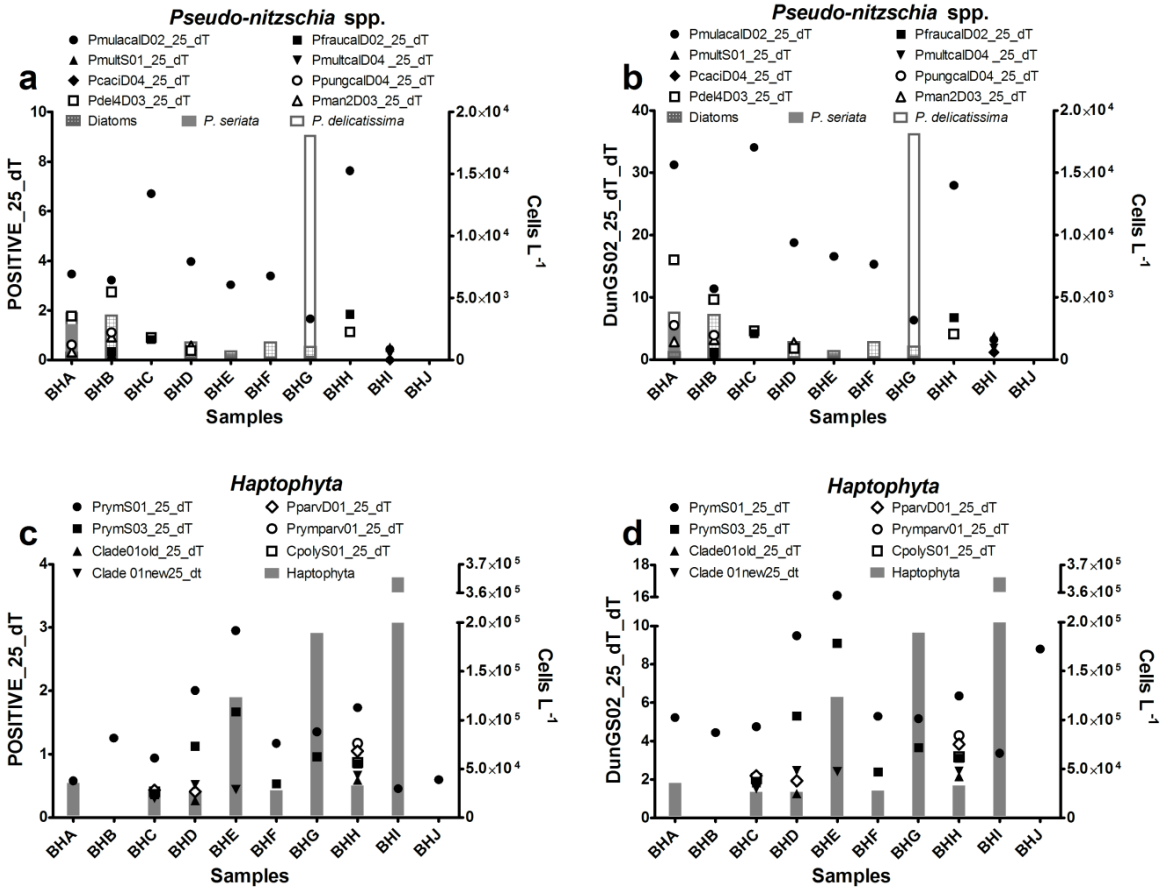

Fig. VI-5. *Pseudo-nitzschia* group and *Haptophyta* microarray results with LM cell counts from Bell Harbour 2011 sampling survey. Signal intensity results from the 3rd generation microarray were normalised against POSITIVE_25_dT (a and c) and DunGS02_25_dT_dT (b and d) probes and plotted with *Pseudo-nitzchia delicatissima* and *P. seriata* groups (a and b) and total Haptophyta species LM counts (c and d).

Other microarray results

Dinophysis family (DphyFS_25_dT) and genus (DphyGS03_25_dT) probes were recorded producing positive microarray S/N ratio values above the threshold level ≥2 in samples BHI and BHJ, however, they were not detected in LM Counts. Similarly, the harmful algae *Chloromorum toxicum* (= cf. *Chattonella* sp.), *Heterosigma akashiwo*, *Karlodinium veneficum* and *Pseudochattonella* species were neither recorded in LM counting but were also not detected by the microarray.

DISCUSSION

During the summer of 2011, maximum cell densities of *P. micans* coincided with the highest temperature and chlorophyll *a* results. This pattern is consistent with previous studies carried out in this survey area (Pybus 1990). Fluorometer total chlorophyll and total pigments were calculated by implementing the slope y = 0.0887x of the corrected fluorescence with total pigment extracts from the spectrophotometer readings across the Bell Harbour sample set. High biomass of *P. micans* blooms are influenced by high levels of dissolved nutrients concentrations (Eker-Devel et al. 2006), although further analysis would be required to determine if this was the case during the 2011 survey. In the study carried out by Pybus (1990) during a 1980/81 survey of the Poulnaclogh Bay area in Bell Harbour, *P. micans* blooms were associated with high levels of dissolve oxygen. The geographic layout of Bell Harbour protects the area from the full impact of both tidal dilution and prevailing westerly winds, preventing the dispersal of *P. micans* blooms and possibly contributing to high cell densities of *P. micans*. They are still capable of developing as is witnessed by observations of 2009 and 2011 sampling surveys (McCoy et al. 2013).

In a study by Chen et al. (2013) which developed rRNA-targeted oligonucleotide probes for fluorescence in situ hybridisation (FISH) to aid detection of *P. micans*, they eluded that due to the hard thecae of *Prorocentrum* with sticky polysaccharides that RNA extraction of such a genus is not promising. This observation was also noted in early microarray generation RNA extraction protocols which were not sufficiently optimized to deal with a whole range of phytoplankton organisms with regards to hard thecae phytoplankton and although cells were observed in LM counts they were not detected by the microarray (Kegel et al. 2013a; Barra et al 2013). However, with RNA extraction methods being subsequently optimized with longer bead beating steps, this allowed for sufficient breaking up of armoured dinoflagellates and diatoms, particularly *P. micans* and *Pseudo-nitzschia* cells, which released their corresponding DNA and RNA for labelling and successful hybridisation and detection by the MIDTAL microarray (Barra et al. 2013; McCoy et al. 2013). Total RNA extract was plotted with total Chlorophyll *a* calculated from processed field samples. This was shown to be significantly correlating, which implies that the total RNA content extracted from the field samples over the sampling period was predominantly made up of chlorophyll *a* producing phytoplankton.

This corresponds to the LM counts and microarray results and indicates that chlorophyll *a* and RNA in field extracts was composed predominantly by *P. micans*.

Initially the main focus of weekly sampling was to investigate the capabilities of the microarray to observe and assess the phytoplankton dynamics and diversity over time, and look more closely at the phytoplankton community. This can be greatly enhanced with the use of microarrays for the processing of bulk samples. Although this study concentrated on the progression of the *P. micans* bloom, which occupied a high percentage of the phytoplankton assemblage particularly between the weeks of 4th July to 27th September, there was also a high biodiversity of phytoplankton taxa observed in LM counts and recorded by the microarray prior to this period. This included the presence of the harmful algal genera *Alexandrium*, *Azadinium*, *Gymnodinales*, *Pseudo-nitzchia* grps and *Haptophyta*.

The species specific microarray probe for *P. micans* was detected in a total of 7 out of 8 samples which contained *P. micans* cells observed in LM counts. The lowest number of cells to be detected being 3800 cells/L^{-1} which corresponds to a detection limit of 1300 cells per filter. However at present the detection limit is only representative for this particular study and as of yet the species specific probe PmicaD02_25_dT for *P. micans* has not been calibrated for the latest 3rd generation MIDTAL microarray. Further experiments would be required to calibrate the microarray signals to cell numbers with pure *P. micans* cultures. The significant correlation observed between the *Dinophyta* probe DinoE12_25_dT and the species specific *P. micans* probe PmicaD02_25_dT may indicate that the DinoE12_25_dT probe is more species specific towards *P. micans* then the other *Dinophyta* probe DinoB_25_dT spotted on the 3rd generation microarray. This could be used as an additional indicator to validate positive *P. micans* probe microarray results for future environmental sample analysis along with LM counts.

Alexandrium was detected to genus level in five samples with only one sample indicating the species level. The detection of the species level probe for toxic *A. tamarense* ribotype (NA group I) was however, not a true microarray result. This is due to both the *A. tamarense* complex probe (AtamaS01_25_dT) and the second *A. tamarense* NA probe (ATNA_D01_25_dT) also spotted on the microarray not recording positive microarray S/N ratio values ≥2 and thereby indicated that this was a false positive microarray signal

for the *A. tamarense* group I ribotype (ATNA_D02_25_dT) probe in sample BHI (Taylor et al., 2013; McCoy et al. 2014a).

McCoy et al. (2014a) have shown that the detection limits of *Alexandrium* to genus level by the microarray required at least 1400 cells per filter to produce a positive microarray signal when analysing field samples taken from the North Channel of Cork Harbour, Ireland. In Bell Harbour survey the microarray detected the presence of *Alexandrium* genus at a cell concentration of 1300 cell/L^{-1}, which infers a detection limit of only 260 cells per filter. These detection limits are a lot lower than those observed in Cork Harbour and further analysis would be required to determine why this is the case, however the microarray must detect to species level to really draw comparisons between detection limits observed from different studies. The only *Alexandrium* species specific probes spotted on the MIDTAL microarray are those for *A. minutum*, *A. ostenfeldii*, and a non-toxic (NA group III), toxic (NA group I) and complex (NA, WE, TA) ribotypes for *A. tamarense*. This may indicate that a separate *Alexandrium* species is present in Bell Harbour and could be a reason for not detecting to species level from the Bell Harbour samples. A study by Touzet et al. (2009) showed the presence of *A. andersoni*, *A. tamutum* and *A. peruvianum* on the West coast of Ireland, which could also be present in Bell Harbour and this may be the *Alexandrium* species that are highlighting the genus level probe.

Toxin analysis from the multi SPR biosensor results did not detect the presences of PSP saxitoxins in samples BHA, BHB, BHC, BHD and BHG, all of which contained *Alexandrium* sp. in LM counts which may support the above possibility of non-toxic *A. andersoni*, *A. tamutum* and *A. peruvianum* strains being present (Anderson et al. 2012b). Further analysis would need to be undertaken to determine if this was the case. However, the ELISA method did detect PSP saxitoxins with in sample BHD to BHJ with the multi SPR also detecting its presence in samples BHE and BHF (Table VI-S1). The ELISA method is more sensitive than the multi SPR method which is supported from earlier studies carried out by McNamee et al. (2013) and McCoy et al. (2014a).

Two *Azadinium* species probes were detected in two different samples and LM counts recorded the presence of *Heterocapsa* species in one of these samples which are morphologically similar to *Azadinium spinosum*, thereby making it somewhat difficult to

identify them by LM counts alone to species level (Hernández-Becerril et al. 2012). *Azadinium spinosum* strains have been identified in Irish waters, and are associated with the closure of shellfish farms due to their production of Azaspiracids (AZP) toxins contaminating shellfish stocks (Salas et al. 2011).

The microarray signal intensity's indicated the presence of *G. catenatum*, however the implementation of the hierarchy file dictated that these were determined to be false positive results due to additional *G. catenatum* (LSGcat0270A24_dT) probe not being highlighted in parallel. The advantage of incorporating a customised hierarchical file allows the elimination of false positives (Metfies et al. 2008; Dittami and Edvardsen 2013), the complete taxonomic hierarchy leading to that species must be highlighted and above the set threshold level (Kegel et al. 2013b). Additionally, *Karenia* species detected in several other samples were also deemed false positives due to higher *Karenia* group probes (KmGcS06_25_dT, KargeD01_25_dT, KareGD01_25_dT) also not being recorded or of a greater signal intensity then the *Karenia* species specific probes for *K. mikimotoi* and *K. brevis*. Although *Gymnodinales* were detected in LM counts, they were not the species indicated above.

Pseudo-nitzschia spp. were detected both on the microarray and LM counts, with the presence of domoic acid toxin detected by ether the multi SPR biosensor or by ELISA for all Bell Harbour samples analysed (Table VI-S1; McNamee et al. 2013). A number of species level probes were highlighted for both *P. seriata* and *P. delicatissima* groups with the higher group *Pseudo-nitzschia* spp. probe (PmulacalD02_25_dT) being recorded alongside. However, for the PmulacalD02_25_dT probe to be regarded as a true positive microarray signal by the hierarchy file, the higher group probes PsnGS02_25_dT and PSN+FRAGS02-25new_dT are required to be producing a S/N ratio above the set threshold limit ≥ 2 as well (Barra et al. 2013; see McCoy et al. 2014b Chapter II.B). Therefore, this PmulacalD02_25_dT probe and any probe below this in terms of hierarchy including species specific probes are considered false positives. This may be due to the low cell numbers present during the survey and the limits of detection were too low to confirm the higher group probes, this may need to be factored into future hierarchical files. Also, these hybridisations were carried out on a 3rd generation microarray prior to the addition of the KREAblock blocking solution step to the latest 3.3 version microarray hybridisations to reduce background noise (Kegel et al. 2013b; see

McCoy et al. 2014b Chapter II.B). Comparisons of before and after the addition of the blocking solution to the hybridisation protocol can be seen in a McCoy et al. (2014b) were similar issues were observed regarding true positive results for the PmulacalD02_25_dT probe. However it must be noted that labelled RNA DoL% were all within the optimal range according to Kreatech Biotechnology nucleic acid labelling kit guidelines, except for samples BHA and BHB which were only just under the lower optimal Dol% range, but still preformed successful hybridisations. Proficient RNA extraction efficiency is one of the most important steps for high quality RNA labelling and optimal DoL% which subsequently leads to successful microarray hybridisations (Metfies and Medlin 2005; 2008).

Dinophysis family and genus probes were highlighted in samples BHI and BHJ by the microarray but were not observed in LM counts. *Dinophysis* spp. may have been overlooked in LM counts due to the dominant presence of *P. micans* during this period of sampling, it has also been observed in previous studies that the presence of *Dinophysis* and *Prorocentrum* commonly co-occur together in phytoplankton assemblages (Reguera et al. 1993). The advantage of the microarray provides the ability to detect to species level, when LM operators may only be able to identify phytoplankton to genus level or may miss their present altogether due to the small volumes used for counting compared to larger volumes filtered for microarray analysis. This is similarly observed for the identification of *Prymnesium* species with the microarray recording the presence of *P. parvum* and *P. polylepis* from the 2011 Bell Harbour samples.

The MIDTAL microarray provides a means of identifying and quantifying a whole range of phytoplankton groups and with the combination of multi SPR biosensors can also simultaneously analysis for multiple marine biotoxins in a single analysis (Campbell et al. 2011; McCoy et al. 2014; McNamee et al. 2013). Their use may also assist in the development of early warning systems in coastal waters and have the potential to be routinely employed by monitoring agencies in compliance with European Union directives (Directive 91/492 and Commission Decision 2002/225).

CONCLUSION

Bell Harbour of Galway Bay has been associated with the bloom forming dinoflagellate *P. micans* for over three decades. Cell densities of *P. micans* blooms appear to be increasing, with exceptionally high numbers recorded in 2011. Total RNA and chlorophyll extraction correlated significantly, indicating the high quality of both extracts. The MIDTAL microarray identified a diverse range of phytoplankton present in Bell Harbour during the sampling period which corresponded with both LM counts and toxin analysis. These finding further enhance the development of the MIDTAL microarray with the main aim of aiding national monitoring agencies with a reliable and fast means of identifying and quantifying the harmful phytoplankton community in natural water samples.

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SUPPORTING INFORMATION

Table VI-S1. Paralytic shellfish poisoning (PSP) saxitoxin (STX), Okadaic Acid + *Dinophysis* toxins (DTXS) and Domoic acid (ASP) biotoxin results of filtered seawater samples from Bell Harbour during the period of the 03rd May to 27th September. Samples were measured by both the multiplex SPR biosensor and commercial CER ELISA and results indicated by the presence or absence of detectable levels of toxin from each method adapted from McNamee et al. (2013).

Station	MA Station code	Sample Date	PSP Toxins (STX)		Okadaic Acid + DTXS		Domoic Acid	
			Multi SPR	ELISA	Multi SPR	ELISA	Multi SPR	ELISA
Bell Harbour 9		3-May-11	-	-	-	-	+	+
Bell Harbour 10		9-May-11	-	-	-	-	-	+
Bell Harbour 11	BHA	15-May-11	-	-	-	-	+	+
Bell Harbour 12		23-May-11	-	-	-	-	+	-
Bell Harbour 13	BHB	29-May-11	-	-	+	-	+	-
Bell Harbour 14	BHC	5-Jun-11	-	-	-	-	-	+
Bell Harbour 15	BHD	13-Jun-11	-	+	-	-	+	-
Bell Harbour 16	BHE	20-Jun-11	+	+	+	-	+	+
Bell Harbour 17	BHF	25-Jun-11	+	+	+	+	+	-
Bell Harbour 18	BHG	4-Jul-11	-	+	+	-	+	-
Bell Harbour 19	BHH	11-Jul-11	-	+	-	-	-	+
Bell Harbour 20	BHI	18-Jul-11	-	+	+	+	+	-
Bell Harbour 21	BHJ	26-Jul-11	-	+	+	-	+	-
Bell Harbour 22		4-Aug-11	+	+	+	+	-	+
Bell Harbour 23		9-Aug-11	-	+	-	-	+	-
Bell Harbour 24		18-Aug-11	+	+	-	-	+	+
Bell Harbour 25		23-Aug-11	-	+	-	-	+	-
Bell Harbour 26		27-Sep-11	-	-	+	+	-	+

Note: Samples highlighted in bold with their corresponding microarray (MA) sample code were the 10 Bell Harbour samples chosen to be analysed by the 3rd generation microarray. Multi SPR: multiples surface plasmon resonance biosensor; ELISA: enzyme-linked immunosorbent assay.

CHAPTER VII

DISCUSSION & CONCLUDING REMARKS

DISCUSSION & CONCLUDING REMARKS

Overview

Europe and across the globe have experienced the harmful effects of algal blooms that have seriously threatened public health, and caused enormous economic losses to fisheries and tourism (Anderson et al. 2012a). Harmful Algal Bloom (HAB) species, with the exception of those generating high biomass, are relatively minor components of the plankton. The natural occurrence of HAB species is often episodic and highly unpredictable. Toxicity can be expressed at relatively low cell abundances, often with a catastrophic impact on the ecosystem as a whole. At such low abundances, identification, isolation and estimation of the species present and the toxins they produce can be very difficult and time consuming and morphology as determined by light microscopy may be insufficient to give definitive species and toxin attribution. As detection is an essential component of any early warning or prediction system, efforts must be directed to the development of technologies to detect cells, blooms, or toxins on scales necessary for early warning strategies to be implemented. Molecular and biochemical methods are now available that offer rapid means of both species and toxin detection and there are strict EU regulatory directives which govern the uses of these biological and chemical methods in accordance with Commission Regulations (EC) No. 2010/477, No. 1664/2006, No. 853/2004 and No. 2074/2005 (Gilmartin and Silke 2009). Molecular probes can detect individual species or even strains of species and their toxins which have been combined with a variety of formats to facilitate the acquisition of data in real-time for monitoring purposes. These include automated sandwich hybridisation assays (SHA) devices (Anderson et al. 2005), flow cytometry (Scorzetti et al. 2009), environmental sample processor (ESP; Doucette et al. 2009), real-time PCR assays (Flannery et al. 2012; Lee 2010), SPATT devises deployed upstream from shellfish farms to monitor algal toxins (Turrell et al. 2007; Fux et al. 2009), immunoassay based methods such as LC-MS (Anderson 2009).

Microarrays offer the most expeditious avenue to obtain high sample throughput with highly accurate species detection afforded by the probes. Existing rRNA probes and antibodies for toxic algal species/strains and their toxins can be adapted and optimized for microarray use to strengthened the EU`s ability to monitor for toxic algae. The testing of microarrays for the detection of the dinoflagellate *Alexandrium* and its toxic species

has already been tested and known as the ALEX CHIP (Gescher et al. 2008). Additionally, the multiplex analysis of Cryptophyte probe sequences on a DNA microarray has also proved applicable and is known as the PHYLOCHIP (Metfies and Medlin 2007). At the heart of the DNA microarray technology is a DNA microchip that contains an array of oligonucleotides, PCR-products, or cDNAs spotted onto a small surface. Target nucleic acids are labelled with a fluorescent dye prior to their hybridisation to the DNA chip. The fluorescence pattern on the DNA-chip after the hybridisation of the target DNA to the probes spotted onto the microchip is then analysed with a fluorescent laser-scanner (Metfies and Medlin 2005; 2008).

In the MIDTAL project we have adapted an RNA approach for species identification, using oligonucleotide probes that specifically target the 18S-28S rDNA domains using a hierarchical probe design strategy such that higher taxonomic levels for genera, family, class are also included on the microarray as a positive control for species level signals. However, the adaptation of routinely used FISH probes to the microarray format must be retested as some can be un-usable due to problems with the secondary and tertiary structure of the ribosomal RNA molecule. Therefore existing probes may need modifying and new probes must be re-designed from either 18S rRNA gene or D1/D2 of the 28S rRNA gene region. At the beginning of this thesis there were four main points we wanted to address before national monitoring agenises could be confident in routinely adopting the MIDTAL microarray.

- Can the MIDTAL microarray reliably detect HAB species using pure cultures and environmental samples?
- Can we use microarrays to further investigate phytoplankton biodiversity and the quantitative changes in the biodiversity with time?
- In terms of quantification, how representative are the limits of detection inferred by the microarray with current national harmful phytoplankton monitoring programmes?

This brings us to the first pressing hypothesis, “Can the MIDTAL microarray reliably detect HAB species using pure cultures and environmental samples?” To answer this the specificity of the existing FISH probes was assessed by Fluorescent *in situ* Hybridisation (FISH) and the interactions between target and non-target probes spotted on subsequent generations of the MIDTAL microarray was determined through microarray hybridisations with labelled RNA from pure *Prymnesium* spp. cultures.

Applicability of microarrays

Testing of probe specificity

FISH analysis

The re-evaluation and testing of existing oligonucleotide *Prymnesium* probes assigned to NUIG, was assessed by whole-cell FISH for higher group *Prymnesiophyta* probes (PRYM01, PRYM02, PRYM03), genus-level (PRYMGL01A, PRYMGL01B), clade-level *Prymnesium* probes (Clade01) and species specifics probes for *P. parvum* (PRYM694) and *P. polylepis* (Cpoly01) using a number of the *Prymnesium parvum* and *P. polylepis* pure culture strains. Observations were analysed under epi-fluorescent inverted microscope and the tested hierarchical probe set accurately identified *Prymnesium* spp. from cultured and spiked field samples from Irish waters without any apparent cross reactivity or inhibitory affects from other algal species (McCoy et al. 2014c). This was consistent with previous studies, who also verified their specificity through dot-blot hybridisation using PCR amplified with either 18S or 28S rDNA fragments of different target and non-target microalgae (Lange et al. 1996; Simon et al. 1997; 2000; Töbe et al. 2006; Eller et al. 2007).

Microarray analysis

To fully assess the interaction, specificity and potential cross reactivity between the hierarchical *Prymnesium* spp. probe set from the numerous other phytoplankton taxa probes spotted on the MIDTAL microarray, a number of microarray hybridisations with labelled RNA from pure *Prymnesium* algal cultures were carried out. The 1st generation chip designed for a specified range of HAB species produced weak signals for several species-probe combinations resulting in low specificity with a high number of cross reactivity with non-specific target sequences for hybridisations performed at 58 °C with pure culture labelled *P. parvum* and *P. polylepis* RNA (McCoy et al. 2014d). These issues were also encountered in previous microarray formats which determined that oligonucleotides routinely used for FISH hybridisations were too short, produced weak signal intensities and probes designed in the second half of the 18S molecule had inaccessible secondary structures to achieve specific hybridisation reactions (Chou et al. 2004; Metfies and Medlin 2008). Medlin 2013 showed that improvements in binding success of labelled RNA can be achieved by simply increasing the probe sequence

amplicon length in 18S rRNA gene, which thereby increase signal intensity's of target probes.

Subsequent generations of the MIDTAL microarray

A 2nd generation microarray was subsequently designed in which probe sequences were extended in length from 18 to 25 nucleotides. Due to these changes and to ensure probe specificity a number of protocol alterations had to be implemented and optimised. This included fragmentation and denaturing steps to improve diffusion rates of labelled RNA, increased hybridisation temperature due to higher melting point temperatures of the extended probes and optimised microarray washing steps to minimise background noise. All of these changes increased the signal intensities of weak *Prymnesium* spp. probes and substantially reduced the number of non-specific binding/false positives observed with the previous generation microarrays and un-optimised protocols on the 2nd generation microarray when hybridised with labelled *P. parvum* and *P. polylepis* RNA.

The length of the poly T spacer linking the probe to the glass slide was increased to a tested optimal length of 25 ploy Ts to address issues relating to the interactions of labelled RNA successfully interacting with its target probe in the 3rd generation microarray (Medlin 2013). Comparing hybridisations with pooled labelled *P. parvum* and *P. polylepis* RNA hybridised to the 2nd and 3rd generation microarray indicated the improvements made with regard to molecule and probe interaction, with increased signal intensities observed with corresponding target probes on the 3rd generation microarray.

Due to the hierarchal approach of the MIDTAL microarray, false positive signals could be eliminated by imposing a customised hierarchical file which dictates that for any species level probe recorded by the microarray, all the higher taxonomic probes for that species must first be highlighting and be above a set threshold limit. This provided the best means for determining if the microarray signals were true positive results. This is especially important when analysing environmental samples which contain an unknown composition of species. This can also be validated by the use of Light Microscopy (LM) techniques to determine the absence or presence of these species or identified to at least genus level from Lugol's preserved field samples. This not only satisfies the first part of the first hypothesis but also indicates the potential of the MIDTAL microarray for analysing HABs from environmental samples, which had to be put to the test through

field trials, this leads us to our second half of the first hypothesis; "Can the MIDTAL microarray reliably detect HAB species using environmental samples?"

Microarray data and HAB events

Microarray field trials

The first field trials were carried out using the 2nd generation microarray from a wide range of ecological niches spanning the south and western coastline of Ireland. Such a wide variety of sites was selected to assess the capabilities of the microarray for detecting a diverse range of the phytoplankton communities. From this study the MIDTAL microarray successfully detect the presence of *Pseudo-nitzschia*, *Alexandrium*, *Dinophysis*, *Karenia*, *Prorocentrum* and *Prymnesium* groups from a total of 13 samples collected between the period of July 2009 to April 2010 which were in general agreement with LM counts (McCoy et al. 2013). However, there were a number of issues which arose from this first field trial. For example, *Pseudo-nitzschia 'seriata'* groups were observed in LM counts but failed to record signal-to-noise ratio values above the set threshold level ≥2 for corresponding *P. seriata* group probes on the microarray. This was also the case for a number of samples which contained *Prorocentrum micans* and *Dinophysis acuta* in LM counts but produced signal intensities too low to be considered positive microarray results and were deemed false negatives. This may have resulted from the cell densities being below the detectable capabilities of the microarray or that the RNA extraction protocol was not stringent enough in relation to breaking up of the cells which thereby prevented the release of corresponding DNA and RNA for labelling, successful hybridisation and detection by the MIDTAL microarray (Barra et al. 2013; McCoy et al. 2013). There was also the case of low labelling efficiencies of field samples which can be a direct reflection of the quality of the extracted RNA, thereby reducing the sensitivity and specificity of target probes. A number of false positives results were also recorded which can be linked to microarray chip washing steps not being stringent enough, which has been shown previously to be responsible for an increased incidents of un-specific binding and cross reactivity. A lot the methodological issues mentioned above where addressed and optimised for the subsequent 3rd generation microarray.

The growth rates of phytoplankton can vary dramatically. Small plankton species such as the Haptophytes which are grouped in nannoplankton size range (2-20 µm) are capable

of doubling their numbers in a single day, whereas in general the larger microplankton (20-200 µm) are relatively slow growing organisms in comparison, with certain diatoms and dinoflagellate genera which may only double every week or two. Therefore, to fully understand the community dynamics of a diverse range of phytoplankton and not to miss out on key events, regular sampling must be undertaken and ideally in the same location. To determine if the MIDTAL microarray can tell us anything about the phytoplankton community structure over time it was decided to concentrate sampling efforts to every week in one particular location during 2011 sampling surveys. Two locations were chosen to achieve this, the North Channel of Cork Harbour and Bell Harbour of Galway Bay. This now brings us to our second hypothesis; "Can we use microarrays to further investigate phytoplankton biodiversity and the quantitative changes in the biodiversity with time?"

Microarrays and phytoplankton biodiversity

During the 2011 sampling surveys there was an extensive bloom of *Alexandrium minutum* in the North Channel of Cork Harbour and *Prorocentrum micans* in Bell Harbour of Galway Bay. The progression of both of these blooms was monitored from initiation to termination under LM and with the 3rd generation microarray (McCoy et al. 2014b; 2014e). The dominant *Alexandrium* species found was the toxin producing *A. minutum* strain (Cosgrove et al., 2014). Both *A. minutum* and *P. micans* species probes were successfully detected by the MIDTAL microarray with the hierarchical file determining them as true positive microarray results due to the corresponding taxonomic higher group probes signal-to-noise ratio values being above the set threshold limit and of greater signal intensity. Additionally, the detection of PSP toxins by the multi SPR biosensor, ELISA and HPLC methods during the *A. minutum* bloom further validated the presence of this species and demonstrated the usefulness of the combination of both MIDTAL microarray and multiplex SPR biosensor for the detection and monitoring of toxic algae and their toxins. Although both the McCoy et al. (2014b) and McCoy et al. (2014e) studies concentrated on the progression of one particular species in bloom, which occupied a high percentage of the phytoplankton assemblage, there was also a high biodiversity of phytoplankton taxa observed in LM counts and recorded by the microarray. This was particular apparent from the Bell Harbour survey with the microarray detecting the presence of *Dinophysis* spp. and *Prymnesium* spp. co-occurring

along with the *P. micans* bloom and also pointed to the demise of *Alexandrium* densities which could have been effected by the rapid increase of *P. micans* cells (McCoy et al. 2014e). The microarray can not only detect what species are co-occurring together in one analysis but can also track the progression of HAB events, giving us great insights into the phytoplankton ecology with the potential to be used as an early warning tool. The MIDTAL microarray also has the great potential to be used as a quantitative tool with both *A. minutum* and *P. micans* probes normalised signal intensity's significantly correlating with cell counts. The most challenging aspect of applying molecular methods is to make them quantitative, which brings us to our final key hypothesis; "In terms of quantification, how representative are the limits of detection inferred by the microarray with current national harmful phytoplankton monitoring programmes?"

Microarrays and quantification

Microarray calibration curves

Microarray calibrations curves were performed on the 2nd and 3rd generation microarrays. The calibration curves showed the relationship between increasing labelled RNA amounts with increasing microarray signal intensities for each species specific probe which correspond to relative cell numbers. In addition, normalised total signal intensities are recommended for comparisons between microarrays and in order to relate signals for specific probes to cell concentrations using external calibration curves (Dittami and Edvardsen 2013). These external calibration curves were achieved with labelled RNA extracts from known cell numbers of the control species *Dunaliella tertiolecta* with normalised signal intensities and were also used for RNA extraction proficiency between partners (McCoy et al. 2012; 2013) By spiking field samples with known concentration of *D. tertiolecta* cells, RNA extraction efficiencies could be determined for field extracts and also be used to infer cell numbers when normalising signal intensity's with specific *Dunaliella* sp. probes spotted on the microarray through analyses with the GPR Analyzer programme developed by Dittami and Edvardsen (2013). Cell numbers can be inferred for all normalised probe signal intensity's from the available calibration data which corresponds to the hybridisation-specific detection limit of numerous species incorporated into the GPR Analyser programme.

Environmental stresses

For the MIDTAL project, a calibration between RNA content and cell number with microarray signal intensity's was one of the main focuses of the project to produce a predictive tool in which numbers of toxic algal cells could be inferred from the microarray signal. In order to produce such a predictive tool, it was necessary to test how RNA content varied under different stress conditions and to see if RNA content per cell varied between treatments and over time. Experiments were carried out using the eukaryote marine phytoplankton *Prymnesium parvum. P.* (= *Chrysochomulina*) *polylepis*, cf. *Chattonella* sp. and *Karlodinium veneficum* cells grown under different stresses of light, temperature, salinity and inorganic nutrient conditions over a 72 hour period with measurements taken every 24 hours (McCoy et al. 2014a). This was done to try and mimic the conditions in the field of which marine organisms encounter many stresses when interacting with their environment. The study showed that total rRNA does not always positively correlate with growth rate, with no significant change in rRNA content over time when exposed to the majority of environmental stresses and that total rRNA content significantly correlated with increasing cell numbers (McCoy et al. 2014a). Similarly, this was also determined to be the case for toxic species *Heterosigma akashiwo* and *Alexandrium* species (Blanco et al. 2013; Taylor et al. 2013; 2014). However, phytoplankton are phylogenetically diverse which means they can have dramatically different physiological capability's and non-significant variation in RNA content under environmental stresses for one particular species and its various strains may not always be the case for other phytoplankton species tested on the microarray. For instance, the testing of *Pseudochattonella* spp. under environmental stresses found several cases of significant variability of RNA content among strains and over time (Dittami and Edvardsen 2012). This then implies that microarrays can only be considered semi-quantitative with respect to cell number for certain species represented on the MIDTAL microarray. Dittami and Edvardsen (2012) also demonstrated that *Pseudochattonella* RNA content and cell number performed equally well as proxy for total biovolume (biomass) which was also similarly shown to be the case in McCoy et al. (2014e) were total RNA extract significantly correlated with total chlorophyll and total pigment per sample which also provides and additional indicator of extraction quality.

It is important to note that the RNA content was related to cell numbers for all the microalgae species strains tested independent of the environmental stress conditions.

Additionally it has been shown that increasing total RNA correlates with increasing signal intensities on a microarray detection platform (Metfies and Medlin 2005; 2008). This allows the conversion of signal intensities to infer cell numbers from calibrations undertaken for each oligonucleotide 18S and 28S gene probe spotted on the microarray therefore, permitting the use of the microarray as not only a detection platform but also as a quantitative tool.

Detection limits

Calibration curves for *Prymnesium* spp. with increasing labelled RNA amounts of 1 ng, 5 ng, 25 ng and 100 ng were performed on both the 2nd and 3rd generation microarrays. These experiments revealed that the detection limit to reliably highlight corresponding species level probes for both *P. parvum* and *P. polylepis* required an RNA amount of 5 ng or greater which equates to 8,800 and 3,800 cells respectively (McCoy et al. 2013; 2014a). However, not all species specific probes spotted on the microarray behave in the same way and all have their own individual detection limits. For instance cf. *Chattonella* and *K. veneficum* species have detection limits as low as 1 ng, which is equivalent to <200 *cf. Chattonella* cells and ~250 *K. veneficum* cells (McCoy et al. 2014a). However, it soon became apartment that detection limits of species specific probes observed from hybridisations with pure culture RNA was a lot lower than those determined from field samples, which could be a result of the potential interferences from non-target RNA found in environmental samples.

National harmful phytoplankton monitoring programmes use the accredited Utermohl methods with LM for the identification and enumeration of harmful phytoplankton species. The method has a detection limit of just 1 cell which is equivalent to 40 cells/L when using a 25 ml Utermöhl counting chamber which is the routine method used in Ireland. In some instances the presence of toxic genus is enough for further action in terms of downward processing of shellfish flesh to determine the presence or absence of toxins. However, the LM approach has a disadvantage in that it cannot always reliably detect to species level and differentiate between toxic and non-toxic strains, whereas specificity is the main strength and advantage of the MIDTAL microarray over these traditional methods.

The best means of explaining the detection limits of the MIDTAL microarray is through a number of examples. Taylor and co-workers demonstrated positive microarray signals

in laboratory based calibration curves were as low as 240 and 270 cells for *A. tamarense* and *A. minutum* probes (Taylor et al., 2013; 2014). However, higher detection limits of 3.900 *A. minutum* cells were reported by McCoy et al. (2014b) during the 2011 Cork Harbour field study, which suggests that there may be interference from other organisms present in the phytoplankton assemblage and additional adjustments may be required when dealing with field samples as opposed to microarray hybridisation results from pure cultures. The Bell Harbour field sampling survey also carried out in 2011, indicated the lowest number of *P. micans* cells to produce a positive microarray signal-to-noises ratio ≥2 was 3,800 cells/L, which corresponds to a detection limit of 1,300 cells (McCoy et al. 2014e). Although, at present this detection limit is only representative for this particular study and as of yet the species specific probe for *P. micans* (PmicaD02_25_dT) has not been calibrated for on the latest 3rd generation MIDTAL microarray.

Calibration curves experiments for *Pseudo-nitzschia multistriata* species on the 2nd generation microarray showed that its corresponding probe signal PmulaD03_25 had a detection threshold of 5 ng of RNA which corresponded to 4,600 cells from pure cultures (Barra et al. 2013). However, this did not correlate with field samples, where signal-to-noise ratio values did not reach the set threshold level, regardless of the presence of *P. multistriata* in LM counts with cell densities of up to 44,000 cells/L (Barra et al. 2013). However it must be noted that improvements have been made since the 2nd generation microarray which have been discussed above but additionally the introduction of ammonium acetate (NH_4Ac) steps to clean up RNA, significantly improved RNA labelling efficiency's and also the addition of KREAblock blocking solution to the hybridisation mix, decreases background noise particularly when analysing field sample on the 3rd generation microarray (McCoy et al. 2014d).

McCoy et al. (2014d) showed the comparison of hybridisations with and without the NH_4Ac RNA clean up step and addition of KREAblock on two versions of the 3rd generation microarray using six field samples from Killary Harbour. The introduction of both of these protocol optimisation steps reduced the number of false positives but also improved the quality and sensitivity of hybridisations thereby increasing positive microarray results on the latest 3.3 version microarray which may have been over looked with previous microarray generations. This also lowered corresponding species specific probe detection limits. An example of this was the detection of *P. multistriata* probe (PmulaD03_25_dT) on the 3.3 version microarray with just 1,400 cell/L of *Pseudo-nitzschia seriata* group species observed in Killary Harbour LM counts, which is a big

improvement of corresponding PmulaD03_25 probe detection limits from the 2nd generation microarray mentioned above (Barra et al. 2013; McCoy et al. 2014d). Also from this McCoy et al. (2014d) study, the detection of *Alexandrium* genus probe (AlexGD01_25_dT) was positively recorded with *Alexandrium* cell density of 260 cells/L which infers a detection limit of 170 cells, were previously this probes detection limit from the North Channel of Cork Harbour field samples was indicated to be 1,400 *Alexandrium* cells (McCoy et al. 2014b). This not only points out the continuing improvements made during the evolution of the MIDTAL microarray but highlights that previously published microarray calibration curves for each species specific probe that used older protocols may now have considerably lower detection limits compared to using the latest 3.3 version optimised microarray protocols and also emphasises the importance of determining the true limits of detection through rigorous field trials.

Concluding remarks

The MIDTAL microarray probe sequence has been patented and the microarray is now commercially available in kit form with all the necessary reagents from Microbia Environnement [(France); http://www.microbiaenvironnement.com)]. To date 14 kits have been sold with a beta test price is €750 for 20 slides (Medlin LK pers comm). It is yet to be seen if national phytoplankton and biotoxin monitoring programmes will adapt this technology to their routine monitoring of HABs as there are some high initial cost in setting up laboratory's with the necessary equipment to undertake such analysis and the use of other molecular techniques such as FISH and quantitative real-time PCR are just recently gaining traction in this area. However, the vision of the project is that the MIDTAL microarray becomes a standardised technology, used in a multifaceted approach along with current technologies such as FISH, qRT-PCR, multi SPR biosensors, HPLC etc. There is still some room for further optimisation in terms of sensitivity and detection limits but it would be very unlikely that the microarray could detect down to just one cell without sacrificing specificity. In monitoring it is more important to be specific, so you can determine if toxic or non-toxic species are present or not, only then does sensitivity become important. Therefore, it is likely that the use of microarrays will advantageously used in monitoring phytoplankton biodiversity over long time scales and additional techniques with lower detection limit capabilities will be used in conjunction with the microarray.

Owing to the success of the MIDTAL project a similar project called MicroAqua (µAQUA) Innovative aquatic biosensor is currently funded by the EU FP7 Framework Programme with 12 partners from Italy, UK, France, Ireland, Bulgaria, Germany, Turkey and Spain. The aim of the project is to develop a universal microarray to simultaneously detect and differentiate major waterborne pathogens (bacteria, viruses, protozoa and cyanobacteria) independently of traditional methods which rely on cultivation, microscopy or biochemical characterisation. A multiplexed sensor-based detection of freshwater toxin contamination such as cyanobacterial toxins will also be developed along with this project. The threat of waterborne disease in Europe are predicted to increase in the future and the µAQUA microarray aim is to have a commercial semi-continuous water quality monitoring tool which will be applied in field trials in five of the countries mention above. Like the MIDTAL project there will be certain steps to optimise and become proficient at to achieve these goals, such as standardisation of sample concentration; enhancement and calibration of microarray signal intensity's.

Although many techniques and technologies have been developed to date for monitoring HAB species, they are quite specialised with many individually funded projects specifically focused on one particular goal with no apparent communication between similar projects. Although we attend conferences to disseminate our latest findings, there is also an air of competitiveness between other scientists in our fields, which possibly relate back to fears of where the next funding opportunity will come from and how to stay ahead of ones piers especially when it comes to competitive calls for future funding opportunity's that will come with Horizon 2020.
Successful funding can often result from successful similar projects, such as the collaborative ongoing MicroAqua (µAQUA) project, it would be exciting to see the combination of high throughput technologies with for example predictive modelling projects. Here, an array of biological data revealing the diverse community dynamics interpreted by the microarray could be incorporated into physical models to further develop our understanding of the habits of HAB events and provide more detailed early warning monitoring tools in the future.

Oligonucleotide microarrays have also been used for analysing gene expression analysis and have a high potential for better insights into reasons for microalgal toxin production

(Anderson et al. 2012b). A study by Yang et al. (2010) used this technology to interpret gene expression analysis for the toxic dinoflagellate *Alexandrium minutum*, where they demonstrated the shift of 14 genes in relation to predator prey interactions with copepod grazers which subsequently led to the increased expression of toxic producing genes thereby resulted in increases of toxin content and giving insights into their possibly involved as a defence mechanism. This study also demonstrated that microarray and qPCR results agreed quantitatively with regard to relative abundances of expression ratios of toxic *A. minutum* strains.

There is also great potential in the use of Next Generation Sequencing (NGS) techniques to assess not only the diversity and structure of the microbial community but also possibly be used to assess the relative abundances in terms of cell numbers by counting environmental gene tags or PCR amplicons under various environmental conditions (Ebenezer et al. 2012). However, their use as quantitative tools is not completely proven due to possible bias from PCR amplification and NGS reads (Ebenezer et al. 2012). A study by Egge et al. (2013), investigate the use of 454 pyrosequencing to assess if the relative read abundances of marine Haptophytes could be related to cell numbers, however they determined that there was no correlation between the two and only a weak correlation was observed with proportional biomass. This study did however indicate the potential of improving the estimates of relative abundances in terms of biomass if different approaches were used, such as DNA as a template instead of cDNA (Egge et al. 2013). NGS is however a relatively new technology and transferring its potential capability's to quantifying marine microalgae are very much in its infancy and there is still a lot of future work to be done in this area of research.

Any technology requires reliable quality control, whether microarray, qRT-PCR or NGS. Ultimately, this will be supplied by conventional microscopy. Nevertheless, this project has shown the growing advantages of molecular techniques to enhance our understanding of phytoplankton communities and their dynamics. With the high sample throughput and robustness of the technique, microarrays will be able to minimise the use of laborious techniques currently used in HAB monitoring.

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APPENDICES I

FIELD TESTING FOR TOXIC ALGAE WITH A MICROARRAY: INITIAL RESULTS FROM THE MIDTAL PROJECT

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NOVEL SENSORS

McCoy, G.R.[1], Raine, R.[1], Medlin, L.K.[2], Chen, J.[2], Kooistra, W.[3], Barra, L.[3], Ruggiero, M.V.[3], Graneli, E.[4], Hagström, J.A.[4], Salomon, P.S.[4], Reguera, B.[5], Rodríguez, F.[5], Escalera, L.[5], Edvardsen, B.[6], Dittami, S.M.[6], Eikrem, W.[6], Lewis, J.M.[7], Berzano, M. [7], Elliott, C.T.[8], Campbell, K. [8] & Pazos, Y.[9]

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ABSTRACT

One of the key tasks in the project MIDTAL (MIcroarrays for the Detection of Toxic ALgae) is to demonstrate the applicability of microarrays to monitor harmful algae across a broad range of ecological niches and toxic species responsible for harmful algal events. Water samples are collected from a series of sites used in national phytoplankton and biotoxin monitoring programmes across Europe. The samples are filtered; the rRNA is extracted, labelled with a fluorescent dye and applied to a microarray chip. The signal intensity from >120 probes previously spotted on the chip is measured and analysed. Preliminary results comparing microarray signal intensities with actual field counts are presented.

INTRODUCTION

Blooms of toxic or harmful microalgae referred to as harmful algal blooms (HABs), represent a significant threat to fisheries resources and human health throughout the world. Since many HABs have significant economic impacts, and the danger to human health posed by the consumption of contaminated seafood, monitoring programmes which measure toxins that have accumulated in shellfish flesh has become a necessity. In Europe, this requirement for monitoring is established in a series of directives in which monitoring of coastal waters for potentially harmful phytoplankton is also mandatory. Traditionally phytoplankton identification and enumeration is carried out using light microscopy (LM). This technique requires a high degree of skill on behalf of the operator, and is time-consuming. Furthermore, the morphological similarity between different species within or even across phytoplankton genera has meant that LM alone is at times insufficient to assess the potential toxicity of a water sample. A variety of methods based on the sequencing of nucleic acids have been developed which have considerably improved our ability to accurately identify organisms to the species level. These have been outlined recently in a new manual for phytoplankton analysis (Karlson *et al*. 2010). Microarrays are the state of the art technology in molecular biology for the processing of bulk samples for the detection of target RNA/DNA sequences. In MIDTAL, existing rRNA (18S, 28S) probes and antibodies for toxic algal species and their toxins have been adapted for use in a microarray format. This paper presents the first field trial results.

MATERIALS AND METHODS

Water samples are taken and a measured volume is filtered through nitrocellulose filters (pore size 1-3 µm). The volume of sample filtered depends on the turbidity of the water: 0.5-2 l is usually filtered up to a point when the filter starts to clog. The filter is then immediately submersed in 1 ml of Tri-Reagent (Ambion, UK) and an aliquot of *Dunaliella tertiolecta* (5 x 10^6 cells) is added as an internal control for the RNA extraction process. The material is then stored at -80 °C. RNA extraction is carried out through cell lysis, sequential extraction with 1-Bromo-3-chloro-propane (BCP) and isopropanol, followed by an ethanol wash. After the final centrifugation step, the pellet is suspended in RNase free water and stored at -80 °C. The RNA is then labelled using a Platinum Bright 647 Infrared Nucleic Acid kit, fragmented and hybridised to a pre-activated epoxysilane-coated microarray chip at a temperature of 65 °C. Unlabelled RNA is removed from the chip surface using 3 washing steps, with different stringency involving EDTA, thereby minimising background noise. The chip, pre-spotted with over 120 oligonucleotide probes corresponding to a taxonomic hierarchy (kingdom, class, genus and species) for harmful algal species, is scanned (Genepix 4000B Axon Inc.) and the fluorescence signal intensity from each probe is measured. Results are then compared with microscopic examination of the original water sample. This ongoing process will be carried out over two years. Preliminary results comparing microarray signal intensities with actual field counts are presented.

RESULTS AND DISCUSSION

RNA extraction efficiency

Good yields of high quality RNA were extracted from *D. tertiolecta* cells when a preliminary standard curve was made (Fig. AP.I-1). The relationship between cell numbers and RNA content was linear with a satisfactory coefficient of determination was obtained from four randomly selected project partners.

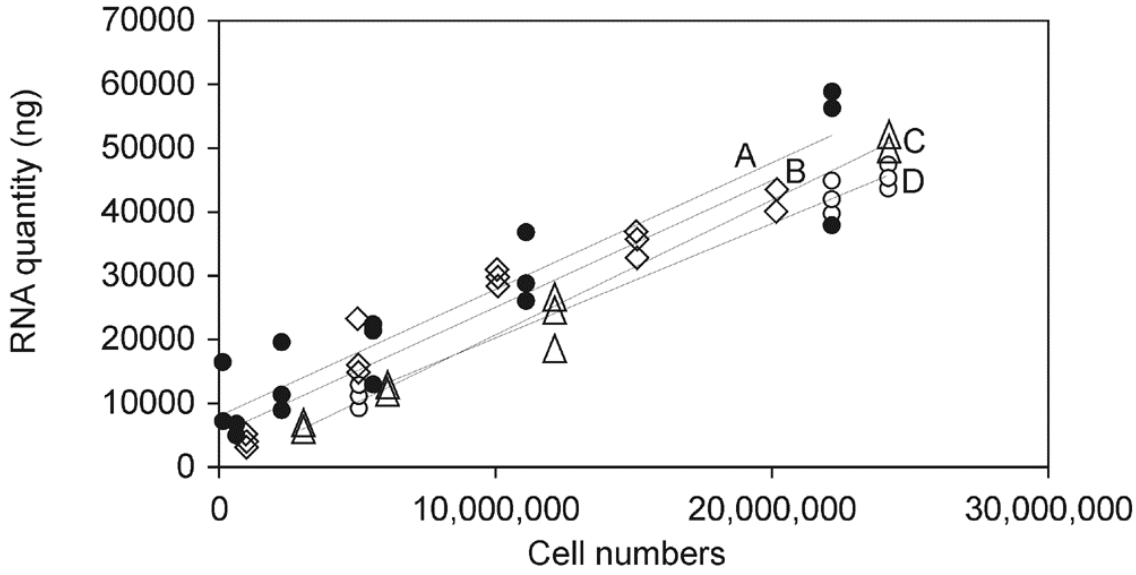

Fig. AP.I-1. Comparison of RNA extraction efficiencies carried out on cultures of *Dunaliella tertiolecta* between four MIDTAL partners A (R^2 = 0.8836), B (R^2 = 0.9243), C (R^2 = 0.9848) and D (R^2 = 0.9912).

Sensitivity of the hybridisations

The sensitivity of hybridisations onto the microarray was investigated by testing a range of probes which should be highlighted by a particular organism growing under different environmental conditions. Fig. AP.I-2 shows results from probes for prymnesiophytes, which were tested on a culture of *Prymnesium parvum.* These probes were adapted for the microarray from those published by Lange *et al*. 1996; Simon *et al*. 1997; 2000; Töbe *et al*. 2006; Eller *et al*. 2007. A NanoDrop Spectrophotometer was used to quantify the RNA after the labelling and RNA clean-up steps to determine the exact labelled RNA amount when approximately 1 ng, 5 ng, 25 ng and 100 ng were hybridised to the chip. A pre-selected signal: noise ratio threshold level was applied so that the limit of quantification was represented by a signal of 2. Thus if the optimum probe for prymnesiophytes (PrymS02_25; Lange *et al*. 1996) is applied, then the microarray can not accurately detect RNA amounts below 5 ng (Fig. AP.I-2a). Example of image intensities is also shown in Fig. AP.I-2b.

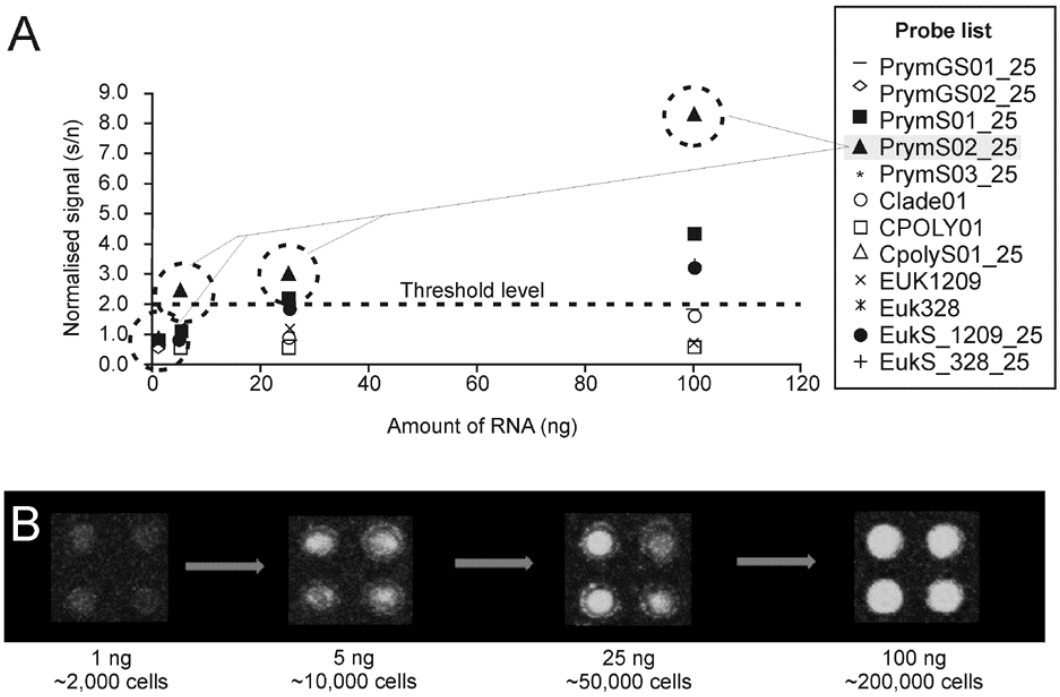

Fig. AP.I-2. Standardisation of the *Prymnesium parvum* signal. (A) Calibration curve of RNA (1ng, 5ng, 25ng and 100ng) against signal intensity for a range of probes. (B) Images of the optimum probe PrymS02_25 when increasing amounts of RNA are hybridised to the microarray. Increasing signal intensity represents increasing cell numbers.

Development of microarray chip

A 1st chip designed for a specified range of HAB species produced weak signals for several species-probe combinations. A 2nd generation chip was subsequently designed in which the probes were increased in length to 25 base pairs. This meant that a higher melting point temperature was required and thus the hybridisation temperature was increased from 58 to 65 °C. This temperature was adopted as standard between all project partners. Hybridisation temperature will be further optimised for the next generation of chip.

Light microscopy and microarray field results

Examples of microarray results are shown in Fig. AP.I-3 and AP.I-4. Fig. AP.I-3 compares data obtained from the 1st and 2nd generation chips. The sample was taken in Bell Harbour on the west coast of Ireland in 2009, when a bloom of *Prorocentrum micans* was occurring at the time. Examination under the light microscope showed that this was the dominant species with a cell density of 360,000 cells l^{-1}.

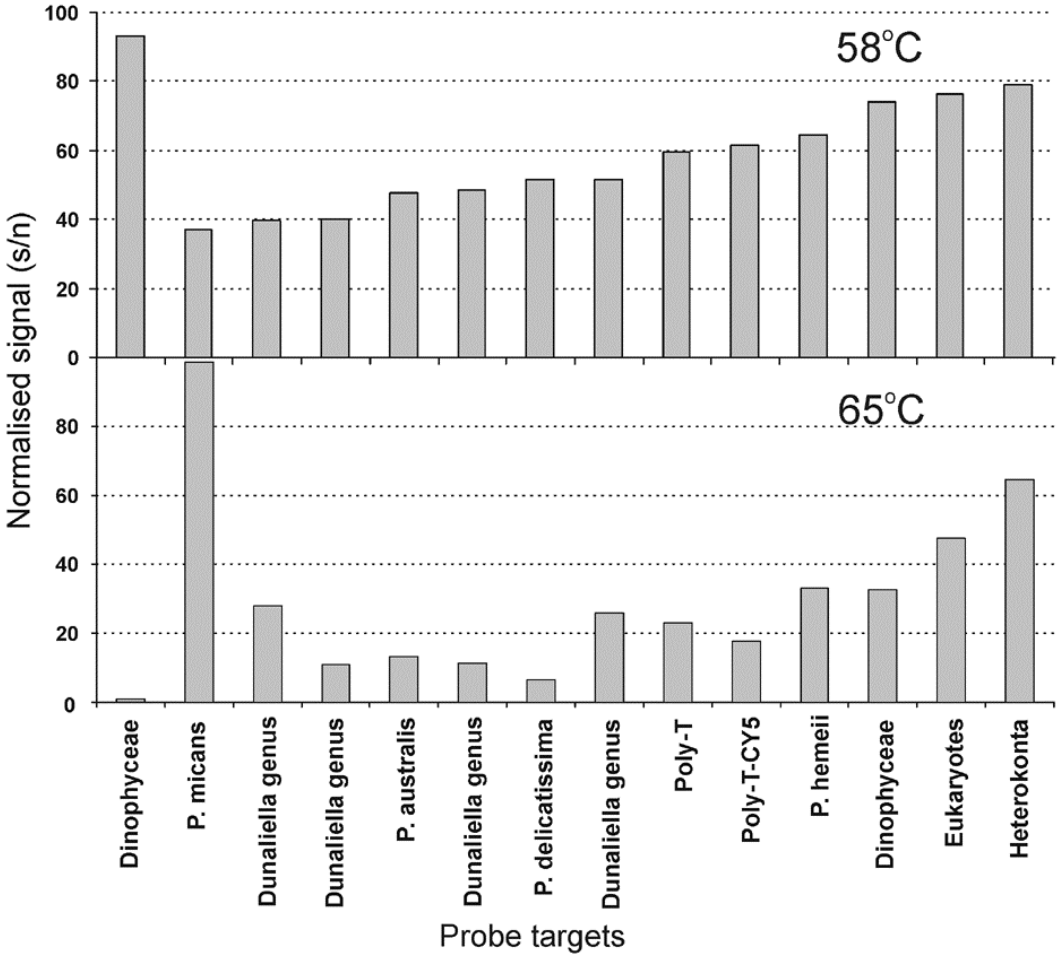

Fig. AP.I-3. Microarray results of 1st and 2nd generation chips both hybridised with the same Bell Harbour field extract (25/08/09) at a temperature of 58 °C and 65 °C respectively. Note the difference between the Pmica02 and PmicaD02_25 probe species specific for *P. micans*.

The *P. micans* probe used on the 2nd generation chip (PmicaD02_25 (98.53 s/n ratio); L.K. Medlin unpublished) gave a vastly stronger signal to its complement (Pmica02 (37.15 s/n ratio)) on the 1st generation microarray, which was seven base pairs shorter. A general agreement between microarray signal results and cell counts was obtained. There is also an elevated signal from the class level probe for Dinoflagellates. The strongest signals in Fig. AP.I-3 signifies eukaryotes, heterokonts, dinoflagellates, as well as the chlorophyte *Dunaliella* and Poly-T-CY5 which were used as controls. Cross-reactivity with *P. heimii* will need to be addressed on the 3rd generation chip because it reacts with many target species.

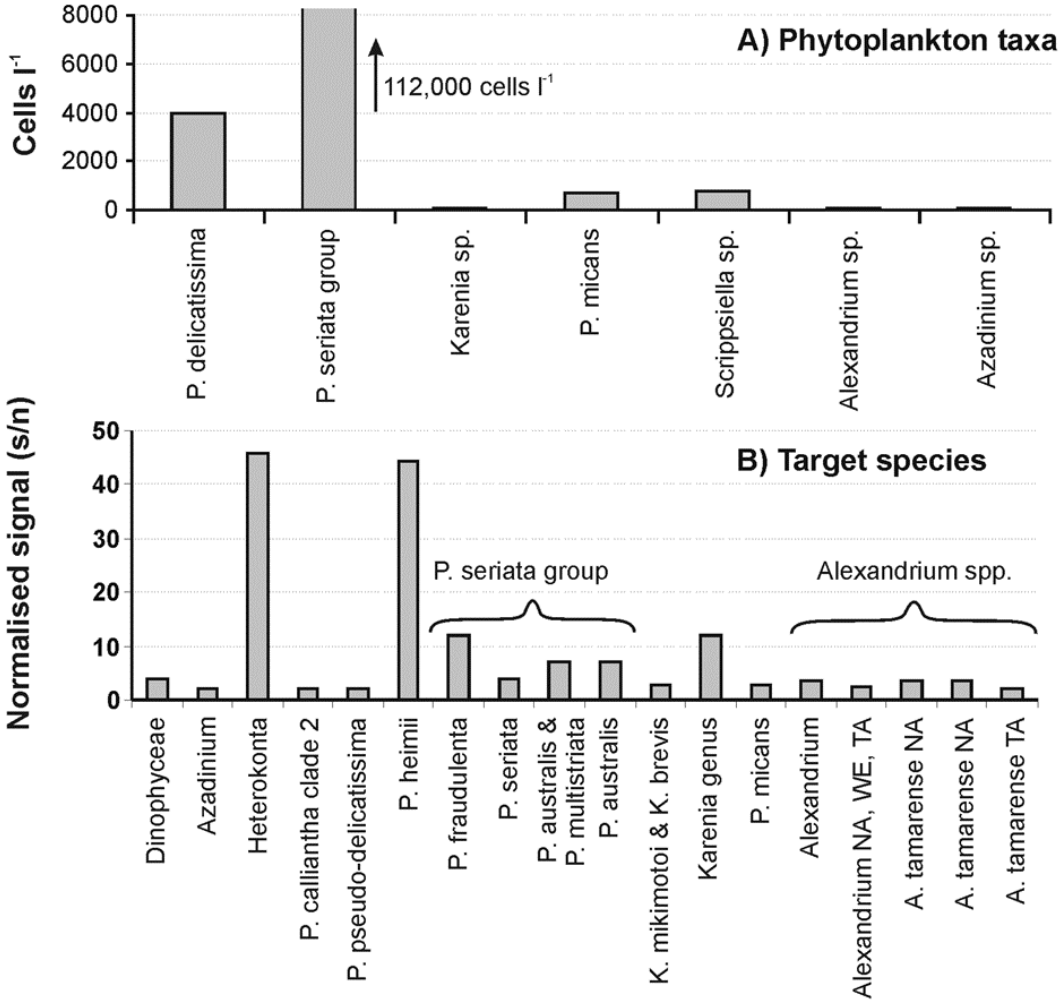

Fig. AP.I-4. (A) Cell counts and (B) 2nd generation microarray chip hybridised with RNA at a temperature of 65 °C from a Killala Bay field extract taken on 15 August 2009.

A second comparison between light microscopy counts and a selection of 2nd generation microarray results from a sample obtained from Killala Bay in August 2009 is shown in Fig. AP.I-4. An assemblage of *Pseudo-nitzschia. seriata* group organisms numerically dominated the sample (112,000 cells l^{-1}) (Fig. AP.I-4a). The microarray data could identify these as *P. fraudulenta, P. seriata, australis*, and *P. multiseries.* A variety of *Alexandrium* probe signals were also evident, which could not be resolved by LM (Fig. AP.I-4b) and require electron microscopy to confirm the species.

CONCLUSIONS

The aim of MIDTAL is to provide a new method to support toxic algal monitoring and reduce the need for the mouse bioassay. Demonstration of its capabilities is the first step towards this goal. These first field results indicate that there remains further development work to be done but point towards the potential successful development of a 'universal' HAB microarray.

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APPENDICES II

SUMMARY OF PROBES ON THE MIDTAL CHIP

The following is a list of the probes on the 3rd generation (3.3 version) MIDTAL chip and also indicates the hierarchical probes present on the chip (Lewis et al. 2012). Table AP.II-1 represents the label code and corresponding names for every probe spotted on the 3.3 version microarray chip surface. Competitor probes are used in case of single mismatch (MM) to the target (Lewis et al. 2012; Kegel et al. 2013).

PROBE LISTING

Dunaliella genus, 3 probes

Higher group and Division level Probes

Eukaryotes 2 probes
Heterokonts
non-Heterokonts
Prymnesiophyta 2 probes

Class level probes

Prymnesiophyceae
Dinophyceae (incl. Apicomplexa) 2 probes

Clade level probes

Pseudo-nitzschia delicatissima complex all clades
Dinophysiaceae (*Dinophysis* + *Phalacroma*)
Dinophysiaceae (*Dinophysis* + *Phalacroma*)
Dinophysisales
Prymnesiales

Genus level probes

Pseudo-nitzschia
Pseudo-nitzschia + *Fragilariopsis* (probe a)
Pseudo-nitzschia + *Fragilariopsis* (probe b)
Pseudo-nitzschia (excluding *P. pungens*)
Pseudo-nitzschia + some *Fragilariopsis*
Alexandrium
Pseudochattonella 5 probes
Dinophysis 3 probes
All *Dinophysis* and *Phalacroma*
Prymnesium
Dinophysis in part
Karlodinium
Karenia 3 probes
Azadinium 3 probes
Azadinium + Karenia mikimoitoi
Prorocentrum

Species Level Probes

Alexandrium (NA=North Atlantic clade, WE=West European clade, TA=Temperate Asian)
Alexandrium minutum
Alexandrium tamarense (NA)
Alexandrium tamarense (NA)
Alexandrium tamarense (TA)
Alexandrium ostenfeldii 2 probes
Prymnesium (= *Chrysochromulina*) *polylepis*
Prymnesium parvum 2 probes
Karenia mikimotoi and some *K. brevis*
Karenia brevis 6 probes
competitor *Karenia brevis*
Karlodinium veneficum 6 probes
Prorocentrum lima 3 probes
Prorocentrum minimum
Gymnodonium catenatum
Dinophysis acuminata+ D. dens + D. sacculus
Dinophysis acuta + D. fortii
Dinophysis acuminata
Dinophysis acuta
Dinophysis norvegica
Phalacroma rotundatum
Pseudo-nitzschia australis
P. australis and *P. seriata*
P. australis and *P. multistriata*
P. australis, P. seriata, P. delicatissima 2 probes
P. australis, P. seriata, P. calliantha
P. fraudulenta, P. subfraudulenta, P. calliantha
P. fraudulenta + P. multistriata
P. caciantha 3 probes

P. calliantha Clade 1
P. mannii 4 probes
P. delicatissima sensu stricto (=*P. delicatissima* Clade2)
P. cf. delicatissima Clade 4, 2 probes
P. dolorosa (= *P. delicatissima* Clade 3 + *P. Micropora*) 2 probes
competitor *P. delicatissima* 3A
P. arenysensis (= *P. delicatissima* Clade 1 + *P. multistriata*)
P. delicatissima sensu stricto (=*P. delicatissima* Clade 2) + *P. calliantha*
P. cf. delicatissima Clade 4
P. galaxiae 3 probes
P. multiseries 2 probes
P. multiseries + *P. calliantha* 4 probes
P. multistriata
P. pseudodelicatissima + *P. cuspidata* (probe a)
P. pseudodelicatissima + *P.*cuspidata (probe b)
P. pungens + *P. calliantha* 3 probes
P. seriata + *P. calliantha*
Chloromorum toxicum 3 probes
Karenia mikimotoi
Gymnodinium catenatum 4 probes
Heterosgima akashiwo 8 probes
Pseudochattonella verruculosa
Pseudochattonella farcimen

Table AP.II-1. List of label, probe names, target species and target groups represented on the 3.3 version MIDTAL microarray available from Microbia Environnement (France).

Label	3.3 Version Probe Names	Targeted Species	Target group
M3001	POSITIVE_25_dT	TATA box protein, as positive control	Control
M3002	NEGATIVE1_25_dT	Negative	Control
M3003	NEGATIVE2_25_dT	Negative	Control
M3004	NEGATIVE3_25_dT	Negative	Control
M3005	Poly-T-CY5	Poly-T (30)-CY5, as spotting control Poly(dA) as blocking probe	
M3006	KbreD04_25c_dT	Competitor *Karenia mikimotoi* and *breve*	Species Level Probe
M3007	DunGS02_25_dT_dT	*Dunaliella* genus, as internal control	Control
M3008	DunGS05_25_dT_dT	*Dunaliella* genus, as internal control	Control
M3009	EukS_328_25_dT	*Eukaryotes*	Higher Group Probes
M3010	EukS_1209_25_dT	*Eukaryotes*	Higher Group Probes
M3013	PrymS01_25_dT	*Prymnesiophyta*	Higher Group Probes
M3015	PrymS03_25_dT	*Prymnesiophyceae*	Class Level Probes
M3016	DinoB_25_dT	*Dinophyceae (incl. Apicomplexa)*	Class Level Probes
M3017	DinoE12_25_dT	*Dinophyceae (incl. Apicomplexa)*	Class Level Probes

M3018	PdeliD02_25_dT	*P.delicatissima* all clades	Clade Level Probes
M3019	DphyexacutaFS01_25_dT	*Dinophysiaceae (Dinophysis + Phalacroma)*	Clade Level Probes
M3020	DphyFS02_25_dT	*Dinophysiaceae (Dinophysis + Phalacroma)*	Clade Level Probes
M3021	Clade 01new25_dT	*Prymnesium* B1 clade	Clade Level Probes
M3022	Clade01old_25_dT	*Prymnesium*	Clade Level Probes
M3023	PsnGS01_25_dT	*Pseudo-nitzschia*	Genus Level Probe
M3024	PsnGS02_25_dT	*Pseudo-nitzschia + Fragilariopsis*	Genus Level Probe
M3025	PSN+FRAGS02-25new_dT	*Pseudo-nitzschia + Fragilariopsis*	Genus Level Probe
M3026	PSN no pungens_25_dT	*Pseudo-nitzschia no pungens*	Genus Level Probe
M3027	PSN + some Frags_25_dT	*Pseudo-nitzschia + some Fragilariopsis*	Genus Level Probe
M3028	KareGD01_25_dT	*Karenia*	Genus Level Probe
M3029	AlexGD01_25_dT	*Alexandrium*	Genus Level Probe
M3030	DphyGD01_25_dT	*Dinophysis* in part	Genus Level Probe
M3031	DphyGD02_25_dT	*Dinophysis*	Genus Level Probe
M3032	PschGS01_25_dT	*Pseudochattonella* (genus)	Genus Level Probe
M3033	PverD01_25_dT	*Pseudochattonella verruculosa*	Species Level Probe
M3034	PmulacalD02_25_dT	*P.multistriata + P. calliantha*	Species Level Probe
M3035	PschGS04_25_dT	*Pseudochattonella* (genus)	Genus Level Probe
M3036	PschGS05_25_dT	*Pseudochattonella* (genus)	Genus Level Probe
M3037	DphyGS01_25_dT	*Dinophysis genus sensu stricto*	Genus Level Probe
M3038	DphyGS02_25_dT	*Dinophysis genus sensu stricto*	Genus Level Probe
M3039	DphyGS03_25_dT	all *dinophysis* and *phalacroma*	Genus Level Probe
M3040	DphyGS04_25_dT	all *dinophysis*	Genus Level Probe
M3041	KargeD01_25_dT	*Karlodinium* genus	Genus Level Probe
M3043	AzaGD01_25_dT	*Azadinium* Genus	Genus Level Probe
M3044	AzaGD03_25_dT	*Azadinium* Genus	Genus Level Probe
M3045	AzaGS01_25_dT	*Azadinium* Genus	Genus Level Probe
M3046	AzaGS02_25_dT	*Azadinium* Genus	Genus Level Probe
M3047	AtamaS01_25_dT	*Alexandrium* NA,WE,TA,	Species Level Probe
M3048	AminuS01_25_dT	*Alexandrium minutum*	Species Level Probe
M3049	ATNA_D01_25_dT	*Alexandrium tamarense* (NA)	Species Level Probe
M3050	ATNA_D02_25_dT	*Alexandrium tamarense* (NA)	Species Level Probe
M3051	ATTA _D01_25_dT	*Alexandrium tamarense* (TA)	Species Level Probe
M3052	AostD01 _25_dT	*Alexandrium ostenfeldii*	Species Level Probe
M3053	AostS02 _25_dT	*Alexandrium ostenfeldii*	Species Level Probe
M3054	CpolyS01_25_dT	*Chrysochromulina polylepis*	Species Level Probe
M3055	PparvD01_25_dT	*Prymnesium parvum*	Species Level Probe
M3056	Prymparv01_25_dT	*Prymnesium parvum*	Species Level Probe
M3057	KbreD03_25_dT	*Karenia mikimotoi* and *breve*	Species Level Probe
M3058	KbreD04_25_dT	*Karenia mikimotoi* and *breve*	Species Level Probe

M3060	KmikiD01_25_dT	*Karenia mikimotoi*	Species Level Probe
M3061	KveneD01_25_dT	*Karlodinium veneficum*	Species Level Probe
M3062	KveneD02_25_dT	*Karlodinium veneficum*	Species Level Probe
M3063	KveneD03_25_dT	*Karlodinium veneficum*	Species Level Probe
M3064	KveneD04_25_dT	*Karlodinium veneficum*	Species Level Probe
M3065	KveneD05_25_dT	*Karlodinium veneficum*	Species Level Probe
M3066	KveneD06_25_dT	*Karlodinium veneficum*	Species Level Probe
M3067	SSKbre1448A25_dT	*K. brevis*	Species Level Probe
M3068	LSKbKm0548A25_dT	*K. brevis + K. mikimoitoi*	Species Level Probe
M3070	PlimaFD01-25_dT	*Prorocentrum lima*	Species Level Probe
M3071	PlimaS01_25_dT	*Prorocentrum lima*	Species Level Probe
M3072	ProroPKD01_25_dT	*Prorocentrum* planktonic clade	Clade Level Probe
M3073	PmicaD02_25_dT	*Prorocentrum micans*	Species Level Probe
M3074	PminiD01_25_dT	*Prorocentrum minimum*	Species Level Probe
M3075	ProroFBS02_25_dT	*Prorocentrum benthic*	Clade Level Probe
M3076	GcateS01_25_dT	*Gymnodonium catenatum*	Species Level Probe
M3077	DacumiD02_25_dT	*Dinophysis acuminatum+ dens+sacculus*	Species Level Probe
M3078	DacutaD02_25_dT	*Dinophysis acuta+D.fortii*	Species Level Probe
M3079	DacumiS01_25_dT	*Dinophysis acuminata*	Species Level Probe
M3080	DacutaS01_25_dT	*Dinophysis acuta*	Species Level Probe
M3081	DnorvS01_25_dT	*Dinophysis norvegica*	Species Level Probe
M3082	ProtuS01_25_dT	*Phalacroma rotundatum*	Species Level Probe
M3083	PausserD01_25_dT	*P. australis and seriata*	Species Level Probe
M3084	PmulausD01_25_dT	*P.australis & P.multistriata*	Species Level Probe
M3085	PcaserausD02_25_dT	*P.australis & P.seriata, deli 2*	Species Level Probe
M3086	PcaserausD03_25_dT	*P.australis & P.seriata, P. caliantha*	Species Level Probe
M3087	PfraucalD02_25_dT	*P.fraudulenta, P.subfraudulenta,P calliantha*	Species Level Probe
M3088	PfarD01_25_dT	*Pseudochattonella farcimen*	Species Level Probe
M3089	PcaciD01_25_dT	*P.caciantha*	Species Level Probe
M3090	PcaciD02_25_dT	*P.caciantha*	Species Level Probe
M3091	PcaciD04_25_dT	*P.caciantha*	Species Level Probe
M3092	Pcal1D01_25_dT	*P.calliantha* clade 1	Species Level Probe
M3093	PmanD01_25_dT	*P.mannii*	Species Level Probe
M3094	Pman2D02_25_dT	*P.mannii*	Species Level Probe
M3095	Pman2D03_25_dT	*P.mannii*	Species Level Probe
M3096	Pman2D05_25_dT	*P.mannii*	Species Level Probe
M3098	Pdel4D01_25_dT	*P.delicatissima* Clade4	Species Level Probe
M3099	Pdel4D02_25_dT	*P.delicatissima* Clade4	Species Level Probe
M3100	Pdel3B_25_dT	*P. delicatissima* clade 3+*micropora*	Species Level Probe
M3101	Pdel3A_25_dT	*P. delicatissima* clade 3+*micropora*	Species Level Probe

M3102	CompPdel3_25_dT	competitor *Pdel3A*	Species Level Probe
M3103	Pdel1D01_25_dT	*P.delicatissima* Clade1	Species Level Probe
M3104	Pcaldel2D01_25_dT	*P.delicatissima* Clade2	Species Level Probe
M3105	Pdel4D03_25_dT	*P.delicatissima* Clade4	Species Level Probe
M3106	PgalaD01_25_dT	*P.galaxiae*	Species Level Probe
M3107	PgalaD02_25_dT	*P.galaxiae*	Species Level Probe
M3108	PgalaD04_25_dT	*P.galaxiae*	Species Level Probe
M3109	PmultS01_25_dT	*Pseudo-nitzschia multiseries*	Species Level Probe
M3110	PmultD02 _25_dT	*P.multiseries*	Species Level Probe
M3111	PmultcalD01 _25_dT	*P.multiseries+ P calliantha*	Species Level Probe
M3112	PmultcalD03 _25_dT	*P.multiseries+ P calliantha*	Species Level Probe
M3113	PmultcalD04 _25_dT	*P.multiseries+ P calliantha*	Species Level Probe
M3114	PcalfrauD04_25_dT	*P.fraudulenta+Pmultistriata*	Species Level Probe
M3115	PmulaD03_25_dT	*P.multistriata*	Species Level Probe
M3116	PpdeD01_25_dT	*P.pseudodelicatissima & P.cuspidata*	Species Level Probe
M3117	PpdeD02_25_dT	*P.pseudodelicatissima & P.cuspidata*	Species Level Probe
M3118	PpungcalS01_25_dT	*Pseudo-nitzschia pungens+caliantha*	Species Level Probe
M3119	PpungcalD02_25_dT	*P.pungens+ P. calliantha*	Species Level Probe
M3120	PpungcalD04_25_dT	*P.pungens+ P. calliantha*	Species Level Probe
M3121	PsercalD01_25_dT	*P.seriata+ P. calliantha*	Species Level Probe
M3122	CtoxS05_25_dT	*Chloromorum toxicum*	Species Level Probe
M3123	KmGcS06_25_dT	*Karenia mikimoitoi, Gymnodinium catenatum, Chloromorum toxicum*	Species Level Probe
M3124	CtoxiS07_25_dT	*Chloromorum toxicum*	Species Level Probe
M3125	CtoxiS09_25_dT	*Chloromorum toxicum*	Species Level Probe
M3126	KbreD03c_25_dT	Competitor *Karenia mikimotoi and breve*	Species Level Probe
M3127	SSGcat0826A27_dT	*G. catenatum*	Species Level Probe
M3128	LSGcat0270A24_dT	*G. catenatum*	Species Level Probe
M3130	SSHaka0193A25_dT	*H. akashiwo*	Species Level Probe
M3131	SSHaka0200A25_dT	*H. akashiwo*	Species Level Probe
M3132	LSHaka0544A25b_dT	*H. akashiwo*	Species Level Probe
M3133	LSHaka0268A25_dT	*H. akashiwo*	Species Level Probe
M3134	LSHaka0544A25c_dT	*H. akashiwo*	Species Level Probe
M3135	LSHaka0548A25_dT	*H. akashiwo*	Species Level Probe
M3136	LSHaka0329A25_dT	*H. akashiwo*	Species Level Probe
M3137	LSHaka0358A24_dT	*H. akashiwo*	Species Level Probe
M3138	ProroFBS01	*Prorocentrum benthic*	Clade Level Probe
M3139	LSKBre0548A25c_dT	*Karenia breve*	Species Level Probe
M3140	PmacuS01	*Prorocentrum maculosum/belizeanum*	Species Level Probe
M3141	PmacuD01	*Prorocentrum maculosum*	Species Level Probe
M3142	PmacuD02	*Prorocentrum maculosum*	Species Level Probe

M3143	PbeliS01	*Prorocentrum belizeanum*	Species Level Probe
M3144	ProroFPS01	*Prorocentrum planktonic*	Clade Level Probe
M3145	PrathD01	*Prorocentrum rathymum/mexicanum*	Species Level Probe
M3146	PrathD02	*Prorocentrum rathymum/mexicanum*	Species Level Probe
M3149	DunGS02_25dT	Test	Control
M3152	EukS_1209_25dT	Test	Higher Group Probes
M3153	DacumiD02_10dT	Test	Species Level Probe
M3154	DacumiD02_15dT	Test	Species Level Probe
M3155	DacumiD02_25dT	Test	Species Level Probe
M3156	KveneD03_10dT	Test	Species Level Probe
M3157	KveneD03_15dT	Test	Species Level Probe
M3158	KveneD03_25dT	Test	Species Level Probe
M3159	CtoxiS05_10dT	Test	Species Level Probe
M3160	CtoxiS05_15dT	Test	Species Level Probe
M3161	CtoxiS05_25dT	Test	Species Level Probe
M3162	SSKbre1448A25c_dT	*Karenia brevis*	Species Level Probe
M3163	PcaldelD03_25_dT	*P.delicatissima* Clade2*+P calliantha*	Species Level Probe

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