

2013

# Factors that impact *Pseudo-nitzschia* spp. occurrence, growth, and toxin production

DOWNES-TETTMAR, NAOMI

<http://hdl.handle.net/10026.1/1588>

---

<http://dx.doi.org/10.24382/1419>

University of Plymouth

---

*All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.*

## **Copyright Statement**

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the authors prior consent.



**Factors that impact *Pseudo-nitzschia* spp. occurrence, growth,  
and toxin production**

By

**Naomi Downes-Tettmar**

A thesis submitted to Plymouth University in partial fulfilment for the  
degree of

**DOCTOR OF PHILOSOPHY**

School of Geography, Earth, and Environmental Sciences

Faculty of Science and Technology

In collaboration with

Plymouth Marine Laboratory

**September 2012**



## Abstract

### **Factors that impact *Pseudo-nitzschia* spp. occurrence, growth, and toxin production**

By

Naomi Downes-Tettmar

This work investigates, for the first time, the *Pseudo-nitzschia* (PN) dynamics in the western English Channel (L4) and the environmental factors impacting on domoic acid (DA) production in these waters. This is combined with laboratory studies examining key environmental factors and the multifactorial impact of multiple macronutrient and micronutrient availability on PN growth and DA production.

An LC-MS method was established, optimised, and compared with ELISA for the accurate and reproducible extraction and determination of particulate and dissolved DA. The method was used to measure the seasonal variation in DA at L4 during 2009 and this was compared to PN seasonal abundance and diversity. Three groups a *P. delicatissima*-group, a *P. seriata*-group, and a *P. pungens/multiseries*-group were identified and were found to have different ecological distributions with the latter two groups significantly correlating with DA concentration. Macronutrients, in combination with other environmental factors, were found to influence PN populations at L4. Multifactorial laboratory culture experiments investigating the availability of nitrate, phosphate, and silicate, confirmed that the interrelatedness of all these nutrients significantly affected the growth, decline, and DA production of *P. multiseries*, and highlight the importance of both phosphate and silicate availability for DA production. When the impacts of both macronutrient (phosphate and silicate) and micronutrient (iron and copper) availability were investigated, limited growth and DA production was observed in *P. multiseries* cultures. Results revealed the complexity and interrelationship of factors affecting both PN growth and DA production. Furthermore, molecular methods were developed to elucidate the PN species present from 2009 Lugol's-preserved L4 samples. DNA was successfully extracted and amplified from these samples which had been stored for up to 2 years. Initial sequence analysis identified the *rbcl* DNA marker as an informative site for future work with a number of L4 sequences closely relating to different *Pseudo-nitzschia* spp.



# List of Contents

<b>Chapter 1: General introduction .....</b>	<b>1</b>
<b>1.1 <i>Pseudo-nitzschia</i> and domoic acid (DA).....</b>	<b>3</b>
1.1.1 <i>Pseudo-nitzschia</i> morphology.....	5
1.1.2 Molecular identification of <i>Pseudo-nitzschia</i> .....	8
1.1.3 The life cycle of <i>Pseudo-nitzschia</i> .....	11
<b>1.2 <i>Pseudo-nitzschia</i> spp. and DA in the western English Channel .....</b>	<b>14</b>
<b>1.3 Domoic acid.....</b>	<b>17</b>
1.3.1 The trophic transfer of DA .....	22
1.3.2 The ecological and physiological role of DA .....	23
<b>1.4 Factors influencing DA production in <i>Pseudo-nitzschia</i> spp. ....</b>	<b>26</b>
1.4.1 DA production and growth cycle.....	26
1.4.2 Macronutrients .....	28
1.4.3 Nitrogen source.....	29
1.4.4 Dissolved organic nutrients .....	29
1.4.5 Micronutrients.....	30
1.4.6 Temperature.....	31
1.4.7 Irradiance and photoperiods .....	32
1.4.8 Photoperiods .....	33
1.4.9 Salinity.....	34
1.4.10 pH .....	34
1.4.11 Carbon dioxide (CO <sub>2</sub> ) .....	35
1.4.12 Bacteria.....	36
<b>1.5 Status of knowledge and direction needed .....</b>	<b>37</b>
<b>1.6 Aims and objectives.....</b>	<b>40</b>
 <b>Chapter 2: Evaluation, refinement and comparison of methods for the extraction and analysis of domoic acid in seawater and <i>Pseudo-nitzschia</i> spp.....</b>	 <b>43</b>
<b>Abstract .....</b>	<b>45</b>
<b>2.1 Analytical methods for domoic acid determination .....</b>	<b>47</b>
2.1.1 Chromatographic methods.....	47
2.1.1.1 HPLC methods .....	48
2.1.1.2 LC-MS methods .....	50
2.1.1.3 Additional chromatographic methods .....	51
2.1.1.4 Using an internal standard.....	52
2.1.2 Solid phase extraction.....	57
2.1.3 Overview of Enzyme-linked ImmunoSorbent Assay (ELISA) .....	60
2.1.4 Objectives .....	65
<b>2.2 Methods.....</b>	<b>66</b>
2.2.1 Chemicals .....	66
2.2.2 Solid Phase Extraction (SPE) Methods .....	66
2.2.2.1 SPE and DA elution .....	66
2.2.2.2 Preparation of particulate DA (pDA) samples .....	67
2.2.2.3 Preparation of dDA sampled .....	67
2.2.2.4 Recovery of authentic DA from spiked seawater.....	68
2.2.2.5 Recovery and reproducibility of pDA and dDA by SPE.....	68
2.2.3 Liquid Chromatography-Mass Spectrometry .....	70
2.2.3.1 LC-MS of DA .....	70
2.2.3.2 Authentic DA calibrations.....	73
2.2.4 Internal standard .....	73
2.2.4.1 Calibration of DA and KA .....	73
2.2.4.2 Response of KA to varying concentrations of DA.....	74
2.2.5 ELISA methods .....	74
2.2.5.1 Preparation of samples for ELISA .....	74



2.2.5.2 Preliminary study.....	75
2.2.6 LC-MS and ELISA analyses of <i>P. multiseri</i> cultures .....	76
2.2.6.1 Preparation and culture maintenance.....	76
2.2.6.2 Experimental cultures.....	76
<b>2.3 Results and discussion .....</b>	<b>77</b>
2.3.1 Solid phase extraction .....	77
2.3.2 LC-MS development .....	79
2.3.3 Use of an internal standard.....	82
2.3.4 ELISA .....	87
2.3.5 Comparisons between LC-MS and ELISA methods.....	91
2.3.6 Conclusions .....	99

## **Chapter 3: Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid in the western English Channel .....**

<b>Abstract.....</b>	<b>103</b>
<b>3.1 Introduction.....</b>	<b>105</b>
<b>3.2 Methods and Materials.....</b>	<b>107</b>
3.2.1 Study area and sampling .....	107
3.2.2 <i>Pseudo-nitzschia</i> abundance and diversity .....	107
3.2.3 Particulate DA (pDA) monitoring.....	108
3.2.4 Environmental parameters.....	109
3.2.5 Statistical analysis .....	111
<b>3.3 Results .....</b>	<b>112</b>
3.3.1 Environmental monitoring .....	112
3.3.2 <i>Pseudo-nitzschia</i> abundance and diversity .....	115
3.3.3. Relating environmental conditions to <i>Pseudo-nitzschia</i> occurrence.....	118
3.3.4 pDA monitoring .....	120
<b>3.4. Discussion .....</b>	<b>123</b>
3.4.1 <i>Pseudo-nitzschia</i> occurrence .....	123
3.4.2 The <i>P. delicatissima</i> -group .....	126
3.4.3 The <i>P. pungens</i> /multiseri-group.....	128
3.4.4 The <i>P. seriata</i> -group .....	129
3.4.5 Analysis of key abiotic factors during water column stratification.....	130
3.4.6 Group level discrimination.....	131
3.4.7 Statistical analysis of key environmental factors .....	134
3.4.8 pDA concentration .....	135
3.4.9 Conclusions .....	138

## **Chapter 4: The growth and decline of *Pseudo-nitzschia multiseri* and domoic acid production under high and low nutrient and trace metal combinations .....**

<b>Abstract.....</b>	<b>143</b>
<b>4.1 Introduction.....</b>	<b>145</b>
<b>4.2 Methods and Materials.....</b>	<b>148</b>
4.2.1 Stock and experimental culture conditions .....	148
4.2.1.1 Macronutrient experiment .....	149
4.2.1.2 Macronutrient and trace metal experiment .....	150
4.2.2 Media preparation in the macronutrient and trace metal experiment.....	152
4.2.3 Cell counts, specific growth rate and nutrient analysis .....	154
4.2.4 Macronutrient and trace metal experiment: FIRE measurements.....	155
4.2.5 DA sampling and extraction.....	155
4.2.6 DA determination using LC-MS .....	156
4.2.7 Experimental design.....	157
<b>4.3 Results.....</b>	<b>158</b>
4.3.1 Macronutrient experiment: Inorganic nutrient concentrations .....	158
4.3.2 Growth and cell numbers .....	158
4.3.2.1 Macronutrient experiment .....	158
4.3.2.2 Macronutrient and trace metal experiment .....	162

4.3.3 Macronutrient and trace metal experiment: $F_v/F_m$ .....	164
4.3.4 DA production .....	165
4.3.4.1 Macronutrient experiment .....	165
<b>4.4 Discussion .....</b>	<b>169</b>
4.4.1 Macronutrient experiment: Growth dynamics .....	169
4.4.1.1 Nutrient ratios and P. multiseria growth dynamics .....	174
4.4.2 Macronutrient and trace metals experiment: Growth dynamics .....	176
4.4.2.1 Photosynthetic efficiency .....	179
4.4.4 Macronutrient experiment: DA production .....	180
4.4.3.1 Redfield ratio and DA .....	184
4.4.4 Conclusions .....	185
<b>Chapter 5: Genetic identification of <i>Pseudo-nitzschia</i> species in archived L4 water samples .....</b>	<b>188</b>
Abstract .....	190
<b>5.1 Introduction .....</b>	<b>193</b>
<b>5.2 Methods and Materials .....</b>	<b>197</b>
5.2.1 Samples .....	197
5.2.2 DNA extraction .....	197
5.2.3 PCR amplification .....	198
5.2.3.1 rbcL .....	200
5.2.3.2 ITS .....	201
5.2.3.3 SSU .....	202
5.2.4 Phylogenetic analysis .....	202
<b>5.3 Results .....</b>	<b>204</b>
5.3.1 Primer development .....	204
5.3.2 Sequences .....	205
5.3.2 Phylogenetic analysis .....	209
<b>5.4 Discussion .....</b>	<b>210</b>
5.4.1 SSU and ITS markers .....	210
5.4.2 rbcL marker and phylogenetic analysis .....	212
5.4.3 An alternative molecular method .....	214
5.4.4 Conclusions .....	215
<b>Chapter 6: Summary, conclusions and future work .....</b>	<b>217</b>
<b>6.1 Overall conclusions and discussion .....</b>	<b>219</b>
6.1.1 Project rationale and aims .....	219
6.1.2 Main findings .....	221
6.1.2.1 Methods for DA determination .....	221
6.1.2.2 Field monitoring .....	222
6.1.2.3 Culture studies .....	225
<b>6.2 Environmental implications .....</b>	<b>227</b>
6.2.1 Monitoring and time series data .....	227
6.2.2 Forecasting .....	229
<b>6.3 Recommendation for future work .....</b>	<b>233</b>
6.3.1 Continuation of monitoring .....	233
6.3.2 Measuring other toxins in the western English Channel .....	233
6.3.3 Additional tools for the determination of toxins at L4 .....	234
6.3.4 Improving <i>Pseudo-nitzschia</i> cell isolation and cultivation .....	235
6.3.5 Culture studies and growth conditions .....	236
<b>6.4 Summary .....</b>	<b>238</b>
<b>References .....</b>	<b>239</b>



## List of Tables

<b>Table 2.1:</b> Summary of literature values for the limits of detection.....	56
<b>Table 2.2.</b> Gradient for LC elution. ....	71
<b>Table 2.3:</b> Percentage recovery of DA.....	77
<b>Table 3.1:</b> Spearman's rank correlations between <i>Pseudo-nitzschia</i> group abundances and environmental variables at L4.....	119
<b>Table 4.1:</b> Composition of the synthetic ocean water (SOW) used in Aquil medium. ....	149
<b>Table 4.2:</b> Initial nitrate, phosphate, and silicate concentrations .....	150
<b>Table 4.3:</b> Trace metal enrichments .....	151
<b>Table 4.4:</b> Composition of vitamin components in Aquil medium.....	153
<b>Table 4.5:</b> Macronutrient and trace metal experimental design .....	154
<b>Table 4.6:</b> Comparison of the mean growth rates of <i>P. multiseries</i> .....	160
<b>Table 5.1:</b> Details of the primer pairs used for the PCR amplification.....	199



## List of Figures

<b>Figure 1.1:</b> SEM images of a variety of <i>Pseudo-nitzschia</i> species frustules illustrating how morphological characteristics are measured .....	6
<b>Figure 1.2:</b> The rDNA operon composed of the small subunit (SSU), internal transcribed spacers (ITS1 and ITS2), 5.8S, and the large subunit (LSU) gene regions. ....	8
<b>Figure 1.3:</b> A representative schematic of the life cycle of the pennate diatom <i>P. multistriata</i> . .....	12
<b>Figure 1.4:</b> Position of the monitoring station L4 in the western English Channel (WEC).....	16
<b>Figure 1.5:</b> Chemical structures for glutamic acid and the DA isomers, epidomoic acid and isodomoic acid. ....	19
<b>Figure 1.6:</b> Schematic diagram of the potential pathway of domoic acid biosynthesis. ....	21
<b>Figure 2.1:</b> Chemical structure of (A) domoic acid, (B) kainic acid, and (C) dihydrokainic acid. ....	55
<b>Figure 2.2:</b> Schematic showing how the functional groups of DA are influenced by pH. ....	59
<b>Figure 2.3:</b> Schematic showing the ELISA immunoassay format. ....	62
<b>Figure 2.4:</b> Diagram summarising preparation of pDA and dDA samples for LC-MS analysis .....	69
<b>Figure 2.5:</b> Example spectra showing (A) the extracted ion count (EIC) for DA ( $m/z$ 312) and (B) the fragmentation pattern ( $MS^2$ ) characteristic of DA ( $m/z$ 266, 248, and 220). ....	72
<b>Figure 2.6:</b> Mean concentrations of replicate pDA and dDA culture samples. ....	78
<b>Figure 2.7:</b> (A) Extracted ion mass chromatogram indicating retention time of DA ( $m/z$ 312, $[M+H]^+$ ) at 5.3 minutes, (B) ESI mass spectrum of DA. ....	80
<b>Figure 2.8:</b> LC-MS calibration curve for DA standard solutions at seven concentrations. ....	81
<b>Figure 2.9:</b> DA calibrations .....	82
<b>Figure 2.10:</b> LC-MS extracted mass chromatogram ( $m/z$ 214 + 312 $[M+H]^+$ ) for KA and DA peaks .....	83
<b>Figure 2.11:</b> LC-MS calibrations for DA and KA standard solutions.....	84
<b>Figure 2.12:</b> Ratio of DA and KA across the calibration curve. ....	85
<b>Figure 2.13:</b> Comparison between the peak areas for five varying duplicate DA concentrations .....	86
<b>Figure 2.14:</b> LC-MS extracted chromatograms .....	87
<b>Figure 2.15:</b> DA calibration curve from the ASP ELISA macro .....	89
<b>Figure 2.16:</b> Total and dissolved DA (dDA) concentration.....	90

<b>Figure 2.17:</b> Comparisons between LC-MS and ELISA determinations for particulate DA (pDA) concentrations .....	91
<b>Figure 2.18:</b> Mean triplicate growth curve for experimental flasks containing <i>P. multiseri</i> ...	93
<b>Figure 2.19:</b> Mean concentrations in both total DA and dDA in <i>P. multiseri</i> cultures .....	94
<b>Figure 2.20:</b> Mean cell abundances of <i>P. multiseri</i> along side the mean cellular DA concentrations, analysed using ELISA.....	95
<b>Figure 2.21:</b> A log plot of the mean cellular DA and dissolved DA (dDA) concentrations .....	96
<b>Figure 2.22:</b> Mean cell abundances of <i>P. multiseri</i> alongside the mean cellular DA concentrations; analysed using LC-MS methods. ....	97
<b>Figure 3.1:</b> Mixed layer depth (MLD) at L4 during 2009 .....	111
<b>Figure 3.2:</b> Nutrient concentrations ( $\mu\text{mol L}^{-1}$ ) and sea-surface temperature ( $^{\circ}\text{C}$ ) at the monitoring station L4 during 2009 .....	114
<b>Figure 3.3:</b> Total diatom abundance ( $\times 10^3 \text{ cells L}^{-1}$ ) and total <i>Pseudo-nitzschia</i> spp. (P-n) abundance ( $\times 10^3 \text{ cells L}^{-1}$ ) at the monitoring station L4.....	116
<b>Figure 3.4:</b> Cell abundance of the <i>Pseudo-nitzschia</i> groups .....	117
<b>Figure 3.5:</b> Particulate domoic acid concentration .....	120
<b>Figure 3.6:</b> LC chromatograms and MS <sup>n</sup> fragmentation profiles for an isomer of domoic acid .....	122
<b>Figure 3.7:</b> Transmission electron microscope images .....	125
<b>Figure 4.1:</b> (A) Microphotograph of QuadraPure <sup>TM</sup> beads; (B) Structure of the polystyrene QuadraPure <sup>TM</sup> metal scavenging beads .....	151
<b>Figure 4.2:</b> Mean log cell abundance per mL of <i>Pseudo-nitzschia multiseri</i> cultures .....	161
<b>Figure 4.3:</b> Mean <i>P. multiseri</i> cell abundances .....	163
<b>Figure 4.4:</b> Maximum quantum yield of photosystem II ( $F_v/F_m$ ) for <i>P. multiseri</i> cultures ..	164
<b>Figure 4.5:</b> (A) Total DA, (B) pDA and (C) cellular DA .....	167
<b>Figure 4.6:</b> Mean dDA concentrations .....	168
<b>Figure 5.1:</b> rbcL PCR products from nine L4 water samples .....	205
<b>Figure 5.2:</b> Maximum likelihood phylogenetic tree .....	206
<b>Figure 5.3:</b> Neighbour Joining phylogenetic tree .....	207
<b>Figure 5.4:</b> Bayesian Inference phylogenetic tree .....	208
<b>Figure 6.1:</b> A diagram representing the three approaches used in this project,.....	232

## Acknowledgements

First and foremost I would like to pay particular thanks to my supervisors Carole Llewellyn, Steve Rowland, and Peter Miller for all of their support and guidance during my PhD. Especially to Carole and Steve for all of their practical help and encouragement during the developmental stages of my PhD, for being a constant source of sound advice, and for sharing my frustrations and successes along the way. Thanks to Peter who helped shape my project during the early stages of my PhD, for feedback on my work, and for helping me explore the application of remote sensing data in my project. I am sincerely grateful for each of their contributions to my project and thank them for helping make this an enriching experience.

Funding for this work was gratefully received from the Natural Environment Research Council, in the form of a studentship, without which, the work herein would not have been possible.

My gratitude to all those at Plymouth Marine Laboratory (PML) who have offered their time and expertise during my PhD, and for those that have generally made working at PML an enjoyable and pleasant experience. Thanks to Peter Croot for being a sounding board and offering advice on trace metals, diatom growth, and toxin production; to Daniel White for initial LC-MS training and guidance in using the experimental design software, Design Expert; to Paul Somerfield and Bob Clarke for lengthy discussions and emails about statistics and PRIMER; to Claire Widdicombe for her interest in my project and her help concerning phytoplankton sampling and identification at L4; and to Steve Archer for training me on the FIRE fluorometer system. I would also like to express thanks to the crew of the Plymouth Quest and core scientists funded on the WCO as part of the UK Natural Environmental Research Council's National Capability.

I am grateful to those at Plymouth University who were so welcoming and accommodating when I was there conducting experimental work and using the University facilities. Thanks to Roy Moates, Pete Bond, and Glenn Harper from the EM unit for their support using TEM to analyse environmental and culture samples, for their enthusiasm, and for inclusion in group tea breaks.

Thank you to Robin Raine for letting me visit his team and labs in Galway, Ireland at the start of my PhD for training in a number of molecular techniques, as well as *Pseudo-nitzschia* enumeration and identification methods. I am grateful to Robin Raine and Evelyn Kready for providing me with *Pseudo-nitzschia* cultures to take back to PML.

I owe many thanks to Rowena Stern at the Marine Biological Association (MBA) who has taught me so much about molecular biology and provided me with an opportunity to explore the application of such methods in my PhD. I am grateful to the MBA for recognising me as an associate researcher and providing funding to facilitate this work and collaboration. On a personal level, thanks to Rowena and Grazyna for their friendship, evening meals on the Barbican, and making the many hours spent at the MBA an absolute pleasure.

Thanks to all the lovely people I have had the pleasure of sharing an office with during my PhD. Thanks in particular to those who have been there since the beginning; to Nicole for showing me around the lab when I first arrived and offering me so much support when I came across difficulties on the LC-MS, for her friendship outside of the lab which made my move to Plymouth from the Northwest not so daunting; to Bonnie



for being a true office matriarch from beginning to end, for her distractions and willingness to be distracted, for her warmth and wisdom, and for all the pottery pots; to Hannah and Helen for being good PhD role models; to Jess for her friendship inside and outside work which was generally food related whether it be pizza express lunches or dinner dates. Thanks also to those students in my cohort for their support, enthusiasm, and shared understanding.

Thanks to Ted and Liz who have been there from the start and have offered me a great deal of support, a getaway to Fort Picklecombe, fantastic meals, and numerous care packages. Thank you to them for their interest in my studies, their care and consideration, and thoughtfulness.

My warmest thanks to Dan for supporting me through the last few months of my PhD, for feeding me and generally making life easy for me so that I was free to focus on writing up, for his calm and logical approach, for his understanding, and for accepting that he cannot read my thesis until it has been finished! Thanks also to Dan and the lovely Beth for sharing me with my PhD on more weekends than I would dare to count.

Finally, a heartfelt thank you to my mum and sister for all of their love, support, humour, and pride. Thanks to my sister for her friendship, generosity, and for being on the other end of the phone if I ever needed to take my mind off my work. Thanks to my mum for having confidence in me, for being there through the ups and downs of life, for all her positive energy, and most importantly for all the shared laughter.

## Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Graduate Committee.

This study was financed with the aid of a studentship from the Natural Environment Research Council and carried out in collaboration with Plymouth Marine Laboratory.

Relevant scientific seminars and conferences were attended throughout the period of study at which research was regularly presented; external institutions were visited for consultation purposes and several papers have been prepared or will be prepared for publication.

### **Publications:**

Downes-Tettmar, N., Rowland, S., Widdicombe, C., Woodward, M., Llewellyn, C. (2012) Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid in the western English Channel. Cont. Shelf Res. 53, 40-49

Downes-Tettmar, N., Rowland, S., Llewellyn, C. (2013) The effects of macro-nutrients on *Pseudo-nitzschia multiseries* growth and domoic acid production: A multifactorial approach. (*In prep*)

Downes-Tettmar, N., Eloire D, Widdicombe C, Miller P, Llewellyn CA (2014) Harmful algae in the western English Channel: A historical perspective. *In Prep*

### **Presentations and conferences attended:**

Plankton 2011 Symposium '*Plankton diversity and global change*', 29<sup>th</sup> September to the 3<sup>rd</sup> October 2011, Plymouth, UK, Attended

Downes-Tettmar, N., Rowland, S., Widdicombe, C., Miller, P., and Llewellyn, C. 'Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid production in the western English Channel' (oral presentation), *University of Plymouth - Biogeochemistry Research Centre Conference* (Dec 2010)

Downes-Tettmar, N., Rowland, S., Widdicombe, C., Miller, P., and Llewellyn, C.  
'Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid production in the western English Channel' (poster), *14<sup>th</sup> International Conference on Harmful Algae*, Crete (Nov 2010)

Downes-Tettmar, N., Rowland, S., Widdicombe, C., Miller, P., and Llewellyn, C.  
'*Pseudo-nitzschia* spp. and domoic acid production' (oral presentation), *University of Plymouth -Biogeochemistry Research Centre Conference* (Dec 2009)

Chemical methods in marine ecology including toxins and plankton interactions, August 2008, MARBEF-Naples, Italy, Attended.

Word Count: 50, 863

Signed.....

Naomi Downes-Tettmar

22<sup>nd</sup> February 2013

## **Chapter 1: General introduction**



## 1.1 *Pseudo-nitzschia* and domoic acid (DA)

There are around 5000 described marine phytoplankton species worldwide, 300 of which are deemed harmful, and of these harmful species around 80 have been reported as toxic (Sournia et al., 1991; Smayda, 2006; Uronen, 2007). One such group of species (genus) is the widely studied chain forming pennate diatom, *Pseudo-nitzschia*, which is considered to be a cosmopolitan plankter due to its occurrence globally in marine environments (Hasle, 2002). The genus comprises 37 species, of which 14 have been found to produce the potent neurotoxin, domoic acid (DA): *P. australis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. galaxiae*, *P. multiseriata*, *P. multistriata*, *P. pungens*, *P. seriata*, *P. turgidula*, *P. cuspidata*, and *P. pseudodelicatissima*, *P. brasiliana*, and *P. granii* (Lelong et al., 2012; Trainer et al., 2012). DA is responsible for amnesic shellfish poisoning in seabirds, marine mammals and humans (Bargu et al., 2011; Trainer et al., 2012). The toxin enters the food chain *via* filter feeders such as molluscan shellfish and is transferred by various vectors (e.g. clams, mussels, crabs, and filter feeding fish (Wekell et al., 1994; Lefebvre et al., 2002a; Lefebvre et al., 2002b)) to higher mammals where the toxin bio-accumulates and can reach concentrations which may prove fatal if ingested (Scholin et al., 2000). The larger and more persistent these blooms are, the greater the ecosystem and socio-economic impacts (Klein et al., 2010). *Pseudo-nitzschia* blooms have mainly been problematic in North America (Alaska, Bay of Fundy, California, Oregon, and Washington) and Canada (Prince Edward Island and British Columbia) (Hallegraeff, 2003). More recently, many areas across Europe have been affected, including: Spain, France, Portugal, Italy, Denmark, Scotland, and Ireland (Miguez et al., 1996; Campbell et al., 2001; Vale and Sampayo, 2001; Cusack et al., 2002; Fehling et al., 2004b; Ciminiello et al., 2005; Klein et al., 2010). *Pseudo-nitzschia* blooms have been found at a small scale (e.g. in thin layers (Rines et al., 2002)), or at a large scale, covering large areas of coastlines (Trainer et al., 2009).

These blooms can be intense and persistent, reaching high densities visible to the naked eye.

*Pseudo-nitzschia* spp. commonly occur in spring and late autumn when conditions are unfavourable for other phytoplankton (e.g. for other diatoms, dinoflagellates and prymnophytes) to grow optimally. Most phytoplankton species respond to warm temperatures and calm stratified waters; however, *Pseudo-nitzschia* spp. typically bloom under lower temperatures and in un-stratified conditions. This allows them to out-compete other phytoplankton species and take advantage of nutrient regeneration in surface waters (Mos, 2001). *Pseudo-nitzschia* spp. are also able to tolerate a wide range of salinities (Thessen et al., 2005) and as such have also been found in estuarine environments (Marshall et al., 2005). However, the environmental conditions that favour the growth of *Pseudo-nitzschia* spp. differ from the conditions that promote the production of DA by the diatom. For instance, along the coast of California *Pseudo-nitzschia* spp. blooms are initiated during spring time by nutrient rich upwelling water but production of DA is at its highest as coastal upwelling subsides and nutrients are depleted (Trainer et al., 2000). Other influential conditions on growth and DA production are temperature (Lewis et al., 1993), pH (Lundholm et al., 2004), and irradiance (Fehling et al., 2005). The environmental cues that promote toxic blooms of *Pseudo-nitzschia* spp. are complex and intricately linked to the external environment. These cues may be unique to the regions in which blooms occur and to the species involved.

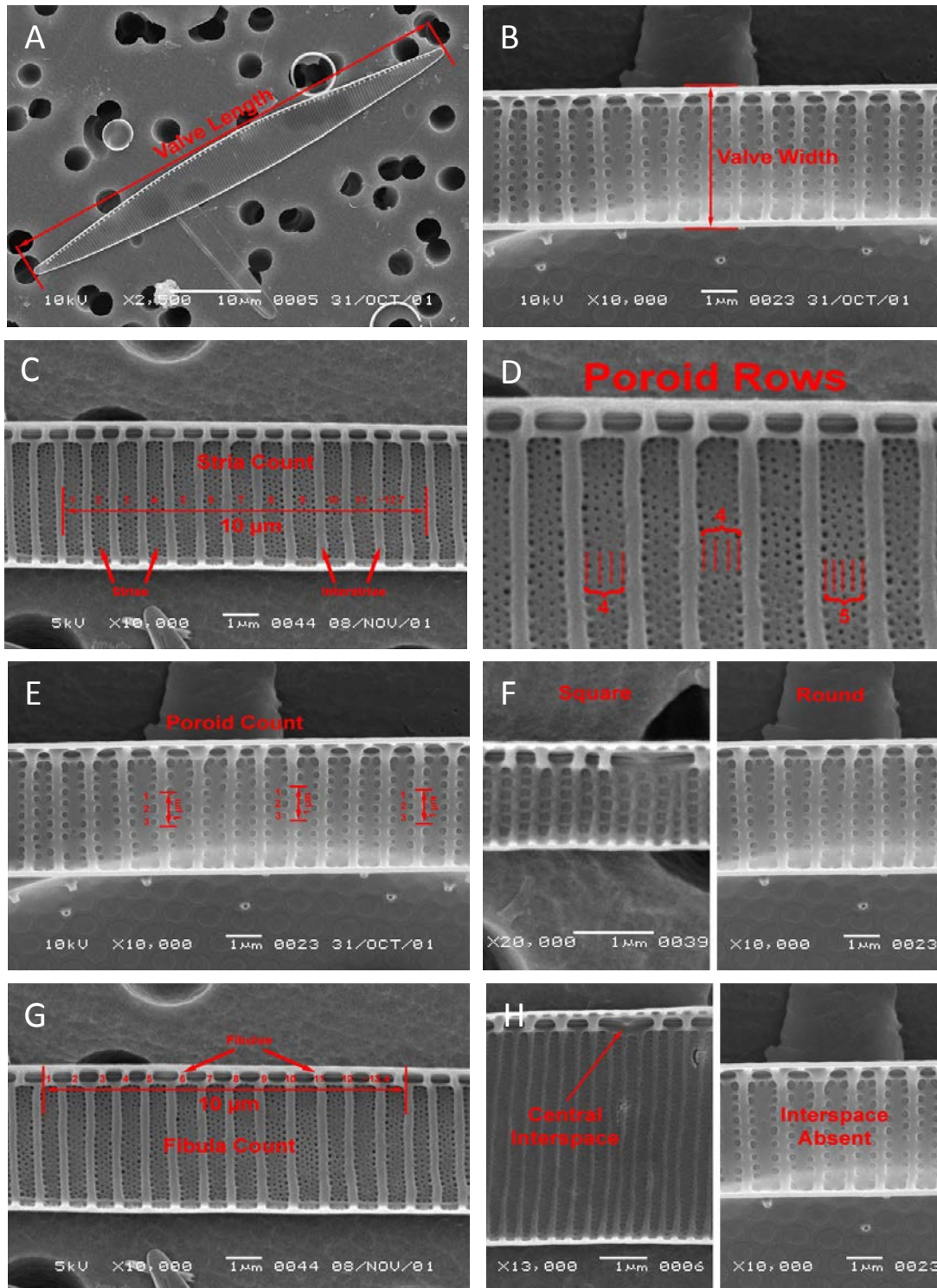
### 1.1.1 *Pseudo-nitzschia* morphology

The pennate diatom, *Pseudo-nitzschia*, is characterised by having an elongated narrow cell that consists of two silica halves or thecae that fit together to form a frustule (Hasle and Syvertsen, 1997). Inside each frustule are intricate patterns of poroids that connect intracellular material with the surrounding environment. These poroids are organised into compartments called striae and separated by interstriae. The number of poroids, striae and interstriae are unique to each species of *Pseudo-nitzschia* and are important distinguishing characteristics used for identification (Hasle and Syvertsen, 1997). The external canal wall of each frustule is characterised by the number of fibulae present and the presence or absence of a central interspace. It is in this external canal wall that the strongly eccentric raphe system can be found that enables chains of *Pseudo-nitzschia* cells to be motile (Hasle and Syvertsen, 1997). *Pseudo-nitzschia* cells often form long chains by the overlapping of their frustules at the cell tip. These long chains have been described as ‘stepped colonies’ made up of hundreds of cells (Bates, 2000).

Morphological characteristics (Fig. 1.1) that are used for the identification of *Pseudo-nitzschia* to species level (Hasle and Syvertsen, 1997):

- Length/width/shape of cell
- Number of fibulae and interstriae per 10  $\mu\text{m}$
- Number of poroids per 1  $\mu\text{m}$
- Number of poroid rows per stria
- Presence/absence of central interspace
- Shape of Poroids- Round or Square
- Length of overlap of cell ends
- Shape of valve/cell ends





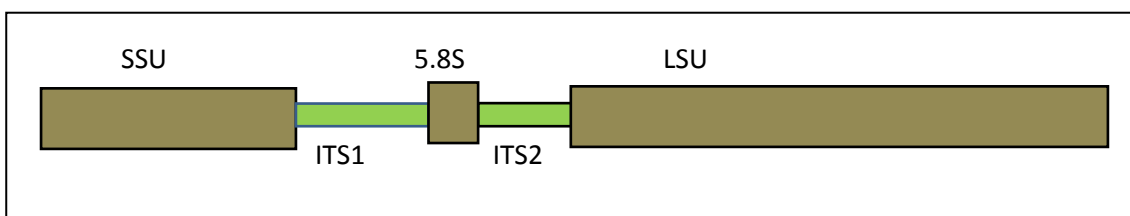
**Figure 1.1:** SEM images of a variety of *Pseudo-nitzschia* species frustules illustrating how morphological characteristics are measured; (A) valve length, (B) valve width, (C) stria and interstria per 10 µm, (D) number of rows of poroids, (E) number of poroids per 1 µm, (F) shape of the poroids, (G) number of fibulae per 10 µm, (H) presence or absence of a central space. Images were taken from the identification program '*Pseudo-nitzschia-lator*' (Ehrman and Kaczmarska, 2004).

Using a light microscope (LM), basic morphological information such as the length, width and shape of *Pseudo-nitzschia* cells can be obtained, but this alone does not allow identification to species level. From this information *Pseudo-nitzschia* cells can be crudely divided into two groups or complexes, the *Pseudo-nitzschia seriata*-group and the *Pseudo-nitzschia delicatissima*-group (Kaczmarska et al., 2007; Quijano-Scheggia et al., 2008). These groups are classified by the width of the cells/frustules in valve view with *Pseudo-nitzschia seriata*-groups containing species with frustule widths of  $> 3 \mu\text{m}$ , and *Pseudo-nitzschia delicatissima*-groups containing species with frustule widths of  $< 3 \mu\text{m}$ . It is only by viewing *Pseudo-nitzschia* cells using electron microscopy (EM) that it is possible to identify cells to species level. Using EM, information on the number of poroids, striae, interstriae, fibulae, and the presence or absence of a central interspace can be determined for each frustule (Fig. 1.1). To determine these morphological characteristics, cells must first be cleaned with an acid solution to remove outer organic matter surrounding the frustules. Once the organic matter has been removed the fine detail of the inorganic silica frustule can be resolved. This is normally achieved using transmission electron microscopy (TEM) to achieve a better image resolution. An electron beam passes through all the spaces in the frustules giving rise to a shadow image displayed in varied darkness which reveals the fine morphological structure of the cells. Another form of EM is scanning electron microscopy (SEM) which requires the cells to be coated in gold and palladium. SEM uses electrons to scan the cells, interacting with the gold coating, and provides a 3D image of the cell surface in fine detail. Due to the structure of *Pseudo-nitzschia* cells TEM provides much finer detail for species identification and therefore is the favoured method of EM. However, due to the variety and similarities in morphologies of some of these *Pseudo-nitzschia* species EM is not an unequivocal method for identification.

More commonly, in conjunction with EM, molecular methods are used for identification purposes.

### 1.1.2 Molecular identification of *Pseudo-nitzschia*

Identification of *Pseudo-nitzschia* from natural samples can often be problematic due to number of morphologically similar species or those species which are considered to be cryptic (genetically distinct but morphologically almost indistinguishable) (McDonald et al., 2007). As a result of this, molecular methods have been developed to facilitate the rapid and precise identification of *Pseudo-nitzschia* species. These methods are largely based on genus-specific primers which have been developed to amplify certain fragments/genes of rDNA (Fig. 1.2) within community DNA. These genes are amplified using a polymerase chain reaction (PCR). rDNA has long been used as a target operon because of its phylogenetic specificity and high copy numbers in the eukaryotic genome. The rDNA operon (Fig. 1.2) is composed of an external transcribed spacer (ETS) gene, a small subunit (SSU, 18S) gene, internal transcribed spacers 1 and 2 (ITS1, ITS2) genes, a 5.8S gene, a long subunit (LSU, 28S) gene and a non-transcribed spacer (NTS) gene.



**Figure 1.2:** The rDNA operon composed of the small subunit (SSU), internal transcribed spacers (ITS1 and ITS2), 5.8S, and the large subunit (LSU) gene regions.

Each of these target genes provides a different level of genetic information depending on the species and it is the variation in this genetic information which enables species identification. For example, *P. cuspidata* was identified using primers designed to amplify the ITS1 region, while the LSU region failed to separate this taxon from *P. pseudodelicatissima* species (McDonald et al., 2007). The ITS1 and ITS2 regions are known to be highly variable at both inter- and intra-specific levels for *Pseudo-nitzschia* species and may be the best molecular marker for elucidating *Pseudo-nitzschia* taxonomy (Orsini et al., 2002; Hubbard et al., 2008; Orive et al., 2010). LSU and SSU regions have been found to be good markers to distinguish among *Pseudo-nitzschia* species but display a lower variability and therefore are less effective at resolving phylogeny than ITS regions (McDonald et al., 2007; Moschandreou et al., 2010). Another marker used in *Pseudo-nitzschia* identification is the *rbcL* (chloroplast Ribisco large subunit) gene. This gene has been used extensively in the study of photosynthetic eukaryotes (Lindstrom and Fredericq, 2003). The *rbcL* region, like the ITS region, is highly variable and therefore a good marker to distinguish between species (McDonald et al., 2007). Due to the differences in the genetic information provided by these target regions, studies frequently use a combination of these markers to increase phylogenetic resolution and method robustness.

Other PCR molecular methods for the rapid identification of *Pseudo-nitzschia* species have also been developed. One of these methods is based on automated ribosomal intergenic spacer analysis (ARISA) which uses PCR primers to amplify DNA fragment lengths from the ITS region for different *Pseudo-nitzschia* species (Hubbard et al., 2008). This region was targeted because ITS sequences and fragment lengths can vary between species and morpho-types. ITS PCR Primers used for ARISA are fluorescently labelled and once the PCR products are analysed, fluorescent peaks representing various

fragment lengths are determined. Sequences and fragment lengths are compared for different *Pseudo-nitzschia* species and variations in them can be used to differentiate between species or individuals within a species. This method can be effective and more rapid than some microscopic approaches but needs to go through rigorous validation. A good understanding of the underlying taxonomy and sequence information is essential (Trainer et al., 2012). Another PCR method is based on high-resolution melt curve analysis and species-specific quantitative PCR (Q-PCR) (Andree et al., 2011). In this method species-specific primers (ITS1, ITS2 and 5.8S) were developed for Q-PCR assays to identify and quantify *Pseudo-nitzschia* species from natural community assemblages (Andree et al., 2011). Species-specific Q-PCR is followed by melt curve analysis which measures the point at which DNA strands disassociate when heated. This measurement varies according to *Pseudo-nitzschia* species and therefore can be used as a tool to discriminate between species and strains. This method was designed to supplement optical microscopy. While this method shows potential for monitoring purposes there have been some discrepancies between Q-PCR and microscopic determinations of *Pseudo-nitzschia* cell concentrations (Trainer et al., 2012). Further investigation is needed into the development of species-specific primers which would make this method more precise and accurate.

As an alternative molecular method to PCR, fluorescently labelled DNA-targeted probes have also been developed to distinguish between *Pseudo-nitzschia* species in culture and natural samples. Oligonucleotide probes have been developed that target specific gene regions of rDNA within *Pseudo-nitzschia* cells (Vrieling et al., 1996). Through the application of whole cell hybridisation with these probes a number of *Pseudo-nitzschia* species have been identified (Scholin et al., 1997; Miller and Scholin, 1998). Whole cell hybridisation allows identification of specific organisms while

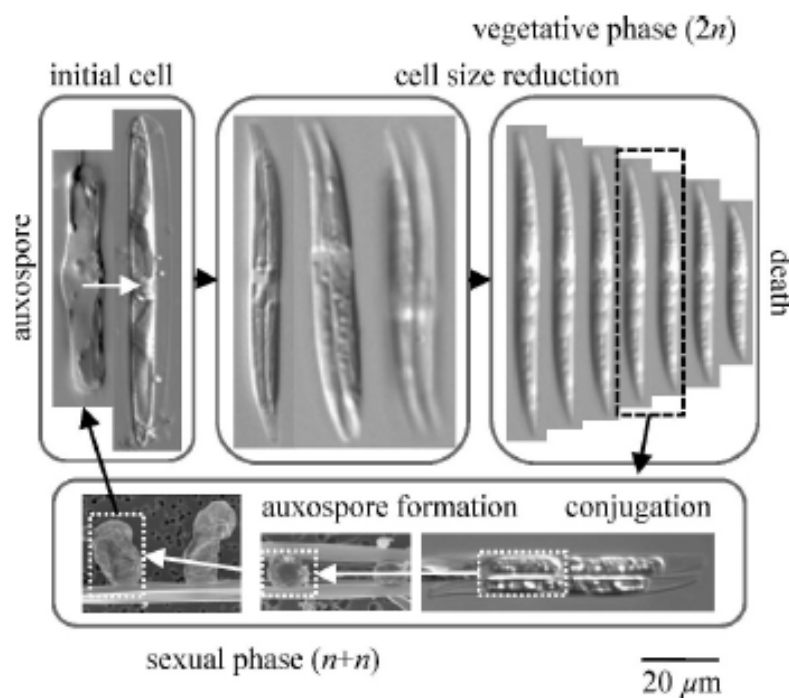
retaining their cellular shape and integrity (Miller and Scholin, 1998). This method requires cells to be fixed to preserve macro-molecular structure, followed by an alcohol rinse to permeabilise the cells. These cells are then exposed to fluorescently labelled oligonucleotide probes either on a microscope slide or by prior mixing. These cells are incubated and then excess probes are rinsed from the samples. Samples are then viewed under an epifluorescent microscope or using flow cytometer.

In contrast to whole cell hybridisation, sandwich hybridisation is another method used alongside fluorescently labelled probes. In this method, samples containing cells are homogenised to release their cell contents, including DNA (Scholin et al., 1997). At this point two separate hybridisation reactions ensue: capture of target DNA using an oligonucleotide probe, and binding of a signal probe to a sequence near that of the capture site. Visualisations of capture probe/rDNA/signal probe ‘sandwiches’ are accomplished enzymatically, yielding colorimetric or chemiluminescent products (Scholin et al., 1997). Both methods have been found to be useful for identification of *Pseudo-nitzschia* species (Rhodes et al., 1998a; Parsons et al., 1999). However, sandwich hybridisation methods have been found to be the most rapid and least labour intensive method to execute especially in the identification of *P. australis* (Scholin et al., 1997).

### **1.1.3 The life cycle of *Pseudo-nitzschia***

Like most other diatoms, the pennate diatom *Pseudo-nitzschia* has a complex lifecycle. For example, cells go through a prolonged period of mitotic division where each daughter cell receives one parent cell theca, as an epitheca, and then forms its own hypotheca (Hasle and Syvertsen, 1997). Through this form of asexual reproduction the

size of the cell frustules gradually decreases with time (Fig. 1.3). Once cells have diminished to ca. 30 % (varies between species) of their original size sexual reproduction occurs to regenerate the large cell size (Davidovich and Bates, 1998). Sexual reproduction involves meiosis, the conjugation of two types of gametes, gamete fusion, and the formation of a specialised zygote, the auxospore (Fig. 1.3). The auxospore expands to form a cell of maximum size. This can be two or three times the size of the parent cells (Quijano-Scheggia et al., 2009).



**Figure 1.3:** A representative schematic of the life cycle of the pennate diatom *P. multistriata*. Taken from D’Alelio et al. (2010). The schematic depicts the progressive reduction in cell size from a large initial cell (auxospore) after several stages of asexual reproduction in the vegetative phase. Once the cell size has reached a threshold (ca. 39-55 % of the original size in *P. multistriata*), below which the population crashes, conjugation occurs of two gametes from compatible cells and these fuse together to form a zygote. The zygote, now called an auxospore, elongates and hosts the formation of a long initial cell.

Once cells have decreased to a threshold size, sexual reproduction can only take place if there are compatible strains present to copulate with. These compatible strains have to be from the opposite mating type, as *Pseudo-nitzschia* species have a heterothallic mating system. This means that separate clones, known as mating types, produce male and female gametes. Successful sexual reproduction occurs when there are lots of *Pseudo-nitzschia* in the water column and therefore a greater chance of cells from opposite mating types being present for conjugation (Amato et al., 2007). This is likely to occur during a bloom event which would lead to a further increase in cell numbers. Another requirement for sexualisation is that cells must be in good physiological condition and usually growing rapidly (Quijano-Scheggia et al., 2010). Under laboratory conditions the whole process of sexual reproduction and production of the auxospore has been found to take 2-4 days. Factors such as temperature, light intensity, and photoperiod have all been found to effect sexualisation (Davidovich and Bates, 1998; Hiltz et al., 2000; Quijano-Scheggia et al., 2009). Evidence has also been found for the release of a type of 'pheromone' that acts as a chemical cue to other sexually active *Pseudo-nitzschia* (Haché, 2000). Filtrates from sexually reproducing clones have been found to increase gamete production in other clones (Haché, 2000).

The toxicity of *Pseudo-nitzschia* species has also been related to sexual reproduction. For instance, cultures of *Pseudo-nitzschia* decrease in size over time and in conjunction with this they lose their ability to produce DA (Bates, 1998). However, in vegetative cultures of *P. multiseries* that have lost their ability to produce DA, once they have gone through sexual reproduction, their offspring are able to produce DA and in some cases are more toxic than the parent cells (Bates et al., 1999).



## **1.2 *Pseudo-nitzschia* spp. and DA in the western English Channel**

Domoic acid (DA) is regularly detected in shellfish samples along the south west coast of England, occurring in areas such as Salcombe, Fowey, Fal, Falmouth, Taw, and Plymouth (Stubbs et al., 2007a; Stubbs et al., 2008). These areas contain classified shellfish harvesting and relaying sites and are monitored for biotoxins by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) on behalf of the Food Standards Agency (FSA). The main shellfish species cultivated in England and Wales are mussels and oysters (Rhodes et al., 1998b). The production of these species equated to 13,930 tonnes in 2010 (Rhodes et al., 1998b). Other cultivated species in England include clams and cockles (34 tonnes in 2010) (Rhodes et al., 1998b). The estimated value of the production (tonnes) of all of these species in 2010 was £9.5 million (Smith and Kitts, 1995). As well as farmed shellfish, scallop fisheries are also an important industry in England and Wales, especially in the south west of England (Smith and Kitts, 1995). Scallop beds are mainly harvested by dredging and can be found along much of the English side of the Channel. Landings from the western English Channel alone account for landings of approximately 6,000 tonnes between 2005 and 2008 with an increase to 8,000 tonnes between 2009 and 2011 (Smith and Kitts, 1995). This increase was largely due to an increase in offshore regions being harvested from 2009 onwards (Smith and Kitts, 1995).

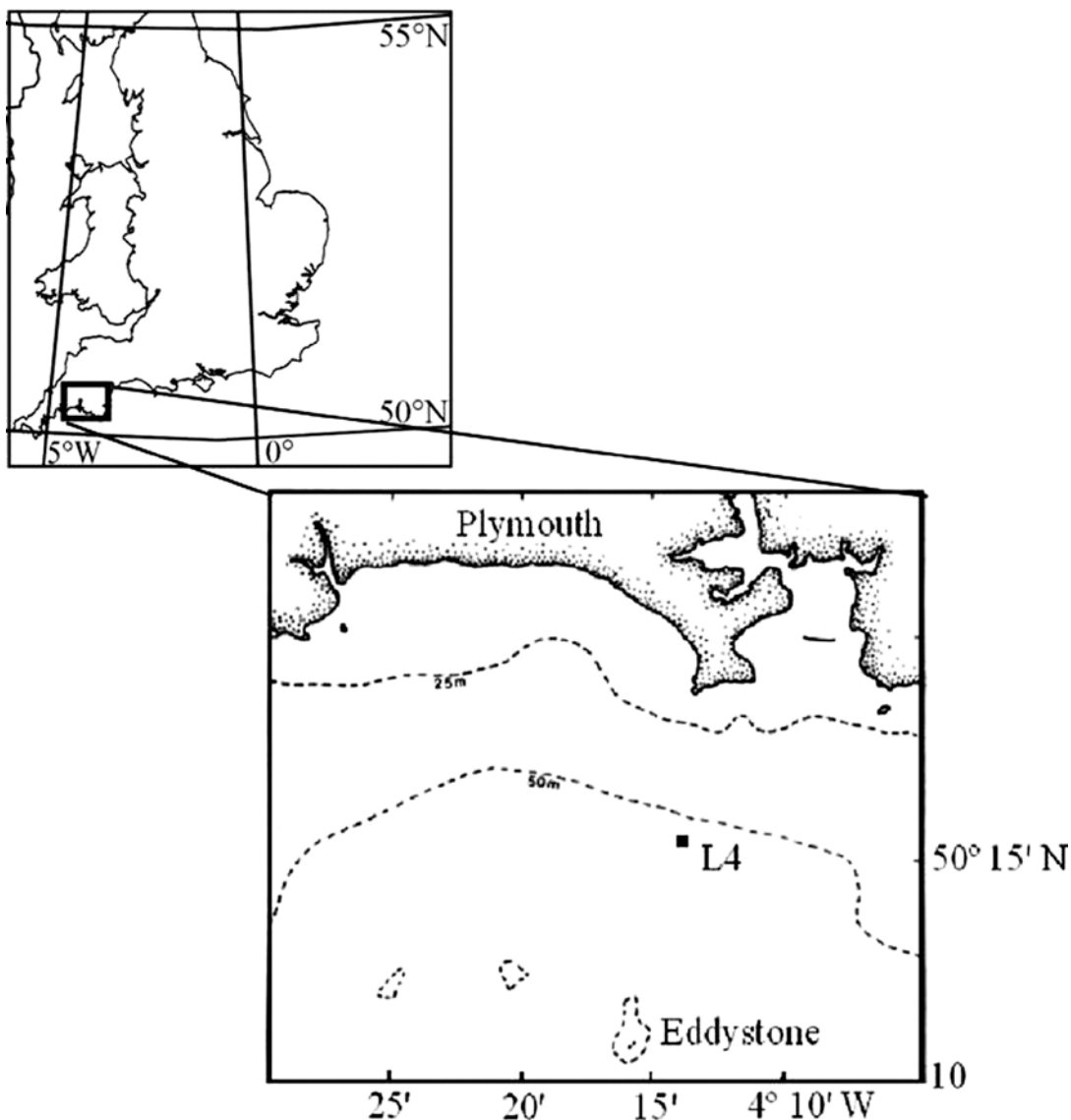
DA and associated amnesic shellfish poisoning (ASP) events have had little impact on the shellfish industry and scallop fisheries in the southwest of England to date (Stubbs et al., 2007b). Despite the detection of DA in some farmed shellfish areas, concentrations have been found to persist below the regulatory levels (20 mg kg<sup>-1</sup>). However, cultivated shellfish and scallop beds remain vulnerable to closures as a result of ASP, which could affect the economic value of these industries as seen in Scotland

and across parts of Europe. Therefore, it is important to monitor DA and improve our understanding of DA production in this region and how it might impact the shellfish industry and scallop fisheries in future years.

The occurrence of *Pseudo-nitzschia* spp. in these areas is also common and cell concentrations have exceeded action levels ( $150 \times 10^3$  cells L<sup>-1</sup>) on a number of occasions (Stubbs et al., 2007a; Stubbs et al., 2008). Studies of *Pseudo-nitzschia* spp. in the UK have shown that thirteen species have been identified in Scottish waters in total (*P. americana*, *P. australis*, *P. caciantha*, *P. cuspidata*, *P. decepiens*, *P. delicatissima*, *P. fraudulenta*, *P. multistriata*, *P. pseudodelicatissima*, *P. pungens*, *P. multiseries*, *P. cf. subpacifica* as well as an unidentified species (Bresnan et al., 2003; Fehling et al., 2006)) and six species have been identified in English waters (*P. australis*, *P. delicatissima*, *P. fraudulenta*, *P. pungens*, *P. pseudodelicatissima* and *P. multiseries* (Stubbs et al., 2007a; b)). However, only a small number of these species have been shown to produce toxins. For example, two species have been confirmed as toxin producers in Scottish waters (*P. seriata* and *P. australis*; (Fehling et al., 2004b) and only one so far from English waters (*P. multiseries*; (Bresnan et al., 2007)).

The research station L4 (50°15'N, 4°13'W) is a long term monitoring site for Plymouth Marine Laboratory situated approximately ten nautical miles south west of Plymouth in the English Channel (Fig. 1.4). It has a water depth of about 55 m and is subjected to the outflow of estuarine water from Plymouth Sound (Southward et al., 2005). The station is influenced by both seasonally stratified and transitionally-mixed stratified waters (Pingree and Griffiths, 1978) and experiences numerous phytoplankton blooms during the spring and summer months. Sampling occurs at this station weekly and has done so since 1992 (Southward et al., 2005). A collection of biological, physical, and

chemical measurements are also taken at this site (Southward et al., 2005). Such data have confirmed that *Pseudo-nitzschia* species persist from spring until late summer each year. These data have also shown that this species has exceeded 'safe' levels ( $150 \times 10^3$  cells  $L^{-1}$  (Stubbs et al., 2005)) at several points over the 16 years. These species were identified by light microscopy and have been separated into two complexes (*P. seriata*-group and *P. delicatissima*-group) and one species (*P. pungens*). However, it is not known whether any of the species found at this monitoring site are toxigenic.



**Figure 1.4:** Position of the monitoring station L4 in the western English Channel (WEC). Taken from Wyatt et al. (2010)

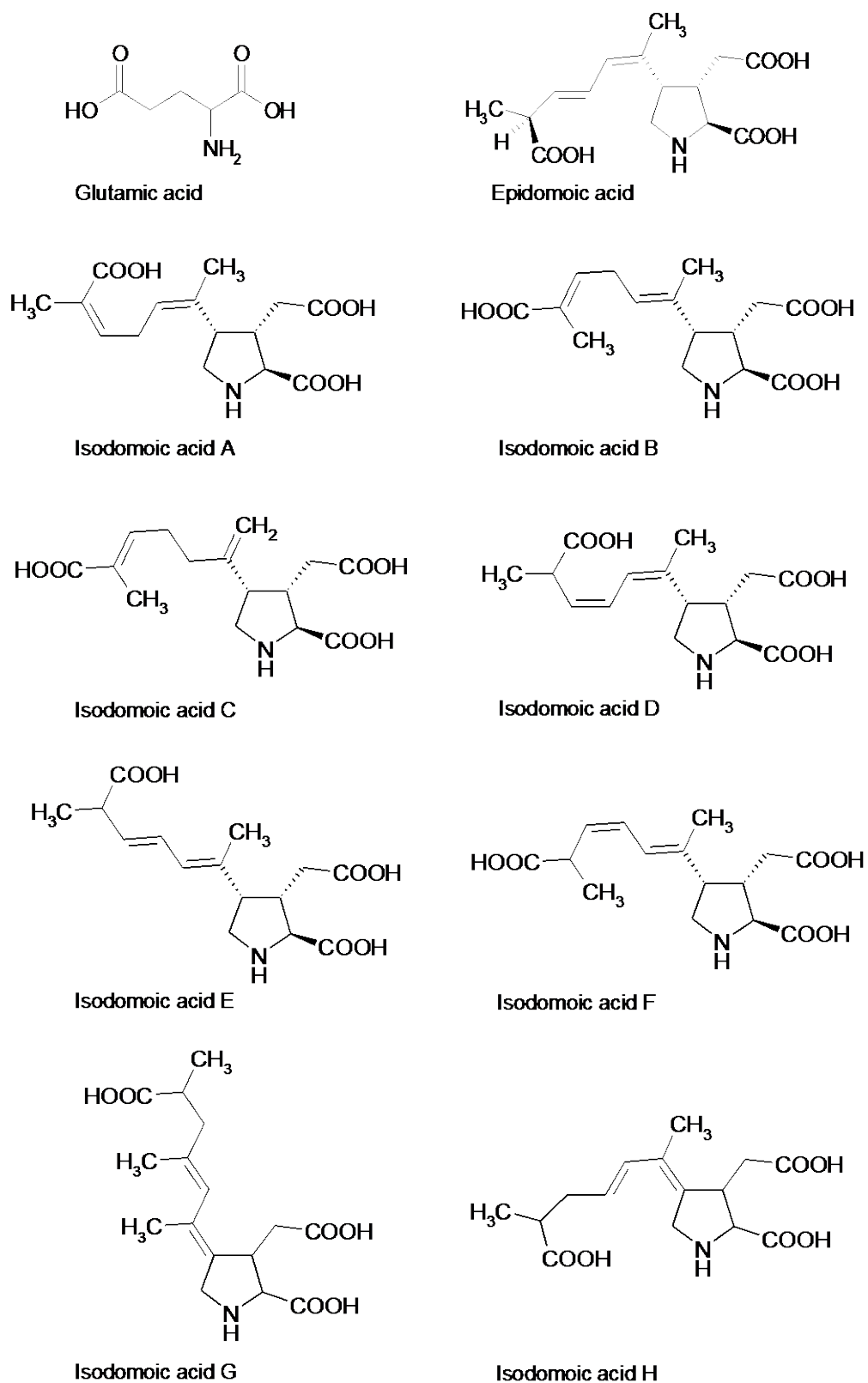
Long term time data series, while relatively rare, have proved an essential resource to study the variation and intensity of harmful phytoplankton and their environment (Hays et al., 2005; Raine et al., 2008). The 20 years of phytoplankton time series data collected at the monitoring station L4 (1992 to 2012) are valuable and a central tool that can be used to look at phytoplankton composition and abundance. Despite indications that harmful algal species are regularly detected in the western English Channel (WEC) and the existence of an extensive time series database at L4, there have been no studies to the author's knowledge to confirm (or otherwise) the occurrence of toxigenic *Pseudo-nitzschia* spp. at this site.

Whilst the monitoring station L4 in the WEC is located away from of any cultivated shellfish areas, there are extensive scallop beds close to this site. Previous DA monitoring by CEFAS has shown that DA concentrations are generally low in this region which may suggests that scallop fisheries are unlikely to be impacted by ASP events. However, this may not always be the case, and as well as impacting on scallops, DA may also impact higher trophic levels such as sea birds and mammals through bioaccumulation in this region. More information is given on bioaccumulation and trophic transfer of DA in the following Section (Section 1.3.1).

### **1.3 Domoic acid**

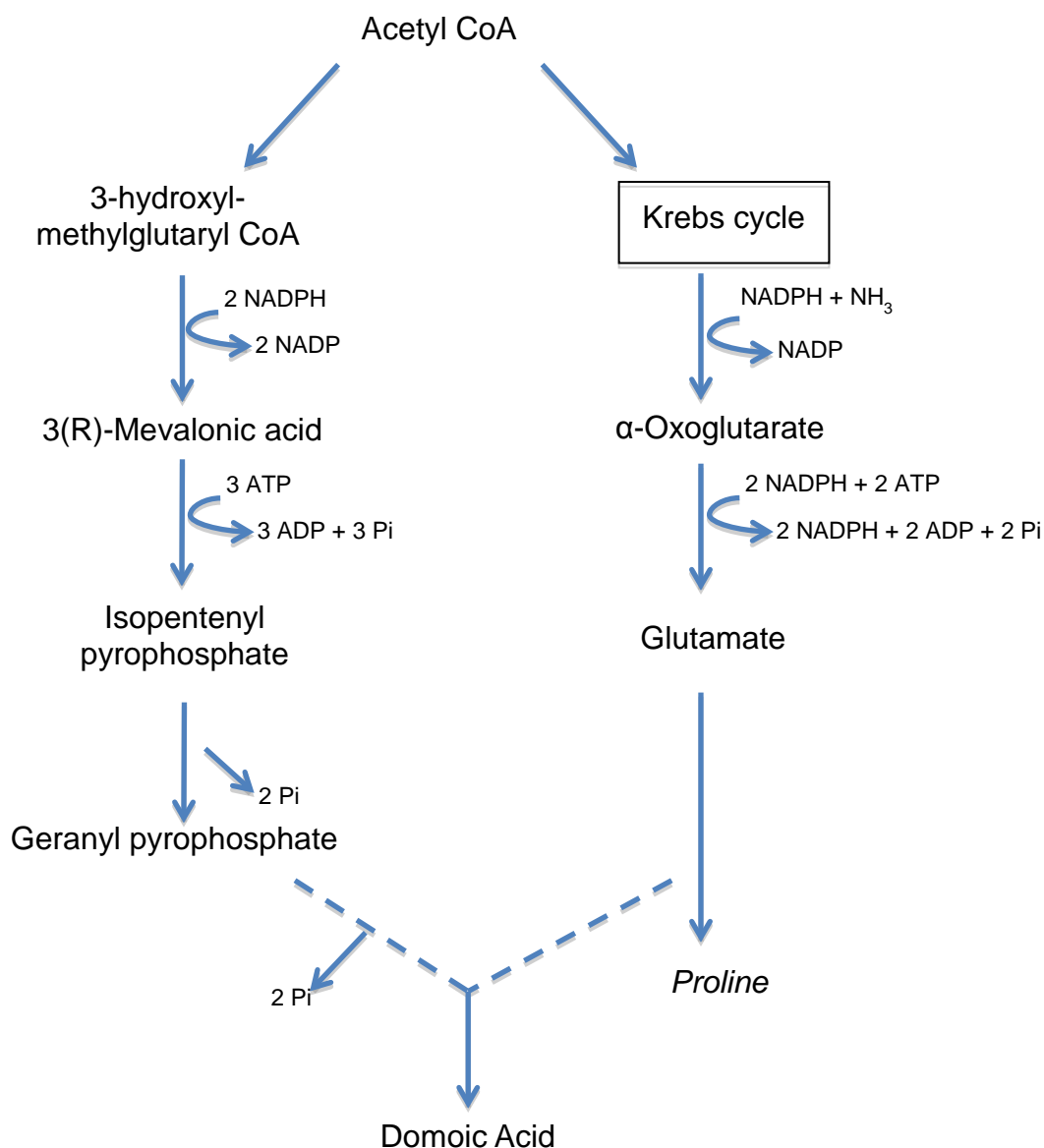
Domoic acid (DA) is a small molecule (molecular weight 311.14 Da) containing three carboxylic groups which make it highly hydrophilic and polar. DA belongs to a group of amino acids called the kainoids, which are neuroexcitatory, and mimic the excitatory neurotransmitter L-glutamic acid (Fig. 1.5). It has several related geometrical isomers that make up the isodomoic acid group (isodomoic acid A, B, C, D, E, F, G, and H) and

the diastereoisomer epidomoic acid (Fig.1.5). Besides being detected in *Pseudonitzschia* spp., DA and its isomers, have been detected in a small number of macroalgae species from the family Rhodomelaceae (i.e. *Chondria armata*) and the diatom *Nitzschia navis-varingia* (Zaman et al., 1997; Kotaki et al., 2005; Romero et al., 2012).



**Figure 1.5:** Chemical structures for glutamic acid and the DA isomers, epidomoic acid and isodomoic acid.

Despite the biosynthetic pathway of DA only being partly resolved, it has been determined that DA is biosynthesised (Fig. 1.6) from the condensation of two precursor units; an activated citric acid cycle derivative and a geranyl moiety (Laycock et al., 1989; Douglas et al., 1992). These precursors are believed to be glutamate from the Krebs cycle and geranyl pyrophosphate from acetyl CoA (Douglas et al., 1992). A number of enzymes are required for the synthesis of these units as well as substantial amounts of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) (Pan et al., 1998). It is understood that the condensation of these precursors forms the proline ring portion of the DA molecule (Laycock et al., 1989; Bates, 1998).



**Figure 1.6:** Schematic diagram of the potential pathway of domoic acid biosynthesis. Redrawn from Pan et al. (1998)

DA strongly binds to glutamate receptors in the human brain where it stimulates nerve cells to transmit impulses continuously until the cells die, causing destructive neuronal depolarisation and short term memory loss (Van Dolah, 2000; Clayden et al., 2005). Other symptoms in humans include dizziness, nausea and vomiting, ultimately sometimes leading to coma and brain damage. Symptoms can occur within 30 minutes to 24 hours after consumption of contaminated shellfish. The isodomoic acid group are considered to be less toxic than DA due to the fact the isomers in this group bind less



strongly to glutamate receptors. The effects of DA were first recorded in 1987 in Canada, where over 100 people became ill and three died after consuming DA contaminated mussels (Wright et al., 1989; Bates, 1998). DA poisoning, sometimes known as amnesic shellfish poisoning (ASP), occurs as a result of a population explosion of toxigenic cells of the *Pseudo-nitzschia* to sufficiently high concentrations for the toxins to be harmful to human health. Outbreaks have also affected marine birds (pelicans and cormorants), and marine mammals (sea lions and blue whales) (Scholin et al., 2000; Mos, 2001; Lefebvre et al., 2002a). DA may be detected and quantified from shellfish, seawater, and culture samples by a number of sensitive analytical methods, which are discussed in detail in Chapter 2.

### **1.3.1 The trophic transfer of DA**

DA has been found to enter the food chain through benthic and filter feeding marine invertebrates. The toxin mainly accumulates in the digestive glands of shellfish, with no observed toxicological effect to the organism. The negative impacts of the toxin come from bioaccumulation in the shellfish which are then consumed by higher organisms. Common shellfish vectors of domoic acid are clams, oysters, mussels, razor clams, sand crabs, cockles, scallops and cuttlefish (Horner and Postel, 1993; Wekell et al., 1994; Vale and Sampayo, 2001; Blanco et al., 2002; Campbell et al., 2003; Costa et al., 2005; Smith et al., 2006). All these species accumulate DA in different glands and for different lengths of times, which could ultimately effect the biotransfer into higher organisms and their overall toxic impact (Costa et al., 2005). Other vectors include copepods, krill, and cephalopods (Lincoln et al., 2001; Bargu and Silver, 2003; Costa et al., 2005). Planktivorous and omnivorous fish can also act as trophic vectors of DA, accumulating toxin in their gastrointestinal tracts (Fritz et al., 1992; Scholin et al.,

2000). Such species include northern anchovy, mackerel, sardines, sole, and turbot (Fritz et al., 1992; Scholin et al., 2000; Vale and Sampayo, 2001; Busse et al., 2006; Vigilant and Silver, 2007).

ASP events have only been found to involve three trophic levels, starting with *Pseudo-nitzschia* spp., then concentration by a bivalve or planktivorous fish and eventually consumption by humans, marine mammals or marine birds. Marine birds, such as brown pelicans and Brandt's cormorants have suffered from ASP events which have led to mass mortalities of these birds (Fritz et al., 1992; Sierra-Beltrán et al., 1997). Two species of planktivorous fish (mackerel and anchovies) were responsible for the transfer of DA in these events (Fritz et al., 1992; Work et al., 1993; Sierra-Beltrán et al., 1997). Surviving birds showed sub-acute ASP symptoms including disorientation and agitation, as well as swimming and flying impairment (Sierra-Beltrán et al., 1997). The transfer of DA to marine mammals has also been the cause of a large number of mass mortalities since the 1990s (Gulland et al., 2002). ASP has been found to cause neurological dysfunctions, such as seizures, disorientation and to produce scratching in populations of Californian sea lions, as well as live strandings and mortality (Gulland et al., 2002). ASP toxins have been found to impact on southern Californian sea otters (Kreuder et al., 2003), long beaked common dolphins (Sierra-Beltrán et al., 1998), humpback whales, and blue whales (Lefebvre et al., 2002a).

### **1.3.2 The ecological and physiological role of DA**

Despite over 20 years of research on toxic *Pseudo-nitzschia* species, the physiological and ecological role of DA still remains unclear (Bates, 1998). Like other harmful algal bloom (HAB) toxins, DA, is considered to be a secondary metabolite. Secondary

metabolites are those compounds that do not appear to participate in growth and development (Plumley, 1997). They are usually specific to species or taxonomic groups and they may have several functions, many unknown. Some secondary metabolites have been shown to have important adaptive significance, aiding organism survival and defence in competitive environments (Croteau et al., 2000). Their role may be intrinsic (e.g. protection from UV light or to store intracellular nutrients) or extrinsic (e.g. toxic to predators, allelopathic substances, or metal scavengers such as sideriophores) (Plumley, 1997).

A number of hypotheses have been proposed and tested for the function of DA specifically:

One simple hypothesis is that DA may serve as a way of dispensing of excess photosynthetic energy when cells are no longer able to grow. This is consistent with the hypothesis that DA is synthesized when photophosphorylated high-energy intermediates such as ATP are not used for primary metabolism (Pan et al., 1996a; Pan et al., 1996b). This also correlates with the fact that the majority of DA is excreted from the cells under these conditions (Bates et al., 1991; Pan et al., 1996a).

The role of DA as an antifeedant has also been studied on zooplankton grazers (e.g. copepods) and more recently in micro-zooplankton grazers (dinoflagellates) (Turner and Tester, 1997; Olson et al., 2008). However, DA in both cases had no functional role in deterring predators from grazing on toxigenic *Pseudo-nitzschia* cells.

Another hypothesis was that DA may act as an allelochemical, which is a compound that is deleterious to other algae and may provide a competitive advantage to the species. This would explain why *Pseudo-nitzschia* spp. blooms have been observed to be long lasting monospecific blooms. This hypothesis has been tested on two diatom species e.g. *Chaetoceros gracilis* and *Skeletonema costatum* (Windust, 1992) and four other algal species *Chrysochromulina ericina*, *Heterocapsa triquetra*, *Eutreptiella gymnastica* and *Rhodomonas marina* (Lundholm et al., 2005). These studies determined that there were no allelopathic effects of ASP toxins to phytoplankton (Windust, 1992; Lundholm et al., 2005).

A number of studies have provided convincing evidence for the role of DA in iron acquisition and alleviation of the toxic effects of copper in the marine environment (Rue and Bruland, 2001; Maldonado et al., 2002; Wells et al., 2005). Due to the similarity in structure of DA to other phytosiderophores it has been suggested that the role of the toxin may be to chelate trace metals, making them more or less bioavailable (Rue and Bruland, 2001; Maldonado et al., 2002). As phytoplankton are normally able to take up only 'free', unbound trace-metal ions, the ligand may help to keep 'excess' trace metals sequestered in a form that is not directly bioavailable (Sunda and Huntsman, 1995; Sunda et al., 2005). However, as the phytoplankton cells take up the free ion, more of the trace metals become bioavailable as they dissociate from the ligand (Sunda et al., 2005). This hypothesis was tested experimentally and studies have demonstrated the DA does indeed bind iron and copper and that the production and active release of this toxin is stimulated under conditions of iron or copper stress during the exponential phase of growth (Maldonado et al., 2002; Wells et al., 2005).

## **1.4 Factors influencing DA production in *Pseudo-nitzschia* spp.**

There have been many laboratory studies focusing on the effects of a variety of individual environmental factors that might trigger DA production in *Pseudo-nitzschia* spp. The study of these toxigenic species in culture has furthered an understanding of the intricate relationships between *Pseudo-nitzschia* spp. and DA production in the environment. It has also strongly influenced the interpretation of many field studies conducted in the last decade. Much of the research to date has focused on a small number of toxigenic *Pseudo-nitzschia* spp. including *P. multiseriata*, *P. seriata*, *P. australis*, *P. delicatissima*, and *P. pseudodelicatissima*. Of these species, *P. multiseriata* has been studied the most extensively and many of the key findings have been extrapolated to other species. This may be because *P. multiseriata* has been known the longest as it was the first species to be identified as a toxin producer and it is also found in most locations around the world. The paucity of laboratory studies on other *Pseudo-nitzschia* spp. has meant assumptions have been made about the timings of DA production in the growth phase without prior testing. Recent studies have shown that there is great amount of variability in DA production between species and that there is variation within species. This reinforces the need for further studies of DA production on other toxic *Pseudo-nitzschia* species.

### **1.4.1 DA production and growth cycle**

Early studies using *P. multiseriata* have shown that, in this species, DA production does not occur in the exponential growth phase but becomes evident towards the onset of the stationary phase (Bates et al., 1991). At this point cell division either slows down or ceases altogether, causing an increase in DA production throughout the stationary

phase. The two triggering mechanisms for DA production appear to be the slowing of division rate and depletion of phosphate and silicate in the media. As *P. multiseri* cultures progress through the stationary phase and as cultures age, increasing amounts of DA are released from the cells into the medium (Bates et al., 1991; Pan et al., 1996a; Pan et al., 1996c).

DA production has also been found in semi-continuous cultures and continuous cultures using *P. multiseri* (Bates et al., 1996; Kudela et al., 2002). Under these conditions DA is produced as a result of nutrient stress, despite the fact the cells are continuing to divide and do not reach a stationary phase. Results show that, under silicate limited continuous culture conditions, the rate of division impacts on the amount of DA produced (Bates et al., 1996). For instance, increased concentrations of DA were detected in those cultures dividing slowly compared to fast dividing cultures (Bates et al., 1996). In a review by Pan et al. (1998) two explanations were given for the effects of nutrient limitation on DA production: *i.* a decrease in primary metabolism making available the necessary precursors and high-energy compounds to be channelled into DA and *ii.* induced gene expression for regulating DA production.

Studies using *P. australis* have mostly shown that DA production is similar to that of *P. multiseri*, whereby toxins are produced in the late exponentially phase of growth (Cusack et al., 2002; Maldonado et al., 2002). However, some studies have found DA production in early exponential cultures (Garrison et al., 1992; Howard et al., 2007). Similarly, DA production in *P. seriata* species has been found to closely match production in *P. multiseri*. While traces of DA have been detected in the exponential phase, most of the production occurs during the onset of stationary phase. As with all these studies, the concentrations of DA produced were dependent on the culture

conditions (Lundholm et al., 1994; Fehling et al., 2004a). To date, only one study has examined DA production in *P. pseudodelicatissima*. This study found that cellular concentrations and net production of DA was highest in early exponential phase cultures (Pan et al., 2001). In contrast to *P. multiseriis* cultures, there was no net production of DA in the stationary phase of growth; instead, increased extracellular DA was measured (Pan et al., 2001).

#### **1.4.2 Macronutrients**

While nutrient depletion is known to trigger the production of DA in stationary phase cultures, the limiting effects of specific nutrients under controlled conditions can either enhance or decrease production. Nitrogen limitation, unlike phosphate or silicate limitation, has been found to be directly unfavourable for DA production by *P. multiseriis* (Bates et al., 1991). Nitrogen is a key structural element that is essential for DA synthesis and it has been demonstrated that nitrogen depleted batch cultures of *P. multiseriis* have failed to produce DA in the stationary phase until supplemented with a source of nitrogen, such as nitrate (Bates et al., 1991; Pan et al., 1998). Phosphate and silicate limitation have both been found to trigger DA production in *P. multiseriis* and *P. seriata* cultures (Pan et al., 1996a; Pan et al., 1996b; Fehling et al., 2004a). Silicate limitation has been found to yield increased concentrations of DA compared to phosphate limitation (Pan et al., 1996b). The impacts of these three nutrients and most importantly the interrelationships between all three will be discussed in further detail in Chapter 4.

### **1.4.3 Nitrogen source**

A number of studies have examined the effects of different nitrogen sources on *Pseudo-nitzschia* growth and DA production. Bates et al. (1993) altered the concentrations of ammonium and nitrate across five different treatments and found enhanced DA production at elevated ammonium concentrations ( $> 200 \mu\text{M}$ ) when compared to cultures exposed to the equivalent nitrate concentration. This may be a response to ammonia toxicity; the latter can cause physiological stress on cells, thereby increasing DA production; or ammonium may be a more energetically favourable form of nitrogen for the synthesis of DA (Pan et al., 1998). The effects of nitrogen source have also been investigated for the species *P. australis*, which was grown in batch cultures with differing levels of nitrate, ammonium, glutamine or urea (Howard et al., 2007; Cochlan et al., 2008). Urea-grown cultures were found to have produced more DA than the ammonium or nitrate grown cultures, even though growth rate was reduced, demonstrating the capabilities of the diatoms to utilise both inorganic and organic forms of nitrogen. When examining the nutritional preference of *P. australis* to different nitrogenous substrates, cultures showed a preference for nitrate; in the absence of this, ammonium is preferred, followed by glutamine and lastly, urea (Cochlan et al., 2008). These results have led to the suggestion that urea and other forms of organic nitrogen substrates in coastal run off are more influential in bloom development and maintenance than previously thought (Howard et al., 2007; Cochlan et al., 2008).

### **1.4.4 Dissolved organic nutrients**

In conjunction with organic forms of nitrogen, the significance of dissolved organic nutrients as a whole (dissolved organic phosphorus and carbon) to the nutrition of *Pseudo-nitzschia delicatissima* has recently been explored by Loureiro et al. (2009b). This has been done through a series of semi-continuous uni-algal culture experiments.



These experiments examined culture growth, cell abundance, cell stoichiometry, and chlorophyll *a* cell concentration in response to L1 media combined with; dissolved organic matter (DOM) and either 1/5 of the normal nitrate concentration or no nitrate, or L1 media without the added DOM (Loureiro et al., 2009b). Results showed that while growth rates were similar across treatments, chlorophyll *a* concentrations reached higher levels in L1 medium without the addition of DOM and with a standard concentration of nitrate (nitrogen replete conditions). In contrast, lower concentrations of chlorophyll *a* were measured in L1 medium with the addition of DOM but no nitrate (nitrogen deficient treatment). This suggested that the nutrients required for culture growth and photosynthesis were acquired from the availability of DOM (Loureiro et al., 2009b). Loureiro et al. (2009a) also examined the uptake rates of *P. delicatissima* to ammonium and urea in culture experiments. It was found that like *P. australis*, *P. delicatissima* more readily acquires ammonium than urea in the absence of nitrate. Overall this study found that DOM positively affects the growth *P. delicatissima*. This was further confirmed in a separate set of field and experimental studies by Loureiro et al. (2009a) in Alfacs Bay, North West Mediterranean Sea. This study was able to show the importance of DOM to microalgal assemblage in the Alfacs Bay and how organic nutrients influence the occurrence and relative abundance of *Pseudo-nitzschia* in this embayment (Loureiro et al., 2009a; Loureiro et al., 2009b).

#### **1.4.5 Micronutrients**

The effects of the micronutrients iron and copper on the growth and DA production of *P. multiseriata*, *P. australis*, and *P. fraudulenta* cultures (batch and semi-continuous) have been studied (Bates et al., 2000; Maldonado et al., 2002; Wells et al., 2005). The purpose of these studies was to establish whether DA is able to bind to iron and copper,

altering their bioavailability (Rue and Bruland, 2001). This will be discussed in further detail in Chapter 4.

The effects of other trace metals on *P. multiseriis* growth and DA production have also been examined (Subba Rao et al., 1998; Zhiming and Subba Rao, 1998). One of these studies investigated the effects of lithium and found that cultures enriched with this trace metal produced substantially more DA (230 fg DA cell<sup>-1</sup>) than controls (135 fg DA cell<sup>-1</sup>) (Subba Rao et al., 1998). It is believed that in Cardigan Bay, where the first domoic acid episode was reported, high levels of lithium were evident in the water as a result of freshwater run-off and could have played a part in enhancing DA production leading to the ASP event in 1987 (Subba Rao et al., 1998). The effects of germanium have also been investigated and results have shown that increasing concentrations of this trace metal can inhibit *P. multiseriis* growth and DA production (Zhiming and Subba Rao, 1998).

#### **1.4.6 Temperature**

Few studies have examined the effects of temperature on toxigenic *Pseudo-nitzschia* spp. In a study by Lewis et al. (1993) batch cultures of *P. multiseriis* were exposed to five different temperature treatments (5 °C to 25 °C). Results showed that growth and DA production decreased with decreasing temperature. Growth rates increased from 0.25 d<sup>-1</sup> at 5 °C to a maximum of 0.65 d<sup>-1</sup> at 20 °C and the maximum DA concentrations occurred at 25°C. One theory for this observation is that high temperatures increase growth of the population thus accelerating the onset of the stationary phase by nutrient limitation (Mos, 2001) or that cells were experiencing physiological stress (Bates, 1998). In batch culture the stationary phase may be longer, the culture ages more, and

more DA is produced. In contrast to *P. multiseriata*, *P. seriata* has been found to produce higher levels of cellular DA at 4 °C than at 15 °C (Lundholm et al., 1994). Furthermore, in these studies morphological differences were observed in frustule formation between cultures grown at different temperatures (Lundholm et al., 1994).

#### **1.4.7 Irradiance and photoperiods**

Irradiance is an important factor for DA production as it influences photosynthetic ability and therefore ability to synthesise DA (Bates, 1998). There are a limited number of studies investigating the effects of varying irradiance levels and photoperiods on *Pseudo-nitzschia* species in batch culture (Bates et al., 1991; Pan et al., 1996c; Bates, 1998; Fehling et al., 2005). Studies have shown that in cultures of *P. multiseriata*, high irradiance has increased the growth of the population and accelerated the start of the stationary phase and culture ageing. In one study, five different irradiance levels were investigated, (53, 250, 410, 815, 1100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and optimal growth was found between 410-1100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , although DA was not measured in this study (Pan et al., 1996c). An earlier study by Bates et al. (1991) which examined the effects of two different irradiance levels (145 and 45  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) on DA production, reported that while DA was produced, there was no difference attributable to differences in irradiance levels. In the same Bates et al. study (1991) DA production ceased when the cultures were placed in the dark. This indicates a close coupling between photosynthesis and DA synthesis (Bates et al., 1991).

The effects of irradiance have also been investigated for *P. australis* with the focus on low irradiance (12  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and high irradiance (115  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) (Cusack et al., 2002). Only trace amounts of DA were produced under low irradiance

conditions. However, in contrast to the previous study on *P. multiseriata*, DA production increased with high irradiance conditions, starting in the late exponential phase and reaching maximum concentrations of 26 pg DA cell<sup>-1</sup> (Cusack et al., 2002). In addition, it has been shown in experiments using *P. multiseriata*, that DA production only decreases once irradiance levels fall below 35  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  (Bates, 1998).

#### 1.4.8 Photoperiods

As well as irradiance, the amount of time that cultures are exposed to the light (photoperiod) has been found to effect the growth and toxin production of *Pseudo-nitzschia* species. Previous laboratory experiments studying the impacts of light intensity on *Pseudo-nitzschia* species have all used variations in photoperiod, i.e. 12:12, 14:10, 16:8 h L:D and continuous light, but only one study has examined the effects of photoperiod on the growth and toxicity *Pseudo-nitzschia* species. Fehling *et al.* (2005) conducted experiments investigating the effects of spring photoperiods (SP 9 h:15 h L:D) and summer photoperiods (LP 18 h:6 h L:D) on *P. delicatissima* and *P. seriata*. In terms of growth (cells mL<sup>-1</sup> d<sup>-1</sup>) both species achieved lower growth under SP. However, *P. delicatissima* had a peak cell density under SP (Fehling et al., 2005). In contrast, *P. seriata* had peak cell density under LP conditions. The DA production was measured for *P. seriata* which showed increased production under LP. In both SP and LP most of the DA produced was released into the medium. When the results were standardised, taking the DA for the whole culture and calculating the DA produced per cell, there was more DA per cell for *P. seriata* under SP conditions (Fehling et al., 2005). This suggests that toxin production is likely to be a function of photoperiod and growth rate (Fehling et al., 2005).

#### **1.4.9 Salinity**

Three studies have examined the effects of salinity on the growth of four different *Pseudo-nitzschia* species: *P. pseudodelicatissima*, *P. delicatissima*, and *P. multiseriis* (Jackson et al., 1992; Bates, 1998; Thessen et al., 2005). *P. delicatissima* was found to be most tolerant to low salinities and *P. multiseriis*, least tolerant (Thessen et al., 2005). All species grew better at higher salinities and growth was optimal at 30-45 psu (Bates, 1998). Some *Pseudo-nitzschia* grew at salinities as low as 6.25 psu (Thessen et al., 2005). Although DA production was not investigated in these studies Thessen et al. (2005) made reference to the fact that commercially grown oysters grow in a salinity range of 10-20 psu, which suggests that toxigenic *Pseudo-nitzschia* species could persist in these areas and production of DA could occur (Thessen et al., 2005).

More recently Doucette et al. (2008) has explored the effects of salinity (10, 20, 30, and 40 psu) on the growth and production of DA by *P. multiseriis*. This study found that growth rates were maximal and comparable at the three highest salinities but decreased by half at 10 psu. The concentrations of DA per cell and the production rates were also similar and maximal at the highest salinities (30 and 40 psu) but declined by approximately 10 fold once cultures had acclimated to 10 and 20 psu. The main conclusion from this study was that the energetic costs of producing DA at low salinity ranges are too high, as more energy is needed to maintain growth and an osmotic balance.

#### **1.4.10 pH**

In a study by Lundholm et al. (2004) 11 species of *Pseudo-nitzschia*, and one *Nitzschia* species, were studied in response to pH drift in batch cultures, different initial pH

values, and pH and cell volume. The growth of most of these species stopped at pH values of 8.8 to 9.1, and reduced growth was found above a pH of 8.6. Only *P. multiseriis* produced DA in these experiments (Lundholm et al., 2004). The production of DA by this species was induced by elevated pH values; the amount of DA produced was comparable to that found in silicate and phosphate limited cultures (Lundholm et al., 2004). These pH values are representative of pH values found in oceanic systems where large blooms of microalgae occur. This shows the potential for high pH to inhibit cell growth and induce DA production in the field (Lundholm et al., 2004).

#### **1.4.11 Carbon dioxide (CO<sub>2</sub>)**

Marine phytoplankton species have been found to be sensitive to increases in the partial pressure of CO<sub>2</sub> (pCO<sub>2</sub>) in seawater causing a reduction in surface water pH (Boyd et al., 2010). This increase in pCO<sub>2</sub> is associated with CO<sub>2</sub> emissions released during the burning of fossil fuels which results in the progressive acidifying of the world's oceans. Recently, the effects of increased CO<sub>2</sub> on *Pseudo-nitzschia* spp. growth and toxin production were investigated in two studies which examined this factor along side nutrient limitation (Sun et al., 2011; Tatters et al., 2012). One of these studies focused on increasing pCO<sub>2</sub> concentrations (220, 400, 750 ppm), in a matrix with two phosphate concentrations (20 and 0.5  $\mu\text{mol L}^{-1}$ , replete and limited respectively), on cultures of *P. multiseriis* (Sun et al., 2011). It was found that DA concentrations were 30-50 times greater in phosphate-limited conditions and this increased again (4 fold) across cultures which were exposed to high pCO<sub>2</sub> concentrations (Sun et al., 2011). The second of these studies focused on pCO<sub>2</sub> (200, 360, 765 ppm) and silicate availability (106.1 and 10.6  $\mu\text{mol L}^{-1}$ , replete and limited respectively) on cultures of *P. fraudulenta* (Tatters et al., 2012). Results showed that, like phosphate limited *P. multiseriis*

cultures, cellular domoic acid concentrations greatly increased when cultures were exposed to high pCO<sub>2</sub> concentrations and low silicate availability. In both studies pCO<sub>2</sub> concentrations were representative of current conditions and projected future pCO<sub>2</sub> concentrations (765 ppm) for the year 2100 (Boyd et al., 2010; Sun et al., 2011; Tatters et al., 2012). It has also been found that in the Californian upwelling system the range of CO<sub>2</sub> concentrations have already been found to encompass concentrations used in these studies (Feely et al., 2008).

#### **1.4.12 Bacteria**

The interaction between bacteria and phytoplankton is increasingly becoming recognised as an important factor in the role of DA production and bloom dynamics (Bates et al., 2004; Stewart, 2008). Many studies using *P. multiseriis* have demonstrated that there is an increase in the production of DA in axenic cultures after bacteria have been re-added (Douglas et al., 1993; Bates et al., 1995; Osada and Stewart, 1997; Bates et al., 2004; Stewart, 2008). In non-axenic cultures treated with antibiotics such as ampicillin or streptomycin in order to eliminate bacteria, the reintroduction of bacteria enhanced DA production by 2 to 115-fold, depending on the *P. multiseriis* and bacterial strains used (Osada and Stewart, 1997; Bates et al., 2004). Previous studies have identified a direct relationship between increased bacterial abundance and increased DA production and *vice versa* (Bates et al., 1995; Hagström et al., 2007). However, the amount of DA produced depends on the particular bacterium added to the *P. multiseriis* culture. For instance, in those cultures with the native bacteria assemblage, DA production exceeded cultures where just a single species was added (Bates et al., 1995; Osada and Stewart, 1997).

Two main genera of bacteria have been associated with *P. multiseri*; *Moraxella* and *Alteromonas* sp. (Stewart et al., 1997). *Alteromonas* sp. has been shown to produce gluconic acid/gluconolactone, in the presence of glucose, which is then released into the medium (Osada and Stewart, 1997; Stewart et al., 1997). The presence of gluconic acid/gluconolactone in culture is expected to stimulate the production and release of DA (Osada and Stewart, 1997; Stewart et al., 1997). Gluconic acid/gluconolactone is thought to be a nutrient scavenger increasing nutrient stress on *P. multiseri* and inducing production of a counter chelating compound thought to be DA (Pan et al., 1998). In addition, the bacterial consortium has been observed to change over long-term cultures of *P. multiseri*, shifting to bacteria that, instead of stimulating DA production, utilize it. Once high concentrations of DA occur, due to nutrient stress in the stationary phase, available bacteria associated with its utilization increase in abundance and cause a decrease of DA in the medium (Stewart, 2008). Therefore at any one time the amount of DA measured is proportionate (amongst the many other factors discussed above), to the amount of DA produced by the diatoms, stimulated by bacteria and bacterial consumption of DA. This relationship can be affected by factors such as culture age and nutrient status and is considered very complex (Stewart, 2008).

## **1.5 Status of knowledge and direction needed**

It is apparent from the foregoing that there is a wealth of information on the production of DA by *Pseudo-nitzschia* species both in the natural environment and in culture studies. Since the discovery of domoic acid as a human health problem in 1987, there have been many studies monitoring the occurrence of *Pseudo-nitzschia* species worldwide. Methods have been developed to rapidly and reliably identify *Pseudo-*



*nitzschia* species using both light and electron microscopy and molecular methods. In the 1980s only 15 *Pseudo-nitzschia* species had been described but this number had risen to approximately 37 species in 2010. The same too can be said for the technologies and methods used for the detection and quantification of domoic acid (*vide infra*, Chapter 2). Developments in identification and quantification tools have allowed a better understanding of the environmental factors influencing *Pseudo-nitzschia* bloom development and toxin production. These have been studied through environmental monitoring programmes and various laboratory studies. Laboratory studies have also been used to understand the mechanisms behind toxin production, biosynthetic pathways and the fate of domoic acid. Toxicology studies have investigated the impacts of DA on ecosystem health, marine organisms and mammal health and human health. Despite the extensive research and progress achieved so far in this field, the unique nature of *Pseudo-nitzschia* and the many environmental complexities offers the potential for further research questions to be explored.

Monitoring programmes and environmental studies have shown that *Pseudo-nitzschia* species are present around the British Isles and English Channel. These programmes have shown that one or a number of these species might be toxigenic due to the detection of domoic acid in either shellfish samples or water samples (Stubbs et al., 2008). While coastal seas around the South West of the UK may not be problematic areas for amnesic shellfish poisoning currently, there is still little known about the *Pseudo-nitzschia* species that occur in these waters, their patterns of occurrence, and whether these species are toxigenic. The species detected in these waters to date have mainly been identified by light or electron microscopy but there have been no published molecular studies of the *Pseudo-nitzschia* species in the western English Channel so far as is known to the author. Furthermore, coastal regions globally are very diverse and

the relationships between environmental factors in these regions and the biology can be very different. Therefore, it is important to understand the regional environmental factors influencing *Pseudo-nitzschia* species and toxin production which is yet unknown in the western English Channel.

There are a number of methods that have been developed to quantify and monitor DA either in laboratory experiments or in the environment. These have been reviewed in Chapter 2. The main analytical methods used are high performance liquid chromatography either with ultra-violet (HPLC-UV) detection or fluorescence detection (HPLC-FD), liquid chromatography mass spectrometry (LC-MS), and Enzyme-linked Immunosorbent Assay (ELISA). These methods offer varying degrees of sensitivity depending on whether they are used to analyse DA in phytoplankton or shellfish tissue samples. Of these methods LC-MS proved to be the most accurate, sensitive, and reliable method to determine DA occurrence and concentration. Despite this, there are still improvements that could be made to existing published methods to increase their robustness and reproducibility. One such improvement would be the incorporation of an internal standard to increase the reliability of DA quantification.

Although much is known about the effects of various individual environmental factors on *Pseudo-nitzschia* species occurrence and DA production, these have largely been studied in the laboratory in isolation; few multifactorial studies have been made. It is known from environmental studies that many factors can impact on biological systems at any one time. Therefore, there is a question as to how multiple factors affect *Pseudo-nitzschia* species growth and DA production. Few studies have investigated the interrelated effects of multiple factors. Those few that have, found that synergisms do occur between certain factors which can translate to increases in cellular toxicity.

However, these relationships are far from clear and require considerable further exploration.

## 1.6 Aims and objectives

The aims of this study were:

- (1) To develop reliable, sensitive, accurate, and reproducible analytical protocols for the determination of domoic acid in seawater and in *Pseudo-nitzschia* spp. cells.
- (2) To explore the interrelationships between multiple environmental factors, *Pseudo-nitzschia* spp. diversity and occurrence, and toxin production at L4 in the western English Channel.
- (3) To investigate the impacts of both macro and micro-nutrients, using a multifactorial approach, on the dynamics of DA production and growth of *Pseudo-nitzschia* spp.

To this end;

- Chapter 2 evaluates the methods used to determine DA and explores the application of two of these methods, liquid chromatography-mass spectrometry (LC-MS) and enzyme-linked immunosorbent assay (ELISA), for DA identification and quantification. The validity of these methods was investigated in conjunction with protocols for sample purification and DA extraction. These methods were used to quantify particulate, cellular and dissolved DA in *Pseudo-*

*nitzschia multiseries* cultures and the precision and accuracy of these methods were compared.

- Chapter 3 investigates *Pseudo-nitzschia* spp. and DA occurrence, along with a range of environmental parameters, at the time-series monitoring site (L4) in the western English Channel from January to December 2009. The intra-annual variability of *Pseudo-nitzschia* species was examined, and using biological, physical, and chemical data the factors driving *Pseudo-nitzschia* spp. occurrence and DA production were explored.
- Chapter 4 examines the interrelationships between the effects of nitrate, phosphate, and silicate availability on *Pseudo-nitzschia multiseries* growth and DA production using statistical multifactorial experimental design. In conjunction with this the possible role of DA as a trace-metal chelator was explored by investigating the effects of macronutrient (phosphate and silicate) and micronutrient (iron and copper) availability.
- Chapter 5 uses molecular techniques to determine *Pseudo-nitzschia* species diversity at four time points during 2009 at L4 in the western English Channel. This involved method development for the appropriate primers used to amplify the *Pseudo-nitzschia* spp. DNA from this region. The use of 454 high throughput sequencing is explored in this chapter.
- Chapter 6 seeks to synthesise the major findings from the experiments and observations carried out within this study in order to augment current understanding of the impacts of environmental factors of *Pseudo-nitzschia* spp. occurrence, growth, and DA production and to identify key knowledge gaps for future study. Finally, the current knowledge gaps regarding coastal ecosystems and harmful algal blooms in a changing world are explored.



**Chapter 2: Evaluation, refinement and comparison of  
methods for the extraction and analysis of domoic acid in  
seawater and *Pseudo-nitzschia* spp.**



## Abstract

An overview of methods currently available for the extraction, concentration, detection and quantification of DA in seawater and algae are presented. These methods have evolved from the first mouse bioassay methods to the more accurate and reliable methods employing liquid chromatography-mass spectrometry (LC-MS) which are now amongst the recognised standard methods (e.g. of the EU marine biotoxins Reference Laboratory). Nonetheless, after reviewing these methods it became apparent that these methods could be improved further making them more robust and precise. Thus, the extraction and isolation of DA from seawater and algal cells using solid phase extraction was examined. To reproducibly quantify DA concentrations in these substrates, a published LC-MS method required further refinement and optimisation, including the selection and use of a suitable internal standard to improve the reliability and precision of the DA determinations. Once an optimised LC-MS method had been established, a systematic comparison was made with results obtained using a published method for DA detection based on Enzyme-linked ImmunoSorbent Assay (ELISA) in cultured *Pseudo-nitzschia multiseries* cells.





## **2.1 Analytical methods for domoic acid determination**

### **2.1.1 Chromatographic methods**

Since the first identification of domoic acid (DA) in blue mussels in Canada in 1987 by high performance liquid chromatography (HPLC) and as countries around the world became more concerned about toxigenic DA-producing algae reaching their coastlines in quantity, there has been a requirement for reliable, reproducible and rapid methods for the detection and quantification of DA in seawater, algae and contaminated shellfish and fish (Wright et al., 1989; Quilliam, 2003; Litaker et al., 2008). The methods used for DA detection and quantification have evolved over the last two decades (Quilliam, 2003). One of first methods used to study DA in shellfish samples was the mouse bioassay (Wright et al., 1989). In this method, mice were injected in the intraperitoneal cavity with extracts from mussels contaminated by ingestion of toxigenic algae. This was followed by an observation period to determine symptoms and time-to-death (Wright et al., 1989). In this particular case, the mice showed signs of scratching, weakening and eventual death, depending on the amount of toxin present in their system (Wright et al., 1989). Following these early test results, DA was instead separated and purified from shellfish extracts using high performance liquid chromatography (HPLC), high voltage paper electrophoresis or ion-exchange chromatography (Wright et al., 1989). DA characterisation was carried out using ultraviolet absorption spectrophotometry, infrared spectroscopy, mass spectrometry, or nuclear magnetic resonance (NMR) spectroscopy (Wright et al., 1989). While the mouse bioassay successfully detected DA, it became apparent that the limit of detection (LOD) for this method ( $40 \mu\text{g DA g}^{-1}$ ) was higher than the regulatory level ( $20 \mu\text{g DA g}^{-1}$ ) set by the Canadian authorities. This meant that the mouse bioassay could not be used for routine monitoring purposes.

### **2.1.1.1 HPLC methods**

The first chemical analytical method used for routine monitoring of DA was HPLC with ultra-violet diode array spectrophotometric detection (HPLC-UV) (Lawrence et al., 1989; Lawrence et al., 1991). Domoic acid, unlike other algal toxins, has a strong chromophore, allowing detection at 242 nm and this, coupled with a solid phase extraction (SPE) clean-up step, provided a reasonable degree of selectivity to the analysis (Quilliam, 2003; Mafra et al., 2009). The detection limit of DA in the extract solution for this method was 10-80 ng DA mL<sup>-1</sup> depending on the sensitivity of the particular UV detector (Quilliam, 2003). In terms of shellfish tissues, the detection limit of DA was 0.5 µg DA g<sup>-1</sup>, more than 100 times better than the LOD for the mouse bioassay (Furey et al., 2001). This method has since been validated by the AOAC international Official Methods Program and is widely accepted for regulatory monitoring (Quilliam, 2003).

The HPLC-UV method was further developed by Pocklington et al. (1990) who first derivatised samples before analysis, in order to reduce DA LODs. The procedure is based on a reaction between DA and 9-fluorenylmethylchloroformate to form the fluorenylmethoxycarbonyl (FMOC) derivative. The samples were then analysed using reversed-phase HPLC with fluorescence detection (265 nm excitation and 313 nm emission) (Pocklington et al., 1990; Quilliam, 2003). This method is highly sensitive, having a detection limit of 15 pg DA mL<sup>-1</sup> and was used to measure trace concentrations of DA in plankton cultures and seawater (Pocklington et al., 1990). However, due to problems with interferences between shellfish tissues and derivatisation reagents (FMOC), this method cannot be used to analyse shellfish samples. Further disadvantages of this method come from the poor selectivity of the derivatisation process and the gradual loss of detector sensitivity over time (Furey et al., 2001; Wang

et al., 2007; Mafra et al., 2009). Furthermore, the derivatisation and clean-up procedure is very time consuming and can cause losses of DA (Mafra et al., 2009).

More recently, other pre-column derivatisation methods have been developed that allow for the successful determination of DA in phytoplankton and shellfish samples. The first of these was developed by Sun and Wong (1999) using the derivatisation reagent 6-aminoquinolyl-N-hydroxysuccinimidyl carbonate (AQC). This method was used to measure DA in phytoplankton samples and had a detection limit of 1 pg DA mL<sup>-1</sup>. The second method used the derivatisation reagent 4-fluoro-7-nitro-1,2,3-benzoxadiazole (NBD-F) and was analysed using isocratic reversed-phase HPLC with fluorescence detection (HPLC-FD) (James et al., 2000). This method proved to be very sensitive with a LOD in phytoplankton samples of < 1 ng DA mL<sup>-1</sup> and in shellfish samples, 6 ng DA g<sup>-1</sup> (James et al., 2000). However, co-eluting matrix compounds have been observed in the chromatograms which might interfere and the reagents used for this method are very expensive. A third method has been developed that uses post-column derivatisation with the reagent 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-CL) followed by fluorescence detection (Maroulis et al., 2008). This method was used to analyse DA in shellfish samples and had a LOD of 37 ng DA g<sup>-1</sup>. It has been argued that a post column derivatisation method requires less sample preparation and clean-up, chromatograms are free from interference and that it is a sensitive, cost effective alternative to pre column derivatisation methods. As with all these HPLC methods, DA is identified based on the coincidence of the retention time of the suspected DA chromatographic peaks in natural samples with those in an authentic DA sample. However, these DA peaks may represent compounds other than DA. Therefore to ensure more confident identification of DA, LC coupled with mass spectrometry (LC-MS) has frequently been used for confirmation purposes (Wang et al., 2007).

### **2.1.1.2 LC-MS methods**

LC-MS methods are considered to be the primary technology for the confirmation of DA and its isomers in shellfish (Quilliam, 2003; Wang et al., 2007). The EU reference laboratory for marine biotoxins has also approved LC-MS methods for routine monitoring of shellfish toxins. LC-MS with an electrospray ionisation (ESI) method have been found to be unambiguous and able to detect very low concentrations of DA as well as providing characteristic fragmentation patterns for DA and its isomers if multistage MS ( $MS^n$ ) or tandem MS is used (Furey et al., 2001; Quilliam, 2003; Wang et al., 2007). The LOD has been reported to be as low as 5-20 pg DA mL<sup>-1</sup> in seawater, based on samples that have gone through various clean-up and concentration steps (De la Iglesia et al., 2008; Wang et al., 2012). In shellfish extracts, using multiple tandem mass spectrometry, the detection limit was found to be better than 0.02 µg DA g<sup>-1</sup> (Furey et al., 2001). One problem associated with LC-MS methods is that salt build can affect the ESI operating source potentially causing ionisation suppression, loss of sensitivity, contamination, and poor signal stability. However, this can be overcome by the removal of salts from samples using SPE methods (Wang et al., 2007; De la Iglesia et al., 2008) and/or diversion of the mobile phase to prevent early eluting matrix compounds from reaching the ESI interface (Furey et al., 2001; Wang et al., 2012).

A study by Mafra et al. (2009) developed a simplified method that used LC-UV in conjunction with LC-MS to analyse DA without the need for prior sample clean-up. This method involved use of acidified sample extracts and a mobile phase with either trifluoroacetic acid (TFA) or formic acid (FA) for LC-UV or LC-MS analysis, respectively. An acidic mobile phase is essential for good chromatographic behaviour of DA on reversed-phase columns (further details are given in Section 2.1.2). A switching valve was also built into the LC injector to divert the salty eluent front from

the analytical column to waste during the first 4.5 min and after 7.5 min of analysis. This allowed for DA detection in the intervening period at 6.1 min. Using this method to analyse seawater and plankton samples the LOD was 42 pg DA mL<sup>-1</sup> for LC-UV and 15 pg DA mL<sup>-1</sup> for LC-MS determinations. This study demonstrated that LC methods can be very sensitive, and represent a faster, more selective, and less complicated alternative to the HPLC-FD (FMOC) methods and some other LC-MS methods.

#### ***2.1.1.3 Additional chromatographic methods***

Other chromatographic methods for analysing DA include thin layer chromatography (TLC), amino acid analysis, and capillary electrophoresis (Quilliam et al., 1998; Quilliam, 2003). TLC can be used as an alternative to HPLC methods but domoic acid can only be analysed semi-quantitatively using this method (Quilliam et al., 1998; Quilliam, 2003). Domoic acid can be recognised in TLC plates (silica gel 60 F<sub>254</sub>) as a weak UV-quenching spot that stains yellow after spraying with a 1 % ninhydrin (Quilliam, 2003). Due to interference with amino acids present in the crude extracts these must be separated from domoic acid. In the case of phytoplankton samples this can be done using 2D TLC (Quilliam, 2003). Shellfish samples have to be cleaned up using strong anion exchange solid phase extraction prior to analysis. The detection limit for DA using TLC is 0.5 µg, which permits detection in shellfish tissues at ca. 10 µg DA g<sup>-1</sup> (Quilliam, 2003). Amino acid analysis can be used to detect DA in crude extracts of plankton. This can be done using the buffer solutions and ion-exchange column normally used for the analysis of protein hydrolytes, DA elutes close to methionine (Quilliam, 2003). Absorbance measurements at 440 nm provide detection of amino acids with primary amine groups, while absorbance at 570 nm detects imino acids such as proline and domoic acid (Quilliam, 2003). Using this method detection

limits are 1  $\mu\text{g DA mL}^{-1}$ . This method, while fairly sensitive, is not effective on samples containing high concentrations of free amino acids and the analysis time is much longer than that of HPLC (Quilliam, 2003). Shellfish extracts can also be analysed using this approach but need to be cleaned up and concentrated appropriately (Quilliam, 2003).

A number of studies have used capillary electrophoresis successfully to analyse shellfish, seawater, and algal samples (Nguyen et al., 1990; Zhao et al., 1997; Piñeiro et al., 1999). However, samples have required two solid phase extraction steps before proceeding with analysis, these have included strong cation exchange (SCX) and strong anion exchange (SAX) (Zhao et al., 1997). Capillary electrophoresis methods are based on a narrow bore fused silica capillary tube filled with buffer which is connected between two liquid reservoirs. After the injection of a small volume of sample into the capillary, a differential voltage (20-30 k V) is applied to the ends of the capillary (Quilliam, 2003). Ionic substances migrate as narrow bands down the capillary, passing by a detector (UV absorbance, fluorescence etc) (Quilliam, 2003). Detection limits for this method were ca. 3 pg DA on column and 150 ng DA  $\text{g}^{-1}$  in shellfish tissue (Zhao et al., 1997).

#### ***2.1.1.4 Using an internal standard***

Internal standards are frequently used to improve the precision and accuracy of quantitative determination of target compounds by HPLC and LC-MS and related methods (Skoog et al., 2007). Ideally an internal standard is added at known concentration to a solution of every sample and blank prior to extraction. After analysis of sample extracts, any variations observed in the concentrations of the internal standard

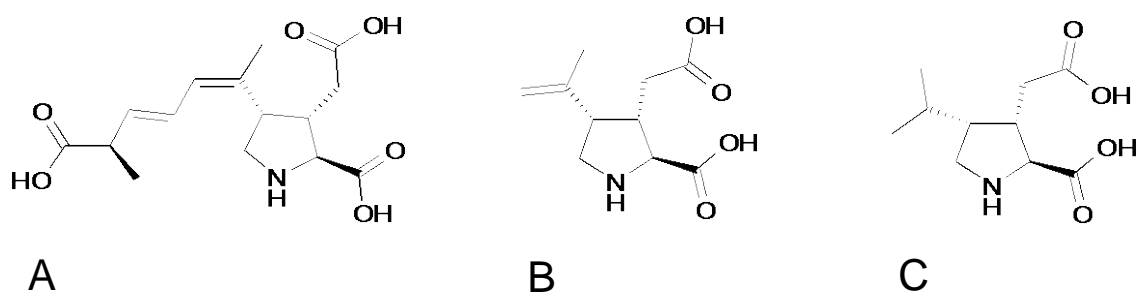
in the samples compared to that of the blank will ideally reflect variations also in the target compound. These variations can be accounted for by adjusting the target compound concentration by a correction factor or percentage. This is done to correct for random and systematic errors which can occur during the preparation of samples, chromatography, and detection using mass spectrometry. The internal standard used needs to be chemically and physically analogous to the analyte, providing a response that is similar to that of the target compound but which is sufficiently different that the two compounds are readily distinguishable (Skoog et al., 2007). Thus the response factor of the internal factor compared to that of the target compound is important. Internal standards can be those compounds that are structurally related, structurally similar or isotopically labeled analogues of the analyte (Skoog et al., 2007).

Relatively few of the chromatographic methods used to analyse DA in either seawater or shellfish samples have incorporated the use of an internal standard. In one study the reason given for this was the lack of commercially available isotopically labelled compounds appropriate for use (De la Iglesia et al., 2008). One of the first and most commonly used internal standards reported in the literature is dihydrokainic acid (DHKA: Fig. 2.1) (Pocklington et al., 1990; Besiktepe et al., 2008; Litaker et al., 2008; Bargu et al., 2011). DHKA was first used in combination with Fmoc derivatised DA and HPLC with fluorescence detection (FD) (Pocklington et al., 1990). As a result of this other studies using this method have also chosen to use this internal standard. DHKA was primarily selected because it had a different retention time to DA, it was commercially available, and it is not known to exist naturally in marine samples.

Another internal standard which has occasionally been used is kainic acid (KA: Fig. 2.1) which has been applied to HPLC-FD (Fmoc) (Maroulis et al., 2008) and to one



LC-MS method (Lawrence et al., 1994). To the knowledge of the present author, KA is the only internal standard reported to be used for LC-MS DA determination and only one study reports its use (Lawrence et al., 1994). Even the latter was unknown to the author at the beginning of the present study. Lawrence et al., (1994) found the compound to be effective in providing more reliable quantitative results for DA in shellfish tissues. One of the prime reasons given for its use as an internal standard was its availability, as the preferred option of isotopically labelled DA was not commercially availability. Lawrence et al., (1994) incorporated KA into their method to compensate for variations in the splitting ratio of LC effluents which was intended to reduce the flow rate of effluents reaching the electrospray interface. Lawrence et al., (1994) found that KA and DA peaks were clearly distinguishable due to their retention times. However, the retention times of both compounds appear to shift according to the type of sample analysed e.g. contaminated razor clams, crab meat, spiked rat urine, or spiked rat serum samples. It is not known whether these shifts are consistent within each sample type or whether they are a result of matrix effects and highly variable. There is no mention in works by Lawrence et al., (1994) as to the reproducibility of using KA as an internal standard and whether the target compound DA affects it. Furthermore, Lawrence et al., (1994) applied the use of KA as an internal standard for quantitative analysis of DA in seafood and biological samples, the application of this method for the analysis of DA in seawater and diatom samples has not been determined. KA has a relative molecular weight of 213 [protonated molecular ion  $m/z$  214,  $M+H$ ]<sup>+</sup> and is structurally very similar, yet distinct, from DA (Fig. 2.1). The use of KA as an internal standard along with LC-MS methods for DA determination will be further explored in this chapter.



**Figure 2.1:** Chemical structure of (A) domoic acid, (B) kainic acid, and (C) dihydrokainic acid.

Method <sup>a</sup>	SPE clean-up <sup>b</sup>	Derivatisation reagent	LOD phytoplankton	LOD shellfish tissue	References
Mouse Bioassay				40 µg g <sup>-1</sup>	Quilliam, 2003
HPLC + UV DAD	SAX-SPE		10-80 ng mL <sup>-1</sup>	0.5 µg g <sup>-1</sup>	Quilliam, 2003
HPLC + FD (FMOC)	SAX-SPE (shellfish only)	9-fluorenylmethylchloroformate	15 pg mL <sup>-1</sup>	0.02-0.03 µg g <sup>-1</sup>	Pocklington et al., 1990
HPLC + FD (AQC)		6-aminoquinoly-N-hydroxysuccinimidyl carbonate	50 pg mL <sup>-1</sup>		Sun and Wong, 1999
HPLC + FD (NBD-F)		4-fluoro-7-nitro-2, 1, 3-benzoxadiazole	1000 pg mL <sup>-1</sup>	0.006 µg g <sup>-1</sup>	James et al., 2000
HPLC + FD (NBD-CL)		4-chloro-7-nitrobenzo-2-oxa-1,3-diazole		0.037 µg g <sup>-1</sup>	Maroulis et al., 2008
LC-MS <sup>n</sup>	SAX-SPE			0.025 µg g <sup>-1</sup> (MS <sup>1</sup> ) 0.008 µg g <sup>-1</sup> (MS <sup>3</sup> )	Furey et al., 2008
LC-UV-MS			42 pg mL <sup>-1</sup> (UV) 15 pg mL <sup>-1</sup> (MS)		Mafra Jr et al., 2009
RRLC-MS/MS	C18 Empore disks		20-60 pg mL <sup>-1</sup>		De la Iglesia et al., 2008
LC-MS <sup>n</sup>	C18 SPE		30 pg mL <sup>-1</sup>		Wang et al., 2007
LC-MS <sup>n</sup>	C18 SPE		5 pg mL		Wang et al., 2012
TLC	SAX-SPE			10 µg g <sup>-1</sup>	Zhao et al., 1997
Amino acid analysis			1 µg mL <sup>-1</sup>		Quilliam et al., 1998
Capillary Electrophoresis	SCX / SAX SPE			0.15 µg g <sup>-1</sup>	Zhao et al., 1997

**Table 2.1:** Summary of literature values for the limits of detection

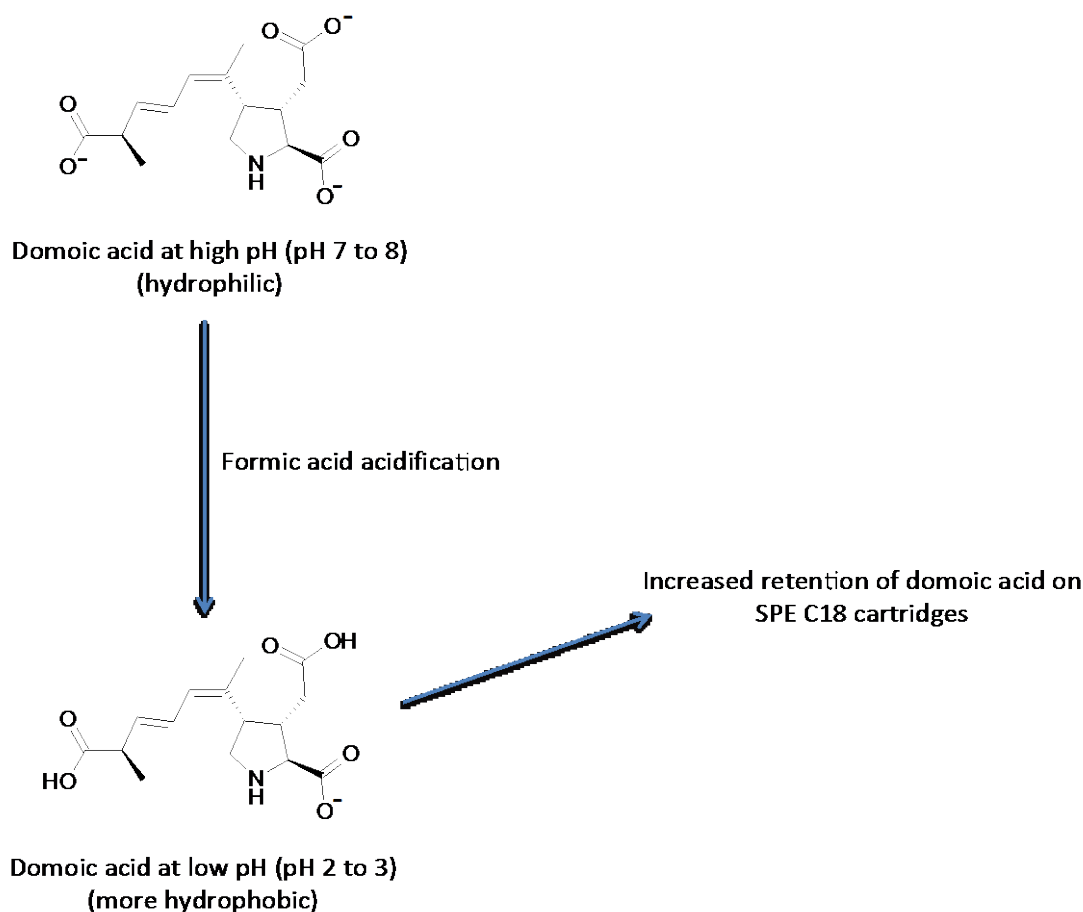
<sup>a</sup>HPLC+UV DAD refers to liquid chromatography with ultra violet diode array detection; HPLC+FD (FMOC) refers to liquid chromatography with fluorescence detection; AQC, NBD-F, and NBC-CL refers to the derivatisation agents used in each method; LC-MS<sup>n</sup> refers to liquid chromatography with multiple tandem mass spectrometry; LC-UV-MS refers to liquid chromatography ultra violet detection coupled with mass spectrometry; RRLC-MS/MS refers to rapid resolution liquid chromatography coupled with tandem mass spectrometry; TLC refers to thin layer chromatography. <sup>b</sup>SAX-SPE refers to strong anion exchange solid phase extraction; SCX refers to strong cation exchange solid phase extraction; C18 refers to octadecylsilane reversed phase sorbent material.

### 2.1.2 Solid phase extraction

Solid phase extraction (SPE) is a common method used to concentrate DA from both seawater and shellfish tissue samples before chromatographic analysis (Quilliam, 2003). In the case of shellfish samples, strong anion exchange (SAX) and cation exchange (SCX) SPE sorbents are used which help reduce the interference between tissue samples and derivatisation reagents (Furey et al., 2001). However, due to the high ionic strength of the SAX sorbents this method cannot be used for seawater samples (De la Iglesia et al., 2008). An alternative clean-up method for seawater samples uses reversed-phase SPE sorbents (Wang et al., 2007; Wang et al., 2012). These sorbents, such as polymeric-like octadecylsilane (C18), have been found to be very effective at extracting DA from seawater (De la Iglesia et al., 2008). When sorbents are not effective, breakthrough of analytes can occur, causing low and highly variable recoveries (De la Iglesia et al., 2008). Successful analyte recoveries largely depend on how the sorbents were initially conditioned, the condition (e.g. pH) of the seawater samples containing the analyte, and the final elution solvents used (Wang et al., 2007; De la Iglesia et al., 2008). The application, development and optimisation of such methods for the recovery of DA in seawater and culture samples, will be the focus of the present section.

Reversed-phase SPE sorbents have been designed to bind hydrophobic analytes and the more hydrophobic the analyte, the stronger it binds to the sorbent. In the case of DA, when this compound is in seawater it is hydrophilic and therefore has no binding capacity to reversed-phase sorbents (Wang et al., 2007). This is because DA has four functional groups; three carboxyl groups and one amino group (Fig. 2.2). These functional groups can influence the overall charged state of DA depending on the pH of the aqueous solution (De la Iglesia et al., 2008). For example, the DA molecule can fluctuate between charged states of -3 to +1. When DA is in seawater (pH 8) these

functional groups are charged causing the compound to be hydrophilic (Wang et al., 2007). To increase the retention of DA on reversed-phase sorbents, acidification of seawater samples is critical (ca. pH 2.7), as this will promote a neutral net charge of the DA molecule and cause it to become hydrophobic (De la Iglesia et al., 2008). Previous studies have used formic acid (0.1 %) to acidify (pH 2.7) both the equilibration solution, used to precondition the sorbent and the seawater sample to achieve optimal DA retention (De la Iglesia et al., 2008). Sometimes a washing step is incorporated after sample loading to eliminate possible interference compounds present in the matrix. However, considerable loss of DA has been reported from this step (De la Iglesia et al., 2008).



**Figure 2.2:** Schematic showing how the functional groups of DA are influenced by pH. The addition of formic acid promotes the molecule to have a neutral net charge, which in turn allows it to bind to reversed-phase SPE sorbents. Adapted from Wang et al. (2012)

Elution of DA is normally carried out using a highly hydrophilic solution. A previous study by De la Iglesia et al. (2008) which examined the success of different elution solvents found that methanol (100 %), while eluting DA, had a negative effect on the retention time and resolution of chromatographic analysis. They found that an elution solvent of acetonitrile: water (1:9 v/v) was more compatible. To elute DA from the reversed-phase sorbent, the pH of this solvent needs to be adjusted so that the charge state of the DA molecule changes (De la Iglesia et al., 2008). In its charged state DA becomes hydrophilic and will elute with the solvent. Highest recovery concentrations of DA are associated with solvents adjusted to pH 8 and buffered with ammonium

formate (0.2 M). Using methods described by De la Iglesia et al. (2008) and Wang et al. (2007) the reproducibility and the recovery of DA from *Pseudo-nitzschia multiseries* cultures using reversed-phase SPE cartridges was explored herein (Section 2.2.2).

### **2.1.3 Overview of Enzyme-linked ImmunoSorbent Assay (ELISA)**

Another method used to detect and quantify DA in shellfish is an immunological Enzyme-Linked ImmunoSorbent Assay (ELISA). This method uses antibodies which recognise specific toxic structures and bind to them (Garthwaite et al., 1998; De la Iglesia et al., 2008). This rapid assay is very useful for screening large sample sizes, it is relatively inexpensive, quick to run, and is able to detect DA concentrations 500 times below the maximum permitted level ( $20 \mu\text{g DA g}^{-1}$ ) allowed in shellfish (Osada and Stewart, 1997; De la Iglesia et al., 2008). There has been a great pressure over the last decade to develop such as assay as an alternative to analytical instrumental methods. While the predominant application of this assay is for toxin determination in shellfish, it has also been applied to the analysis of phytoplankton and seawater samples (Rhodes et al., 1998b; Howard et al., 2007; Schnetzer et al., 2007). The main benefit of such an assay compared to analytical methods is that it can provide cost effective real-time monitoring on-site (Kleivdal et al., 2007b). In terms of DA in shellfish, this assay provides a significant preventative measure against poisoning incidents or product recalls and adds predictability to the shellfish industry (Kleivdal et al., 2007a). For DA in phytoplankton or seawater samples, it reportedly enables confirmation and early warning of DA in the marine environment (Kleivdal et al., 2007a).

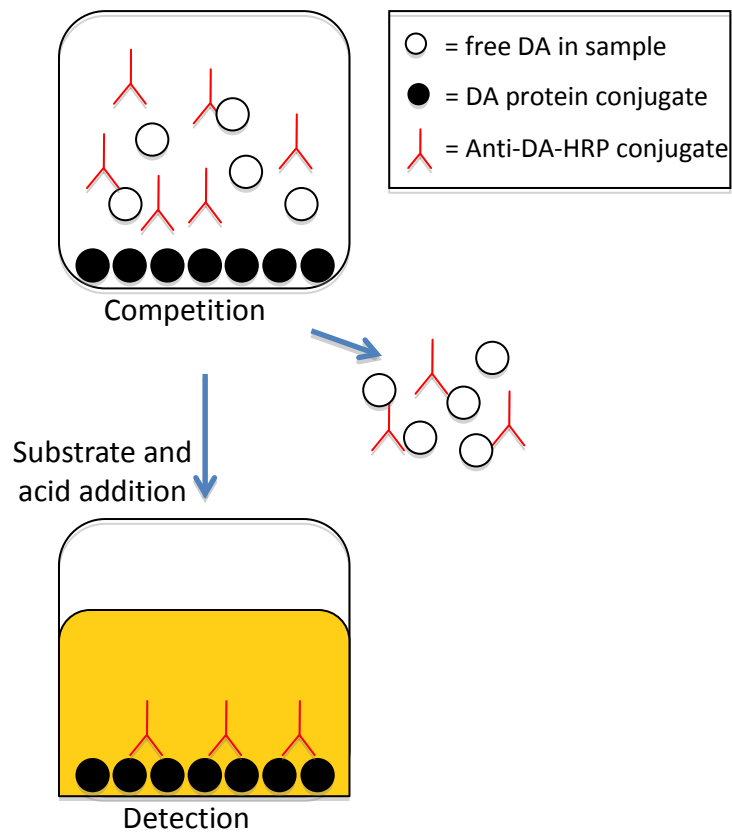
Several groups have developed immunological assays, using different antibodies, for the determination of total DA (DA and the less toxic iso- and epi-domoic acid) in

shellfish and seawater samples (Newsome et al., 1994; Osada et al., 1995; Smith and Kitts, 1995; Kawatsu et al., 1999). Due to the lack of collaborative validation data (Kleivdal et al., 2007b), and problems concerning antibody specificity (Garthwaite, 2000), these assays have not been appropriate for development as international reference methods. In contrast, an indirect competitive (cELISA) method developed by Garthwaite et al. (1998) was identified as being suitable for the use as an alternative to the current LC-UV reference method (Kleivdal et al., 2007b). This assay uses polyclonal ovine anti-DA antibodies which are highly specific for domoic acid. The method developed by Garthwaite et al. (1998) was reformatted by the AgResearch Toxinology group (Hamilton, New Zealand) who used the same antibodies but changed the assay from being an indirect cELISA to a rapid, user-friendly, direct version (Kleivdal et al., 2007a).

The method initially developed by Garthwaite et al. (1998) and later by AgResearch relies on free DA in the sample competing with DA-protein conjugate, which is coated on plastic wells, for binding to anti-DA antibodies free in the solution (Kleivdal et al., 2007b). The ovine anti-DA antibodies are conjugated to horseradish peroxidases (HRP) (Fig. 2.3). Samples diluted in buffer are incubated in the wells with the anti-DA-antibody-HRP conjugate (Kleivdal et al., 2007b). After washing, the amount of conjugate remaining bound to the wells is measured by incubation with a substrate that gives a blue product upon reaction with the HRP enzyme (Kleivdal et al., 2007b). Addition of acid stops the reaction and changes the product colour from blue to yellow (Fig. 2.3). The colour intensity is then measured spectrophotometrically on a plate-reader at 450 nm, and is inversely proportional to the concentration of DA in the sample solution (Kleivdal et al., 2007b). The assay is calibrated using dilutions of a DA



calibration solution supplied with the kit. Each kit can analyse 36 samples including calibration solutions, controls, and blanks.



**Figure 2.3:** Schematic showing the ELISA immunoassay format. Boxes represent individual microwells which are supplied in strips (x12 wells) with the ELISA kit. The wells are coated with DA protein conjugate which competes with free DA in the sample to bind anti-DA-HRP. Conjugates not bound to the wells are removed during the rinsing steps and those that are bound are measured once substrate and acid have been added to stop the reaction. Adapted from the user manual supplied with the ELISA kit (ASP ELISA protocol-Biosense Laboratories)

The newly developed direct cELISA method was subsequently subjected to a single-laboratory validation study at Biosense (Biosense Laboratories, Bergen, Norway) which indicated that the assay was accurate and reliable for toxin determination in shellfish (Kleivdal et al., 2007a). A collaborative study, which gathered validation data from 16 different laboratories, concluded that inter-laboratory precision (relative standard deviation (RSD) range 10-21 %) and recovery (RSD range 88-122 %) of DA determinations from shellfish were acceptable according to the guidance provided by the Association of Analytical Communities (AOAC) for analytical methods. Both of these studies compared cELISA with LC-UV methods for DA determination in shellfish tissue and found that, while there was generally a good agreement between methods, cELISA slightly overestimated DA concentrations (slope at 1.29) (Kleivdal et al., 2007b). The suggested explanation for this overestimation is that the antibodies in the cELISA assay detect DA isomers in addition to DA and epi-DA. In the case of the collaborative validation, overestimations were also attributed to a lower recovery (mean recovery 87 %, n=4) of DA from shellfish tissues leading to an underestimation of toxin concentrations by LC methods (Kleivdal et al., 2007b). Despite these slight overestimations, the high recovery and precision of the Biosense cELISA method has meant that it has been officially approved by AOAC International for regulatory detection of DA in shellfish (Kleivdal et al., 2007b). The detection limits for the application of cELISA on shellfish tissue was 0.003  $\mu\text{g DA g}^{-1}$  and on mammal body fluids was 300 pg DA  $\text{mL}^{-1}$  (Kleivdal et al., 2007b). Only one limit of quantification has been reported for phytoplankton samples, which was approximated at 200 pg DA  $\text{mL}^{-1}$  (Kleivdal et al., 2007a).

There has been a lot of emphasis on the use of cELISA for determining toxin concentrations in shellfish (Smith and Kitts, 1995; Kleivdal et al., 2007b). The method

has gone through a rigorous validation process to provide evidence for its reliability, accuracy, and precision (Kleivdal et al., 2007a). The application of cELISA for phytoplankton and seawater samples has not gone through this same process. However, a number of studies have used the immunoassay in culture experiments (Rue and Bruland, 2001), biological samples (Maucher and Ramsdell, 2005; Goldstein et al., 2008), and on environmental samples (Baugh et al., 2006; Doucette et al., 2009; Sekula-Wood et al., 2009). In these studies, with the exception of that of Rue and Bruland (2001), ELISA was the sole method used to determine DA. A study by Rue and Bruland (2001) compared ELISA determinations with those using a competitive ligand equilibration/adsorptive cathodic stripping voltammetric technique (CLE-ACSV) and found that there was good agreement between both methods. Despite this, there are concerns in the literature of false positives (Quilliam, 2003; Mafra et al., 2009) and matrix effects (Kleivdal et al., 2007a) associated with this method.

Another competitive ELISA kit also available commercially was jointly developed by the National Centers for Coastal Ocean Science, National Ocean Service, and the Northwest Fisheries Science Center, together with an industrial partner Mercury Science, Inc., Durham North Carolina (NOAA/MSI) and utilises a high avidity monoclonal antibody (mAb). It works on a similar basis to the previous Biosense cELISA kit which uses polyclonal antibodies. A fixed number of anti-DA mAb binding sites are incubated with DA in the sample followed by the addition of a DA-horseradish peroxidase (HRP) conjugate. Anti-DA mAb molecules are affixed to the surface of micro-plate wells and a micro-plate reader measures the HRP derived colour change.

Initial validation of this method was conducted by Litaker et al. (2008) who primarily compared NOAA/MSI ELISA with HPLC-UV results for the analysis of shellfish

samples and HPLC-FD (FMOC) results for the analysis of phytoplankton samples. A subset of phytoplankton samples were also analysed using LC-MS methods for confirmatory purposes. Further validation was conducted on intertidal barnacle, limpet, and snail samples which were analysed using HPLC-FD, NOAA ELISA, and Biosense ELISA methods. Validation results showed that there was good agreement in DA determinations between all methods for all samples. However, these results are based on one study and there is no evidence in the literature for the application of the NOAA/MSI ELISA method by other laboratories. After further rigorous validation producers of this method aim to seek approval as another regulatory tool for the analysis of DA in biological samples. To this end, use of this method was explored herein for application to DA determination in cultured diatom samples. Biosense ELISA was used to detect and quantify DA in intracellular and extracellular *Pseudo-nitzschia multiseries* culture samples and results were compared to DA determinations by an improved LC-MS method incorporating an internal standard.

#### **2.1.4 Objectives**

- To develop extraction protocols, using solid phase extraction, for the concentration of DA from algal culture and seawater samples prior to LC-MS analysis.
- To apply and optimise published LC-MS methods for the detection and quantification of DA in culture and seawater samples.
- To assess kainic acid as a potential internal standard to improve the reproducibility and reliability of DA quantification.
- To explore an alternative (ELISA) method for DA determination in toxigenic *Pseudo-nitzschia* spp. culture samples.

- To compare ELISA and LC-MS DA determinations in a culture of *P. multiseri* over an experimental period of 25 days.

## **2.2 Methods**

### **2.2.1 Chemicals**

CRM-DA-e, domoic acid certified reference standard ( $103.3 \mu\text{g mL}^{-1}$ ) was purchased from the National Research Council, Halifax, Canada. Acetonitrile, methanol, and water were LC-MS grade and purchased from Sigma-Aldrich, UK. Formic Acid was purchased from VWR International. The internal standard, kainic acid, was purchased from Sigma-Aldrich, UK.

### **2.2.2 Solid Phase Extraction (SPE) Methods**

#### **2.2.2.1 SPE and DA elution**

Reversed phase polymeric sorbents (Strata X 200 mg, 6 mL; Phenomenex) were used to de-salt seawater and culture samples and in the case of dissolved DA ( $\text{ng mL}^{-1}$ ) they were used for concentration. SPE cartridges were mounted on a filtration manifold connected to a vacuum pump. The cartridges were first conditioned with 10 mL methanol (MeOH) then 10 mL HPLC grade water spiked with 0.1 % formic acid. No vacuum was applied during the MeOH conditioning step to ensure optimal conditioning of sorbents. Vacuum was kept at approximately -5 kPa (roughly 2 drops  $\text{s}^{-1}$ ) during the water (0.1 % formic acid) conditioning step and sample loading. DA elution was carried out with 3 mL of 10 % acetonitrile: water (1:9 v/v) which was buffered with 0.2

M ammonium formate and adjusted to pH 8 using sodium hydroxide (NaOH). The supernatant (3mL) was collected in 5 mL glass centrifuge tubes, an aliquot of which was then transferred to a 1.5 mL screw cap LC amber glass vial for analysis by LC-MS (Section 2.2.3). This method was based on that described by De la Iglesia et al. (2008).

#### ***2.2.2.2 Preparation of particulate DA (pDA) samples***

Samples of *P. multiseriis* culture were first filtered through glass fibre 25 mm Whatman filter papers to separate the cells from the medium. The filtrate was collected in 50 mL plastic centrifuge tubes for clean-up and concentrating using SPE columns (Phenomenex, UK) (Section 2.2.2.3). The particulate matter on the filter was extracted in 2 mL of 50 % methanol: water and sonicated for 1 min before being centrifuged for 15 min at 15 °C and a speed of 3000 rpm. Sample clean-up was performed by re-suspending 1 mL of the supernatant in 50 mL of distilled water spiked with 1 % formic (pH 2.8) acid and loading onto an SPE cartridge (Section 2.2.2.1). Elution of pDA is detailed in Section 2.2.2.1.

#### ***2.2.2.3 Preparation of dDA sampled***

Filtrate (50 mL) samples containing dDA were spiked with 0.1 % formic acid before being passed through the SPE cartridges. Details of sample loading and DA elution are given in Section 2.2.2.1.

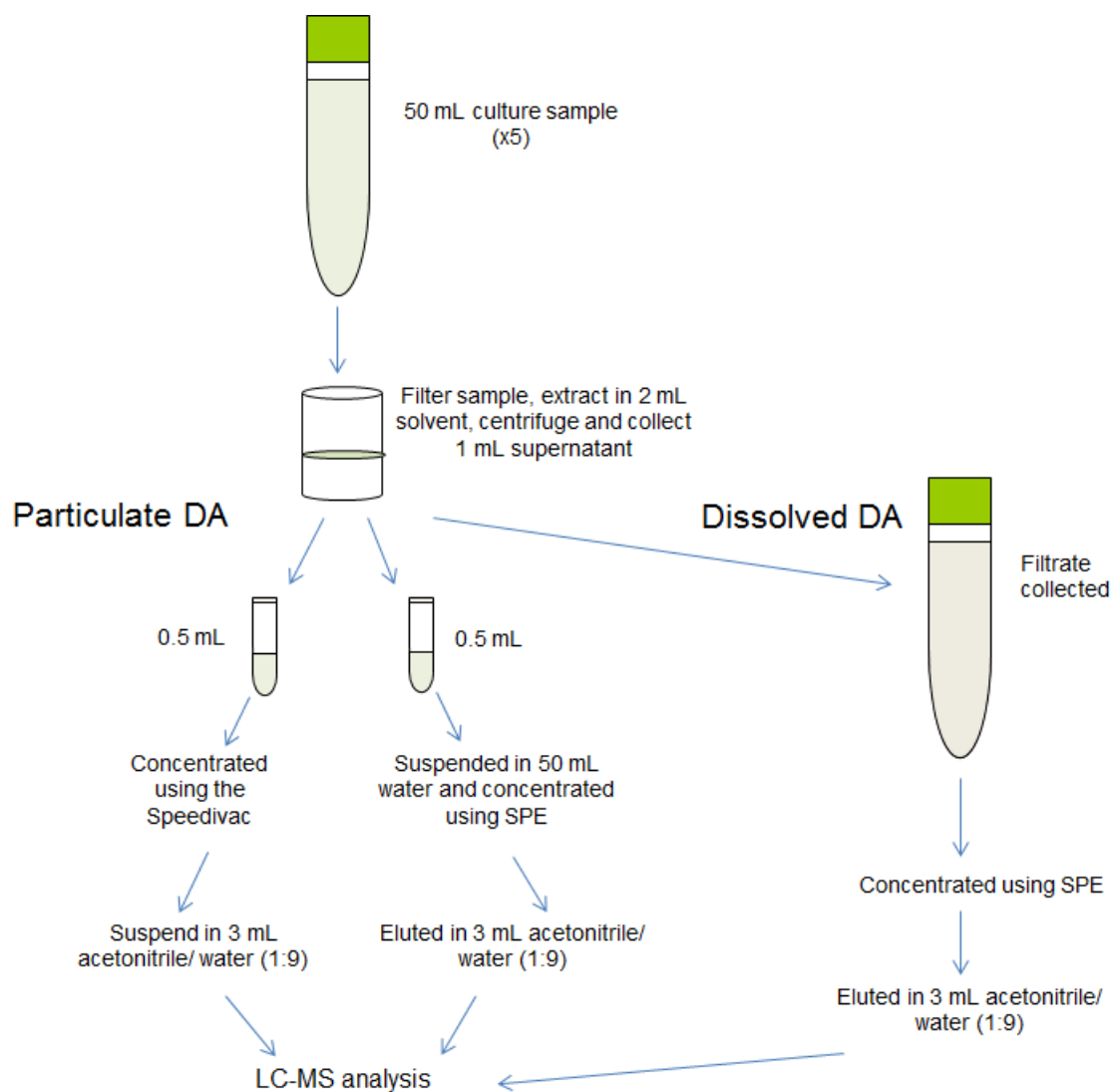
#### **2.2.2.4 Recovery of authentic DA from spiked seawater**

The determination of authentic DA recovery from spiked seawater samples was performed by adding 103 ng of DA standard to 50 mL of filtered seawater in a 50 mL disposable screw-cap centrifuge tube in triplicate. The extraction and elution of DA were performed according to Section 2.2.2.1 and analysis was by LC-MS.

#### **2.2.2.5 Recovery and reproducibility of pDA and dDA by SPE**

Five 50 mL samples of *P. multiseri* culture (cells plus medium) were used to determine the recovery and reproducibility of the reversed-phase SPE method for pDA and dDA concentration and clean-up. The samples were taken from a mature culture of *P. multiseri* during mid-stationary phase ca. day 14. pDA ( $\text{ng mL}^{-1}$ ) in these samples was firstly separated from dDA by filtration onto GFF glass fibre filters as described in Section 2.2.2.2. These particulate samples were extracted in 2 mL of 50 % methanol: water, sonicated and then 1 mL of the supernatant from each of the five samples was placed into a separate Eppendorf tube. Each of these 1 mL samples was further separated into 0.5 mL aliquots (Fig. 2.4). This was done because while SPE is capable of desalting particulate samples, the process actually further dilutes pDA as 1 mL of supernatant is later eluted into 3 mL acetonitrile: water. This is not ideal when determining trace concentrations of DA. Therefore, the effects of adding a concentration step needs to be assessed as follows. One set of 0.5 mL (x 5) aliquots were each re-suspended in 50 mL of distilled water spiked with 1% formic acid and loaded onto the SPE cartridges (Fig. 2.4). The other set of 0.5 mL aliquots was concentrated by evaporation using a SpeediVac (GeneVac, Suffolk, UK) at 40 °C for 2 hours until dry. Both sets of aliquots were re-suspended in 3 mL acetonitrile: water (1:9, v/v) buffered with 0.2 M ammonium formate and adjusted to pH 8. All samples

were examined using LC-MS (injection volume of 50  $\mu$ L). An aliquot of each sub-sample was then transferred to a 1.5 mL screw cap LC amber glass vial for analysis by LC-MS (Section 2.2.3.1). dDA samples were desalted and concentrated using methods described in Section 2.2.3.1 before analysis using LC-MS.



**Figure 2.4:** Diagram summarising preparation of pDA and dDA samples for LC-MS analysis (Section 2.2.2.5).



## 2.2.3 Liquid Chromatography-Mass Spectrometry

### 2.2.3.1 LC-MS of DA

LC-MS was performed using an Agilent binary pump 1200 series (Agilent technologies) coupled with a 6330 ion trap mass spectrometer. DA was ionised using an electrospray ionisation (ESI) operating source. Analysis of DA was performed on a 150 mm x 4.6 mm, 3  $\mu$ m particle, Phenomenex (UK) Luna C<sub>18</sub> reversed-phase analytical column. The mobile phase was prepared from an aqueous solution of 50 nanomolar (nM) formic acid (VWR international) in 10 % methanol (Sigma-Aldrich, UK), 90% water (Solution A), and 50 nM formic acid in 5:95 (v/v) water-acetonitrile (Sigma-Aldrich, UK) with 10 % methanol (Solution B) at a flow rate of 1 mL min<sup>-1</sup> (Table 2.2). This method was based on that previously described by Hummert et al. (2002). After a series of experimental runs using different mobile phases, run times, and optimisation parameters some developments were made to the original method in order to improve chromatography and the MS signal. For instance, the method described by Hummert et al. (2002) used a quaternary pump. However, the LC-MS used in the present study used a binary pump system therefore the mobile phase was adapted for use on a binary system by splitting one of the solutions containing 100 % methanol in the original method into two separate solutions containing 10 %.

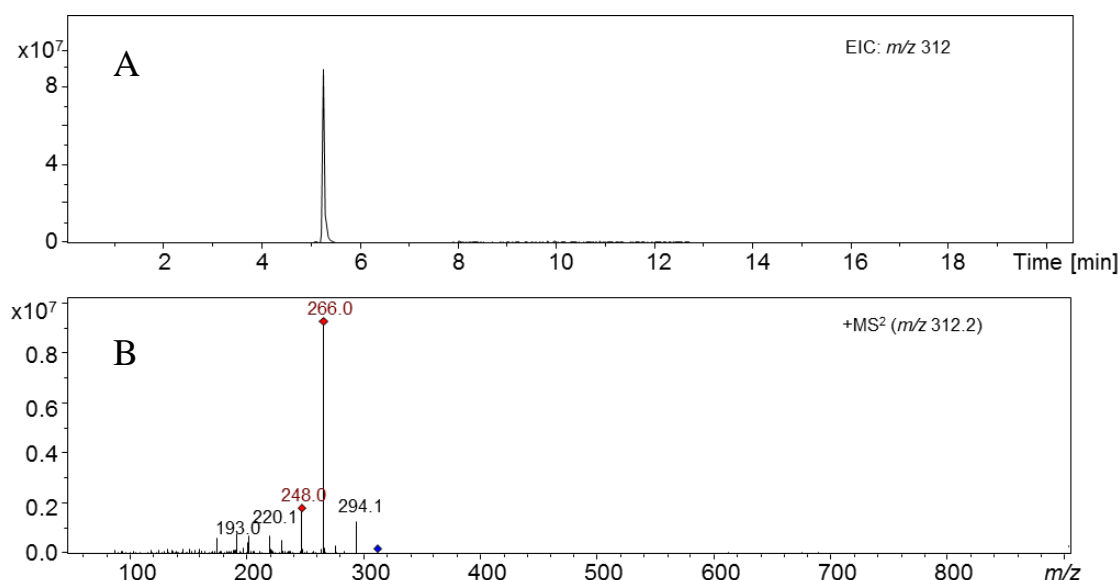
Another modification made to the method was the elimination of ammonium formate from the mobile phase, which as a result, improved the signal sensitivity of the MS. The method published by Hummert et al. (2002) using ammonium formate was used on the LC-MS in the present study and it was found there was an increased signal to noise ratio in the LC chromatography and MS signal. Using the optimisation settings on the LC-MS Chemstation software and injecting domoic acid straight into the MS it was anticipated that the noise could be reduced by targeting domoic in its concentrated form

(1.038  $\mu\text{g mL}^{-1}$ ). While this certainly improved the MS signal it was not until the mobile phase itself was re-made without the addition of ammonium formate that clear DA peaks were observed in the chromatography. It was thought that the ammonium formate was forming false adducts and interfering with the detection of DA. The original run times used in Hummert et al. (2002) were 30 min. This was reduced to 20 min as 10 minutes at the end of each run used the same mobile phase gradient (94.4 % Water/MeOH and 5.6 % Acetonitrile/water/MeOH). This was required to flush the column at the end of each sample run to avoid the build up of salts and compounds retained on the column. However, in the present study only 20 samples were run approximately at one time therefore the risk of salt and compound build up on the column was reduced. Also, the samples were cleaned using SPE methods before each run thus ensuring their purity. There were no negative effects observed in the LC chromatograms and analyte concentrations as a result of reducing the run time. This allowed for shorter run times across multiple samples and reduced the amount of mobile phase required.

Minutes	Solution A (%)	Solution B (%)
0	94.4	5.6
5	62.2	37.8
6	22.2	77.8
19	11.1	88.9
20	94.4	5.6

**Table 2.2.** Gradient for LC elution.

Quantification was performed by integrating the extracted chromatogram peak of the protonated DA molecular ion  $[M+H]^+$   $m/z$  312 (relative molecular weight of DA 311) at a retention time of 5.3 min and determining the peak areas using Chemstation software (Agilent Technologies, Cheshire, UK). The peak area of the DA  $[M+H]^+$  ion was then compared to calibration data, which represented the peak areas for a range of known DA authentic solution concentrations (e.g. 5-100 ng DA on the column) injected onto the column, and a corresponding DA concentration on the column was determined. The ratios of three fragment ions characteristic of DA ( $m/z$  266, 248, and 220) were also monitored to confirm DA identity by comparison with the spectrum of authentic DA (Fig. 2.5).



**Figure 2.5:** Example spectra showing (A) the extracted ion count (EIC) for DA ( $m/z$  312) and (B) the fragmentation pattern ( $MS^2$ ) characteristic of DA ( $m/z$  266, 248, and 220).

### **2.2.3.2 Authentic DA calibrations**

Using the above method, the linearity and precision of LC-MS DA determination was examined. Several concentrations of a DA standard solution were injected, ranging between 5 and 100 ng DA on column. The purchased DA standard was first diluted 1/100 (final stock solution concentration of  $1.038 \mu\text{g mL}^{-1}$ ) before being used for calibration purposes. This avoided overloading the column and reduced the concentration of toxin being handled. Each concentration was injected in duplicate and the standard error calculated.

It was also important to verify whether or not the linearity and precision of the DA calibrations changed over time. DA calibrations were observed over four time points spanning a month of operation. For these calibrations the stock DA standard was diluted 1/10 and had a final concentration of  $10.38 \mu\text{g mL}^{-1}$ . The limit of detection for DA, using the above method, was estimated by injecting five DA concentrations in duplicate onto the system and using a signal to noise ratio of 5:1 (Skoog et al., 2007). The lowest concentration of DA that could be detected was determined (Hummert et al., 2002). The limit of quantification was determined using the same method except the signal to noise ratio 10:1 was used (De la Iglesia et al., 2008).

### **2.2.4 Internal standard**

#### **2.2.4.1 Calibration of DA and KA**

KA was diluted to the same concentration as the DA standard solution (1/10 dilution  $10.38 \mu\text{g mL}^{-1}$ ) and combined 1:1 in a 2 mL glass amber vial (Chromacol Ltd, UK). Five concentrations were injected onto the column in duplicate, adopting developed LC-MS methods (Section 2.2.3.1). The protonated molecular ion peak areas for both

compounds ( $m/z$  214, 312) were measured at each concentration and plotted, and the ratio between the compounds was determined.

#### ***2.2.4.2 Response of KA to varying concentrations of DA***

In a separate set of analyses, varying concentrations of DA (50 to 260 ng DA on column) were mixed with a constant concentration of KA (250 ng KA on column) to determine whether the internal standard response altered according to DA concentration.

### **2.2.5 ELISA methods**

#### ***2.2.5.1 Preparation of samples for ELISA***

An ASP ELISA (Biosense<sup>TM</sup>) kit was purchased directly from GlycoMar, UK. Total and dDA concentrations were determined using ELISA and pDA concentrations were determined by subtracting dDA concentrations from the total. For total DA, a 3 mL culture sample was taken and sonicated for 2 min on ice and then filtered through a 0.2  $\mu$ m acrodisc filter. For dDA, a 3 mL culture sample was taken and filtered through a glass fibre 25 mm Whatman filter paper and the filtrate was collected in a 15 mL polypropylene conical based tube. Aliquots of both total and dDA samples were then analysed with an appropriate dilution factor according to the ASP ELISA kits user's manual. The colour intensity of the final substrate was measured using a spectrophotometer micro-plate reader at 450 nm. The colour intensity is inversely proportional to the concentration of DA in the sample solution. To quantify DA, spectrophotometer data (absorbance values) were input into an Excel® 'macro' which

was included with the ELISA kit. The ‘macro’ contained a calibration function and a conversion formula advisory for determination of DA concentration in algal samples ( $\text{pg mL}^{-1}$ ). Each time the kit was used a calibration was determined for a series of solutions with known DA concentrations to ensure that subsequent determinations fell within the working limits of the ELISA kit (Fig. 2.15).

#### **2.2.5.2 Preliminary study**

Three experimental flasks were inoculated with *P. multiseriis* stock culture (donated by Dr. E. Kready at the Marine Institute, Galway, Ireland) and maintained at  $15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  under a light intensity of  $92\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$  (16:8 h light: dark cycle). Cultures were gently mixed every 24 h. Flasks contained filtered seawater (30 kDa) enriched with f/2 nutrients with the addition of silicate (Guillard, 1975). Triplicate samples (R1-3) were taken from these experimental flasks at Day 10 of the growth cycle as cultures were entering the stationary phase and prepared for ELISA analysis. A sample was also taken from a stationary phase (Day 16) stock culture of *P. multiseriis* (G1) and was prepared for ELISA analysis. An additional sample was taken for analysis from a newly established culture of *P. fraudulenta* (C3) isolated from L4 in the Western English Channel. All these samples were prepared and analysed using the ELISA method (described in Section 2.2.5.1) and by a calibrated, LC-MS method (Section 2.2.3.1). LC-MS analysis was completed on pDA samples which had been concentrated using the SpeediVac system. Samples had not been de-salted using SPE methods, as these methods had not yet been developed. This is also the reason why no comparison was made at this juncture between ELISA and LC-MS filtrate samples.

## **2.2.6 LC-MS and ELISA analyses of *P. multiseri* cultures**

### **2.2.6.1 Preparation and culture maintenance**

Cultures of *P. multiseri* were maintained in sterile tissue culture flasks containing nutrient replete seawater media f/2 with silicate (Guillard, 1972), and sub-cultured every two weeks. Flasks were maintained at  $15\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$  and exposed to 16:8 h light: dark regime from a series of lights fitted vertically on the opposite wall ( $92\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ). Cultures were agitated every 24 h by gentle shaking to homogenise the fluid medium.

### **2.2.6.2 Experimental cultures**

Triplicate 2 L glass conical flasks containing f/2 media were inoculated with *P. multiseri* cells ( $1 \times 10^3\text{ cells mL}^{-1}$ ) and maintained under the same conditions as above. Samples were harvested every two days up until day 15 and then sampled every four days after that. For cell counts, 2 mL samples were taken from the cultures. In addition to this, sub-samples were taken for ELISA analysis (6 mL) and for LC-MS analysis (50 mL). The LC-MS samples were first ‘cleaned-up’ using SPE methods (Section 2.2.2), or in the case of the dDA, they were concentrated using SPE methods and then analysed according to the methods described in Section 2.2.5. ELISA samples were prepared and analysed according to the previous section (Section 2.2.5). Comparisons were drawn between cellular DA concentrations (pDA concentrations normalised to cell abundance) and dDA concentrations.

## 2.3 Results and discussion

### 2.3.1 Solid phase extraction

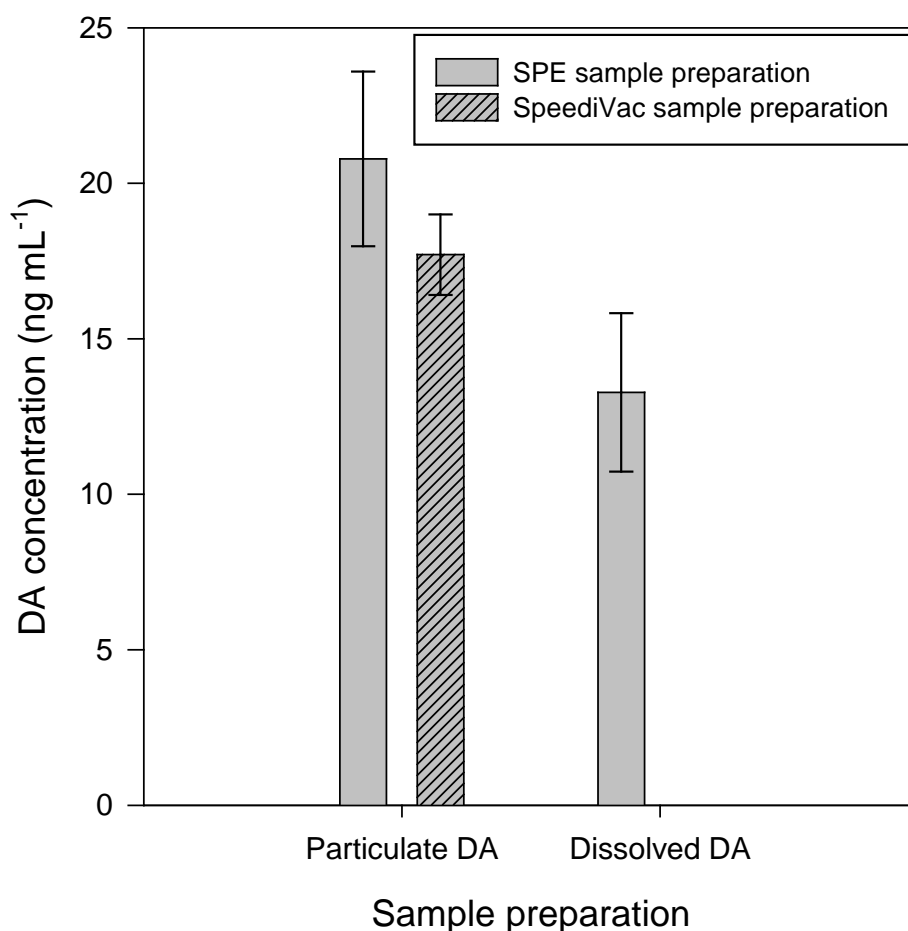
Reversed-phase SPE was successfully applied to recover a known quantity of authentic DA (103 ng) from spiked seawater samples. Recovery of DA from triplicate seawater samples was  $90.6 \pm 1.5$  % (Table 2.3). This is comparable to recovery values of 85 %, and 94 % reported in the literature using similar SPE methods (Wang et al., 2007; De la Iglesia et al., 2008; Wang et al., 2012).

Spiked Conc.	Replicates	Recovery %
103 ng DA	R1	92
	R2	89
	R3	91

**Table 2.3:** Percentage recovery of DA from three replicate (R1-3) seawater samples spiked with 103 ng DA.

The reproducibility and precision of SPE methods was further investigated with five replicate, unspiked, culture samples for pDA and dDA. pDA was concentrated using vacuum evaporation (Fig. 2.6). Figure 2.6 shows that there was variation between replicates for pDA and dDA. Percentage co-variance for these concentrations showed that there was a greater variation between dDA (19 %) determinations than pDA (13 %) determinations. Samples concentrated using the Speedivac had the least variation (7 %). This variability may be due to matrix effects or loss of DA associated with sample preparation. If this were the case then using an internal standard would allow for correction of any variation caused as a result of method preparation.





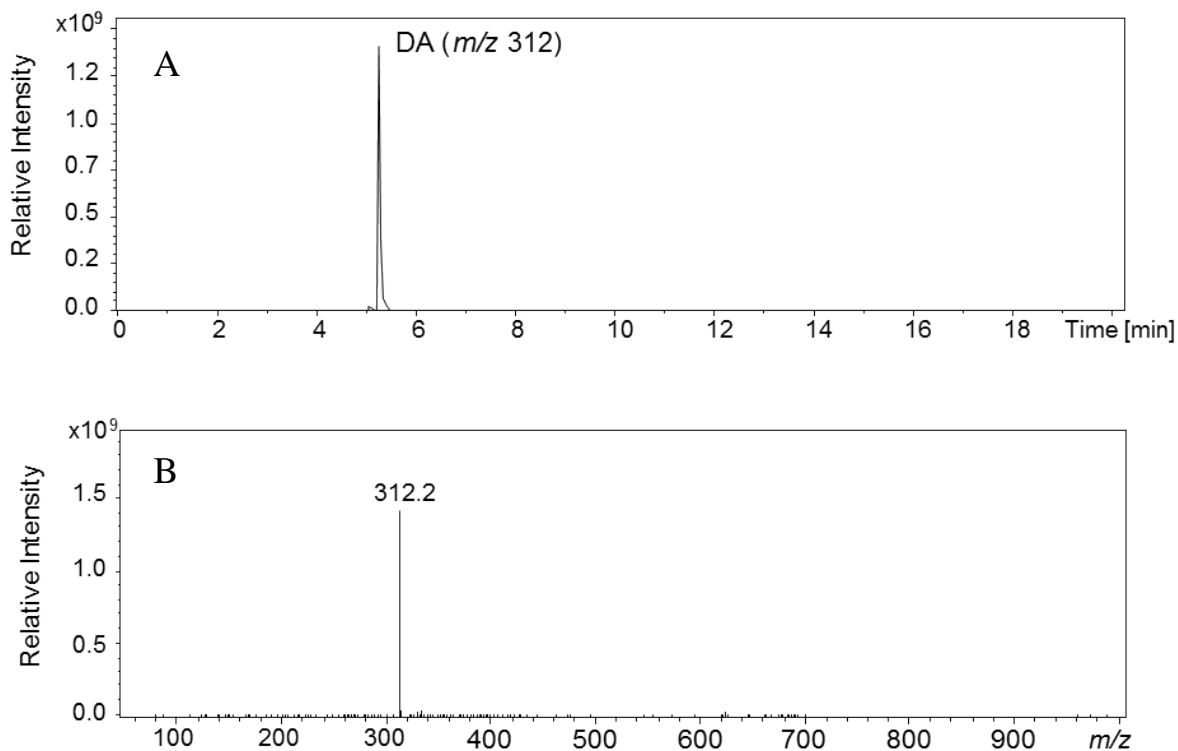
**Figure 2.6:** Mean concentrations of replicate pDA and dDA culture samples. The pDA samples were prepared using SPE methods or SpeediVac. The dDA samples were prepared using SPE methods. Error bars show the standard deviations from the mean (n=5).

No significant difference (t-test,  $P < 0.05$ ) was found between pDA concentrations that were prepared using either SPE methods or concentrated using a SpeediVac system, despite their being a larger variation associated with the former treatment (Fig. 2.6). This suggests that pDA samples can be cleaned-up using SPE methods and can then be concentrated by evaporation using a SpeediVac system with no differential loss of DA. In contrast, a previous study by Wang et al. (2007) reported that evaporative techniques not only caused loss of DA but that these losses were highly variable, ranging from

between 10-50 %. Variable loss was still evident when the drying temperature was reduced and evaporation time increased. Wang et al. (2007) concentrated samples using a turbo-evaporator under nitrogen gas. However, there was no mention of the drying temperature or evaporation time used by Wang et al. (2007) so it is difficult to make a direct comparison between protocols in order to try to understand the differences found in DA recoveries. The data shown in Figure 2.6 also demonstrate that there is no difference in the MS response to samples that were desalted using SPE methods (pDA) compared with those samples not desalted but were instead concentrated using evaporation techniques (pDA using SpeediVac system). If the salts from these samples had affected the MS response a suppression of the MS signal might have been observed. This is more likely to happen during the analysis of a large amount of samples that are processed continuously with no adequate flushing of the column and system to remove built up salt products.

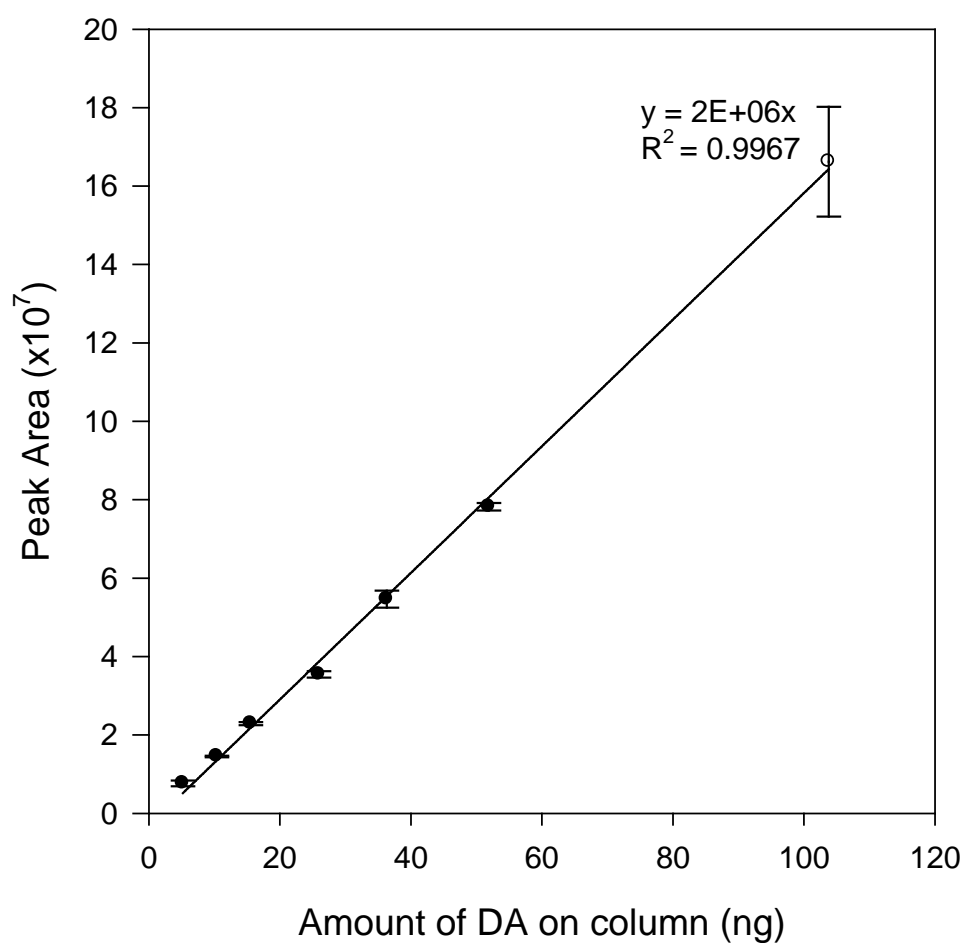
### **2.3.2 LC-MS development**

An LC-MS method for the determination and quantification of DA (Fig. 2.7) was successfully adopted and further developed, based on that described by Hummert et al. (2002). This method was mainly simplified by adapting the method for a binary system, reducing the run time from 30 to 20 mins (Table 2.2), and by eliminating ammonium formate from the mobile phase. These method alterations corresponded to an improved limit of detection for DA compared to 15 ng DA of the column reported by Hummert et al (2002). The LOD for DA on this system was 0.1 ng DA on the column, or 0.04 ng DA mL<sup>-1</sup> seawater. The LOQ for DA on the system was 0.2 ng DA on the column, or 0.08 ng DA mL<sup>-1</sup> seawater.



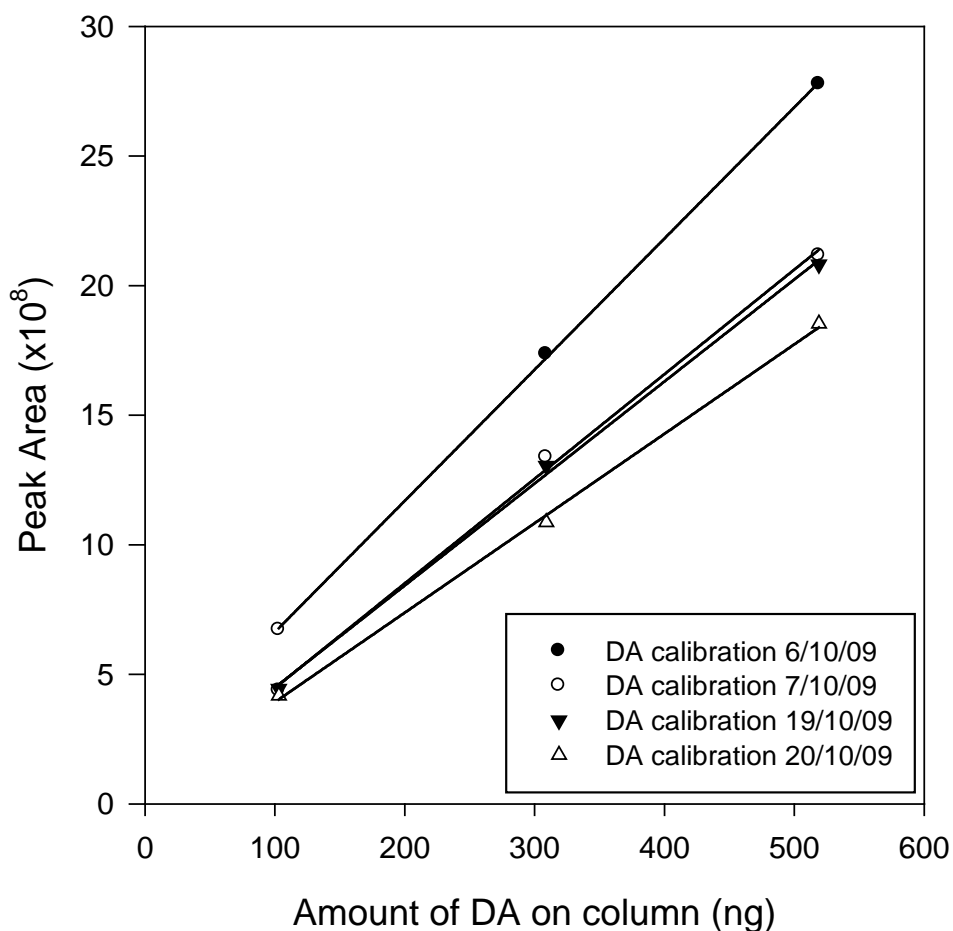
**Figure 2.7:** (A) Extracted ion mass chromatogram indicating retention time of DA ( $m/z$  312,  $[M+H]^+$ ) at 5.3 minutes, (B) ESI mass spectrum of DA. Analysis was conducted using an authentic DA solution.

Using this newly developed method it was found that the signal intensity of the chromatographic peak due to the protonated molecular ion of DA ( $m/z$  312) increased linearly with increasing DA concentrations injected onto the LC column within the range 5-105 ng on the column (Fig. 2.8). It was also observed that, within the same analysis, the linearity and precision remained constant in the range 5-50 ng on column. At the highest DA concentration (105 ng on the column) a greater variation between peak areas for the duplicate injections was observed (Fig. 2.8). However, in subsequent measurements of high DA concentrations ( $> 105$  ng on column) using the LC-MS the precision between replicates has been good (Fig. 2.10).



**Figure 2.8:** LC-MS calibration curve for DA standard solutions at seven concentrations. Error bars represent the standard error (SEM) about the mean (n=2).

While the signal intensity increased linearly within each analysis, signal response altered through time (Fig. 2.9). This highlights the importance of running calibration standard solutions with every batch of samples analysed using LC-MS if an external calibration method is deployed. Thereby, differences in the MS signal between runs can be accounted for so as not to bias DA determinations.



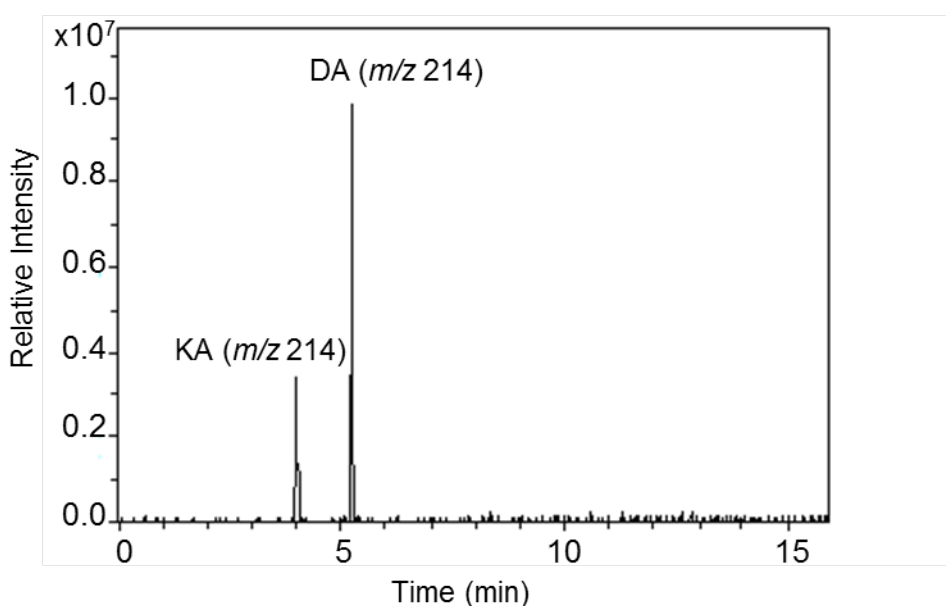
**Figure 2.9:** DA calibrations at four time points based on the peak area of DA at three increasing concentrations.

### 2.3.3 Use of an internal standard

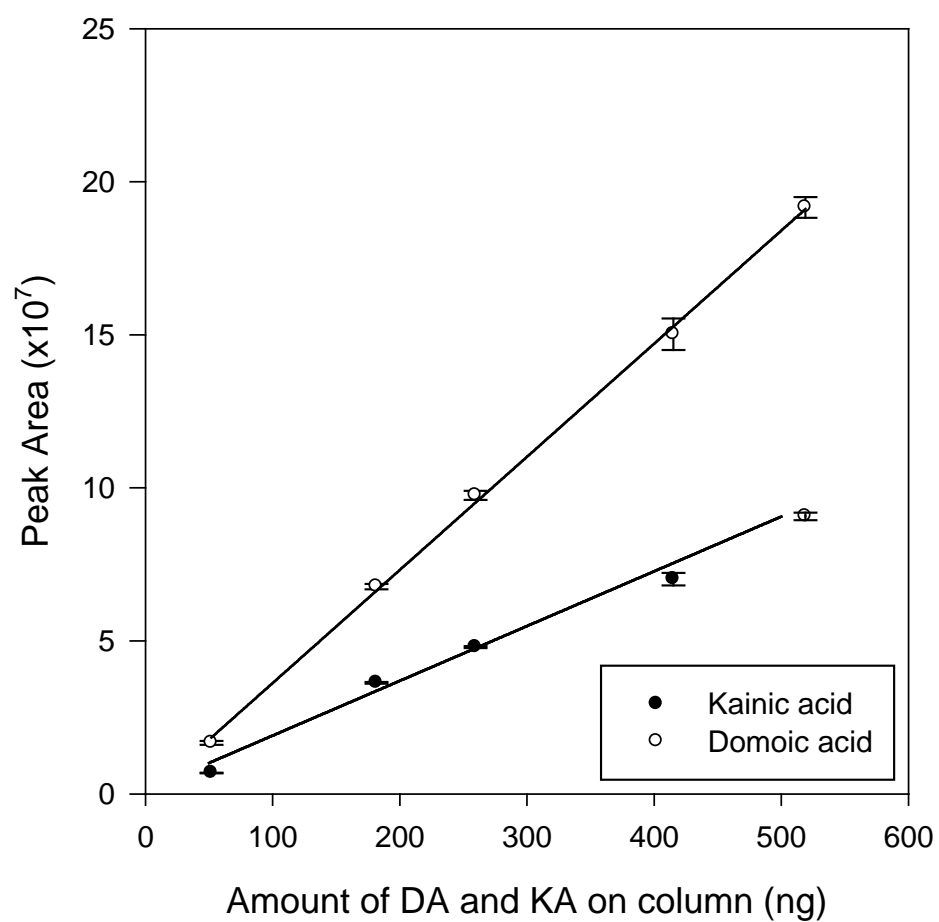
Reliable analytical determinations by methods such as LC-MS routinely deploy use of an internal standard which is often an analogue of the analyte which can be added at known concentrations to the unknown solution prior to extraction and analysis (Skoog et al., 2007). In this way, variations in recoveries and signal response over time (cf Figure 2.9) can be automatically adjusted for. Suitable internal standards must therefore have properties as similar as possible to the analyte in question, but which can still be

differentiated from the analyte. The summed LC-MS extracted mass chromatogram ( $m/z$  214 + 312) produced from analysis of a mixture of authentic kainic acid (KA) and DA in figure 2.9, shows that while DA and KA are structurally similar, they are clearly distinguishable chromatographically under the conditions deployed herein. KA had a retention time of 4.1 minutes and DA a retention time of 5.3 minutes.

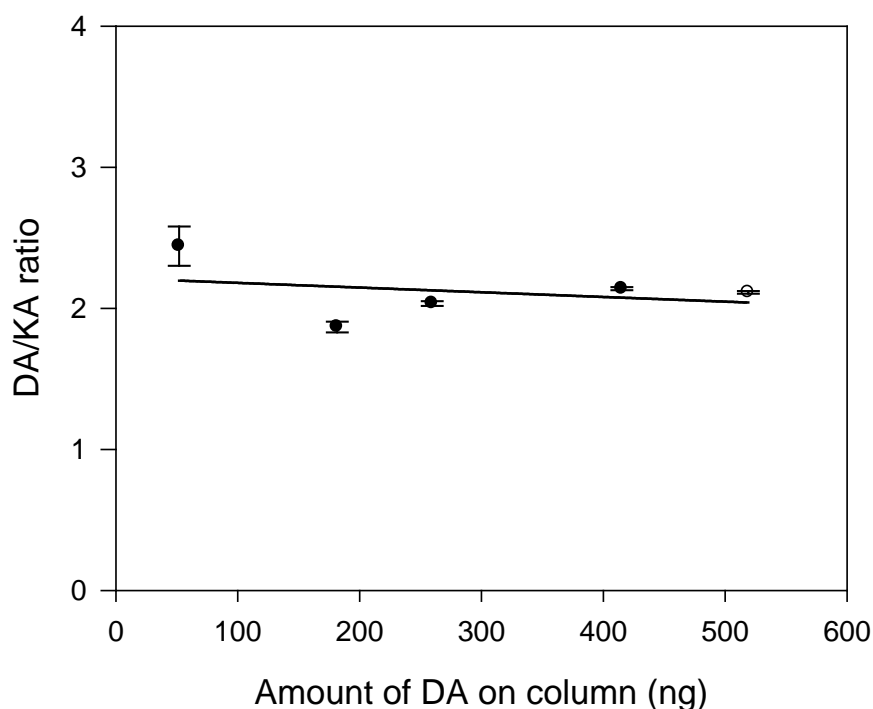
Examination of solutions of 100-500 ng of KA and DA on column by LC-MS extracted ion mass chromatography ( $m/z$  214, 312; Fig. 2.10, 2.11) showed that each compound exhibited a linear response with increasing concentration on column. The MS response to duplicate injections of these compounds was precise (Figure 2.11). As expected, it was clear that the signal intensity for each ion was different but due to the constant and linear responses, the ratio between the two compounds remained constant (Fig. 2.11, 2.12). At each of the five concentrations injected, the DA signal intensity was approximately twice that of KA ( $2.1 \pm 0.2$ ).



**Figure 2.10:** LC-MS extracted mass chromatogram ( $m/z$  214 + 312  $[M+H]^+$ ) for KA and DA peaks. Injection volume, 50  $\mu$ L, containing KA and DA at a ratio of 1:1.



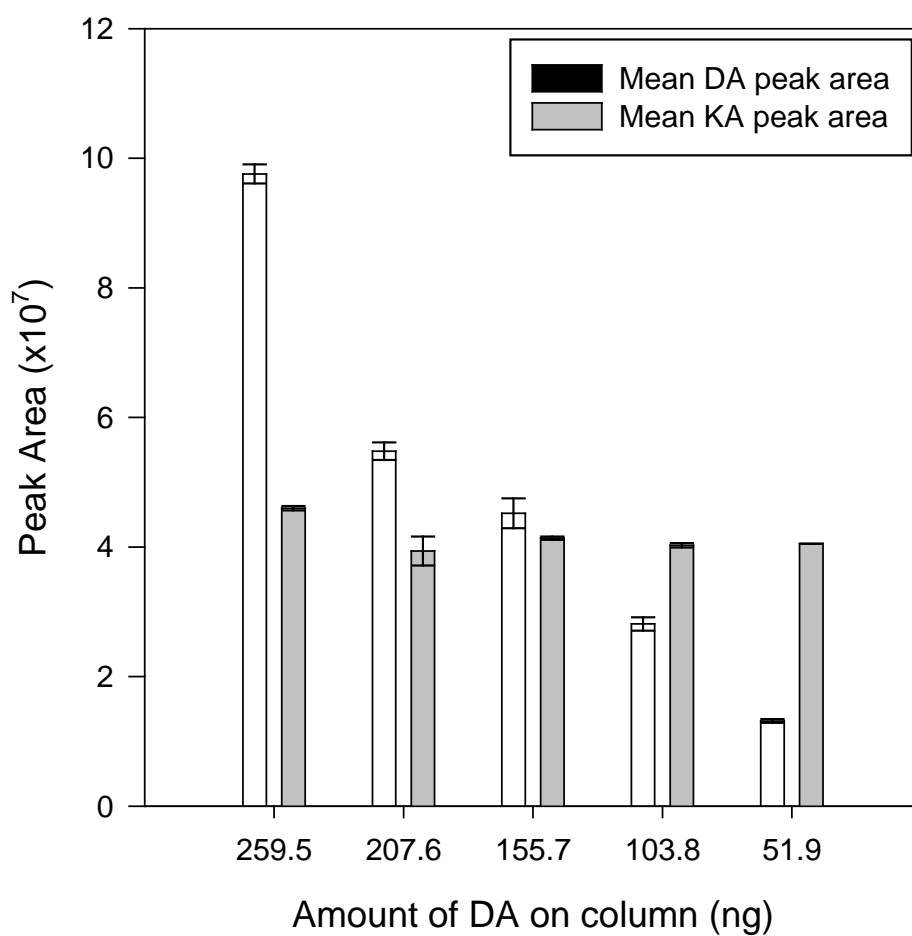
**Figure 2.11:** LC-MS calibrations for DA and KA standard solutions based on the peak area for each compound at five increasing concentrations. Error bars represent standard errors about the mean (n = 2).



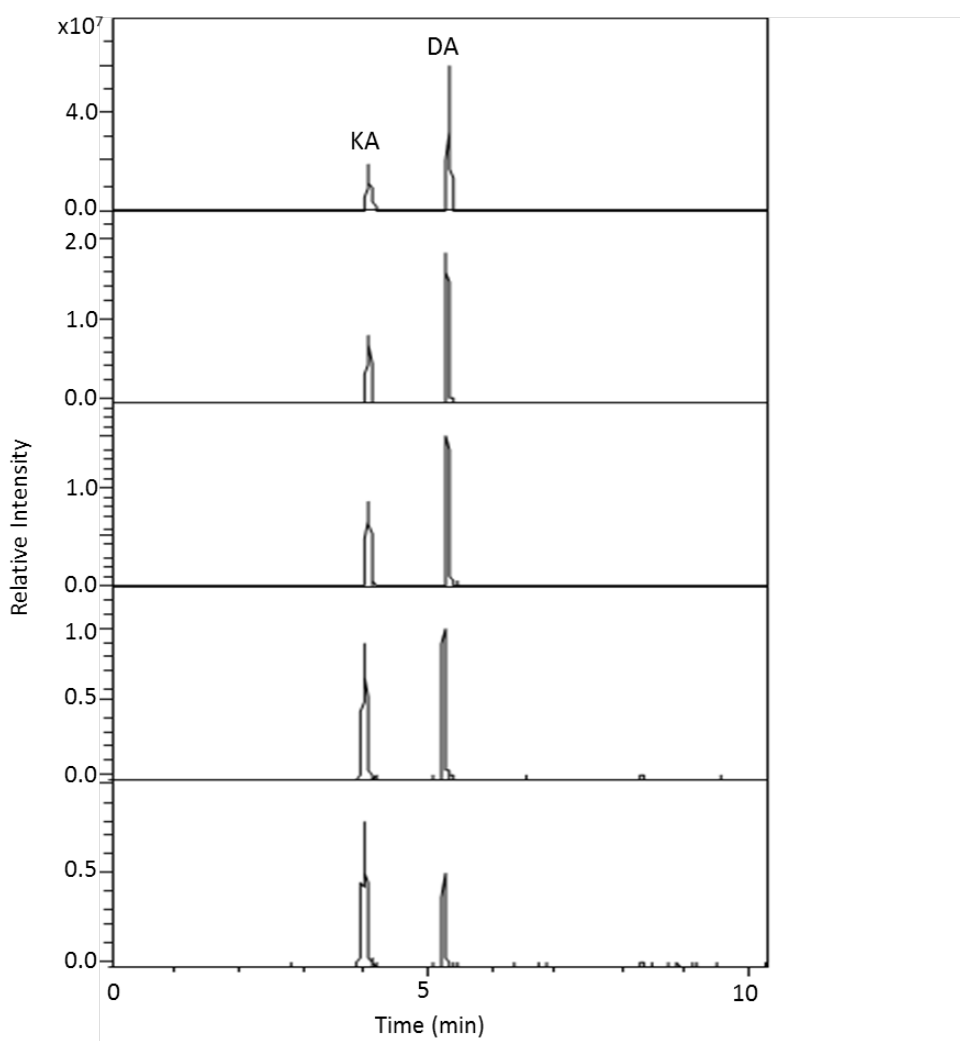
**Figure 2.12:** Ratio of DA and KA across the calibration curve. Error bars represent standard errors about the mean ( $n = 2$ ).

Additionally it was found that the response of KA remained stable over varying DA concentration ranges (Fig. 2.13, 2.14). Therefore, any changes in KA concentrations would be representative of external factors, such as sample preparation or the LC-MS system, rather than interference with DA. Both these results indicate that KA is a reliable internal standard. It is distinguishable both chromatographically and spectroscopically from DA, is stable over the useful concentration ranges and stable with varying DA concentrations. KA was therefore chosen as an internal standard to improve accuracy in all subsequent analyses. Subsequently it was discovered that one other group of workers had deployed KA in a somewhat similar fashion previously (Lawrence et al., 1994) although they were not using KA in conjunction with seawater or diatom samples and did not investigate, to the authors knowledge, how the signal response of KA varied according to DA concentrations.





**Figure 2.13:** Comparison between the peak areas for five varying duplicate DA concentrations injected onto the column in combination with a constant duplicate KA concentration (250 ng on column). Error bars represent standard error about the mean ( $n = 2$ ). Analysis was conducted using LC-MS and standard solutions of DA and KA.

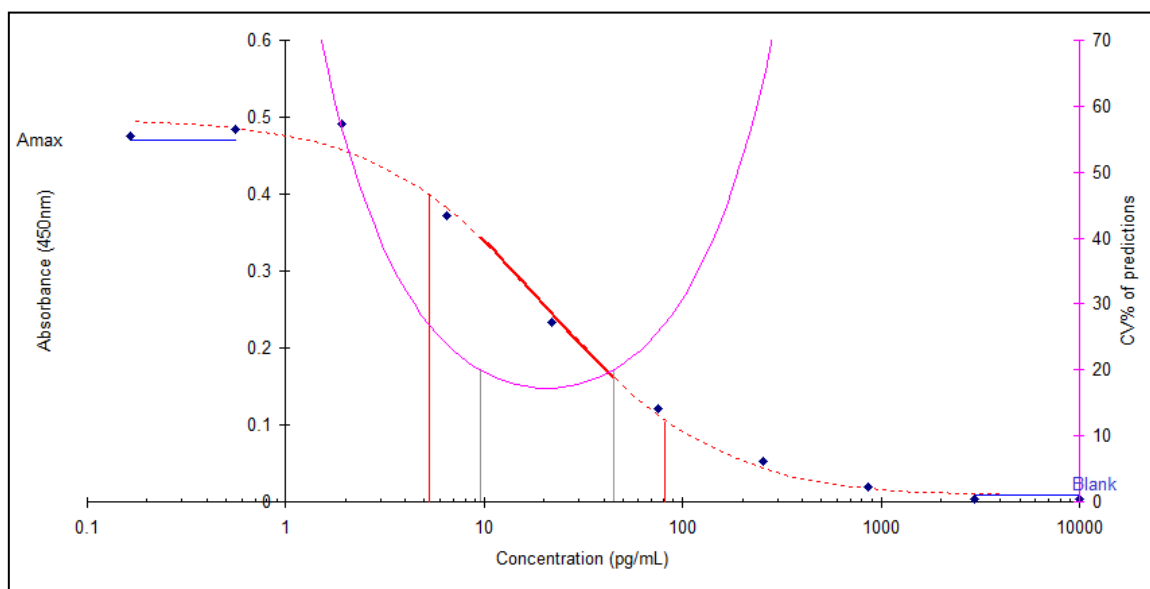


**Figure 2.14:** LC-MS extracted chromatograms of KA (left peak) and DA (right peak) at varying DA concentrations (detailed in Fig. 2.12) and a constant KA concentration (250 ng on column).

### 2.3.4 ELISA

ELISA methods were successfully applied to *Pseudo-nitzschia* culture samples and total and dDA concentrations determined in three different samples (R1-3, G1, C3) (Fig. 2.15, 2.16). For replicate *P. multiseri* (R1-3) culture samples, DA was detected by ELISA in all three replicates and total and dDA concentrations determined. The results showed that at day 10, total DA concentrations were approximately  $7.0 \pm 3.0 \text{ ng mL}^{-1}$  and dDA concentrations were approximately  $1.1 \pm 0.7 \text{ ng mL}^{-1}$ . However, there was a large percentage covariance associated with these values both between duplicate

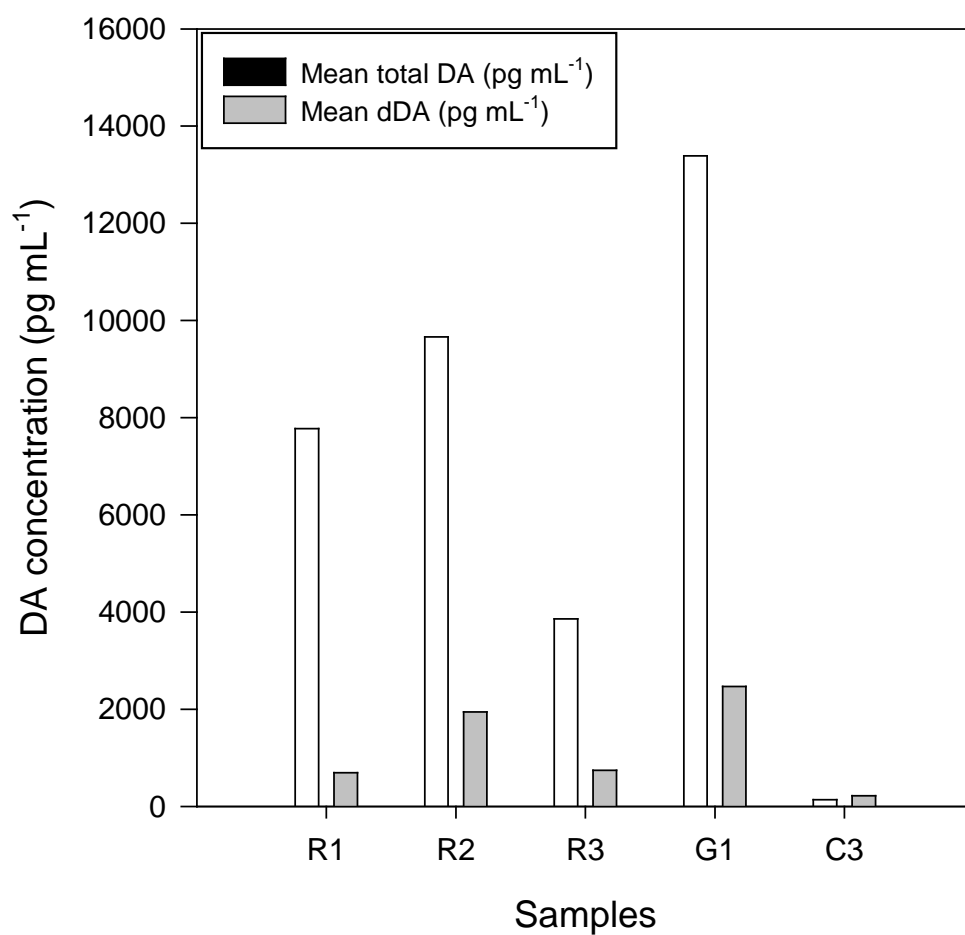
measurements of the same sample and between the triplicate samples measured (R1-3). The percentage covariance in this instance was for the whole method was > 20 % which indicates that DA concentrations are unreliable and the analysis should be repeated. DA was also detected in the late stationary phase *P. multiseriis* stock culture (G1), and as expected concentrations were found to be higher in both total ( $13.4 \pm 1.4 \text{ ng mL}^{-1}$ ) and extracellular ( $2.4 \pm 0.02 \text{ ng mL}^{-1}$ ) fractions as DA production is known to increase across the stationary phase of growth (Bates, 1998). ELISA results for the control sample (C3), taken from a culture containing non-toxic *P. fraudulenta*, estimated low concentrations of DA present in the sample (Fig. 2.16). For this sample the dDA concentration was found to be slightly greater than the total DA measured in the whole sample, which is clearly impossible. The ELISA results for sample C3 are questionable as DA has not been detected before for this species using LC-MS methods, and there is no report of *P. fraudulenta* producing DA from different areas around the British Isles. The detection of DA in this sample is likely to be due to a false positive which possibly highlights the importance of using other (e.g. mass spectrometric) methods for DA confirmation.



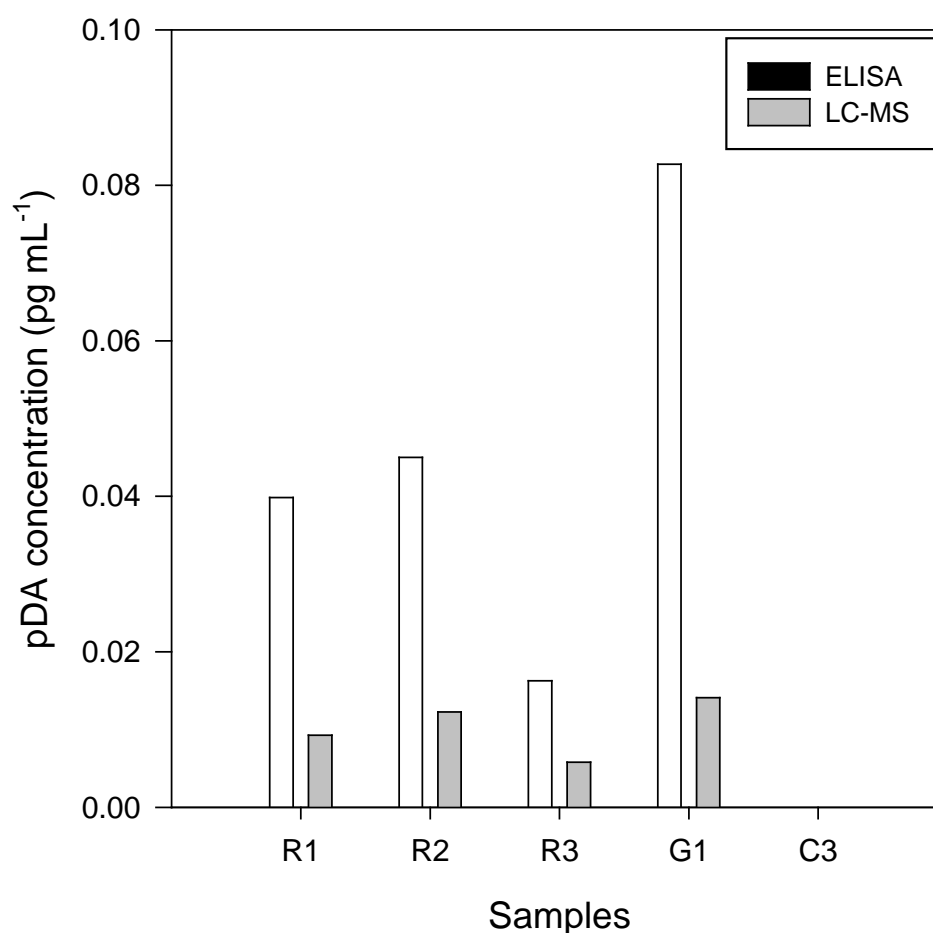
**Figure 2.15:** DA calibration curve from the ASP ELISA macro showing a sigmoid curve alongside working range limits and % coefficient of variation of predictions.

ELISA results were also compared to pDA concentrations determined by LC-MS (Fig. 2.17). On average the ELISA pDA concentrations were four times higher than the LC-MS results and in the case of G1, eight times higher. No pDA was detected in sample C3 using LC-MS methods suggesting that the ELISA result may have been a false positive. Use of the Excel® ‘macro’ provided with the ELISA kit to calculate the DA concentrations, showed that percentage covariance between samples and replicates was high ( $> 20\%$ ). This indicated that the ELISA results should be treated with caution. High percentage covariance was found between duplicate measurements of the calibration solutions measured, this causes a suboptimal fit of the calibration curve which in turn affects the working ranges of the ELISA kit. Thus providing unreliable DA concentrations. High percentage co-variance can be a symptom of poor pipetting technique or uneven rinsing of wells between steps in the method. With this in mind and the fact that ELISA was used for the first time in this study, ELISA was used to

monitor DA occurrence over an entire *P. multiseri*s growth cycle and results were compared to calibrated LC-MS methods for the same samples.



**Figure 2.16:** Total and dissolved DA (dDA) concentrations in replicate early stationary phase *P. multiseri*s samples (R1-R3), a stock culture of *P. multiseri*s in late stationary phase (G1) and non-toxic *P. fraudulent*a culture (C3), all determined by ELISA.

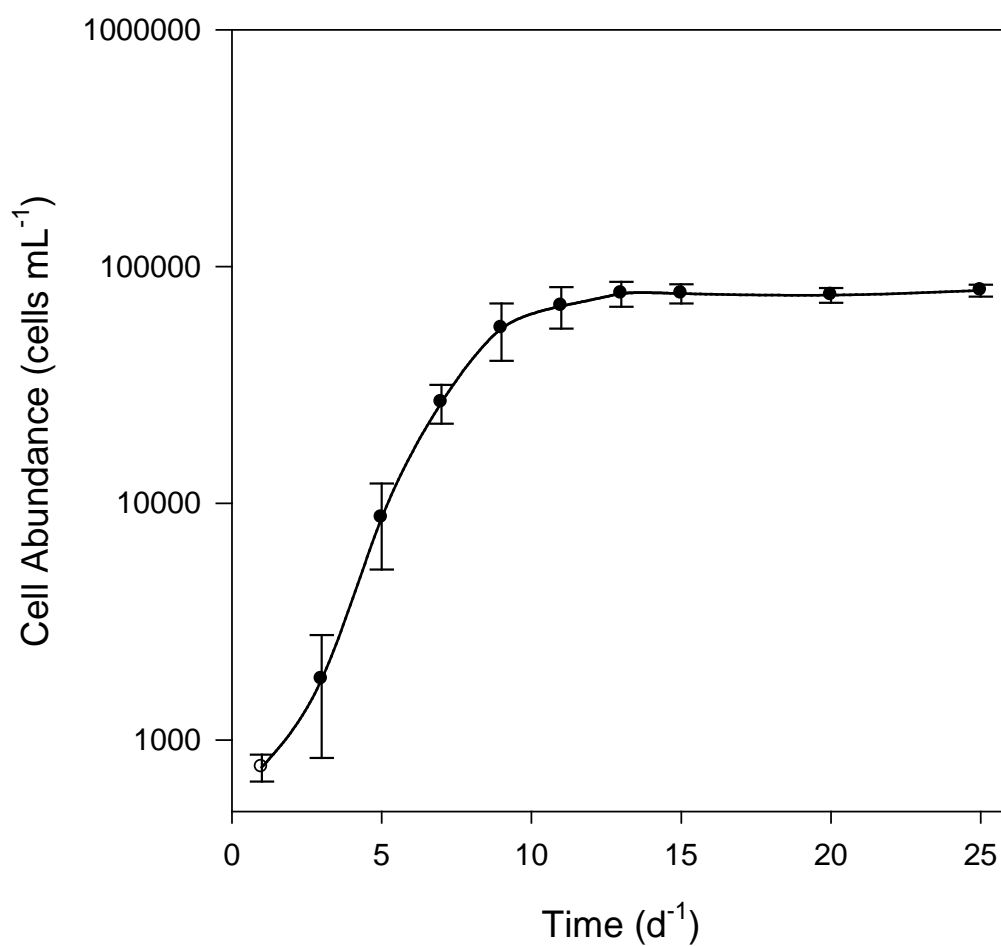


**Figure 2.17:** Comparisons between LC-MS and ELISA determinations for particulate DA (pDA) concentrations in replicate early stationary phase *P. multiseri* samples (R1-R3), a stock culture of *P. multiseri* in late stationary phase (G1) and non-toxic *P. fraudulent* culture (C3).

### 2.3.5 Comparisons between LC-MS and ELISA methods

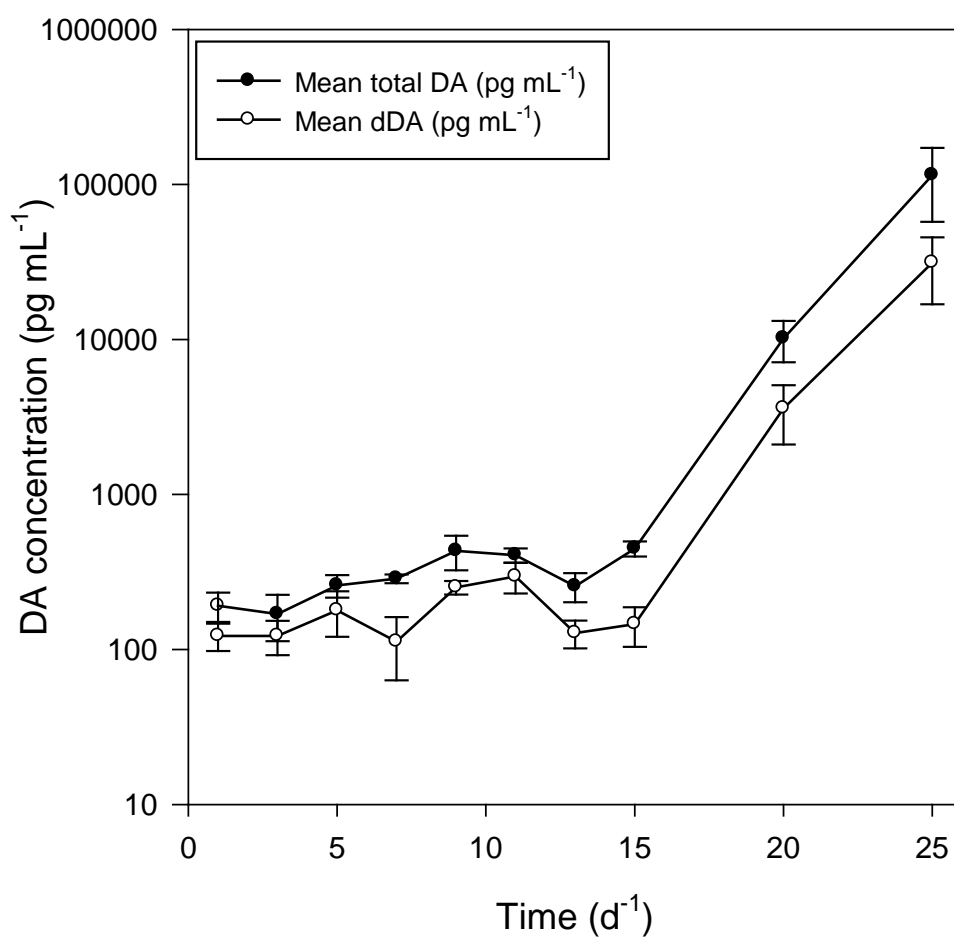
The growth of *P. multiseri* followed a typical growth curve reaching stationary phase at around day 7-8 (Fig. 2.18). The cultures reached a maximum mean cell abundance of  $17 \times 10^4$  cells mL<sup>-1</sup>, with a growth rate of 0.56 cell d<sup>-1</sup>. These values for maximum cell numbers and growth rates compare well to previous literature findings (Bates, 1998). For example, cell numbers have been reported to reach between  $10^4$  and  $10^5$  cells mL<sup>-1</sup> and growth rates are typically between 0.20-0.65 cells mL<sup>-1</sup> d<sup>-1</sup> depending on the growth conditions (Lewis et al., 1993; Pan et al., 1996c; Bates, 1998).

The ELISA results (Fig. 2.19, 2.20) showed that DA was produced throughout the growth curve, reaching high concentrations in the late stationary phase. The concentrations found in the total, dDA, and cellular DA (pDA normalised to cell abundance) fractions (Fig. 2.19, 2.20) were much lower in this experiment than previously reported in the literature, where  $\mu\text{g DA cell}^{-1}$  concentrations have been found (Bates, 1998). However, it is recognised in the literature that toxin production by *Pseudo-nitzschia* spp. can be variable, both within the same species and across species (Kudela et al., 2004). Furthermore, the total DA concentrations in this experiment were lower than the DA concentrations determined in the previous ELISA analyses at Day 10 for the same *P. multiseriata* culture (Section 2.3.4). Differences between these two ELISA results may correspond to differences in the growth rate and cell abundance of cultures in these experiments.



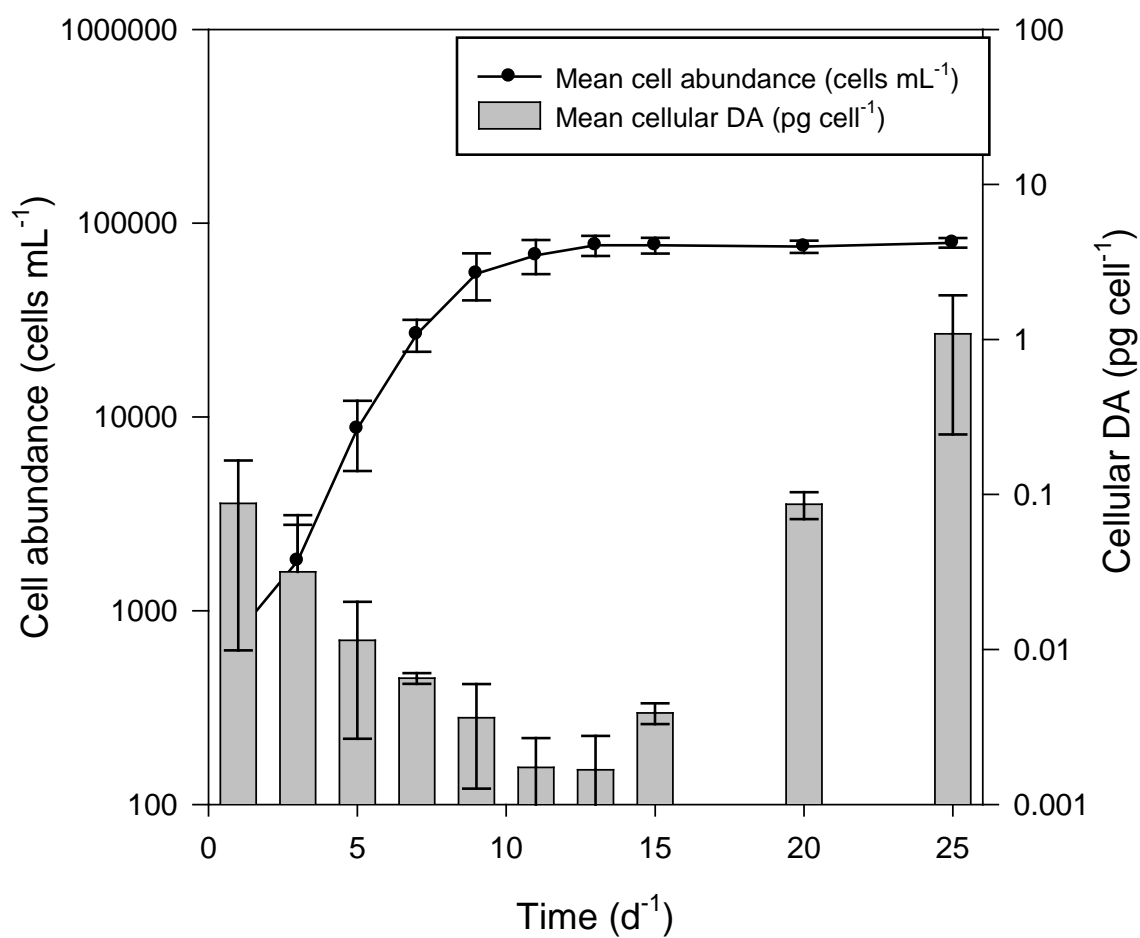
**Figure 2.18:** Mean triplicate growth curve for experimental flasks containing *P. multiseri*. Error bars represent the standard deviation from the mean ( $n = 3$ ). Growth rate was  $0.56 \text{ cells mL}^{-1} \text{ d}^{-1}$ .





**Figure 2.19:** Mean concentrations in both total DA and dDA in *P. multiseri* cultures measured in triplicate over 25 days by ELISA (pg mL<sup>-1</sup>). Error bars represent the standard deviation from the mean (n = 3).

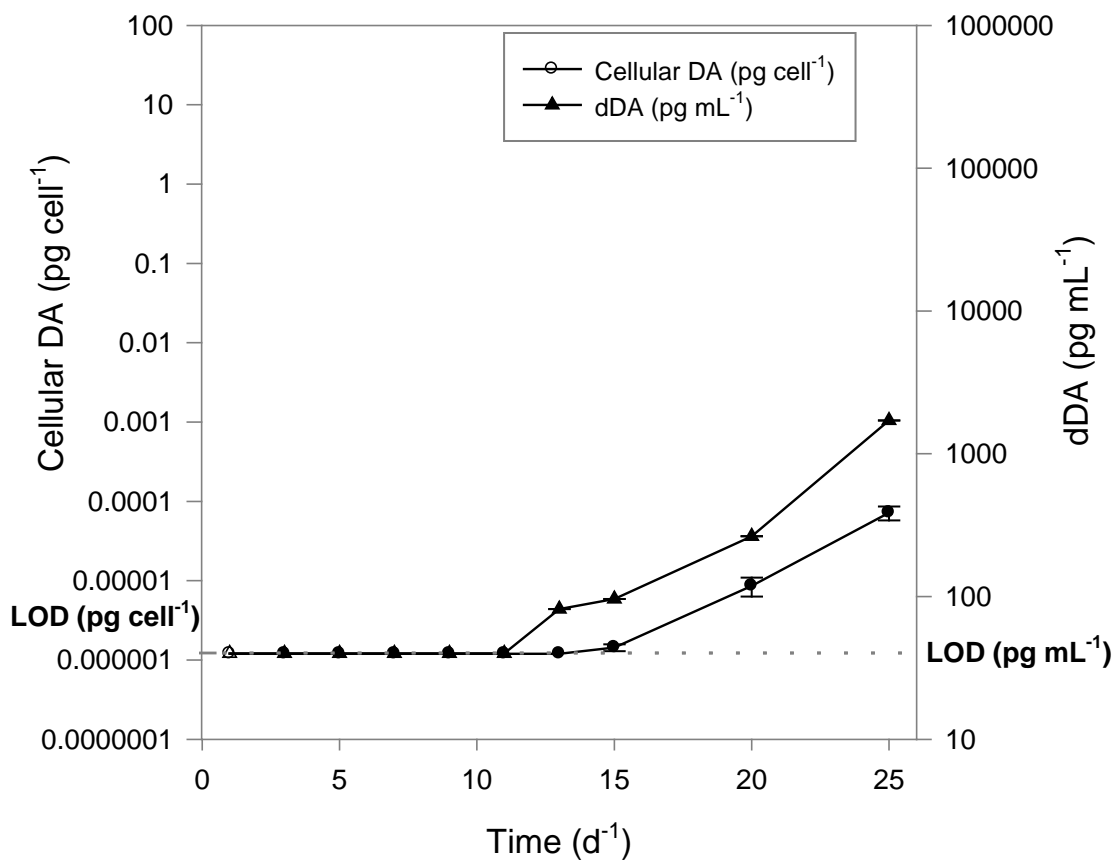
The ELISA results for cellular DA concentrations showed that high concentrations were evident at the start of the growth curve and during the stationary phase (Fig. 2.20). Detection of cellular DA at the start of the growth curve might be the result of residual DA from newly inoculated cells, taken from a late exponential phase stock culture, that were producing DA when transferred. However, cultures were given an initial acclimation period to avoid this occurring, and only a small amount of stock culture is transferred to the experimental cultures.



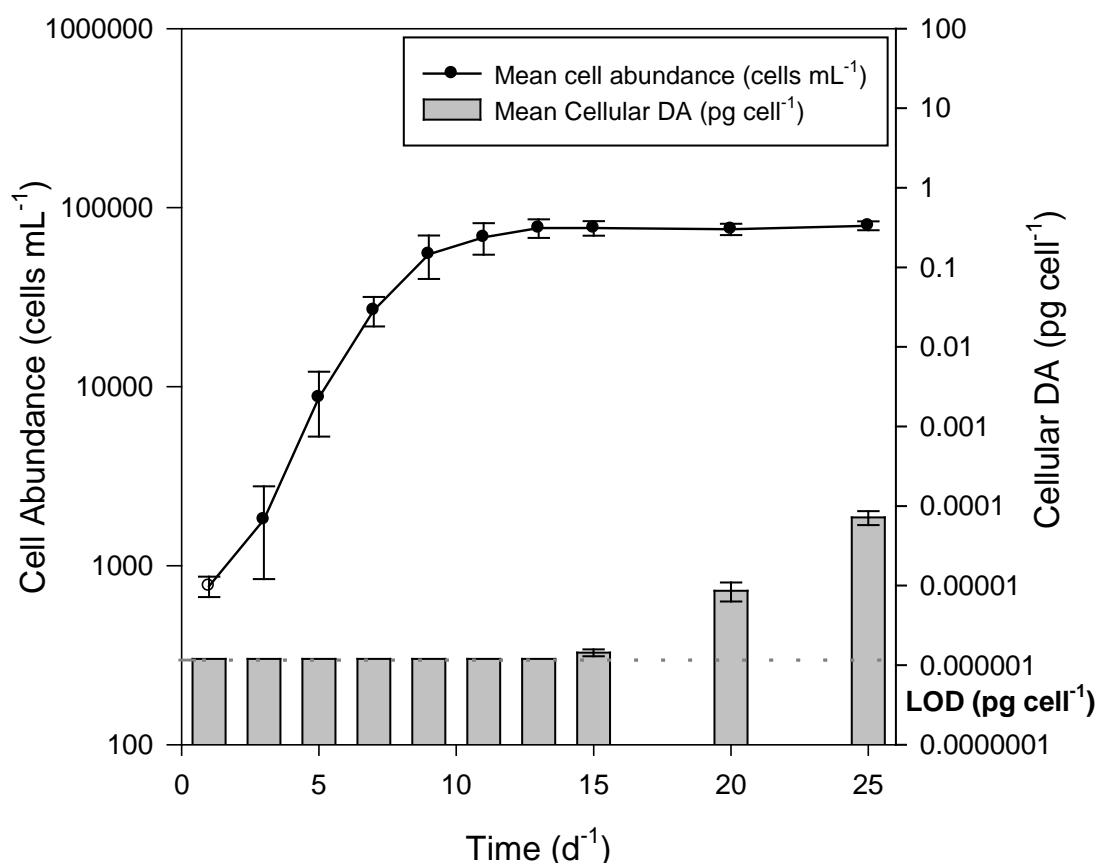
**Figure 2.20:** Mean cell abundances of *P. multiseri* along side the mean cellular DA concentrations, analysed using ELISA. Error bars represent the standard deviation from the mean (n = 3). Growth rate was 0.56 cells mL<sup>-1</sup> d<sup>-1</sup>.

Results from the ELISA macro determined that, according to the analysis of the DA calibration solutions, percentage co-variance was within an acceptable range and therefore results were reliable and within the working ranges of the kit. Despite this, a much lower concentration of DA was determined in the same samples by LC-MS (Fig. 2.19, 2.20, 2.21, 2.22). LC-MS DA determinations were approximately six times lower, in the case of maximum dDA, and 10,000 times lower, in the case of maximum cellular DA (Fig. 2.21). Another difference between these methods was that, using LC-MS, cellular and dDA were not detected in experimental cultures prior to day 13 (Fig. 2.21). This result agrees with previous findings that DA is commonly produced by *P.*

*multiseries* in late exponential/ early stationary phase growth (Bates, 1998) and not during the early phases of growth as suggested by the ELISA results. There also appears to be less variation between those LC-MS DA determinations which were concentrated and cleaned up using SPE methods (Fig. 2.21, 2.22).



**Figure 2.21:** A log plot of the mean cellular DA and dissolved DA (dDA) concentrations in triplicate cultures of *P. multiseries* using LC-MS. Error bars represent the standard deviation from the mean ( $n = 3$ ). The dotted line marks the limit of detection  $1.2 \times 10^{-6} \text{ pg cell}^{-1}$  (Cellular DA) on the left axis and  $40 \text{ pg mL}^{-1}$  (dDA) on the right axis.



**Figure 2.22:** Mean cell abundances of *P. multiseriis* alongside the mean cellular DA concentrations; analysed using LC-MS methods. Error bars represent the standard deviation from the mean ( $n = 3$ ). Growth rate was  $0.56 \text{ cells mL}^{-1} \text{ d}^{-1}$ . The dotted line marks the limit of detection which is  $1.2 \times 10^{-6} \text{ pg cell}^{-1}$  (Cellular DA  $\text{pg cell}^{-1}$ ).

In the literature, few studies actually compare cELISA results with other analytical methods; they usually favour one method and often measure DA in shellfish samples as opposed to phytoplankton samples. Validation studies for Biosense cELISA methods have generally shown that there is good agreement between cELISA and HPLC-UV results, with slight overestimations found depending on the samples analysed (Kleivdal et al., 2007a; Kleivdal et al., 2007b). There has been little focus on rigorous validation for cELISA analysis of phytoplankton samples although, where tested, method comparisons have generally corroborated with some slight overestimations evident (Kleivdal et al., 2007a). Similarly, a comparative study was conducted on DA concentrations in different seafood products (e.g. fresh, frozen, canned, and boiled

shellfish) using Biosense cELISA and the mouse bioassay and HPLC-UV methods and found that there were inconsistencies with cELISA both over- and under-estimating toxin concentrations (Garet et al., 2010). While these studies show that cELISA can overestimate DA it does not explain the large discrepancies observed in the experimental work herein comparing cELISA and LC-MS methods.

A recent study which provided single laboratory validation for cELISA methods using monoclonal antibodies (NOAA/MSI cELISA) found good agreement across cELISA and HPLC-UV results with no apparent overestimations. This study also compared results for phytoplankton samples using HPLC-FD (FMOC), NOAA/MSI cELISA, and Biosense cELISA and again found good agreement across methods. However, the NOAA/MSI cELISA method has not been used in any other field or lab study since this initial validation. Taking into consideration the findings from these various validation studies and even though cELISA methods have not been widely used on phytoplankton samples or validated against LC-MS methods, it is difficult to understand why there is so much disparity between the Biosense cELISA results and the present LC-MS analyses of the same samples. Extraction protocols were similar to those used in other studies and cELISA methods were carried out in accordance with manufacturer guidelines. Due to the acceptable level of co-variance during the present analysis, the results obtained were shown to be reproducible within batches analysed. It is possible that matrix effects could have impacted on the cELISA analysis, causing overestimations in DA determinations. The only way to get a better understanding of this would be to send samples for analysis by multiple laboratories. At this point, in the present study ELISA was discarded as an analytical tool until further information could be gained about the differences in results found between the methods. LC-MS is in any event, the favoured approved regulatory method of, for example the EU Marine

Biotoxin Reference Laboratory ([www.aesan.msps.es/en/CRLMB/web/home.shtml](http://www.aesan.msps.es/en/CRLMB/web/home.shtml)) and the method developed herein incorporating an internal standard proved to be reproducible, precise and accurate for DA quantification in both spiked (e.g. Fig. 2.11) and unspiked (e.g. Fig. 2.21) samples over the relevant concentration ranges expected in *Pseudo-nitzschia* from previous studies (Bates, 1998).

### **2.3.6 Conclusions**

The extraction, clean-up, and concentration of DA from seawater and culture matrices using solid phase extraction (SPE) methods was explored and shown that SPE was a reproducible method, providing good recovery (ca. 91 %) of 103 ng DA spiked in seawater samples. A published LC-MS method was further developed for the detection and quantification of DA in seawater and algal culture samples. This method was shown to be consistent, precise and highly sensitive when the use of an internal standard, kainic acid, was included. The results showed that KA was a reliable internal standard that improved the precision of all subsequent LC-MS analyses. An alternative method for DA quantification, based on a competitive enzyme-linked immunoSorbent assay (cELISA), was assessed and found to be very variable, providing significant overestimations of DA concentrations as determined by LC-MS for comparison. Due to the observed unreliability of this method, at least in the hands of the present author, ELISA was not pursued as an alternative analytical tool for future work, despite the attractions of high speed and lower costs. The methods developed herein served as an important tool for the accurate and precise detection and quantification of DA in culture experiments and environmental monitoring in subsequent studies, as described in the following chapters.



### **Chapter 3: Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid in the western English Channel**





## Abstract

The seasonal and intra-annual variation of *Pseudo-nitzschia* species and particulate domoic acid (DA) concentrations, at a long term monitoring site (L4) in the western English Channel, is reported over an annual cycle (January to December 2009). To determine what drives *Pseudo-nitzschia* spp. occurrence and DA production at L4 we relate results to a range of physical and chemical environmental parameters. *Pseudo-nitzschia* spp. occurred throughout the year ranging from 40 cells L<sup>-1</sup> and 250 x 10<sup>3</sup> cells L<sup>-1</sup> with two peaks in abundance occurring in June/July and in August. In August *Pseudo-nitzschia* spp. accounted for 100 % of the total diatom community. Three distinct groups or categories of species were enumerated according to morphology and size; those resembling *P. delicatissima* cell types, those resembling *P. seriata*, and those resembling *P. pungens/multiseries*. The *P. delicatissima*-group was responsible for the high abundance in August. A comparison of the resemblance matrices for these groups using PRIMER and the BEST analysis showed that the three groups were ecologically different with a number of environmental parameters influencing the abundance of the groups. The *P. delicatissima*-group was significantly influenced by the physical environmental factors of temperature, hours of light, rainfall, as well as phosphate, and salinity, whereas *P. pungens/multiseries*-group were significantly influenced by macronutrients. The *P. seriata*-group was significantly influenced by temperature and nitrate. Particulate DA was detected over a five week period from May to July with a maximum in June (0.4 ng DA L<sup>-1</sup>). When DA was present the surface waters were limited by silicate and nitrate. DA was significantly correlated with the presence of the *P. seriata*-group and the *P. pungens/multiseries*-group ( $p < 0.05$ ). This is the first time that DA has been detected at the long term monitoring site in the western English Channel and results suggest that for this site silicate plays an important role in its production.



### 3.1 Introduction

The occurrence of *Pseudo-nitzschia* species and the production of DA not only have a significant impact on coastal ecosystems, but also a socio-economic impact in terms of shellfish farming, and harvesting (Klein et al., 2010). Understanding the factors that trigger high *Pseudo-nitzschia* abundances and toxin production is essential. However, these factors are often intricately linked to the environment and remain largely unclear (Kaczmarek et al., 2007). Recent studies have shown the importance of both macro- and micro-nutrients (Maldonado et al., 2002; Fehling et al., 2004a; Wells et al., 2005) on the growth dynamics of *Pseudo-nitzschia*, as well as the effects of increasing temperature (Lewis et al., 1993), salinity (Thessen et al., 2005), pH (Lundholm et al., 2004), and irradiance (Fehling et al., 2005). The influence of these factors on DA production has also been explored suggesting that, in the case of nutrients, when these are limiting, DA is produced as part of a cell stress response (Pan et al., 1998; Trainer et al., 2012). This is except for nutrients containing nitrogen, as nitrogen is required for DA synthesis (Bates et al., 1991; Bates, 1998). Furthermore, DA production has also been correlated with increasing temperature (Lewis et al., 1993), decreasing temperature (Lundholm et al., 1994), irradiance (Fehling et al., 2005), and pH (Lundholm et al., 2004).

Most current knowledge of DA production and *Pseudo-nitzschia* spp. is based on cultured *Pseudo-nitzschia* species and regional ASP events (Klein et al., 2010). However, there is a lot of variability between these regions (e.g. upwelling areas, estuarine areas) in which toxic events occur and the species found in these areas also differ. Thus, it is difficult to unravel some of the main findings associated with DA production. Moreover, while *Pseudo-nitzschia* species are considered to be cosmopolites, not all strains within a species are able to produce DA, and indeed those

strains that can, do not produce DA all the time or during the same growth phase (Garrison et al., 1992; Fehling et al., 2004a). Therefore, it is imperative to consider regions in isolation and to collect detailed system information. Only then can the effects of environmental factors on *Pseudo-nitzschia* population dynamics and toxin production, in a particular area, be better understood.

For this purpose, the seasonal variation of *Pseudo-nitzschia* groups and particulate DA occurrence, along with several environmental parameters, were monitored at a time-series site (L4) in the western English Channel over one year (January-December 2009). L4 is comparable with other temperate coastal waters, despite being influenced by nearby estuarine outflows, by being well mixed during autumn and winter months and weakly stratified during the spring and summer months (Rees et al., 2009). Typically, stratification starts in May and persists until the end of October (Smyth et al., 2010). Nutrients are relatively abundant during well mixed periods and decline once waters become more stratified. It is during these periods of stratification that phytoplankton blooms occur (Holligan and Harbour, 1977; Rees et al., 2009). *Pseudo-nitzschia* spp. is a genus recorded annually at L4 (Widdicombe et al., 2010); however, there have been no studies to identify the species present or the occurrence of DA at this station. Therefore, the present study aims to describe the seasonal and intra-annual variability of *Pseudo-nitzschia* at L4, and to use biological, physical, and chemical data to determine what factors drive *Pseudo-nitzschia* spp. occurrence and DA production.

## **3.2 Methods and Materials**

### **3.2.1 Study area and sampling**

Samples were collected weekly from January to December 2009 at a long term monitoring station L4 (50°15'N, 4°13'W) in the western English Channel (Fig. 1.4) situated approximately ten nautical miles south west of Plymouth in the English Channel (<http://www.westernchannelobservatory.org.uk>). Station L4 has a water depth of about 55 m and is subjected to the outflow of estuarine water from the Tamar estuary (Southward et al., 2005).

### **3.2.2 *Pseudo-nitzschia* abundance and diversity**

Water samples for total phytoplankton identification were, as part of the long term monitoring programme, collected at a depth of 10 m using a 10 L Niskin bottle. Two sub-samples were taken from this; 200 mL was removed and preserved in 2 % Lugol's iodine solution and 200 mL was preserved in 4 % neutral formaldehyde. Prior to analysis preserved water samples were acclimatised to room temperature and gently rotated to resuspend and separate cell material. Volumes of between 10 and 100 mL (depending on cell density) of the Lugol's iodine preserved sub-sample were transferred to a plankton settling chamber and settled for approximately 16 hours per cm of sample and based on Utermöhl's method (Utermöhl, 1958) all cells  $> 2 \mu\text{m}$  were identified and counted using a Leica DM IRB inverted light microscope (Widdicombe et al., 2010). Cell enumeration was carried out at either x 200 or x 400 magnification (Widdicombe et al., 2010). Cells were identified to species level where possible and assigned to different functional groups. The abundance of each taxa were recorded according to the number of cells per unit volume of the sample ( $\text{cells mL}^{-1}$ ). The identification of the

taxa at L4 was carried out by Claire Widdicombe (PML) as part of the long-term time series and these samples were then re-analysed for the purpose of this study.

*Pseudo-nitzschia* spp. cells were assigned to one of two *Pseudo-nitzschia* groups based on size and morphology: the *P. delicatissima*-group (width < 3 µm) and the *P. seriata*-group (width >3 µm) (Hasle and Syvertsen, 1997). Cells resembling *P. pungens* were counted separately to the other group species, although *P. pungens* commonly falls in the *P. seriata*-group based on cell width. The *P. seriata*-group can be divided into a further two groups based on cell morphology (Hasle, 1965). Group one within the *P. seriata*-group includes: *P. fraudulenta*, *P. seriata*, *P. australis*, and *P. subpacifica*. Group two within the *P. seriata*-group includes: *P. pungens* and *P. multiseriata* (Hasle, 1995). Although *P. pungens* cells were distinguishable from both *P. seriata*- and *P. delicatissima*-group species due to their morphology it is not possible to confidently discriminate between *P. pungens* and *P. multiseriata* unless using an electron microscope. Therefore, for the purpose of this study, three groups were determined: The *P. delicatissima*-group, the *P. pungens/multiseriata*-group, and the *P. seriata*-group.

### 3.2.3 Particulate DA (pDA) monitoring

Water samples collected from a depth of 10 m, which corresponded to the depth of phytoplankton samples, were divided into 1 litre subsamples (in triplicate) and particulates were filtered onto Whatman GF/F filter disc (25 mm) and stored frozen at -20 °C until analysed. pDA was extracted from the particulates in 2 mL of 50 % methanol: water and sonicated for one minute before being centrifuged for 15 minutes at 15 °C at a speed of 3000 rpm. pDA concentrations were determined using liquid chromatography coupled with mass spectrometry (LC-MS). The method used was

adapted from Hummert et al. (2002). DA certified reference material, CRM-DA-e ( $103.3 \mu\text{g mL}^{-1}$ ), was purchased from the National Research Council (Halifax, Canada) and was used for calibration. Further details of LC-MS methods are given in Chapter 2, Section 2.2.3.1.

DA was detected using an electrospray ionisation (ESI) operating source. Separation of DA was performed by injecting a  $50 \mu\text{L}$  extract onto a  $150 \text{ mm} \times 4.6 \text{ mm}$ ,  $3 \mu\text{m}$  particle, Phenomenex (UK) Luna  $\text{C}_{18}$  reversed-phase analytical column. The mobile phase was prepared from an aqueous solution of  $50 \text{ (nM)}$  formic acid (VWR international) in  $10 \%$  methanol (Sigma-Aldrich, UK),  $90 \%$  water (Sigma-Aldrich, UK) (Solution A), and  $50 \text{ nM}$  formic acid in  $5:95 \text{ (v/v)}$  water-acetonitrile (Sigma-Aldrich, UK) with  $10 \%$  methanol (Solution B) at a flow rate of  $1 \text{ mL min}^{-1}$ . The retention time of authentic DA was  $5.3$  minutes.

Quantification was performed by integrating the extracted chromatogram peak of the DA ion  $[\text{M}+\text{H}]^+$   $m/z$   $312$  and determining the peak area using Chemstation software (Agilent Technologies, Cheshire, UK). The ratios of three molecular fragment ions ( $m/z$   $266$ ,  $248$ , and  $220$ ) were also monitored to confirm DA identity by comparison with the spectrum of authentic DA (Fig. 2.5).

### **3.2.4 Environmental parameters**

Weekly analysis of the major dissolved inorganic nutrients (e.g.  $\text{NO}_3$ ,  $\text{NO}_2$ ,  $\text{PO}_4$ ,  $\text{NH}_4$ , and  $\text{Si(OH)}_4$ ) present in L4 surface waters were conducted as part of the WEC programme at PML using recognised analytical techniques detailed in Woodward and Rees (2001). Water samples for nutrient analysis were collected, as in previous years,

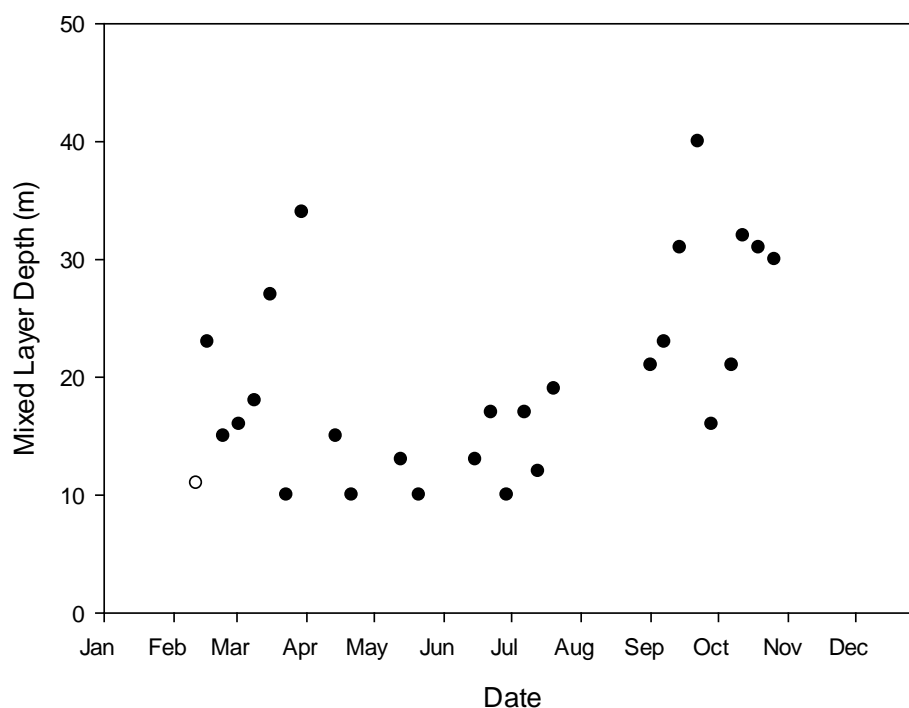


from surface waters (nominal depth of 3 m). This was by either a cleaned pumped supply or by water collected in a CTD rosette bottle. Phosphate was analysed based upon the production of the phospho-molybdenum-blue complex by reaction with molybdate and ascorbic acid, and the catalyst of potassiumantimony tartrate (Zang and Chi, 2002). Silicate was analysed based on the reaction of inorganic silicate with the ammonium molybdate to form silicomolybdic acid. This is reduced by ascorbic acid to form a silico-molybdenum blue complex (Kirkwood, 1989). Nitrate analysis calculates the sum of the total nitrate and nitrite ions. The reaction based on the reduction of nitrate to nitrite, using a copper/cadmium column, in an ammonium chloride solution (pH= 8.5). The nitrite ions react with an acidic sulphanilamide solution to form a diazo compound. This is then reacted with the N-1-naphthylethylenediaminedihydrochloride (NEDD), to form a reddish purple azo dye. The concentration of nitrate is determined by subtracting the nitrite concentration from the combined concentration (Brewer and Riley, 1965). Nitrite concentrations are determined using the same method adopted in the analysis of nitrate (Grasshoff, 1976). The analysis of ammonia is based upon the production of the indophenol-blue complex (Mantoura and Woodward, 1983).

Sea-surface temperature, salinity, and chlorophyll *a* measurements were all taken from surface waters (nominal depth of 3 m). Sea surface temperature and salinity are measured on board the research vessel using water collected from a CTD rosette. Chlorophyll *a* is analysed using reversed phase high performance liquid chromatography (HPLC) on filtered and extracted water samples collected using a CTD rosette (Llewellyn et al., 2005).

Whilst it is recognised that there is a mismatch in sampling depth between the environmental parameters and the phytoplankton data, the mixed layer depth (MLD:

taken as the depth at which there was a 0.125 density change from surface waters) throughout the year at L4 was at a depth of  $\geq 10$  m, Figure 3.1. Further information on the nutrients, sea-surface temperature, salinity, irradiance, chlorophyll, and meteorological measurements are given by Smyth et al. (2010).



**Figure 3.1:** Mixed layer depth (MLD) at L4 during 2009. MLD is calculated by the depth for which the density difference with the surface is over 0.125.

### 3.2.5 Statistical analysis

The relationships between the environmental parameters at L4 and *Pseudo-nitzschia* group species occurrences were investigated using the statistical software PRIMER 6.1 (Clarke and Gorley, 2006). All data were transformed as appropriate to approximate normal distribution and, in the case of the environmental data, normalised so that the data were on a common scale. Bray-Curtis resemblance matrices were calculated for *Pseudo-nitzschia* group data and Euclidean Distance resemblance matrices were

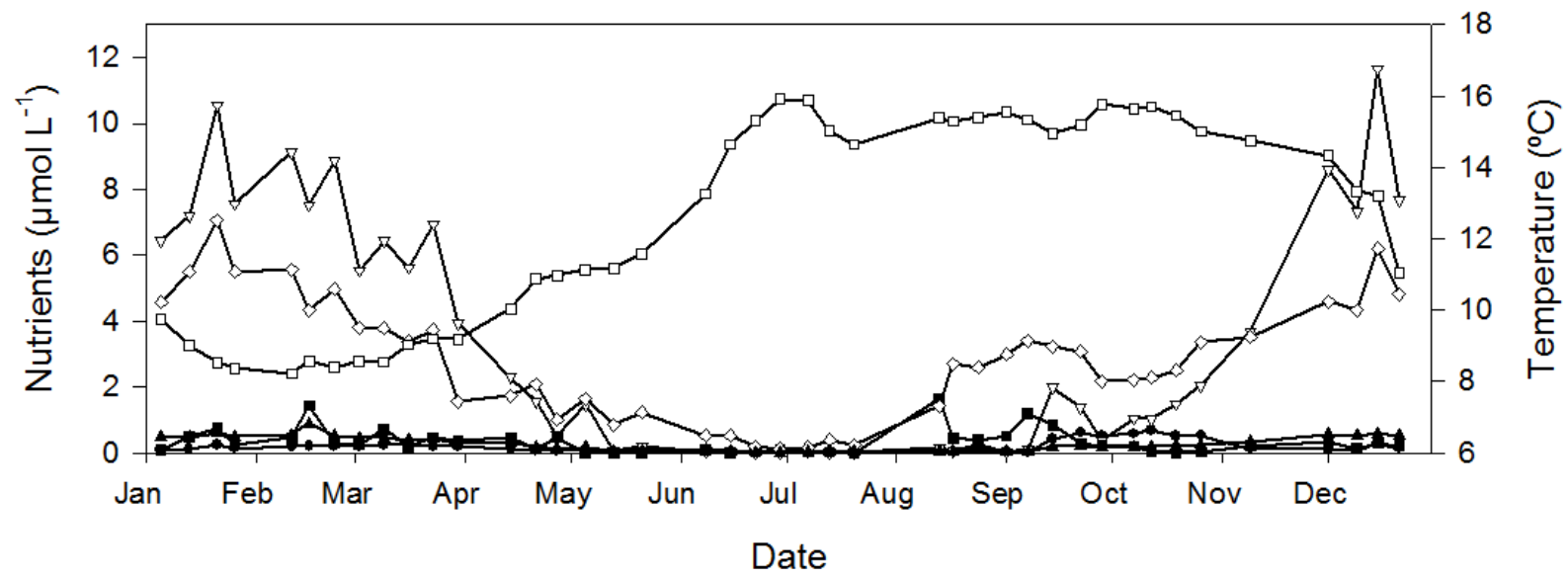
calculated for the environmental data (Clarke and Ainsworth, 1993). The matrices examine the similarities between pairs of samples (*Pseudo-nitzschia* group abundance) and pairs of variables (environmental data) (Clarke and Ainsworth, 1993). BEST analysis was used to compare the assemblage patterns for each of the *Pseudo-nitzschia* resemblance matrices to the environmental parameters (Clarke and Ainsworth, 1993). BEST analysis works by relating the similarity matrices for the biotic data to the environmental distance matrices (Heino, 2008). Using a rank correlation, BEST analysis calculates the combination of environmental variables which have a similarity matrix that best explain that of the biotic data (Clarke and Ainsworth, 1993). Correlations were calculated using Spearman's rank and significance of the correlations between matrices was based on random permutations of the data (999 permutations) (Heino, 2008). Additionally, a separate table was created of Spearman's rank correlations between transformed *Pseudo-nitzschia* group abundance data and environmental variables using PRIMER.

### **3.3 Results**

#### **3.3.1 Environmental monitoring**

Sea surface temperatures (Fig. 3.2) during the sampling period (Jan-Dec 2009) varied between 8.2 °C (Feb) and 15.9 °C (Jul), and salinity was  $34.9 \pm 0.4$  psu. Nutrient concentrations (Fig. 3.2) at L4 showed clear seasonal patterns, generally increasing over the winter months (well mixed) and decreasing to trace concentrations during the summer months (where there is stratification and a typical thermocline depth of 20 m (Smyth et al. 2010). Nitrate concentrations varied between undetected ( $< 0.02 \mu\text{mol L}^{-1}$ ) in June and  $11.7 \mu\text{mol L}^{-1}$  in December. Phosphate concentrations ranged from undetected ( $< 0.02 \mu\text{mol L}^{-1}$ ) in June to  $0.9 \mu\text{mol L}^{-1}$  in February. Silicate

concentrations varied between  $0.1 \mu\text{mol L}^{-1}$  in June and  $7.0 \mu\text{mol L}^{-1}$  in January. Ammonia concentrations ranged from undetected ( $< 0.05 \mu\text{mol L}^{-1}$ ) in June to  $1.7 \mu\text{mol L}^{-1}$  in August. Nitrite concentrations varied between undetected ( $< 0.02 \mu\text{mol L}^{-1}$ ) in June and  $0.67 \mu\text{mol L}^{-1}$  in October. Despite low concentrations of nutrients between May and August there were clear spikes where small increases in nutrients were observed. Chlorophyll-a concentrations showed the highest values ( $6.8 \text{ mg m}^{-3}$ ) in May, August, and October and the lowest ( $0.4 \text{ mg m}^{-3}$ ) from November through to April. The maximum hours of light were 16.6 h and the maximum light level reached was  $47.0 \text{ E m}^{-2} \text{ d}^{-1}$  during the summer months June (late) and August, respectively. In winter, the lowest hours of light were 8.3 h and minimum light levels were  $3.9 \text{ E m}^{-2} \text{ d}^{-1}$ .

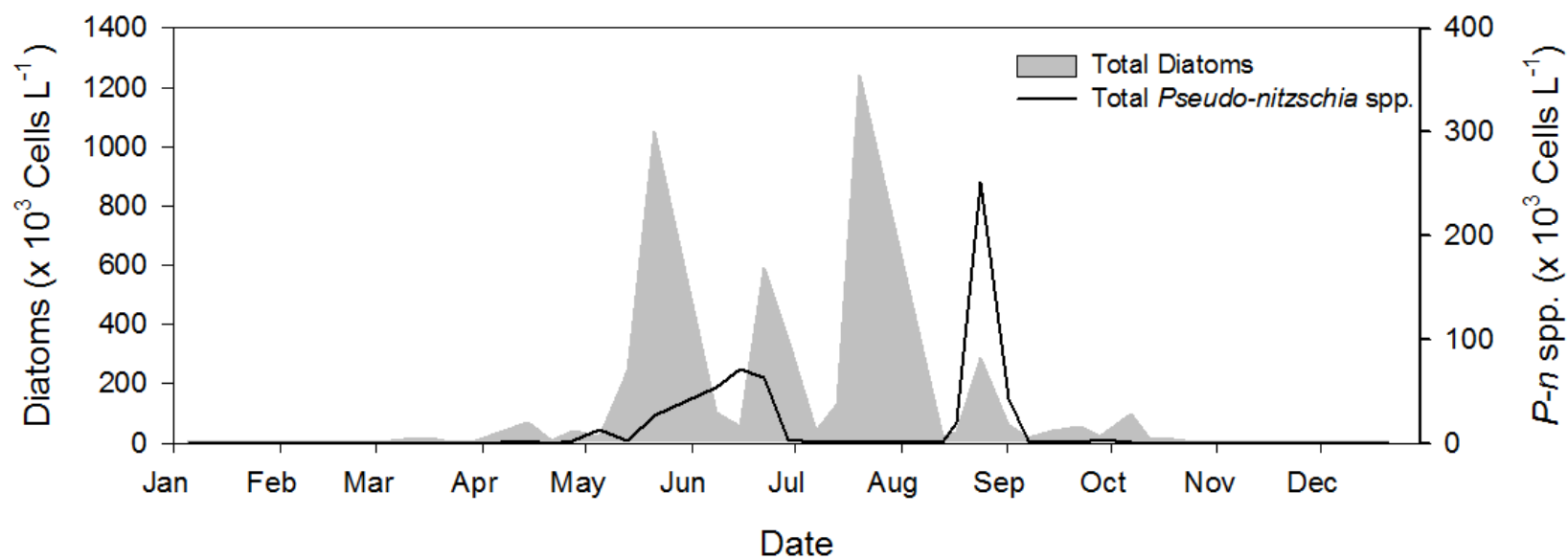


**Figure 3.2:** Nutrient concentrations ( $\mu\text{mol L}^{-1}$ ) and sea-surface temperature ( $^{\circ}\text{C}$ ) at the monitoring station L4 during 2009: nitrite (●); nitrate (▽); ammonium (■); silicate (◇); phosphate (▲); and temperature (□) data collected as part of the WEC programme at PML.

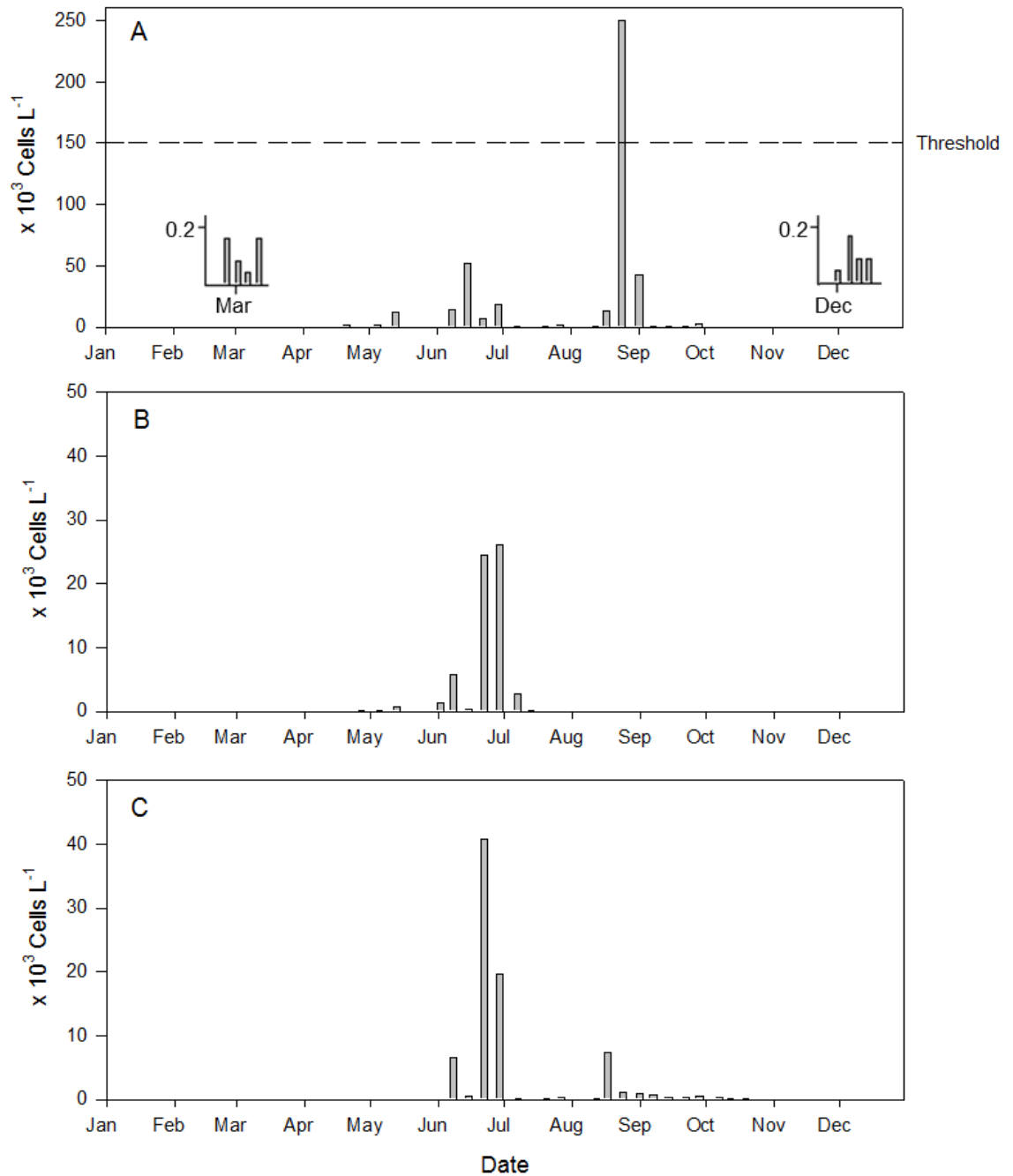
### 3.3.2 *Pseudo-nitzschia* abundance and diversity

The genus *Pseudo-nitzschia* was present throughout the year with abundances ranging between 40 cells L<sup>-1</sup> to 250 x 10<sup>3</sup> cells L<sup>-1</sup> (Fig. 3.3 and 3.4). The main occurrence of this genus did not co-occur with other diatom species (Fig. 3.3), with the highest *Pseudo-nitzschia* spp. abundance in late August accounting for 100 % of the diatom community.

Three morphology and cell size-based groups of the *Pseudo-nitzschia* genus were identified by light microscopy and therefore enumerated; a *P. delicatissima*-group (Fig. 3.4A), a *P. pungens/multiseries*-group (Fig. 3.4B), and a *P. seriata*-group (Fig. 3.4C). All three groups contributed to the high levels of *Pseudo-nitzschia* spp. present during the summer months (Fig. 3.3). Within the total species assemblage, the smaller cell (< 3 µm width) *P. delicatissima*-group formed the main background abundances throughout the year (Fig. 3.4A). The *P. delicatissima*-group co-occurred with the *P. pungens/multiseries*-group (spring/summer), and the *P. seriata*-group (summer/autumn) (Fig. 3.4 A-C). The *P. delicatissima*-group reached the highest abundance of all the groups and was responsible for high cell numbers (250 x 10<sup>3</sup> cells L<sup>-1</sup>) in August (Fig. 3.4A).



**Figure 3.3:** Total diatom abundance ( $\times 10^3$  cells  $L^{-1}$ ) and total *Pseudo-nitzschia* spp. (P-n) abundance ( $\times 10^3$  cells  $L^{-1}$ ) at the monitoring station L4 during 2009.



**Figure 3.4:** Cell abundance of the *Pseudo-nitzschia* groups present at the monitoring station L4 from January to December 2009; (A) *P. delicatissima*-group with two insets displaying cell abundances ( $< 0.2 \times 10^3 \text{ cells L}^{-1}$ ) in March and December 2009, (B) *P. pungens/multiseries*-group, and (C) *P. seriata*-group. Dashed line in (A) represents the threshold cell abundance ( $150 \times 10^3 \text{ cells L}^{-1}$ ) used by monitoring programmes above which samples are analysed for toxins.



### 3.3.3. Relating environmental conditions to *Pseudo-nitzschia* occurrence

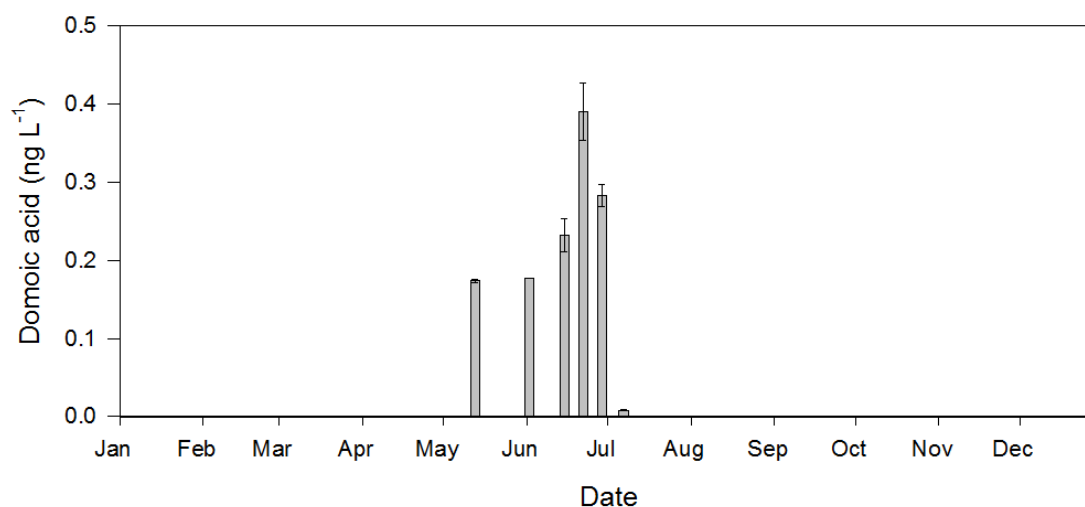
The environmental data and *Pseudo-nitzschia* group assemblage data were compared using BEST to determine which variables best explained/matched the species group data (Table 3.1). Results showed that the three groups identified were ecologically different. The *P. delicatissima*-group significantly ( $p < 0.02$ ) correlated to hours of light, phosphate, salinity, temperature, and rainfall. With the exceptions of phosphate and rainfall, all correlations were positive. The *P. pungens/multiseries*-group was significantly ( $p < 0.01$ ) negatively correlated to concentrations of all of the main nutrients (nitrate, phosphate, silicate, and ammonia) measured at L4, and the *P. seriata*-group were significantly ( $p < 0.01$ ) negatively correlated to nitrate and positively correlated to temperature.

Environmental Variables	<i>P. delicatissima</i> -group	<i>P. pungens/multiseries</i> -group	<i>P. seriata</i> -group
Temperature	<b>0.54</b>	0.23	<b>0.75</b>
Maximum light	0.51	0.46	0.32
Hours of light	<b>0.67</b>	0.71	0.49
Salinity	<b>0.56</b>	0.52	0.54
Nitrate	-0.71	<b>-0.64</b>	<b>-0.69</b>
Nitrite	-0.49	<b>-0.68</b>	-0.24
Phosphate	<b>-0.69</b>	<b>-0.58</b>	-0.69
Ammonia	-0.23	<b>-0.54</b>	-0.06
Silicate	-0.57	<b>-0.7</b>	-0.49
Total Chlorophyll-a	0.48	0.26	0.4
Rainfall	<b>-0.22</b>	0.15	0.03

**Table 3.1:** Spearman's rank correlations between *Pseudo-nitzschia* group abundances and environmental variables at L4 during 2009. Bold correlations are significant  $p < 0.02$  as defined by BEST analysis.

### 3.3.4 pDA monitoring

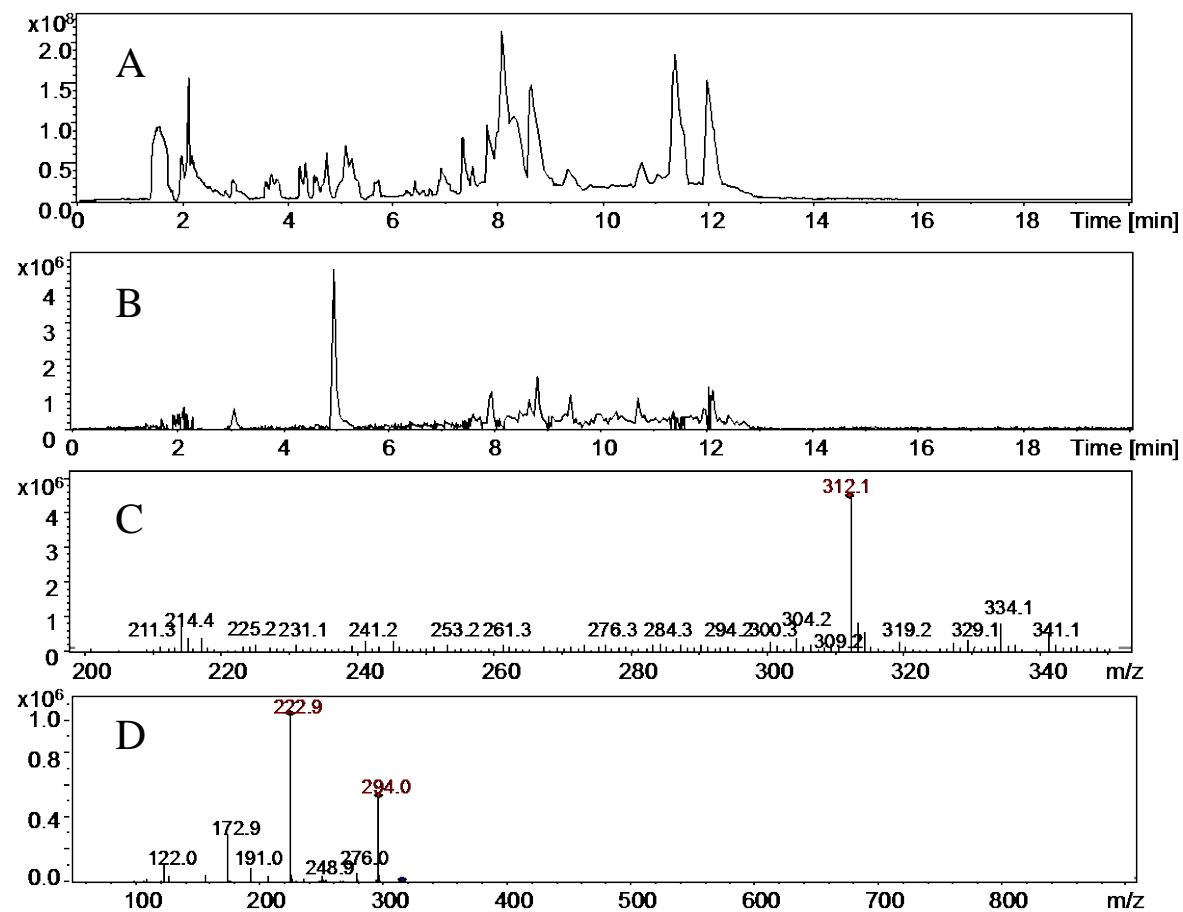
LC-MS analysis of L4 water samples during the sampling period showed that pDA was present from May to August (Fig. 3.5), albeit at low concentrations ( $0.2$  and  $0.4 \text{ ng L}^{-1}$ ) with the highest concentrations in June. The highest concentrations of pDA were measured when all three *Pseudo-nitzschia* groups were present between June and July. pDA was also measured during May when only two *Pseudo-nitzschia* groups were present, the *P. delicatissima*-group and *P. pungens/multiseries*-group. pDA concentrations peaked as total *Pseudo-nitzschia* spp. abundance started to decline at the end of June. The nutrient availability during this period (May to July) declined to either trace or undetectable concentrations in the case of nitrates, nitrites, and phosphates.



**Figure 3.5:** Particulate domoic acid concentration ( $\text{ng L}^{-1}$ ) at the monitoring station L4 during 2009. Error bars show the standard deviation from the mean (N replicates = 3).

A second DA compound was detected in L4 water samples collected on the 1<sup>st</sup> September 2009 (Fig 3.6) at a concentration of  $0.07 \pm 0.002 \text{ ng L}^{-1}$ . This compound had a similar mass to charge ratio ( $m/z$  312.1) to that of authentic DA and DA previously detected at L4. However, the MS<sup>2</sup> fragmentation pattern was different with none of the

usual signature ions, used for identification purposes, being present. This compound also eluted at a slightly earlier time interval (5.0 min) than authentic DA (5.3 min) suggesting it was DA isomer. It was only detected once during the study period Jan-Dec 2009.



**Figure 3.6:** LC chromatograms and MS<sup>n</sup> fragmentation profiles for an isomer of domoic acid recovered from *Pseudo-nitzschia* spp. on the 1<sup>st</sup> of Sept. 2009: (A) chromatogram of total ion counts, (B) chromatogram of extracted ion count at  $m/z$  312.1, (C) mass spectra of DA and (D) MS<sup>n</sup> fragmentation profile for  $m/z$  312.1.

Aside from the detection of a DA isomer, pDA was not measured again in the 2009 time series despite the presence of *P. delicatissima*- and *P. seriata*-group species in August and September. To further relate the associations between pDA occurrence and the assemblage of *Pseudo-nitzschia* species, a BEST analysis was conducted. This determined that pDA was significantly ( $p < 0.05$ ) positively correlated to the presence of the *P. seriata*-group in June and July, and the *P. pungens/multiseries*-group from May through to July (Fig. 3.4B and C). This would suggest that it was unlikely that species within the *P. delicatissima*-group produced toxins at L4 during this period.

### **3.4. Discussion**

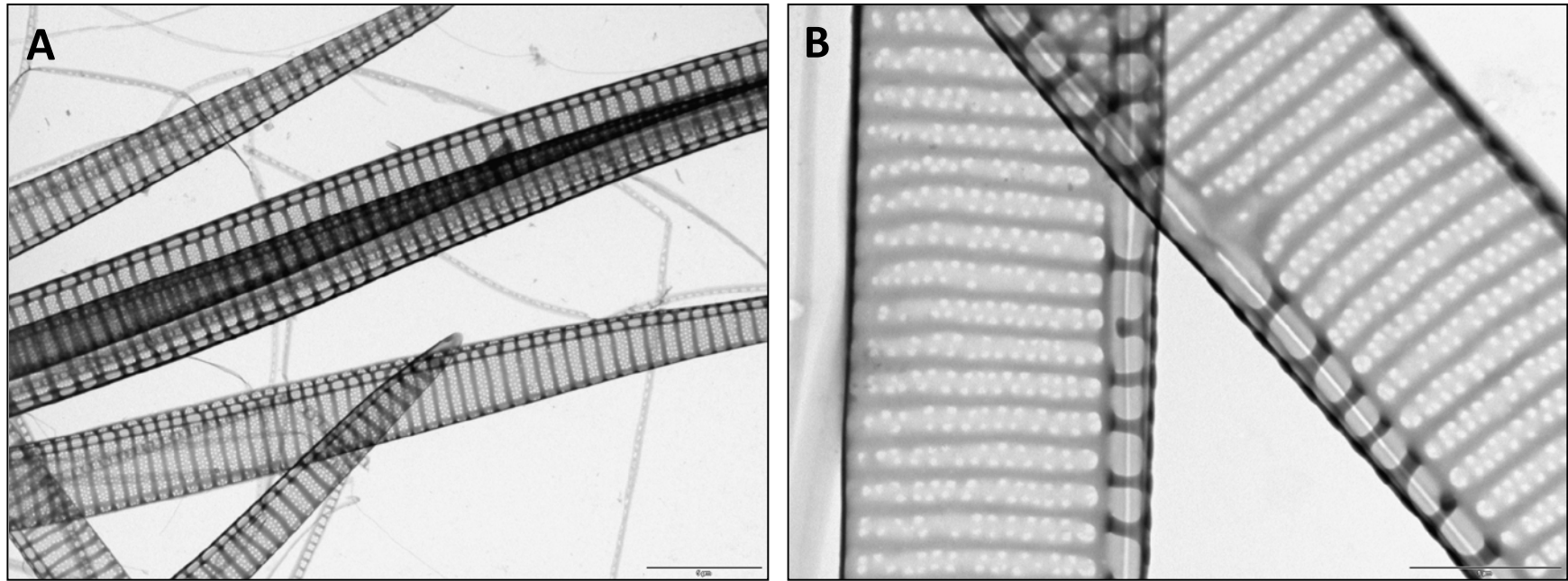
This study is the first assessment of *Pseudo-nitzschia* group succession and pDA occurrence at the monitoring station L4 in the western English Channel. We have shown that there are at least three groups of *Pseudo-nitzschia* taxa present and that one or more of the species within these groups produces DA. The seasonal variation of these groups has been explored along with the environmental conditions influencing *Pseudo-nitzschia* group dynamics.

#### **3.4.1 *Pseudo-nitzschia* occurrence**

The patterns in occurrence of *Pseudo-nitzschia* groups during the study period, suggested that there was a degree of seasonal separation between the three groups of *Pseudo-nitzschia* monitored. This degree of separation is a likely consequence of the strong seasonal patterns in environmental parameters at this site. Seasonal differences were mainly found between the timing of the *P. pungens/multiseries*-group and the *P.*

*seriata*-group during the year. However, the *P. delicatissima*-group was the most dominant group of *Pseudo-nitzschia* at L4 due to their presence throughout the year. The *P. delicatissima*-group increased in abundance between May and September and peaked again in late August. Temporal differences between the *P. delicatissima*-group and the *P. seriata*-group have been observed in previous studies (Fehling et al., 2006; Kaczmarska et al., 2007), showing the benefit of this type of discrimination.

A morphological analysis of the L4 samples was attempted using transmission electron microscopy (TEM) to gain further detail on the *Pseudo-nitzschia* species within the groups identified. However, the samples from L4 did not contain a high enough concentration of *Pseudo-nitzschia* cells to begin with and as a result did not stand up to the rigorous process of centrifugation and acid exposure required to clean the cells of organic matter for TEM. These samples had also been fixed (Lugol's Iodine) and stored prior to analysis which may have caused the cells to become more susceptible to deterioration during acid washing steps. A number of attempts were made to isolate individual *Pseudo-nitzschia* cells from L4 water samples for identification purposes and to confirm toxin production. Of the species successfully cultured *P. delicatissima* and *P. fraudulenta* were identified. Neither of which were found to produce DA.



12  
5

**Figure 3.7:** Transmission electron microscope images (JEOL 1200 TEM, Herts, England) of (A) *Pseudo-nitzschia fraudulenta* (scale bar represents 5 μm) and (B) *Pseudo-nitzschia delicatissima* (scale bar represents 1 μm) isolated and cultured from L4. Cells were identified using the ‘*Pseudo-nitzschia*-lator’ identification key (Ehrman and Kaczmarska, 2004). Frustules were cleaned by incubating a concentrated cell sample in a saturated potassium permanganate solution (KMnO<sub>4</sub> in distilled water) overnight. Cells were then heated with concentrated hydrochloric acid until the solution turned opaque and rinsed by centrifugation and several washing steps with distilled water.



### 3.4.2 The *P. delicatissima*-group

The results of the BEST analyses indicated that the *Pseudo-nitzschia* groups identified in this study were significantly correlated with different sets of environmental parameters. This may explain the observed seasonal separation between the three groups of *Pseudo-nitzschia* monitored at L4. For example, the significant parameters correlating to *P. delicatissima*-group abundance, with the exception of phosphate and salinity, were physical factors such as hours of light, temperature, and rainfall. The factors hours of light, salinity, and temperature all positively correlated with the abundance of *P. delicatissima*-group species. When examining the occurrence of this group, it was evident that whilst being present throughout the year, the *P. delicatissima*-group reached higher abundances when these factors increased (e.g. hours of light, temperature, and salinity). Phosphate and rainfall were found to negatively correlate to the *P. delicatissima*-group.

The negative relationship between the *P. delicatissima*-group and phosphate suggests that the abundance of this group increased with decreasing concentrations of phosphate. This relationship represents the requirement of phosphate for cell growth as evidenced in the time series where depletion of this nutrient through May and June corresponds to an increase in group-species abundance. As well as utilising residual phosphates in the water column in spring, the *P. delicatissima*-group also responded to pulses of phosphates in the surface waters, as observed during the summer bloom of this group. In addition to phosphate, small increases in nitrate, silicate, and ammonia concentrations were also evident during the spring/summer months at L4. When examining the ratio of nutrients (N:Si:P), despite these small increases in the nitrate, phosphate, and silicate concentrations, these nutrients remained limiting. This corresponds with previous suggestions that the *P. delicatissima*-group are effective

scavengers in low nutrient conditions (Fehling et al., 2006), owing to the fact the cells are smaller than other *Pseudo-nitzschia* group species having a larger surface area to volume ratio (Raven, 1998; Wells, 2003) and are able to utilise other forms of nitrogen such as ammonia (Fehling et al., 2006). This may have been a contributing factor influencing the growth of *P. delicatissima*-group species, in late summer, at L4. With regards to rainfall negatively correlating with the *P. delicatissima*-group abundance at L4, the main occurrence of this group in spring and summer corresponds to periods where there has been little (2 mm) or no rainfall (data not shown). Although a negative relationship was found between rainfall and the *P. delicatissima*-group abundance there was some evidence of rainfall prior to these blooms which corresponded to small increases in nutrient concentrations. A recent study at L4 has shown the significance of rainfall in stimulating blooms of other phytoplankton species at this site due to increased estuarine outflow supplying nutrient-rich waters (Rees et al., 2009). This has also been evidenced in other regions where heavy rainfall has been associated with *Pseudo-nitzschia* blooms as a result of nutrient rich freshwater inputs. (Trainer et al., 2000; Quijano-Scheggia et al., 2008; Klein et al., 2010)

Previous laboratory and field studies have identified the importance of hours of light (photoperiod) (Fehling et al., 2005; Fehling et al., 2006), temperature (Lewis et al., 1993; Bates, 1998), and salinity (Doucette et al., 2008) on *Pseudo-nitzschia* spp. and growth. For instance, a laboratory study by Fehling et al. (2005), which mimicked Scottish west coast photoperiod conditions, found that the species *P. delicatissima* achieved a greater cell density during short photoperiods indicative of spring conditions than *P. seriata* which responded better to long photoperiods. In a later field study by Fehling et al. (2006) which examined the seasonality of *Pseudo-nitzschia* groups (*P. delicatissima*- and *P. seriata*-group) in Scottish waters, photoperiod was the most

significant environmental factor influencing species occurrence having a positive influence. In relation to L4, the most substantial bloom of the *P. delicatissima*-group was observed in late August during long photoperiods. However, unlike other *Pseudo-nitzschia* groups found at L4, the *P. delicatissima*-group was more prevalent throughout the year during periods of reduced hours of light. Regarding temperature and salinity, previous studies have suggested a positive relationship between these two environmental factors and the growth of *Pseudo-nitzschia* spp. under laboratory conditions (Lewis et al., 1993; Thessen et al., 2005). Furthermore, field studies have shown how these factors have been linked to seasonality in *Pseudo-nitzschia* spp. (Quijano-Scheggia et al., 2008).

### **3.4.3 The *P. pungens*/multiseries-group**

BEST analysis revealed that, in contrast to the *P. delicatissima*-group, the *P. pungens*/multiseries-group was significantly negatively correlated with chemical parameters such as macronutrients (nitrate, nitrite, silicate, phosphate, and ammonia). Similar to the *P. delicatissima*-group, a negative correlation with major nutrients at L4 suggests that these nutrients are utilised by these species during the main growth season. A similar negative relationship between *Pseudo-nitzschia* groups and macronutrients has been reported by Fehling et al. (2006) in Scottish waters. In the present study, the *P. pungens*/multiseries-group only occurred during late spring/early summer, along with the *P. delicatissima*-group and the *P. seriata*-group, during which all the nutrients declined to either, undetected, or trace concentrations. The ratio of nutrients (N:Si:P) during this time indicated that, despite low nutrient concentrations, nitrates were consistently limiting (Geider and La Roche, 2002). This was the case throughout the spring and summer months, May to September. The decline in the *Pseudo-nitzschia*

group species in June coincided with a total depletion of nitrates from the surface waters. In conjunction, silicate also became limiting and continued to be limiting until mid August. During this time there was no significant *Pseudo-nitzschia* presence. Unlike the *P. delicatissima*-group and *P. seriata*-group, a subsequent second occurrence of the *P. pungens/multiseries*-group did not occur in August. At this time, silicates were no longer limiting but phosphates were. This may suggest that the *P. pungens/multiseries*-group species are less able to compete with other *Pseudo-nitzschia* spp. when phosphate concentrations are low. Previous work by Kaczmarska et al. (2007) has identified the importance of phosphate concentrations for *P. pungens* occurrence in Bay of Fundy. They hypothesised that *P. pungens* would be more likely to grow in abundance once phosphate concentrations become elevated. Unfortunately, Kaczmarska et al. (2007) did not record *P. multiseries* at their sampling stations during 2003 so therefore no comparison can be drawn between *P. pungens* and *P. multiseries* species and their ecological preferences. However, these species are genetically closely related and are likely to inhabit similar ecological niches (Lundholm et al., 2010).

#### **3.4.4 The *P. seriata*-group**

BEST analysis for the *P. seriata*-group indicated that the abundance of this group was significantly correlated with temperature and nitrate concentrations. The occurrence of this group coincided with a substantial increase in temperature from around 11 to 14 °C in June. Subsequent sea surface temperature was around 15 °C until the end of November, which encompassed the second occurrence of the *P. seriata*-group in the time series. The *P. seriata*-group in this study appeared to be more restricted by temperature than the other species mentioned. For example, the *P. pungens/multiseries*-group occurred in water temperatures ranging between 10.9 and 15.9 °C, and the *P.*

*delicatissima*-group in temperatures ranging between 8.5 and 15.9 °C. This compares with findings by Fehling et al. (2006) in Scottish waters where temperature was the main correlating factor for the *P. seriata*-group when relating environmental variables to *Pseudo-nitzschia* groups.

The negative relationship between the occurrence of the *P. seriata*-group and nitrate concentrations is similar to that of the other *Pseudo-nitzschia* group species at L4. For instance, the *P. seriata*-group first occurred in June during which the main nutrients were present at low concentrations and declining. The *P. seriata*-group occurred again in late summer but at a much lower abundance compared to the larger bloom of *P. delicatissima*-group species. During this period both nitrates and phosphates were available but remained limiting. These observations, along with the results for the BEST analysis, suggest that the *P. seriata*-group responded mainly to nitrate concentrations in surface waters. However, this group had limited growth, which may have been a result of phosphate limitation and/or competition by the smaller cell *P. delicatissima*-group species. Likewise, a negative relationship has been reported between *P. seriata*-group species growth and nitrate availability in Scottish waters due to the utilisation of this nutrient in surface waters (Fehling et al., 2006).

#### **3.4.5 Analysis of key abiotic factors during water column stratification**

In conjunction with the analysis of L4 results on a yearly basis, the key explanatory environmental factors and *Pseudo-nitzschia* occurrence were explored during the main period of water column stratification from May to October during 2009. This was completed as before (Section 3.2.5) using PRIMER and BEST analysis. The purpose of this complementary analysis was to increase the resolution of the data set by focusing

on a specific date range which also encompassed the main phytoplankton growth period. Analysis showed that the occurrence of species in the *P. delicatissima*-, *P. seriata*-, and *P. pungens/multiseries*-groups were correlated to all the environmental factors monitored at L4. There were no significant key explanatory variables identified from the BEST analysis. This contrasts to the BEST analysis for results from the whole year. This may be due to the fact that during the period of seasonal stratification at L4 *Pseudo-nitzschia* species from all groups were regularly present and their occurrences overlapped. For instance, while the abundance of the *P. delicatissima*-group dominated the diatom composition in August, the *P. seriata*-group also occurred but at a significantly lower cell concentration. Furthermore, during the period of seasonal stratification at L4, the environmental variables, such as the main nutrients, largely stabilise within this stratified layer. The overlapping of the *Pseudo-nitzschia* groups in this narrow date range, as well as the similarity in environmental conditions across the period of seasonal stratification, may prevent increased elucidation of the key environmental factors at L4 during this time frame.

#### **3.4.6 Group level discrimination**

To date, *Pseudo-nitzschia* group level discrimination has been used in a number of studies (Newsome et al., 1994; Fehling et al., 2006; Kaczmarska et al., 2007; Martin et al., 2009; Sahraoui et al., 2009). This type of discrimination has been found to be useful in characterising *Pseudo-nitzschia* populations in certain regions. Fehling et al. (2006) reported that the two main groups identified (*P. delicatissima*- and *P. seriata*-groups) in Scottish waters had different temporal and ecological distributions. Through cluster analysis they were able to show how this type of discrimination was favourable for separating *Pseudo-nitzschia* populations. Kaczmarska et al. (2007) also showed

statistical separation of *Pseudo-nitzschia* populations according to the main taxonomic groups in the Quoddy region, Bay of Fundy. They were also able to show how *P. pungens* separated from the *P. seriata*-group in terms of ecological distribution and was the most dissimilar species of this group. Kaczmarek et al. (2007) compared results from species level and group level analyses for important environmental factors influencing *Pseudo-nitzschia* populations. They found that both types of analyses showed similar separations between species and groups based on valve morphology. Additionally, a study by Marić et al. (1994) examined *Pseudo-nitzschia* species at group level over a four decadal period in the north-eastern Adriatic and found that there was a clear shift in the abundance and frequency of the *Pseudo-nitzschia* groups present (*P. delicatissima*- and *P. seriata*-groups). This was of particular significance to the region as it favoured the *P. delicatissima*-group which has previously been associated with toxin production in Croatian coastal waters (Newsome et al., 1994). These studies and the one presented here show that group level discrimination can provide important fundamental information on *Pseudo-nitzschia* populations and the environmental factors that are most influential to the occurrence and toxicity of the species within the *Pseudo-nitzschia* groups.

While group level discrimination of *Pseudo-nitzschia* populations can provide valuable information (Fehling et al., 2012), a number of studies that have monitored individual *Pseudo-nitzschia* species have shown that important ecological information can be lost (Almandoz et al., 2007; Klein et al., 2010; Lundholm et al., 2010). These studies have found that *Pseudo-nitzschia* species respond differently to the environment and can form corresponding groups that are not necessarily based on valve morphology. For instance, Klein et al. (2010) found that in the eastern English Channel *Pseudo-nitzschia* species separated into four groups according to the environmental factors they were

associated with. These groups were made up of species from both *P. seriata*- and *P. delicatissima*-groups. Another study exploring a 100 year record of *Pseudo-nitzschia* spp. in a sill-fjord in Denmark found that there was a pronounced shift in species composition within this genus during the 1900's (Lundholm et al., 2010). Such shifts could be missed as a result of monitoring *Pseudo-nitzschia* species at group level. This could have important implications on the perceived toxigenic potential of *Pseudo-nitzschia* blooms.

From these studies, which investigate either *Pseudo-nitzschia* groups or individual species and influential environmental factors, it seems that how well *Pseudo-nitzschia* dynamics can be explained, due to the type of taxonomic discrimination, depends largely on the region in which this genus is studied. It is only through the comparison of both types of taxonomic discrimination (species and group level) over time that you can understand how representative group level discrimination is. While this approach may help inform monitoring programmes in certain regions it is evident from detailed morphological and molecular research over the last decade that the genus *Pseudo-nitzschia* is very complex and diverse (Lundholm et al., 2003; Amato et al., 2007; Churro et al., 2009). For instance, molecular approaches have allowed for the identification of morphologically similar species (pseudo-cryptic species) and those species which are considered to be genetically distinct but morphologically identical (cryptic species) (McDonald et al., 2007; Quijano-Scheggia et al., 2009). By targeting specific regions of the genome a number of *Pseudo-nitzschia* complexes have been further elucidated and shown to comprise of a number of species not previously considered that are well separated in terms of their morphology, phylogeny, and mating compatibilities (Lundholm et al., 2003; Amato et al., 2007; Hubbard et al., 2008; Quijano-Scheggia et al., 2009). It is clear from these studies that more detailed



information is needed for the reliable identification of *Pseudo-nitzschia* spp. and that morphological information alone is not adequate. As such, the integration of molecular tools into monitoring systems is essential in understanding *Pseudo-nitzschia* population structures and associated physiological parameters.

#### **3.4.7 Statistical analysis of key environmental factors**

Many studies which have related *Pseudo-nitzschia* species/group species occurrence to key environmental factors have used multivariate statistical software packages to create ordination plots. These ordination plots cluster variables that have a high degree of similarity and those that are dissimilar have a greater distance between them. Two methods commonly used to create ordination plots are principle component analysis (PCA) using PRIMER (Martin et al., 2009; Macintyre et al., 2011) and canonical component analysis (CCA) using CANOCO (Ter Braak and Šmilauer, 2002). Of these two methods CCA has been used the most extensively in studies of *Pseudo-nitzschia* species dynamics (Almandoz et al., 2007; Kaczmarska et al., 2007; Sahraoui et al., 2009; Klein et al., 2010). These studies have created biplots by comparing biotic and abiotic data in a single matrix and analysing them with a common measure of similarity. This can be a very effective way of presenting complex environmental data. However, due to assumptions made by CAA this type of analysis was not appropriate in the present study. For instance, CAA makes assumptions of multinomial (counts which are independent arrivals) for species abundance and the *Pseudo-nitzschia* abundance data from L4 is not multinomial. Furthermore, when creating ordination biplots, the patterns in the relatedness between variables in these plots can be dominated by either the environmental data or the biotic data and it is difficult to determine which or to what effect. Instead, the environmental data and *Pseudo-nitzschia* data were dealt with

separately in our study, being analysed with different measures of similarity and then the matrices were compared using BEST analysis to identify key explanatory environmental variables. BEST analysis was therefore the most appropriate for the 2009 L4 time-series data. A similar approach has been used in other studies (Quijano-Scheggia et al., 2008; Macintyre et al., 2011).

### 3.4.8 pDA concentration

Our study is the first to record DA at the L4 monitoring station in the western English Channel. Analysis of *Pseudo-nitzschia* spp. and pDA data using BEST analysis allowed us to conclude that the pDA concentrations recorded during spring and early summer were more likely associated with the occurrence of the *P. seriata*-group and *P. pungens/multiseries*-group. However, during the second occurrence of the *P. seriata*-group in August, with the exception of the DA isomer, no pDA was detected. This could be due to a shift in species composition within this group between their first occurrence in June and second in August/September. Although the *P. seriata*-group was significantly correlated with pDA presence, it may have been that *P. pungens/multiseries*-group species alone were responsible. If indeed species within the *P. pungens/multiseries*-group were producing DA, it is more likely to be as result of *P. multiseries*, as *P. pungens* has mostly been found to be non-toxic although toxic strains are present in some regions (Trainer et al., 1998; Baugh et al., 2006). To our knowledge, *P. pungens* has not been found to be toxic in waters across the British Isles. Another possibility for the presence of pDA from May through to July but not in August/September when the *P. seriata*-group were present for a second time, could be that the environmental conditions were not favourable for toxin production during this time. These factors highlight some of the limitations of group level identification, as we

cannot be certain of the underlying *Pseudo-nitzschia* species dynamics within any group and how this relates back to the environment and toxin production.

pDA concentrations increased over a two month period reaching highest concentrations in late June ( $0.4 \text{ ng L}^{-1}$ ). However, this concentration was low compared to other reported pDA concentrations in seawater, which have been as high as  $1401 \text{ ng DA L}^{-1}$  from phytoplankton samples in Luanda Bay, Angola (Blanco et al., 2010) or  $8000 \text{ ng DA L}^{-1}$  from a bloom of *Pseudo-nitzschia* in the Gulf of Mexico in 2005 (Macintyre et al., 2011). The pDA concentrations at L4 coincided with the highest abundances of the *P. seriata*-group and the *P. pungens/multiseries*-group of  $40 \times 10^3$  and  $26 \times 10^3 \text{ cells L}^{-1}$  respectively, and at a time when nitrates and silicates were both limiting. It is well known that DA production is often stimulated by nutrient stress, a consequence of either phosphate, or silicate limitation (Fehling et al., 2004a). Therefore, considering the environmental conditions at the time pDA was measured, silicate limitation may be a driving factor in the production of DA at L4. However, the concentrations of pDA were low, which may have been due to the fact that nitrates were either limiting or absent during this period, restricting the production of DA which is a nitrogen containing molecule (Bates, 1998; Hagström et al., 2011). Likewise, the abundances of the *P. seriata*-group and *P. pungens/multiseries*-group were low in comparison to other *Pseudo-nitzschia* spp. abundance studies where counts have exceeded  $10^6 \text{ cells L}^{-1}$  (Trainer et al., 2012). As a result, the abundance of cells and the concentration of DA in surface waters during 2009 were not enough to cause a harmful event.

In addition to DA at L4, a low concentration of a second DA compound, likely to be an isomer of DA, was detected in water samples at one time point in the study period. The DA isomer had a similar mass to charge ratio ( $m/z$  312.1) to that of authentic DA but

eluted off the LC column slightly earlier and had different signature ions in the fragmentation profile (MS<sup>n</sup>). This isomer was detected across six replicate water samples taken on the 1<sup>st</sup> September 2009. In conjunction with this, a separate set of samples containing the DA isomer were prepared for LC-MS analysis and spiked with authentic DA. This revealed that the DA isomer co-eluted with authentic DA. The fragmentation profile of the DA isomer differed to that monitored in authentic DA, *Pseudo-nitzschia* cultures, and seawater DA determinations. There was also no evidence, to the author's knowledge, of this fragmentation profile from previously published studies. DA isomers, such as the one detected in the present study, can be the result of photodegradation or in the case of epidomoic acid, heat degradation (Bouillon et al., 2006; McCarron and Hess, 2006). To determine the origin of the DA isomer detected at L4, a series of experiments need to be conducted which monitor the break down products of DA over time. A number of photodegradation studies already exist in the literature but these studies have only monitored the degradation of DA over a maximum of 24 h and only investigated the production of three photoisomers (Bates et al., 2003; Campbell et al., 2005; Bouillon et al., 2006; Bouillon et al., 2008). The detection of the DA isomer in the present study demonstrates that DA and closely related isomers can be present in water samples. These isomers might otherwise be missed if using HPLC or ELISA methods for DA determination. While isomers of DA are generally found to be less toxic than DA, this result shows the benefit of using LC-MS for toxin determination so that compounds are not wrongly assigned.

Current programmes monitoring phytoplankton at shellfish aquaculture sites around the south west coast of England have established a threshold level which triggers a requirement for toxin analysis in shellfish (Stubbs et al., 2005; Hinder et al., 2011). For example, for *Pseudo-nitzschia* spp., if cell abundance exceeds  $150 \times 10^3$  cells L<sup>-1</sup> then

samples are analysed for DA (Stubbs et al., 2007a). At L4, the *P. seriata*-group and the *P. pungens/multiseries*-group were below this threshold limit. However, they were persistent in the water column for some weeks, as was DA. The impacts of persistent low intensity toxin-producing *Pseudo-nitzschia* blooms and the potential for pDA accumulation in the food chain over time are poorly understood and needs further study. In comparison, the *P. delicatissima*-group reached a cell abundance of  $250 \times 10^3 \text{ cells L}^{-1}$  but was not found to be associated with pDA. Across a 17 year time series data set (data not shown) at L4, the *P. delicatissima*-group bloomed in high numbers at least once per year and regularly exceeded  $150 \times 10^3 \text{ cells L}^{-1}$ , reaching a cell abundance of  $250 \times 10^3 \text{ cells L}^{-1}$  in one year (further details at <http://www.westernchannelobservatory.org.uk/>). Likewise, the *P. seriata*-group, but not including *P. pungens/multiseries*-group, also exceeded  $150 \times 10^3 \text{ cells L}^{-1}$  on two occasions (data not shown) during the time series, reaching a cell abundance of  $500 \times 10^3 \text{ cells L}^{-1}$  during one year. This highlights the potential for the *P. seriata*-group to bloom in high numbers at L4 and also to produce significant concentrations of DA in this region.

### 3.4.9 Conclusions

This study monitored the seasonal patterns in occurrence of three categories of the diatom *Pseudo-nitzschia* in the western English Channel over one year. Each *Pseudo-nitzschia* group at this site appeared to respond differently to the external environment. The *P. delicatissima*-group was mostly influenced by physical environmental parameters, whereas the *P. pungens/multiseries*-group was mostly influenced by chemical parameters. The *P. seriata*-group was influenced by a combination of the two. Not just one environmental parameter influenced *Pseudo-nitzschia* spp., but several.

This emphasises the need for multifactorial experiments that assess the impacts of a combination of factors on *Pseudo-nitzschia* populations.

Although the *Pseudo-nitzschia* spp. at L4 produced only low concentrations of pDA there is a theoretical potential for toxic events to occur at this site. pDA mainly occurred during June when water temperatures had increased and surface waters were subject to silicate limitation. These findings indicate that during periods and conditions such as these, toxin production is more likely in this region. One or more species within the *P. seriata*-group are likely candidates for toxin production. However, further investigation is needed to gain an improved understanding of the different *Pseudo-nitzschia* spp. at L4 and to establish which species are toxin producers.

In summary, this study, conducted over one year shows how important detailed high-resolution sampling of the environment is to the understanding of *Pseudo-nitzschia* dynamics and toxin production. The information obtained is useful for phytoplankton monitoring programmes and the eventual inclusion into for forecasting toxic events. This is becoming increasingly important for improving our understanding and prediction of harmful algal bloom processes and toxicity.



**Chapter 4: The growth and decline of *Pseudo-nitzschia multiseries* and domoic acid production under high and low nutrient and trace metal combinations**





## Abstract

The growth and decline of *Pseudo-nitzschia multiseries* and production of domoic acid (DA) were studied under low and high nutrient and trace metal combinations. Experimental batch cultures using eight combinations of high and low nutrients (determined using response surface methodology and set up in triplicate) were grown over a 40 and 22 day period. In macronutrient experiments, growth rates across all cultures varied between 0.36 and 0.41 d<sup>-1</sup>. Significant positive effects of phosphate availability ( $P = 0.0014$ ) were found on cell abundance as cultures progressed into the stationary phase. By the end of the experimental period all the nutrients (nitrate, phosphate and silicate) were significantly ( $P < 0.0001$ ) affecting cultures causing a decline in the number of viable cells during the late stationary phase. Total, particulate (pDA) and dissolved DA (dDA) concentrations were detected from Day 14 and increased up until Day 40. pDA was highest (2.0 ng DA mL<sup>-1</sup>) when cultures were exposed to high nitrate and silicate ( $P < 0.0001$ ). Cellular DA (pDA normalised to cell abundance) concentrations were significantly affected by phosphate ( $P = 0.005$ ) and silicate ( $P < 0.0001$ ). dDA concentrations were highest (3000 pg DA mL<sup>-1</sup>) when nitrate and silicate ( $P = 0.0001$  and  $P = 0.0004$  respectively) availability was low. In macronutrient and trace metal experiments, *P. multiseries* cultures had very limited growth in response to the experimental media to which cultures were exposed. This was evident from the low cell concentrations observed throughout the growth period of 22 days and the reduced growth rates, including in replete nutrient conditions. Photosynthetic efficiency of cells decreased over the experimental period and DA production was negligible. Despite exhaustive attempts to determine the cause of the lack of growth, further optimisation of the growth media and purification methods are required for future trace metal studies. We concluded that the availability of nitrate, phosphate, and silicate all significantly affect the growth, decline and DA production of *P. multiseries*, and our results highlight the importance of both phosphate and silicate availability for DA production.



## 4.1 Introduction

The mechanisms that control the growth and abundance of *Pseudo-nitzschia* spp. and the production of DA are complex and often intricately linked to the external environment (Kaczmarska et al., 2007). Many laboratory culture studies have attempted to investigate these complex mechanisms and to determine the environmental factors which trigger toxin production. These factors have included temperature (e.g. Lewis et al., 1993), irradiance (e.g. Pan et al., 1996c), photoperiod (e.g. Fehling et al., 2005), pH (e.g. Lundholm et al., 2004), salinity (e.g. Thessen et al., 2005; Doucette et al., 2008), availability of macro-nutrients (e.g. Bates et al., 1993; Fehling et al., 2004a; Cochlan et al., 2008). Many of these factors have been found to increase toxin production during the stationary phase of *Pseudo-nitzschia* spp. growth.

Generally, as the stress factor increases (e.g. increase in temperature), so does the concentration of toxin produced (Bates, 1998). However, most of the environmental factors have been studied as single variables in isolation and the focus has been on the effects of one, or at most two, factors independently. Whilst it is certainly important to understand the effects of individual environmental factors on growth and toxin production, it is also essential to consider the effects of multiple, perhaps interrelated, factors. In the marine environment one factor rarely impacts phytoplankton species in isolation, as exemplified by recent field studies where multiple factors have been found to be important for both the occurrence and abundance of certain *Pseudo-nitzschia* species and toxin production (Fehling et al., 2006; Kaczmarska et al., 2007; Klein et al., 2010; Macintyre et al., 2011).

The effects of individual macro-nutrient availability on *Pseudo-nitzschia* spp. have been studied extensively (Bates et al., 1991; Pan et al., 1996a; Pan et al., 1996b; Howard et

al., 2007). When limited, these nutrients have all been found to impose a physiological stress on growing cells and with the exception of nitrates, cause an increase in DA production (Bates, 1998; Pan et al., 1998). When nitrate, which is an important source of nitrogen essential for DA synthesis, is limited, DA production is either ceased or greatly reduced (Bates et al., 1991). In a study by Fehling et al. (2004), which unusually examined the effects of two variables, phosphate and silicate limitation, they found that silicate rather than phosphate limitation increased production of DA by *P. seriata* and was thus more significant for the potential to cause ASP events.

As well as macro-nutrient stress, the limitations of the micro-nutrients iron and copper have been found to stimulate DA production. It is thought that, in the case of iron and copper limitation, DA is produced and excreted in order to bind and make available low concentrations of these trace metals (Rue and Bruland, 2001). Due its chemical structure, DA, with its three carboxyl groups, has been compared to other like compounds capable of binding metal ions (i.e. chelators). Iron and copper, like other trace metal micronutrients, are essential for cell growth and metabolism. They play critical roles in photosynthesis and the assimilation of macronutrients (Sunda et al., 2005). Of these two nutrients, iron is the most important and is involved in chlorophyll synthesis, nitrogen assimilation, and photosynthetic electron transportation (Sunda et al., 2005). Copper, while being toxic at high concentrations, is also an important component of respiratory electron transport and the high-affinity iron transport system (Sunda et al., 2005).

Recent studies have provided compelling evidence for the physiological role of DA as a chelating agent (Bates et al., 2000; Rue and Bruland, 2001; Maldonado et al., 2002; Wells et al., 2005). For example, in a study by Rue and Bruland (2001) the binding

capacity of DA to both iron (Fe III) and copper (Cu II) was examined and it was found that DA does indeed bind to these trace metals. Furthermore, using ambient iron and copper concentrations taken from the coast of California, Rue and Bruland (2001) were able to show that high concentrations of dissolved DA could potentially compete with other naturally occurring ligands and modify the availability of these trace metals in seawater for the benefit of toxigenic diatoms. Studies by Maldonado et al. (2002) and Wells et al. (2006) extended this research and investigated the impacts of varying iron and copper availabilities on the growth and DA production of three *Pseudo-nitzschia* species (*P. australis*, *P. multiseriata*, *P. fraudulenta*). These studies found that *Pseudo-nitzschia* growth rates and overall biomass were affected by conditions of iron and copper limitation, as well as copper toxicity. They found that DA was released into the medium as a result of metal stress and this in turn increased the uptake rates of iron by actively growing *Pseudo-nitzschia* cells. According to Maldonado et al. (2002), during such periods of metal stress, 95% of intracellular DA is actively transported outside the cell.

Findings made by Rue and Bruland (2001), Maldonado et al. (2002), and Wells et al. (2006) have shown that DA production by *P. multiseriata*, *P. australis*, and *P. fraudulenta* is a function of bioactive metal stress, suggesting the metabolic role of DA lies outside the cell. This contrasts to findings in macro-nutrient studies (Bates, 1998) which showed that intracellular DA concentrations increased and accumulated during phosphate and silicate limitation, which could suggest that DA plays a role within the cell. The combination of these observations would imply that intracellular DA would not accumulate in the cells if they were experiencing metal stress simultaneously to silicate or phosphate stress. Therefore, a greater amount of DA would be actively released from cells, from accumulated DA pools and newly enhanced synthesised DA,

into the surrounding waters, which would likely affect the overall toxicity of *Pseudo-nitzschia* blooms.

The present study firstly examined simultaneously the interrelationships between the effects of three nutrients (nitrate, phosphate, and silicate) on *Pseudo-nitzschia multiseries* growth and particularly, DA production, using a statistical multifactorial experimental design. Secondly, experiments were designed to test the hypothesis that *P. multiseries* would produce and release greater concentrations of DA under combined macronutrient and trace metal limitation, compared to the independent effects of these treatments. This was achieved by measuring the growth response and production of DA by the toxigenic diatom *Pseudo-nitzschia multiseries* under trace metal stress (iron and copper) and macronutrient stress (phosphate and silicate). Experiments were conducted using batch cultures. The effects were assessed using response surface methodology (RSM) in order to minimise the number of combination experiments required whilst still allowing systematic and efficient identification of the significant factors and interaction effects between the response parameters.

## **4.2 Methods and Materials**

### **4.2.1 Stock and experimental culture conditions**

A culture of *P. multiseries* was obtained from The Marine Institute, Galway, Ireland. Cultures were maintained in sterile tissue culture flasks containing seawater enriched with f/2 nutrients with the addition of silicate (Guillard, 1975). Flasks were maintained at  $15^{\circ}\text{C} \pm 1^{\circ}\text{C}$  under a light intensity of  $92 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  (16:8 h light: dark cycle). Cultures were gently swirled by hand every 24 h.

#### 4.2.1.1 Macronutrient experiment

For experimental cultures, the artificial seawater medium Aquil was prepared (Table 4.1) and enriched with f/2 nutrients modified to achieve two different initial nitrate, phosphate, and silicate concentrations (high and low concentrations). Low concentrations were approximately eight times lower than f/2 concentrations (Table 4.2). Nutrients were added from stock solutions with known concentrations (nitrate ( $\text{NaNO}_3$ )  $75 \text{ g L}^{-1}$ , phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )  $5 \text{ g L}^{-1}$ , silicate ( $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ )  $20 \text{ g L}^{-1}$ ). Trace metal and vitamin components were added according to f/2 concentrations (Guillard, 1975). Batch culture experiments were carried out in triplicate using 1 L Erlenmeyer flasks and were maintained under the same temperature and light conditions as the stock cultures with daily agitation.

Salts	Concentration in final medium (M)
<i>Anhydrous Salts</i>	
Sodium Chloride	$4.20 \times 10^{-1}$
Sodium Sulphate	$2.88 \times 10^{-2}$
Potassium chloride	$9.39 \times 10^{-3}$
Sodium bicarbonate	$2.38 \times 10^{-3}$
Potassium bromide	$8.40 \times 10^{-4}$
Boric acid	$4.85 \times 10^{-5}$
Sodium fluoride	$7.15 \times 10^{-5}$
<i>Hydrous Salts</i>	
Magnesium chloride	$5.46 \times 10^{-2}$
Calcium chloride	$1.05 \times 10^{-2}$
Strontium chloride	$6.38 \times 10^{-5}$

**Table 4.1:** Composition of the synthetic ocean water (SOW) used in Aquil medium.



Treatment	Replicates	DIN ( $\mu\text{M}$ )	DIP ( $\mu\text{M}$ )	DSI ( $\mu\text{M}$ )
1	3	110	4.5	13.3
2	3	110	36.2	13.3
3	3	110	36.2	106
4	3	110	4.5	106
5	3	880	36.2	13.3
6	3	880	4.5	13.3
7	3	880	4.5	106
8	3	880	36.2	106

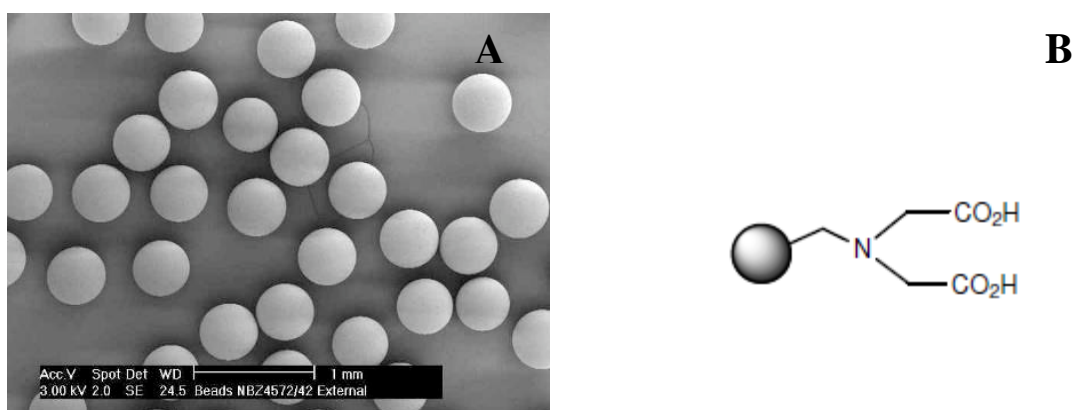
**Table 4.2:** Initial nitrate, phosphate, and silicate concentrations in each experimental culture of *Pseudo-nitzschia multiseries*. These concentrations corresponded to eight different nutrient combinations in triplicate.

#### 4.2.1.2 Macronutrient and trace metal experiment

For experimental cultures, *P. multiseries* was transferred to and acclimated in a modified version of the synthetic seawater medium Aquil (Table 4.3) which was adjusted according to the eight nutrient conditions investigated (Table 4.5) (Sunda et al., 2005). Experimental *Pseudo-nitzschia multiseries* cultures were grown in acid washed 1 L polycarbonate Erlenmeyer flasks cultures were acclimated to the eight investigated experimental conditions prior to the start of the experiment.

Element	Concentration in final medium ( $\mu\text{M}$ )
<i>Basal Media</i>	
EDTA	11.7
Magnesium	0.12
Zinc	0.08
Cobalt	0.05
Nickel	0.01
Vanadium	0.01
Chromium	0.001
Molybdenum	0.09
Selenium	0.01
<i>Iron and Copper-sufficient</i>	
Iron	0.15
Copper	0.01
<i>Iron and Copper-limited</i>	
Iron	0.001
Copper	0.000001

**Table 4.3:** Trace metal enrichments in the synthetic seawater medium used in the present experimental work, taken from Maldonado et al. (2002) and Sunda et al. (2005). Trace metal concentrations are given for basal medium and iron-sufficient or limited and copper-sufficient or limited medium was made by modifying only the concentrations of either iron or copper, respectively.



**Figure 4.1:** (A) Microphotograph of QuadraPure™ beads; (B) Structure of the polystyrene QuadraPure™ metal scavenging beads showing the functional group iminodiacetic acid. Both images were taken from the QuadraPure™ users guide found at the following web address <http://www.sigmaaldrich.com/chemistry/chemical-synthesis/technology-spotlights/reaxa/quadrapures.html>.

#### **4.2.2 Media preparation in the macronutrient and trace metal experiment**

Synthetic seawater media was purified of trace metals by batch processing using macroporous (450-600  $\mu\text{m}$  in diameter) polystyrene metal scavenging QuadraPure<sup>TM</sup> beads (Fig. 4.1). Beads were added (5 g per carboy) to the carboys containing synthetic seawater and gently agitated, keeping the beads in suspension, for 78 h on a rotation table. Prior to the addition of the QuadraPure<sup>TM</sup> beads they were first cleaned and the acid functional group (Fig. 4.1B) was regenerated in 2 M HCL. Media were then filtered to collect the QuadraPure<sup>TM</sup> beads using Teflon polytetrafluoroethylene membrane filters (Sterlitech Corporation, Washington) with a porosity of 20  $\mu\text{m}$  and a diameter of 47 mm. Filtered medium was then sterilised by microwaving (700 Watt) in acid washed 1 L polycarbonate bottles (Keller et al., 1988). Once the medium had cooled it was enriched with filter sterilised (0.2  $\mu\text{m}$  Acrodisc) macronutrient, EDTA, trace metal (Table 4.3), and vitamin (B12, thiamine, and biotin, Table 4.4) solutions. Macronutrients were added at initial concentrations of 880  $\mu\text{M}$  nitrate and either 36.2  $\mu\text{M}$  phosphate and 106  $\mu\text{M}$  silicate (sufficient), or 4.5  $\mu\text{M}$  phosphate and 13.3  $\mu\text{M}$  silicate (limited) according to Table 4.5. The macronutrients (nitrate, phosphate and silicate) were prepared as 1,000-fold concentrated stock solutions in Milli-Q water and treated with the QuadraPure beads to remove contaminant trace metals. Macronutrient stocks were made up in 50 mL acid washed polypropylene tubes and combined with 1 g of QuadraPure beads before being agitated for 78 h on a rotation table. Stocks containing the beads were then filtered as described earlier.

Vitamins	Concentration in final medium (M)
B <sub>12</sub>	3.96 x 10 <sup>-10</sup>
Biotin	2.50 x 10 <sup>-9</sup>
Thiamine	2.96 x 10 <sup>-7</sup>

**Table 4.4:** Composition of vitamin components in Aquil medium.

For trace metals, firstly EDTA (11.7 µM) was added to the medium followed by iron and copper solutions, to create iron and copper ‘sufficient’ or ‘limited’ conditions, according to Table 4.5. Secondly, all the other trace metals (copper, zinc, magnesium, cobalt, selenium, molybdenum, vanadium, chromium, and nickel) were added according to the Aquil medium recipe (Table 4.3) (Sunda et al., 2005). Trace metal stocks were prepared in Milli-Q water containing 0.01 M HCL and stored in acid washed 50 mL polypropylene tubes. The iron and copper stocks were prepared and stored similarly. Bottles containing media were allowed to chemically equilibrate for 48 h in the dark before transfer to experimental flasks. The pH was adjusted to 8.1 for each of the eight triplicate experimental flasks.

To minimise trace-metal contamination, all media manipulations, culture transfers, and samplings were performed in a sterile, dust-free class-100 laminar flow, metal free hood. Trace metal-clean protocols and aseptic techniques were used to the fullest extent possible. All plastic ware, materials, and pipette tips associated with experimental work were first acid washed or soaked prior to use

Treatment	Nitrate ( $\mu\text{M}$ )	Phosphate ( $\mu\text{M}$ )	Silicate ( $\mu\text{M}$ )	Iron ( $\mu\text{M}$ )	Copper ( $\mu\text{M}$ )
1	880	36.2	106	0.15	0.01
2	880	36.2	106	0.0015	0.000001
3	880	36.2	106	0.15	0.000001
4	880	36.2	106	0.0015	0.01
5	880	4.5	13.3	0.15	0.01
6	880	4.5	13.3	0.0015	0.000001
7	880	4.5	13.3	0.15	0.000001
8	880	4.5	13.3	0.0015	0.01

**Table 4.5:** Macronutrient and trace metal experimental design showing nutrient concentrations across eight treatments. Flasks were set up in triplicate. Nutrients were supplied at two initial concentrations (high or low) with the exception of nitrate which was always replete. Phosphate and Silicate were classed as a single factor and was jointly either supplied at high or low concentration. Iron and copper were classed as separate factors.

#### 4.2.3 Cell counts, specific growth rate and nutrient analysis

Cell growth was monitored by the abundance of viable cells using light microscopy. A 2 mL sample was collected every 48 h. This occurred over the entire 40 day experimental period for macronutrient experiments and 24 days for macronutrient and trace metal experiments. Cells were fixed with Lugol's iodine (2 % final concentration) and counted using a Sedgwick-Rafter chamber (Graticules Ltd., Tonbridge, Kent, UK) and light microscope to determine the abundance of cells. Specific growth rates ( $\mu$ ,  $\text{d}^{-1}$ ) were determined by regressing the natural log ( $\ln$ ) of cell numbers versus time, over the exponential growth phase.

For nutrient analyses in the macronutrient experiment, 15 mL samples of culture were filtered (Millipore 47mm 0.45 $\mu\text{m}$  membrane filters) for each treatment at the beginning

(Day 2) and end (Day 40) of the experimental period and the filtrates stored ( $-20^{\circ}\text{C}$ ) in acid-washed Nalgene bottles (Fisher Scientific, Loughborough, Leicestershire, UK) to await analysis. After thawing, samples were analysed for nitrate, phosphate and silicate concentrations using a nutrient auto-analyser (Branne and Luebbe, AAIII; SPX Flow Technology Ltd., Brixworth, Northampton, UK) employing standard methods (Brewer and Riley, 1965; Woodward and Rees, 2001; Zang and Chi, 2002). Stock solutions were also analysed prior to experimental setup.

#### **4.2.4 Macronutrient and trace metal experiment: FIRE measurements**

As a proxy for iron stress, fluorescence induction and relaxation measurements were performed on cultures throughout the growth curve. A 3 mL sample was taken from each flask every 48 h in a 15 mL polypropylene tube, of which 1 mL was analysed using a FIRE fluorometer system (Satlantic, Halifax, Nova Scotia). Prior to each measurement, samples were acclimated in the dark at room temperature for 20 min. Analysis was conducted in darkness to prevent light interference. Using instrument software, the ratio ( $F_V/F_M$ ) of variable fluorescence ( $F_V$ ) to maximum fluorescence ( $F_M$ ) was determined, which gives an indication of photosynthetic efficiency for experimental cultures in response to various trace-metal/nutrient conditions.

#### **4.2.5 DA sampling and extraction**

Preliminary growth experiments have showed that initial production of DA in batch culture has occurred when cells reached the stationary phase at ca. 14 days (Fig. 2.21). Therefore, for macronutrient experiments, samples for DA determination were taken on

Day 14 and every sixth day thereafter. For macronutrient and trace metal experiments, previous studies have indicated that iron and copper stress have caused *Pseudo-nitzschia* spp. to produce DA earlier, during the exponential phase of growth, than studies investigating macronutrient stress (Maldonado et al., 2002). On this basis, samples for DA determination in this study were taken on Day 2 and every fourth day thereafter. DA for this particular experiment has not been reported herein due to concentrations being negligible as a result of limited culture growth across treatments.

At these sampling time points, 50 mL samples (cells plus medium) were collected in 50 mL polypropylene conical based tubes. Samples were filtered (25mm GFF, Whatman) under low vacuum and the filters placed in 2 mL amber tubes and stored at -80 °C and retained for particulate DA (pDA) determination. For dissolved DA (dDA), filtrate was collected in 50 mL polypropylene conical based tubes and extracted according to a method described by Wang *et al.* (2007). In brief, the filtrate was concentrated using the STRATA X solid phase extraction (SPE) columns (Phenomenex, UK). Details of SPE columns, conditioning steps, and DA elution are given in Chapter 2 Section 2.2.2. Before particulate DA samples could be analysed, filters containing *P. multiseriata* cells were extracted in 2 mL of 50 % MeOH: water and sonicated for 1 min before being centrifuged for 15 minutes at 15°C at 3000 rpm. The supernatant was collected and analysed by liquid chromatography-mass spectrometry (LC-MS).

#### **4.2.6 DA determination using LC-MS**

DA was detected using liquid chromatography ion trap mass spectrometry (LC-MS) with an electrospray ionisation (ESI) operating source. Chromatography was performed on a 150 mm x 4.6 mm, 3 µm particle, Phenomenex (UK) Luna C<sub>18</sub> reversed-phase

analytical column. The volume of sample injected onto the column was 50  $\mu$ L. Details of the LC-MS method used in the present study are given in Chapter 2 Section 2.2.3.

Quantification was performed by integrating the extracted mass chromatogram peak of the ion  $m/z$  312 assigned as  $[M+H]^+$  for DA and determining the peak area using Chemstation software (Agilent Technologies, Cheshire, UK). The ratios of three fragment ions in the spectra ( $m/z$  266, 248, and 220) were also monitored to confirm DA identity by comparison with the spectrum of authentic DA.

#### **4.2.7 Experimental design**

Experimental design and data analysis software (Design-Expert® V.7.1.5, Stat-Ease Inc., Minneapolis, USA) was used to design a multifactorial experiment utilising response surface methodology to examine the effects of macronutrient availability as well as macronutrient and trace metal availability at two levels (high and low concentrations) on *P. multiseri* growth and DA production. A total of 24 experimental runs, based on 8 triplicate treatments, were used to compile the models. The analysis of variance (ANOVA) for the experimental responses, model coefficients, and formulation of two-dimensional contour plots for visual observation of trends and interactive effects of nutrients were carried out using the same software. Highest order polynomials, chosen to best model data, were based on sum of squares, lack of fit tests and agreement between adjusted and predicted  $R^2$  values.



## 4.3 Results

### 4.3.1 Macronutrient experiment: Inorganic nutrient concentrations

Concentrations of nitrate in the experimental medium were 110  $\mu\text{M}$  (low concentration) and 880  $\mu\text{M}$  (high concentration) at the start of the experiment, which then decreased to  $5 \pm 2.8 \mu\text{M}$  and  $658 \pm 150.0 \mu\text{M}$  respectively by algal utilisation and uptake. Initial phosphate concentrations were 4.5  $\mu\text{M}$  (low concentration) and 36.2  $\mu\text{M}$  (high concentration) which by Day 40 reduced to  $2 \pm 0.8 \mu\text{M}$  and  $19 \pm 5.5 \mu\text{M}$  respectively (Table 4.2). Silicate was added to the experimental medium to achieve starting concentrations of 13.3  $\mu\text{M}$  (low concentration) and 106  $\mu\text{M}$  (high concentration) but due to technical problems the initial and final silicate concentrations were not determined. Analysis of the seawater medium before various inoculations of nutrients determined that silicate was at a trace concentration, 0.68  $\mu\text{M}$ , and the silicate stock solution was 109  $\mu\text{M}$ .

### 4.3.2 Growth and cell numbers

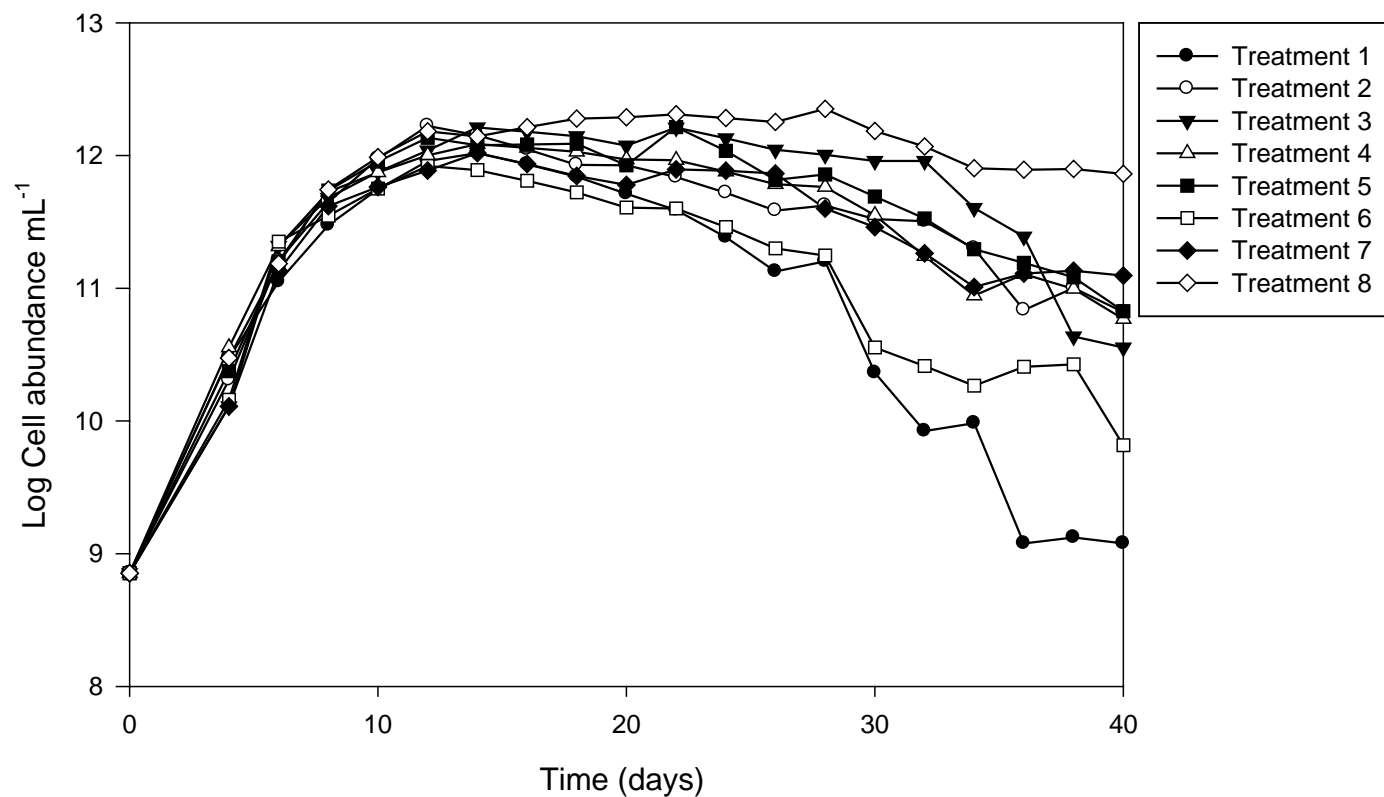
#### 4.3.2.1 Macronutrient experiment

Experimental cultures were inoculated with approximately  $7 \times 10^3 \text{ cells mL}^{-1}$  across all treatments and reached abundances between  $1.5 \times 10^5$  and  $2.3 \times 10^5 \text{ cells mL}^{-1}$ . The growth responses of *P. multiseriis* cultures can be seen in Figure 4.2. Exponential growth occurred between Days 1-6 after which experimental cultures went through a transition from exponential phase through to stationary phase between Days 8-14. This transition was characterised by a period of slower growth between Days 6-12 where growth rates varied between  $0.07 \pm 0.04 \text{ d}^{-1}$  and  $0.14 \pm 0.03 \text{ d}^{-1}$  (Table 4.6). The stationary phase was followed by a rapid decline in cell abundances from approximately

Day 20 onwards (Fig 4.2). This decline varied according to treatment and resulted in an increase in non-viable cells which were those cells that had no cytoplasm. Figure 4.2 shows that Treatment 1, which had low initial concentrations of nitrate, phosphate and silicate, had the shortest stationary phase and the greatest decline in viable cells. This is also evident from the rate of decline which was  $-0.15 \pm 0.01 \text{ d}^{-1}$  compared to a decline of  $-0.03 \pm 0.00 \text{ d}^{-1}$  in Treatment 8 where concentrations of nitrate, phosphate and silicate were all high (Table 4.6). Moreover, cultures in treatment 8 had the longest stationary phase, closely followed by Treatment 3 (low nitrate, high phosphate, and high silicate) which only showed signs of cell decline from Day 30 onwards. Cultures in treatment 6 (high nitrate, low phosphate, and low silicate) showed a similar pattern in the decline of viable cells to those in Treatment 1

	Treatment	Days 1-6 SGR (d <sup>-1</sup> )	± SD	Days 8-12 SGR (d <sup>-1</sup> )	± SD	Cell decline (d <sup>-1</sup> )	±SD	Day 14 (cells mL <sup>-1</sup> )	±SD
160	1	0.37	0.02	0.12	0.01	-0.15	0.01	165,833	31,383
	2	0.38	0.02	0.14	0.03	-0.06	0.02	189,583	7,217
	3	0.40	0.02	0.09	0.04	-0.08	0.02	201,250	8,197
	4	0.41	0.00	0.07	0.05	-0.07	0.02	177,500	5,449
	5	0.39	0.00	0.10	0.06	-0.06	0.01	175,833	26,051
	6	0.40	0.01	0.10	0.07	-0.09	0.01	145,833	14,913
	7	0.36	0.03	0.07	0.04	-0.05	0.01	165,833	24,442
	8	0.39	0.01	0.11	0.03	-0.03	0.00	187,917	22,020

**Table 4.6:** Comparison of the mean growth rates of *P. multiseri*s at two different time frames during exponential growth, mean decline in viable cells during the late stationary phase, and mean cell numbers as cultures went into stationary phase across eight triplicate nutrient treatments. SD represents one standard deviation from the mean.



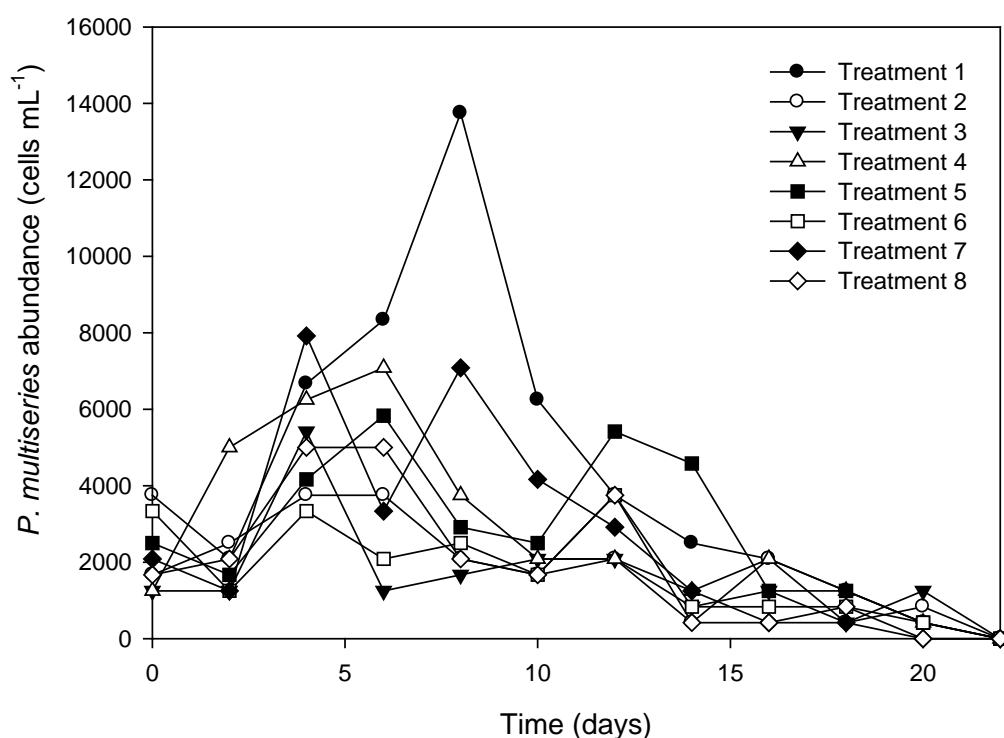
**Figure 4.2:** Mean log cell abundance per mL of *Pseudo-nitzschia multiseries* cultures over eight nutrient treatments during a 40 day response period (details are given in Table 4.21). All results are means of triplicates. Treatment 1= low nitrate (110  $\mu\text{M}$ ), low phosphate (4.5  $\mu\text{M}$ ), low silicate (13.3  $\mu\text{M}$ ); Treatment 2= low nitrate (110  $\mu\text{M}$ ), high phosphate (36.2  $\mu\text{M}$ ), low silicate (13.3  $\mu\text{M}$ ); Treatment 3= low nitrate (110  $\mu\text{M}$ ), high phosphate (36.2  $\mu\text{M}$ ), high silicate (106  $\mu\text{M}$ ); Treatment 4= low nitrate (110  $\mu\text{M}$ ), low phosphate (4.5  $\mu\text{M}$ ), high silicate (106  $\mu\text{M}$ ); Treatment 5= high nitrate (880  $\mu\text{M}$ ), high phosphate (36.2  $\mu\text{M}$ ), low silicate (13.3  $\mu\text{M}$ ); Treatment 6= high nitrate (880  $\mu\text{M}$ ), low phosphate (4.5  $\mu\text{M}$ ), silicate (13.3  $\mu\text{M}$ ); Treatment 7= high nitrate (880  $\mu\text{M}$ ), low phosphate (4.5  $\mu\text{M}$ ), high silicate (106  $\mu\text{M}$ ); Treatment 8= high nitrate (880  $\mu\text{M}$ ), high phosphate (36.2  $\mu\text{M}$ ), high silicate (106  $\mu\text{M}$ ). The standard deviations in cell numbers have been omitted for clarity but are given in Table 4.1.

Specific growth rates across all cultures varied between 0.36 and 0.41 d<sup>-1</sup> (Table 4.6), but despite this variation there were no significant ( $P > 0.05$ ) effects of nutrient availability on the growth rates of *P. multiseri*. However, there were significant effects of nitrate, phosphate, and silicate availability on the rates of cell decline ( $P < 0.0001$ ) during the senescence phase, as well as an interactive effect ( $P < 0.0001$ ) of these three nutrients. In terms of cell abundances, there was a significant effect of phosphate concentration ( $P = 0.0014$ ) between Days 10-14 (Fig. 4.2) during the late exponential phase/early stationary phase. Phosphate was found to have a significant positive effect during this period. Cultures that were exposed to a higher concentration of phosphate (36 µM) reached greater cell densities as the cultures went into stationary phase than those exposed to lower concentrations (4.5 µM). On Day 20, silicate, as well as phosphate, availability became a statistically significant factor (significance for both silicate and phosphate  $P < 0.0001$ ) having a positive effect on cell abundance. By Day 26, the presence of nitrate ( $P = 0.003$ ), phosphate ( $P < 0.0001$ ), and silicate ( $P < 0.0001$ ) were all having a significant positive effect on cell abundance. As a result, where initial nutrient concentrations were high, it followed that cell numbers were also greatest and more sustained across the stationary phase. By Day 26, cell numbers across all cultures were declining but those cultures provided with higher initial concentrations of either nitrate, phosphate, or silicate had higher cell numbers in comparison (Fig. 4.2).

#### **4.3.2.2 Macronutrient and trace metal experiment**

The starting concentration of *P. multiseri* cells in the experimental flasks was 2 - 4 x 10<sup>3</sup> cells mL<sup>-1</sup> depending on the cell concentrations in acclimated cultures. Cultures did not exceed 8 x 10<sup>3</sup> cells mL<sup>-1</sup> across the response period of 22 days (Fig. 4.3). The

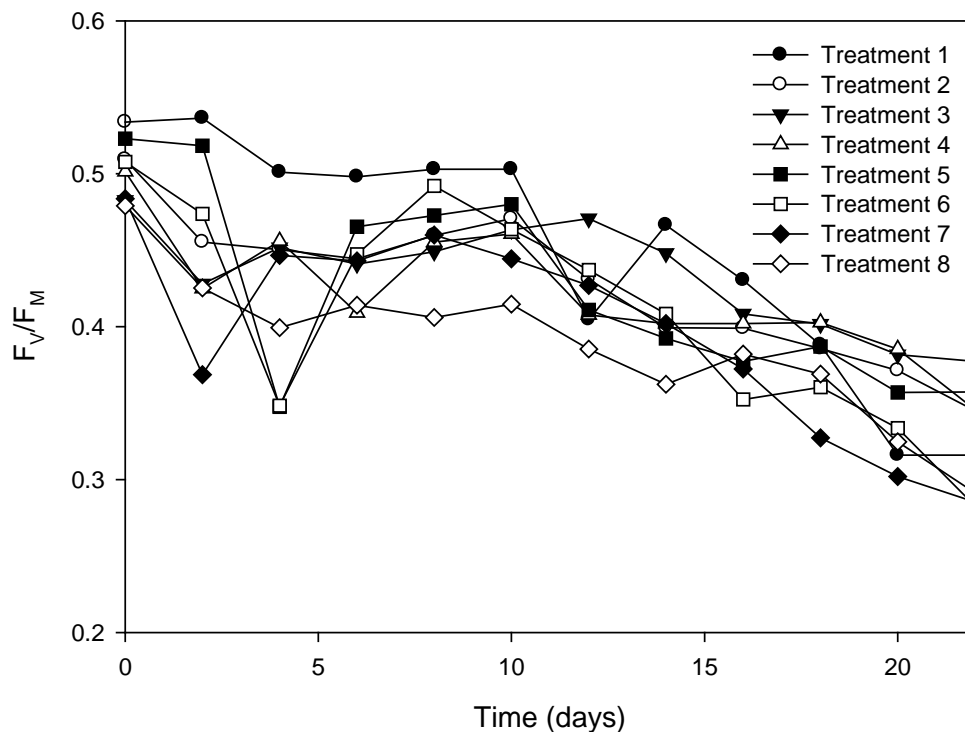
exception to this was Treatment 1 (high phosphate, silicate, iron and copper) which reached an average cell abundance of  $13.8 \times 10^3 \text{ cells mL}^{-1}$ . The majority of cultures decreased in cell numbers within the first two days of the experiment before increasing in abundance. Cultures in treatments 2, 4, and 8 increased in cell abundance during the first two days of the experimental period (Fig. 4.3). With the exception of Treatment 1 on Day 8 ( $P < 0.001$ ), there was no significant ( $P = 0.391$ ) difference between the cell abundances for each treatment across the experimental period. Due to the variability in cell numbers during the experimental period and the lack of exponential growth in some flasks, not all cultures could be assigned a growth rate. Where growth rates could be determined they averaged  $0.30 \pm 0.07 \text{ d}^{-1}$  and no significant ( $P > 0.05$ ) difference was found in growth rates between nutrient treatments.



**Figure 4.3:** Mean *P. multiseriis* cell abundances (cells mL<sup>-1</sup>) over eight nutrient treatments during a 22 day experimental period. All results are means of triplicates. Table 4.5 gives details of the iron, copper, silicate, and phosphate nutrient concentrations for each treatment 1-8.

### 4.3.3 Macronutrient and trace metal experiment: $F_V/F_M$

The maximum quantum yield of photosynthesis expressed as  $F_V/F_M$  decreased over the course of the experimental period from a ratio of approximately 0.5 to 0.35 (Fig. 4.4). At the start of the experimental period  $F_V/F_M$  ratios were highest for those treatments that were supplied with high iron and copper concentrations (Treatment 1 and 5). Treatments 7 and 8 had the lowest  $F_V/F_M$  ratios during the time period. These treatments were supplied with low phosphate, low silicate, and either high iron and low copper, or low iron and high copper, respectively. Most of the treatments showed a decrease in  $F_V/F_M$  ratios at either Days 2 or 4 before increasing again (Fig. 4.4). Significant differences were found in  $F_V/F_M$  ratios at the beginning ( $P = 0.02$ ) of the experimental period (Day 2 and 4) and at Day 14 ( $P < 0.001$ ) with Treatment 1 predominantly having higher ratios compared to other treatments.



**Figure 4.4:** Maximum quantum yield of photosystem II ( $F_V/F_M$ ) for *P. multiseri* cultures over eight nutrient treatments during a 22 day experimental period. All results are means of triplicates. Table 4.5 gives details of the iron, copper, silicate, and phosphate nutrient concentrations for each treatment 1-8.

### 4.3.4 DA production

#### 4.3.4.1 *Macronutrient experiment*

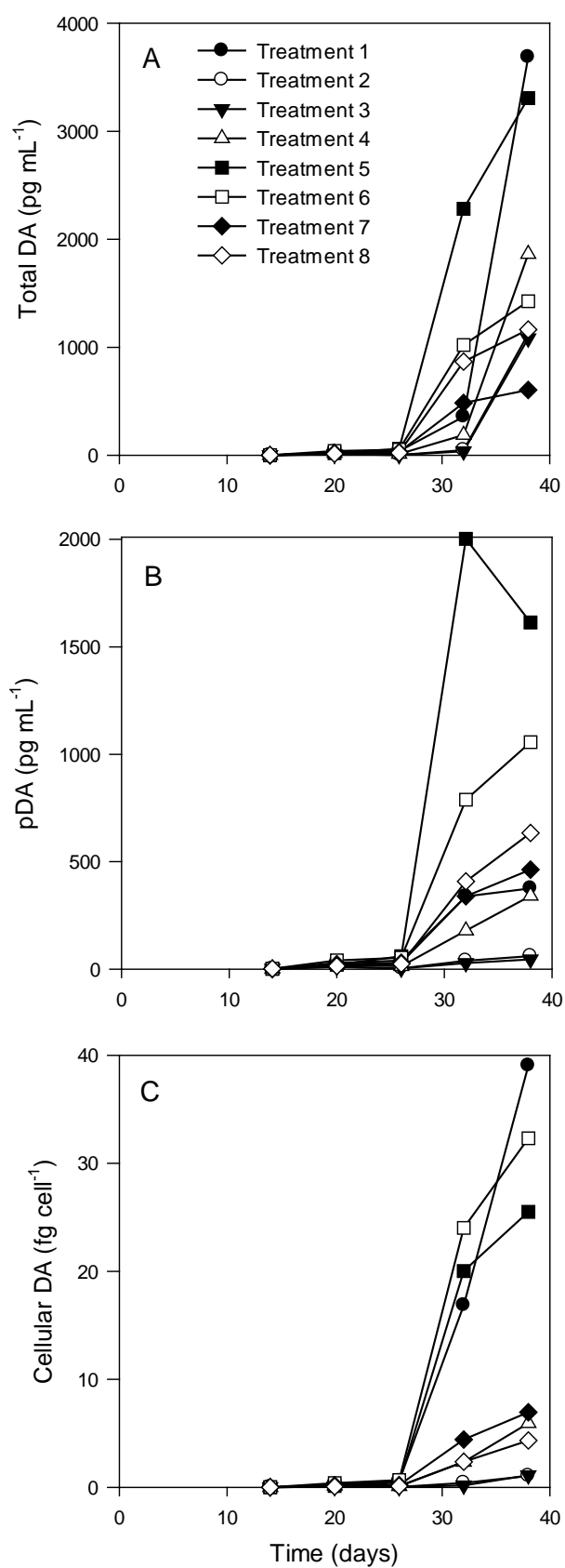
Figure 4.5A represents the total DA determined in experimental cultures across the eight nutrient treatments described in Table 4.2. Total DA is the sum of particulate DA (pDA) and dissolved DA (dDA) concentrations. Total DA concentrations started off low (ca.  $1.5 \text{ pg mL}^{-1}$ ) and rapidly increased from Day 26 across the majority of treatments. Maximum concentrations were observed for all treatments at the end of the experimental period (Day 38). Experimental cultures exposed to Treatments 1 and 5 had the highest concentrations of total DA ( $> 3000 \text{ pg DA mL}^{-1}$ , Day 38). These cultures were supplied with low nitrate, phosphate, and silicate concentrations and high nitrate, phosphate and low silicate concentrations, respectively. Cultures exposed to Treatment 7 (high nitrate, low phosphate, high silicate) had the lowest total DA concentrations ( $< 1000 \text{ pg DA mL}^{-1}$ , Day 38). All other treatments (Treatments 2, 3, 4, 6, and 8) fell between this range with total DA concentrations of  $1000 - 1500 \text{ pg DA mL}^{-1}$ . Total DA was strongly influenced by high dissolved DA concentrations from Day 32.

Figure 4.5B shows that pDA concentrations, like total DA concentrations, started off low and increased from Day 26. All treatments provided with high initial concentrations of nitrate (Treatments 5-8) produced greater concentrations of pDA than those cultures provided with low nitrate concentrations (Treatments 1 to 4; Fig. 4.5B). Cultures in Treatment 5, containing high nitrate, high phosphate, and low silicate concentrations, yielded the highest concentrations ( $1614 \text{ pg DA mL}^{-1}$  on Day 38). This was followed by Treatment 6 (high nitrate, low phosphate, low silicate), Treatment 8 (high nitrate, high phosphate, high silicate), and Treatment 7 (high nitrate, low phosphate, high silicate) (Fig. 4.5B). Treatments 2 (low nitrate, high phosphate, low silicate) and Treatment 3 (low nitrate, high phosphate, high silicate) produced the

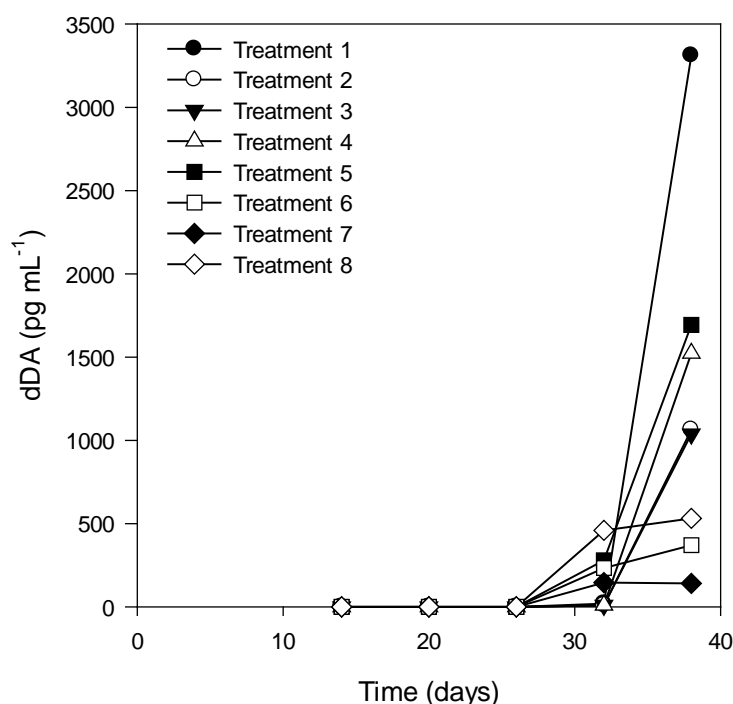


lowest concentrations ( $61 \pm 19.6$  pg DA mL<sup>-1</sup> and  $44 \pm 11.5$  pg DA mL<sup>-1</sup> respectively; Fig. 4.5B).

Figure 4.5C shows cellular DA concentrations (pDA concentrations normalised to cell abundances) in *P. multiseriis* cultures across the response period of 40 days. Initial cellular DA concentrations were low ( $0.01$  fg DA cell<sup>-1</sup>) and increased exponentially from Day 26. This corresponds with the rapid decline in cell abundances from this time point of the growth curve. Figure 4.5C shows that cellular DA concentrations and associated treatments are divided into roughly two groups: those with high final cellular DA concentrations (Treatments 1= low nitrate, low phosphate, low silicate; Treatment 6= high nitrate, low phosphate, low silicate; and Treatment 5= high nitrate, high phosphate, low silicate) and those with low cellular DA concentrations (Treatments 7= high nitrate, low phosphate, high silicate; Treatment 4= low nitrate, low phosphate, high silicate; Treatment 8= high nitrate, high phosphate, high silicate; Treatment 3= low nitrate, high phosphate, high silicate; and Treatment 2= low nitrate, high phosphate, low silicate). Increased cellular DA concentrations in Treatments 1 and 6 ( $39 \pm 10.5$  fg DA cell<sup>-1</sup> and  $32 \pm 8$  fg DA cell<sup>-1</sup> respectively) related to those cultures that underwent the most rapid decline in cell numbers and were exposed to low initial nutrient concentrations, namely silicate. Low cellular DA concentrations varied between  $1 \pm 0.5$  fg DA cell<sup>-1</sup> in Treatment 2 and  $7 \pm 1.2$  fg DA cell<sup>-1</sup> in Treatment 7. The common factor amongst these treatments was high initial silicate concentrations, with the exception of Treatment 2.



**Figure 4.5:** (A) Total DA, (B) pDA and (C) cellular DA over eight nutrient treatments during a 40 day response period (details are given in Table 4.2). All results are means of triplicate flasks. Other explanations as in Fig. 4.2.



**Figure 4.6:** Mean dDA concentrations ( $\text{pg mL}^{-1}$ ) over eight nutrient treatments during a 40 day response period (details are given in Table 4.2). All results are means of triplicate flasks. Other explanations as in Fig. 4.2.

Analysis of variance showed that there were no significant ( $P > 0.05$ ) effects of nutrient availability on total DA, pDA or cellular DA concentrations at Day 14. By Day 20, the availability of both phosphate ( $P < 0.0001$ ) and silicate ( $P = 0.0061$ ) was having a significant negative effect on total DA and pDA concentrations. Nitrate availability was having a significant positive effect ( $P = 0.015$ ). That is, low concentrations of phosphate and silicate produced the highest concentrations of total DA and pDA, but only where nitrate concentrations were high. A similar significant relationship was found between nutrient availability and cellular DA concentrations, except that nitrate availability had no significant effect ( $P > 0.05$ ). Furthermore, no statistically significant interactive effects were found of nitrate, phosphate, and silicate availability on pDA or cellular DA concentrations over the 40 day time course of the experiment.

dDA concentrations (Fig. 4.6) across experimental cultures were below the limit of detection ( $0.04 \text{ ng DA mL}^{-1}$ ) from 14 to 32 days. On Day 32, dDA was detected in some but not all treatments (Treatments 1, 5, 6, 7, and 8) and by Day 38 dDA was detected across all treatments (Fig. 4.6). Maximum dDA concentrations ( $3310 \pm 361 \text{ pg DA mL}^{-1}$ ) were measured in Treatment 1 (low nitrate, low phosphate, low silicate), followed by Treatments 5 (high nitrate, high phosphate, low silicate)  $1693 \pm 39 \text{ pg DA mL}^{-1}$ , Treatment 4 (low nitrate, low phosphate, high silicate)  $1522 \pm 573 \text{ pg DA mL}^{-1}$ , Treatment 2 (low nitrate, high phosphate, low silicate)  $1063 \pm 128 \text{ pg DA mL}^{-1}$ , Treatment 3 (low nitrate, high phosphate, high silicate)  $1038 \pm 165 \text{ pg DA mL}^{-1}$  (Fig. 4.6). With the exception of Treatment 5, increased dDA concentrations occurred when initial nitrate concentrations were low (Fig. 4.6). The lowest concentrations of dDA were measured in Treatment 8 (high nitrate, high phosphate, high silicate)  $532 \pm 297 \text{ pg DA mL}^{-1}$ , Treatment 6 (high nitrate, low phosphate, low silicate)  $371 \pm 245 \text{ pg DA mL}^{-1}$ , and Treatment 7 (high nitrate, low phosphate, high silicate)  $142 \pm 193 \text{ pg DA mL}^{-1}$ . These lower concentrations corresponded with high initial nitrate concentrations (Fig. 4.6). On an individual nutrient basis, dDA concentrations were shown to be significantly negatively affected by nitrate ( $P < 0.0001$ ) and silicate ( $P = 0.0004$ ) availability. Thus, where high concentrations of nitrate and silicate were applied, dDA concentrations were reduced.

## 4.4 Discussion

### 4.4.1 Macronutrient experiment: Growth dynamics

In this study the growth dynamics of *P. multiseriis* and the production of DA were monitored for 40 days under eight combinations of high and low nitrate, phosphate, and silicate concentrations. Our results showed that cultures initially responded in the same

way, with a limited lag phase and an almost parallel progression through the exponential growth phase. Statistical comparison of the growth rates (Days 1-6) showed that there was no significant difference ( $P > 0.05$ ) between the eight treatments used in this study. After Day 6 cultures started to deviate from one another and although they proceeded into the stationary phase at a similar time they did so at different maximum cell abundances (Days 8-14). Phosphate availability was the significant factor positively affecting cell abundances during this period (Day 10-14). Consequently those cultures provided with high concentrations of phosphate reached greater cell abundances (i.e. Treatments 2, 3, 5, and 8; Fig. 4.2). Thereafter, both phosphate and silicate availability had a significant positive effect on cell abundances (Day 20). This suggests that initially phosphate availability was the most important factor determining cell abundances but as cells progressed through stationary phase and became more limited, silicate availability also became important.

Often algal cultures grown in batch culture, like the experimental cultures in the present study, can experience light limitation in the early stages of culture growth. This can restrict photosynthesis and as a result reduce phytoplankton growth rates. In one study by Bates et al. (1991) which examined the effects on irradiance levels on *P. multiseriis* growth and toxin production found that culture growth was accelerated under high irradiance conditions ( $145 \mu E m^{-2} s^{-1}$ ) compared with low irradiance conditions ( $45 \mu E m^{-2} s^{-1}$ ). These cultures reached the stationary phase at different times, with those cultures experiencing high irradiance reaching it 10 d earlier (Bates et al. 1991). The *P. multiseriis* growth rates in the present study are in line with growth rates found by Bates et al. (1991) when cells were grown under lower light levels ( $45 \mu E m^{-2} s^{-1}$ ). This may indicate that cultures were light limited during this early stage of growth resulting in lower growth rates and a prolonged exponential phase (1-10 d). Light limitation can

also occur as a result of self shading in cultures which have undergone many divisions and increased in density. This can then slow cell division rates due to a reduction in photosynthesis causing cells to progress into the stationary phase of growth. If cells were light limited due to self shading in the present study it would be expected that the *P. multiseriis* cell abundances in each treatment would be indistinguishable. However, that was not the case and differences were seen in cell numbers during this time indicating that cultures were responding in part to the different nutrient conditions they were exposed to. Nevertheless, a close coupling has been found between nutrient condition and irradiance which could result in both factors impacting on growth during this period.

The effects of nutrient availability were most apparent as cultures approached the late stationary/senescence phase, causing the number of viable *P. multiseriis* cells to decrease markedly in some treatments. An interesting feature of the decline in cell numbers, not often evident in other studies, was the variability observed between treatments. This may be due to the length of the experimental period. Most other studies have had a shorter duration and therefore have not captured the decline in cell numbers. Possibly the growth conditions used in these studies facilitated an extended stationary phase, instead of loss of cells. Results showed that cultures in Treatment 8 (high nitrate, phosphate, silicate) had the lowest rate of decline of viable cells (Table 4.6). In comparison to the other treatments, cultures in Treatment 8 experienced higher cell abundances over a more sustained period of time (Fig. 4.2). Contrastingly, cultures in Treatment 1 (low initial nitrate, phosphate, and silicate) had the highest rate of cell decline (Table 4.6) and all other treatments were between these extremes (Fig. 4.2). The growth responses of Treatments 3 and 6 most closely followed those of Treatments 8 and 1, suggesting that the next most influential nutrient combinations contained either

high phosphate and silicate or low phosphate and silicate concentrations. Furthermore, throughout this period of cell decline, small, stepped increases in cell abundance were observed in many of the cultures after Day 20 (Fig. 4.2). This suggests that despite nutrient exhaustion, *P. multiseriis* cells were probably able to utilise small amounts of recycled nutrients (e.g. silicate) released by senescent cells. It has been suggested previously that dissolution of silica can occur from empty *Pseudo-nitzschia* spp. frustules, which is then rapidly taken up by new cells (Fehling et al., 2004a).

Results for the growth response of *Pseudo-nitzschia multiseriis* are in accordance with those reported in other studies for this genus. For example, previous studies examining the effects of individual nutrient availability, namely phosphate and silicate, on the early growth stages of *Pseudo-nitzschia* spp. have found that measurements are often similar during exponential growth and deviate once cultures enter the stationary phase (Bates et al., 1991; Pan et al., 1996b; Fehling et al., 2004a). This has been seen in experiments using *P. multiseriis* (Pan et al., 1996b; Kudela et al., 2004), *P. australis* (Kudela et al., 2004), and *P. seriata* species (Fehling et al., 2004a). Typical reported values for *P. multiseriis* growth have ranged between 0.20 d<sup>-1</sup> and 0.60 d<sup>-1</sup> (Pan et al., 1996b; Fehling et al., 2004a; Kudela et al., 2004). Other studies have monitored the growth response of *P. multiseriis* to individual nutrient availability but these experiments have been conducted using chemostats set to maintain a specific population growth rate (Bates et al., 1996; Pan et al., 1996a; Kudela et al., 2004; Hagström et al., 2010).

More commonly the effects of nutrient availability on cell abundances are reported for this genus and are usually associated with silicate availability. In early studies which examined the independent effects of phosphate and silicate availability on *P. multiseriis*, silicate concentration was believed to be the controlling factor, causing a

decrease in maximum cell yield where limiting (Bates et al., 1991; Pan et al., 1996b). However, in the present study phosphate availability was the most significant factor influencing cell yields. The significance of phosphate availability on cell concentrations has also been reported in a recent study by Fehling et al. (2004) who directly compared the availability of silicate and phosphate on the species *P. seriata* and found that phosphate availability had the greatest impact on cell abundances. However, in our study we were additionally able to demonstrate that in concert nitrate, phosphate, and silicate availability were all significant in influencing cell abundances and the length of the stationary phase as cultures progressed through the growth cycle. Few studies have examined the effects of nitrate availability on the growth response of *Pseudo-nitzschia* species. Of these studies Bates et al. (1991), using *P. multiseriis*, found that nitrate limitation caused a decrease in maximum cell concentrations. In contrast to this, Hagström et al. (2010) who examined nitrate and phosphate limitation on two *P. multiseriis* strains, found no impact of nutrient condition on cell abundance for either strain.

Phosphate and silicate availability play an integral role in diatom cell metabolism and therefore the growth and overall success of diatom populations (Pan et al., 1998). Silicate is required by diatoms for frustule formation and also cell cycle progression (Brzezinski et al., 1990; Pan et al., 1998). When cells experience silicate limitation, DNA synthesis can be inhibited, and this in turn impedes the cell division cycle (Pan et al., 1998). Consequently, growth ceases and cells are arrested at specific phases of the growth cycle (Brzezinski et al., 1990). Likewise, phosphate is essential for diatom metabolism and plays a key structural role in the formation of cell membranes, cell bioenergetics, and the synthesis of lipids and nucleotides (Cembella et al., 1984; Lombardi and Wangersky, 1991). Phosphate limitation, like silicate limitation, can



impede the progression of the cell division cycle through the inability of cells to form membranes and to synthesise DNA for cell division. As a result of this, it has been argued that phosphate limitation may have a greater negative impact on cell metabolism than silicate limitation (Pan et al., 1998).

#### **4.4.1.1 Nutrient ratios and *P. multiseri* growth dynamics**

In the present study the effects of absolute nutrient concentrations on *P. multiseri* growth were examined. An alternative to examining absolute nutrient concentrations is to consider the effects of the ratio of these nutrients (N:P, P:Si, N:Si) in each treatment using the redfield ratio. The redfield ratio describes the optimal nutrient ratio for plankton growth and is based on the ratio of C:N:P (105:16:1) (Geider and La Roche, 2002). The ratio of N:P is often used to determine either nitrogen limitation ( $N:P < 16$ ) or phosphate limitation ( $N:P > 16$ ) based on molar ambient dissolved nutrient concentrations (Geider and La Roche, 2002). The optimal ratio of silicate for plankton growth has since been incorporated into the redfield ratio Si:N:P (16:16:1) (Flynn, 2010). Using the redfield ratio to explore the nutrient availability across the eight treatments used in the present study, it can be determined that Treatments 1, 5, 6, 7, and 8 were all phosphate and silicate limited. Treatments 2 and 3 were limited by both nitrate and silicate and Treatment 4 was limited by phosphate only. The ratio of these nutrients (N:P, P:Si, N:Si) varied across treatments depending on the concentration of nutrients used. This suggests that the yield-limiting nutrient in the majority of treatments was phosphate and silicate. Moreover, according to the redfield ratio only two of the treatments were nitrate limiting.

Despite the majority of treatments being limited by both phosphate and silicate, differences were observed in the cell abundances of *P. multiseriis* cultures across these treatments. For instance, as mentioned above Treatment 1 and 8 were supplied with low and high nutrients, respectively, and this was reflected in the cell abundances whereby cells in Treatment 8 reached a higher concentration and maintained this over a longer period of time than Treatment 1. However, considering the redfield ratio, these treatments were limited by the same nutrients and by the same nutrient ratios notwithstanding the fact that absolute concentrations of these nutrients were eight times lower in Treatment 1 than Treatment 8. This would suggest that, in terms of cell abundance, experimental cultures responded to the absolute nutrient concentrations they were initially supplied with rather than the ratio of these nutrients. Alternatively, the ratio of these nutrients may not have been important in this study due to the fact that one or more of these nutrients may not have been supplied at concentrations that were limiting to growth (Davidson et al., 2012).

The values used in the redfield ratio to represent the elemental composition of plankton and their requirement for nutrients has been challenged in recent years by a number of scientists (Flynn, 2010). Values were originally based on the average elemental composition of marine organisms and the concentration of the inorganic nutrients in the deep sea (Geider and La Roche, 2002). These values have since been questioned with regards to their simplicity and inflexibility as well as the fact they do not take into account the optimal ranges of these nutrients in relation to each other (Geider and La Roche, 2002; Flynn, 2010). Furthermore, the importance of nutrient ratios compared to the absolute concentration of these nutrients has been questioned (Flynn, 2010). It has been argued that it is the concentration of nutrients, not the ratios, which is experienced by phytoplankton and therefore it is this that is most important in terms of resource

acquisition (Flynn, 2010). Whether it is the concentration or the ratios of nutrients that are important it is clear that nutrient availability and its estimation in both the field and in laboratory cultures is complex and needs further elucidation.

#### **4.4.2 Macronutrient and trace metals experiment: Growth dynamics**

In this study, *Pseudo-nitzschia multiseries* had very limited growth in response to the experimental media to which cultures were exposed. This was evident from the low cell concentrations observed throughout the growth period of 22 days and the reduced growth rates. Moreover, cultures did not have clear exponential and stationary growth phases commonly seen in phytoplankton batch cultures. Maximum cell concentrations were approximately  $14 \times 10^3$  cells mL<sup>-1</sup>, which contrast to measurements in earlier experimental work where  $150 \times 10^3$  cells mL<sup>-1</sup> had been recorded (macronutrient experiment, this chapter). In the macronutrient experiment, the media used was based on Aquil but with the addition of standard f/2 nutrient (macro- and micro- nutrients, and vitamins) concentrations. For these experiments the media did not need to be purified and cell growth was comparable to that of enriched natural seawater. It was apparent that due to the chemical alterations made for the purpose of this study, the synthetic seawater was no longer favourable for growth of *P. multiseries*. This includes the purifying of the synthetic seawater and changes in the concentrations of micronutrient additions. Similarly, Maldonado et al. (2002) reported reduced growth rates of between 66 - 80 % for *P. multiseries* and *P. australis* cultures grown in modified synthetic seawater as opposed to enriched natural seawater. These growth rates were decreased again by a further 50 % when exposed to conditions of iron and copper stress (Maldonado et al., 2002).

Maldonado et al. (2002) reported maximum cell concentrations for the exponentially growing *P. multiseriis* between  $5\text{-}10 \times 10^3$  cells mL<sup>-1</sup>, and for *P. australis* between  $1\text{-}50 \times 10^3$  cells mL<sup>-1</sup> across three trace metal treatments (iron sufficient, iron limited, high copper). These cell concentrations are similar to findings in the present study (in the Maldonado study, cell counts were only reported for the first 2-9 days of culture growth). Contrasting results were found by Bates et al. (2000), who monitored *P. multiseriis* growth over three iron treatments and found that exponential growth rates ( $\sim 0.8$  d<sup>-1</sup>) were similar and cell concentrations fluctuated between  $90\text{-}200 \times 10^3$  cells mL<sup>-1</sup> with only slight differences. The main difference between these studies, which could account for the dissimilarities between culture growth and cell numbers, was that cells used by Bates et al. (2000) were acclimated for six days prior to the start of the experimental period, rather than over several cell generations (Maldonado et al., 2002). Therefore, the iron stress experienced by cultures in these studies was different and may have been important in determining the growth response of *Pseudo-nitzschia* spp. It is apparent in these trace metal studies that growing *Pseudo-nitzschia* spp. under these growth conditions is both complicated and challenging. This is also the case for achieving the correct balance between trace metal limitation and depletion, as found in Bates et al. (2000). Maldonado et al. (2002) and Wells et al. (2005), used culture media optimised both within and between studies in order to attain improved cell growth: despite these modifications cell concentrations were low and growth rates still greatly reduced.

Due to the lack of *P. multiseriis* growth in this study, even in control treatments, cultures may have been limited or stressed by factors other than iron, copper, phosphate and silicate availability. This could have arisen from the process of medium purification which used QuadraPure™ beads to remove trace metals. These beads

scavenge a number of metals, for instance: Cu, Al, Ga, In, V, Pb, Ni, Zn, Cd, Be, Mn, Sr, Ba, Co, and Fe. Whilst the essential trace elements for diatom growth are reintroduced to newly purified seawater it is not known whether the removal of other non-essential trace elements could have a detrimental effect on species growth. Alternatively, the efficiency of the resin at removing existing trace metals could cause limitation in itself and may explain why those trace metal studies using Chelex 100 (Bates et al., 2000; Maldonado et al., 2002; Wells et al., 2005) resins managed to establish *Pseudo-nitzschia* growth somewhat more successfully. Furthermore, although the beads were washed and the resin regenerated, it is not known if the beads may then release any previously bound compounds into the purified seawater that may be toxic or deleterious to *P. multiseriis* growth.

At low cell concentrations, cells are more susceptible to light stress when combined with conditions of varying nutrient stress. Observations of the *P. multiseriis* cells during the first 4-6 days of the experimental period indicated that cells were losing pigment, vacuolar spaces were evident, and cells appeared to be chlorotic. Chlorosis, having largely been associated with conditions of nutrient stress or extreme physical stress, can be a result of interrelated factors which can alter the overall optical, biochemical, and photosynthetic characteristics of the cell. Considering this and our own observations related to the growth and condition of cells, it may be that cultures were co-limited, which may explain why cultures including the control, displayed limited growth. Furthermore, studies have reported that light intensities of ca. 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  are required to sustain both growth and DA production in *Pseudo-nitzschia* cultures, beyond which light is limiting (Bates et al., 1991; Bates, 1998). The light intensity employed in the present study is likely to be just below that required to saturate growth and this, in conjunction with iron stress, at a time when cells are

demanding more iron, is likely to be unfavourable for cell growth. Alternatively, if cells are experiencing stress due to other factors and therefore have greater metabolic demands, there may be insufficient excess photosynthetic energy available to meet growth requirements.

#### ***4.4.2.1 Photosynthetic efficiency***

Measurements of the maximum photosynthetic efficiency ( $F_V/F_M$ ) of algal cells have been used in previous studies as an indicator of cell health and the impairment of photosystem II. This complementary approach is often used as fluorescence can be a good indicator of nutrient stress (McKay et al., 1997; Kudela et al., 2004; Marchetti et al., 2006) and in one study has been correlated to DA production in *Pseudo-nitzschia* (Kudela et al., 2004). In our study, despite low *P. multiseriis* cell concentrations, the  $F_V/F_M$  ratios were initially relatively high ( $0.50 \pm 0.01$ ) across all treatments before decreasing ( $0.33 \pm 0.04$ ) across the growth period. Maximum  $F_V/F_M$  ratios reported in the literature for healthy cultures of *P. multiseriis* are approximately 0.50 (Bates et al., 2000) and 0.65 (Kudela et al., 2004; El-Sabaawi and Harrison, 2006). These values decreased when limited by either iron or silicate to ratios of 0.2 (Bates et al., 2000) and 0.3, respectively (Kudela et al., 2004). Results from our study are thus similar to findings of other studies and demonstrate that initially culture health was relatively good and decreased over time. However, there was little difference in the  $F_V/F_M$  ratios between nutrient treatments, which contrasts to findings in other studies where there have been clear differences between treatments. This finding may be indicative of the fact that culture growth was limited and other factors were also influencing the *P. multiseriis* cells. Under these circumstances, fluorescence based measurements as a

proxy for cell health, may not be very informative and perhaps should be used in conjunction with other biochemical markers for better determination.

#### **4.4.4 Macronutrient experiment: DA production**

In the present study, DA produced by *P. multiseriis* was evident across all nutrient treatments and increased throughout the stationary phase of growth (Days 14-26). Although, DA concentrations in our study were low, with maximum total DA concentrations of 4000 pg DA mL<sup>-1</sup>, pDA concentrations of ca. 2000 pg mL<sup>-1</sup> and cellular DA concentrations of 39 fg cell<sup>-1</sup>, they were comparable to findings in some other studies of *P. multiseriis*. For example Kudela et al. (2003), found that cellular DA concentrations ranged between 0 and 2500 fg DA cell<sup>-1</sup> for nitrate limited chemostat cultures and 0 to 2800 fg DA cell<sup>-1</sup> for silicate limited chemostat cultures, depending on the growth rates. Other studies examining macronutrient limitation using *P. multiseriis* have found higher cell concentrations (Pan et al., 1996a; Pan et al., 1996b; Fehling et al., 2004a; Hagström et al., 2010). However, these studies used an alternative method for DA quantification based on that by Pocklington et al. (1990), which uses high performance liquid chromatography of a fluorenylmethoxycarbonyl (FMOC) derivative with ultraviolet absorption spectrometry as the detection method. Few studies have used an LC-MS based approach to quantify DA. Differences in methodology may therefore partly account for the differences in concentrations reported across the different studies. No comparison of the analytical methods appears to have been made. Nonetheless, it is clear that there is a lot of variability in the reported DA concentrations both between species and for different strains of *Pseudo-nitzschia*. This variation is observed even when cultures are grown under apparently identical conditions (Kudela et al., 2004; Thessen et al., 2009).

The relationships between absolute nutrient concentrations and total DA, pDA, cellular DA, and dDA were examined in this study. DA concentrations across these fractions were different depending on the nutrient treatments experimental cultures were exposed to. The main difference between high and low total DA was the concentration of silicate cultures were supplied with. Those cultures with the highest concentrations of total DA were supplied with low silicate concentrations. This was further evidenced in the Design Expert ANOVA results which showed that silicate was a significant factor ( $P < 0.0001$ ) having a negative impact on total DA concentrations. This relationship was also observed for cellular DA concentrations showing that silicate ( $P = 0.001$ ) was the main influencing factor effecting cellular DA production. ANOVA results for cellular DA concentrations showed that phosphate availability was also a significant factor ( $P = 0.008$ ) having a negative effect. The significance of these nutrients on DA production has been reported in other studies, with the general consensus that phosphate limitation as well as silicate limitation can stimulate toxin production (Bates, 1998). One study by Fehling et al. (2004), which compared phosphate and silicate limitation, found that silicate limitation had the greatest effect on total DA, cellular DA, and dDA in cultures of *P. seriata* during late stationary phase cultures.

Our results confirm that phosphate and silicate availability are important determinants of toxin production in *P. multiseriata*. This relates to the role of these nutrients in cell metabolism. For instance, when these nutrients are limiting, cell cycle progression is hindered and the cells can become physiologically stressed. In the case of phosphate, the specific metabolic triggers of DA production are largely unknown (Bates, 1998). However, in the case of silicate, DNA synthesis can be inhibited and cells become arrested in certain phases of the cell cycle (Pan et al., 1998). It has further been



suggested that toxin production happens at certain points across the growth cycle and is not constant. Therefore, if stationary phase cells become arrested in a specific phase of the growth cycle due to silicate limitation, and these stages have previously been associated with toxin production, then DA production could progress maximally (Bates, 1998; Pan et al., 1998).

The highest concentrations of pDA were measured in cultures initially supplied with high nitrate concentrations (Fig. 4.5B). The opposite relationship was observed in cultures supplied with low initial concentrations of nitrate, resulting in the lowest concentrations of pDA. Similar findings have been found in other studies that have examined the effects of nitrate concentration as a single variable on DA production in *P. multiseriis* (Kudela et al., 2004; Hagström et al., 2010). For example, even at low concentrations of nitrate, *P. multiseriis* cultures were able to produce DA, despite nitrogen being an essential element required for DA synthesis (Kudela et al., 2004; Hagström et al., 2010). These findings contradict earlier beliefs that DA production would cease during nitrogen limitation (Bates et al., 1991). Our results confirm that nitrogen availability is important for increased DA production in *P. multiseriis* but also that DA production continues at the lowest nitrate concentration tested (110  $\mu$ M).

The release of DA into culture medium is commonly observed in *Pseudo-nitzschia* cultures as cells start to senesce. In previous studies, dDA has been detected in the exponential phase, albeit in low concentrations, however it is usually found in post exponential/stationary phase cultures (Bates, 1998; Fehling et al., 2004a). In the present study, dDA concentrations were detected across all treatments in the late stationary /senescence phase (Day 32 onwards); concentrations prior to this were below the limit of detection (Fig. 4.6). dDA like cellular DA concentrations seem to form roughly two

groups, which include those with higher dDA concentrations and those with lower dDA concentrations. The main difference between these two groups is the concentration of nitrate that these cultures were supplied with, as was the case with pDA concentrations. However, this time nitrate availability had the opposite effect. For instance, high dDA concentrations are associated with those cultures initially supplied with low concentrations of nitrate, with the exception of Treatment 5. This suggests that those cultures exposed to low nitrate availability released more DA into the culture media than those exposed to high nitrate availability. ANOVA results showed that both nitrate and silicate were significant factors having a negative effect on extracellular DA concentrations where these nutrients were in excess.

Generally, dDA has been associated with decaying cells as cultures progress through the stationary phase (Hagström et al., 2007). According to the literature, increased dDA has been associated with both phosphate and silicate limitation and the extent to which seems to depend on the *Pseudo-nitzschia* species used and the nutritional status of the media (Hagström et al., 2007). Of these two nutrients silicate has been found to have the greatest impact on dDA concentrations. In a study by Fehling et al. (2004) 67 % of the total DA was released into the media under silicate limitation compared to 23 % in phosphate limited cultures. In the present study, high proportions (> 81 %) of the total DA were released into the media in Treatments 1- 4 from Day 32. In contrast to findings by Fehling et al. 2004 where silicate limitation was the key factor, these cultures were all supplied with low nitrate concentrations. This decreased to ca. 48 % in Treatments 5 and 8 and 25 % in Treatments 6 and 7 which all experienced high nitrate concentrations.

Nitrate availability is a factor not usually associated with increased concentrations of dDA. The main studies that have examined the effects of nitrate availability on *P. multiseriis* have reported little to no information about the impacts of this nutrient on dDA concentrations. These studies tend to focus on the effects of nitrate on pDA concentrations and the mechanisms which would cause the release of toxins from the cells exposed to low nitrate and silicate remain unknown. In the case of low phosphate availability, it is thought that the formation of cell membranes is impaired, as cells require this essential nutrient for lipid bilayer development. Consequently, this allows cells to become more permeable to DA causing it to leak out into the surrounding media. This level of permeability is an important factor when considering a bloom event, as the extent to which toxins are released or stored in the cells affects the overall toxicity of a bloom.

#### **4.4.3.1 Redfield ratio and DA**

As noted in Section 4.4.2 according to the redfield ratio (N:P, P:Si, N:Si) the majority of treatments in the present study were silicate and phosphate limited. Despite this, differences were observed in total DA, pDA, and cellular DA concentrations across treatments. These differences appeared to correlate to the concentration of nutrients experimental cultures were supplied with. An exception to this was Treatments 2 and 3 which according to the redfield ratio were nitrate limited ( $N:P < 16$ ). While high nitrate concentrations were found to positively impact pDA concentrations in this study, those cultures that were apparently nitrate limited (Treatment 2 and 3) produced the lowest concentrations of pDA and cellular DA. It was found that cultures exposed to Treatments 2 and 3 in general had similar concentrations of DA (Total DA, pDA, cellular DA, and dDA) from Day 14 onwards. It might have been that the concentration

and ratio of nitrate to the other nutrients in these treatments was sufficiently low to limit the production of DA. In contrast, the ratio of nutrients in the other treatments may not have been important due to the concentration of these nutrients potentially not being limiting.

#### **4.4.4 Conclusions**

The present study was separated into two main experiments, the first investigated the combined effects of nitrate, phosphate, and silicate availability on *P. multiseri* growth and DA toxin production has been conducted. The second aimed to investigate the combined impacts of macro- and micro-nutrient stress on *Pseudo-nitzschia multiseri* growth, photosynthetic efficiency, and DA production. In the macronutrient experiment, using response surface methodology, it was determined that phosphate availability was the yield-limiting nutrient for cell numbers as cultures progressed into stationary phase growth. DA production occurred across all treatments during the stationary phase and concentrations increased as cells became progressively nutrient stressed. pDA concentrations were highest when cultures were exposed to increased nitrate concentrations and low phosphate and/or silicate concentrations. However, when pDA concentrations were normalised to the cell numbers (cellular DA), silicate and phosphate availability became important factors. In contrast to pDA, dDA concentrations were highest when nitrate and silicate concentrations were low. In conclusion, our results suggest that interrelated nutrient availability significantly affects the growth response and DA production by *P. multiseri* and highlights the importance of simultaneous phosphate and silicate availability.

In the macronutrient and trace metal experiment, a model system was created which examined the impacts of macro- and micro-nutrient stress, but due to alterations made in the chemical composition of the medium used this was no longer favourable for *P. multiseriis* growth: growth rates and cell numbers were limited across all treatments, including in replete nutrient conditions. This experiment highlighted the challenge in growing toxigenic *Pseudo-nitzschia* species, especially in a modified growth medium. It was concluded that the light intensity and the iron concentrations used for experimental work potentially limited the growth of the *P. multiseriis* cultures. Alternatively, purification of the synthetic seawater may have removed an essential nutrient required for *P. multiseriis* growth that was not later reintroduced. Despite exhaustive attempts to determine the cause of the lack of growth, further work would still be needed to optimise the growth media required to carry out such experiments. This would involve altering the macro- and micro-nutrient concentrations to increase the growth of *P. multiseriis* and to establish whether any of these nutrients are limiting. Culture growth in the new media and under higher light intensities should be examined. Alternative methods for seawater purification should be assessed, as these may be more compatible for experimental purposes.



**Chapter 5: Genetic identification of *Pseudo-nitzschia* species  
in archived L4 water samples**





## Abstract

To investigate the *Pseudo-nitzschia* species present at the monitoring station L4 in the western English Channel a molecular method was developed using existing primers for the identification of *Pseudo-nitzschia* spp. in Lugol's iodine preserved L4 water samples from 2009. Multiple primer sets were tested, using polymerase chain reaction, for their compatibility with L4 samples. This study used a combination of three DNA target regions (ITS, partial SSU, partial rbcL) to investigate *Pseudo-nitzschia* genotypes in four preserved environmental water samples from spring and summer in 2009 (with and without recorded DA). DNA was successfully extracted and amplified from preserved L4 samples that had been stored for up to two years. Sequence analysis showed that, while only bacteria and fungi sequences were detected using 454 on ITS and SSU markers, a number of diatom and *Pseudo-nitzschia* spp. sequences were detected for the rbcL marker. This suggests that the rbcL region is an informative site from which a small number of diatom species and *Pseudo-nitzschia* spp. could be distinguished in preserved archived water samples. However, without additional sequence information from other DNA markers, *Pseudo-nitzschia* species can only be inferred to group level based on cell morphology and size.





## 5.1 Introduction

The identification of *Pseudo-nitzschia* to species level is becoming increasingly important due to the cosmopolitan nature of this marine diatom and the fact that some species or strains produce the neurotoxin domoic acid (Bates, 1998; Hasle, 2002; McDonald et al., 2007). Studies investigating *Pseudo-nitzschia* taxonomy have revealed a high level of complexity and diversity between species and strains within this genus (Lundholm et al., 2003; Amato et al., 2007; Quijano-Scheggia et al., 2009). This makes species level identification very challenging which has led to the increasing use of molecular techniques as traditional methods, using light microscopy and electron microscopy, do not provide sufficient detail for species-level discrimination in some cases (Lundholm et al., 2003; Hasle and Lundholm, 2005; Lundholm et al., 2006). For instance, studies that have combined molecular and morphological approaches have established that *Pseudo-nitzschia* consists of several different species-complexes, with pseudo-cryptic and cryptic species being increasingly described for this genus (Lundholm et al., 2003; Orsini et al., 2004; Lundholm et al., 2006; Quijano-Scheggia et al., 2009). Although our understanding of *Pseudo-nitzschia* population structure has improved over the last decade, the taxonomy of this genus is ever evolving and has implications on our understanding of relationships between *Pseudo-nitzschia* spp., toxin production, and the environment.

Molecular techniques can provide a rapid and reliable solution to the challenges encountered when attempting to resolve *Pseudo-nitzschia* taxonomy. One such technique uses DNA markers to identify *Pseudo-nitzschia* spp. These DNA markers include the large ribosomal subunit (LSU) (Amato et al., 2007; McDonald et al., 2007; Moschandreu et al., 2010), the ribosomal internal transcribed spacer units 1 and 2 (ITS1 and ITS2) (Lundholm et al., 2003; Orsini et al., 2004; Lundholm et al., 2006;

Amato et al., 2007; Andree et al., 2011), the large subunit of ribulose-1,5-bisphosphate carboxylase oxygenase (rbcL) (Amato et al., 2007) and the small ribosomal subunit (SSU) (Manhart et al., 1995; Cusack et al., 2002). DNA markers can identify different species groups depending on their resolution and the LSU, ITS (ITS1-5.8S-ITS2) and rbcL markers can identify down to the species-level although, *P. pseudodelicatissima* and *P. calliantha* species groups show different clustering with ITS and rbcL (Amato et al., 2007). LSU shows similar species groupings except cannot distinguish *P. cuspidata* and *P. pseudodelicatissima*. This is likely due to the mating compatibility between *Pseudo-nitzschia* strains. For example, phylogenetic studies have shown that strains can cluster within a highly supported clade but analysis of the ITS2 has revealed that there are subtle base changes among the strains (Davidovich and Bates, 1998; Lundholm et al., 2003; Amato et al., 2007; Moschandreu et al., 2010). These base changes have been correlated to mating compatibility. As a result of this the ITS2 has been identified as a good region for studies of species delineation and can provide further elucidation of the *Pseudo-nitzschia* complexes that are not resolved using other gene regions.

Using ITS (ITS1-5.8S-ITS2), LSU, and rbcL DNA markers, new *Pseudo-nitzschia* species have recently been identified, *P. hasleana*, *P. fryxelliana*, *P. arenysensis* (Quijano-Scheggia et al., 2009; Lundholm et al., 2012), and in other instances, morphologically identical species have been found to belong to new genetic groups or species (Lundholm et al., 2003; Lundholm et al., 2006; Amato et al., 2007). Using ITS (ITS1-5.8S-ITS2 and ITS4) and LSU and rbcL DNA markers, it has been revealed that the *P. pseudodelicatissima* morphospecies is in fact a group made up of five genetically different species (*P. calliantha*, *P. cuspidata*, *P. calliantha2*, *P. caciaantha*, and *P. pseudodelicatissima*) whilst the *P. delicatissima* morphospecies is a group of three genetic types (*P. delicatissima*, *P. delicatissima2*, and *P. dolorosa*) (Lundholm et al.,

2003; Lundholm et al., 2006; Amato et al., 2007). These genetically different species have also been found to be reproductively isolated from other group members (Amato et al., 2007). In addition to morphospecies, there are also species within the *Pseudo-nitzschia* genus which are genetically and morphologically distinguishable. For example, *P. pungens* has been separated into three subspecific taxa (variants or clades), *P. pungens* var. *pungens*, *P. pungens* var. *cingulate*, and *P. pungens* var. *aveirensis*, based in ITS and rbcL rDNA sequences and differences in frustule ultrastructure (Casteleyn et al., 2010). Each of these variants differ in terms of their geographic distribution occurring in the temperate waters of the Atlantic and Pacific oceans, North Eastern Pacific, and tropical to warm-temperate waters of the Atlantic and Pacific oceans, respectively (Villac and Fryxell, 1998; Churro et al., 2009; Casteleyn et al., 2010). These findings indicate that *P. pungens* variants are biogeographically separated which has led to allopatric speciation (Casteleyn et al., 2010).

The *Pseudo-nitzschia* genus, has been recorded for over 17 years at the monitoring station L4 in the western English Channel (WEC) where it is preserved in a 2% Lugol's iodine solution (Widdicombe et al., 2010; Downes-Tettmar et al., 2013). To date, the *Pseudo-nitzschia* species present at L4 have only been identified to group level: a *P. delicatissima*-group, a *P. seriata* group, and a *P. pungens/multiseries*-group. A study of these groups during 2009 at L4 revealed that they have different seasonal distributions and that one or more of these group species have been found to produce domoic acid (DA) (Downes-Tettmar et al., 2013). While group level identification can provide valuable insight into *Pseudo-nitzschia* population dynamics, the lack of species information at L4 means that little can be determined about the intra-annual variation of *Pseudo-nitzschia* species. Similarly, it is not possible to further elucidate the potentially toxigenic *Pseudo-nitzschia* species present at L4. So far electron microscopy has been

used on preserved L4 water samples to identify *Pseudo-nitzschia* species. Using this method two species were determined, *P. delicatissima* and *P. fraudulenta* (Downes-Tettmar et al., 2013). However, this method was largely unsuccessful due to the deterioration of samples during acid washing steps to remove the organic matter from cells. Alternatively, molecular methods could be used on these preserved samples to discern between *Pseudo-nitzschia* species at L4.

While studies have successfully used molecular techniques to identify single cell and cultures of *Pseudo-nitzschia* spp., few studies have identified *Pseudo-nitzschia* from Lugols-preserved archived water samples (McDonald et al., 2007; Moschandreu et al., 2010). Studies which have used molecular techniques to identify microalgae species from preserved samples, using cell fixatives such as Lugol's iodine and formalin, have found fixative can destroy or alter DNA structure (Godhe et al., 2002; Srinivasan et al., 2002). Very little literature exists on the effectiveness of Lugols in extended storage, but it is known that it can cause changes in cell size and species-specific degradation (Leakey et al., 1994; Menden-Deuer et al., 2001). The recommended storage time of a sample in Lugols is 6 months below 5 °C (Moncheva and Parr, 2010). The L4 samples used in this study were kept at room temperature, in the dark, and stored for 2 years. In these circumstances, the development of appropriate molecular methods can often be a challenging process. The establishment of successful primer sets can be dependent on the state of preservation of samples, the size of the DNA region interrogated and the quantity of cells of interest. Therefore, different combinations of primers and the size of amplified product need to be tested and optimised. It is important that these primers amplify the correct target region of the genome consistently and over a multitude of samples. Therefore, this study aims to develop a molecular method, using existing primers, for the identification of *Pseudo-nitzschia* spp. in Lugol's Iodine preserved L4

samples from 2009 that may overcome problems posed when analysing samples using light and electron microscopy. This study used a combination of three target regions (ITS, partial SSU, partial *rbcL*) to identify *Pseudo-nitzschia* genotypes of four preserved environmental water samples from spring and summer in 2009 with and without recorded DA. The SSU amplicons were investigated using 454 sequencing (Medlin and Kooistra, 2010) to obtain deeper sampling of genotypes present in these samples.

## **5.2 Methods and Materials**

### **5.2.1 Samples**

Water samples containing phytoplankton were collected weekly at the monitoring station L4 from January to December 2009 as part of the Plymouth Marine Laboratory L4 time series. Samples were collected at a depth of 10 m and preserved using 2 % Lugol's Iodine (Chapter 3). All phytoplankton species, including three *Pseudo-nitzschia* groups (*P. delicatissima*-group species, *P. seriata*-groups species, and *P. pungens/multiseries*-group species), were identified and enumerated from these water samples (Chapter 3).

### **5.2.2 DNA extraction**

Of the water samples collected and preserved from L4, nine samples (this was later reduced to four for sequencing) were chosen for analysis from varying time points over the main *Pseudo-nitzschia* spp. growth period (May to September). Samples were centrifuged at 3000 x g to collect cell pellets. DNA was extracted from these cell pellets and purified using a MasterPure™ complete DNA and RNA purification kit



(Epicentre, Madison, Wisconsin). Samples were lysed by adding 150  $\mu$ L of 2X T&C Lysis solution and 1  $\mu$ L Proteinase K to each microcentrifuge tube containing 150  $\mu$ L of L4 sample. These were thoroughly mixed and then incubated at 65 °C. The samples were then vortexed every 5 min during the incubation and then placed on ice for 3-5 min. Nucleic acids were then precipitated by adding 175  $\mu$ L of MPC Protein Precipitation Reagent to 300  $\mu$ L of lysed L4 samples. After being vortexed, the cell debris from the samples was then pelleted by centrifugation at 4 °C for 10 min (10,000 x g). The supernatant, containing nucleic acids, for each sample was transferred to a clean microcentrifuge tube and 500  $\mu$ L of isopropanol was added to precipitate the nucleic acids. Tubes were inverted 30-40 times before nucleic acids were pelleted by centrifugation at 4 °C for 10 min. The isopropanol was removed by pipette leaving the pelleted nucleic acids. The pellet was rinsed twice with 70 % ethanol for each sample. Nucleic acids were then resuspended in 35  $\mu$ L of TE Buffer.

### **5.2.3 PCR amplification**

For each PCR, 2  $\mu$ L of genomic DNA was added to a PCR mixture containing: 1X GoTaq FlexiBuffer (Promega, Southampton, UK), 2.5 mM MgCl<sub>2</sub> (Promega), 2.25 mM dNTPs (Promega), 1 unit GoTaq DNA Polymerase (Promega), Nuclease-free water (Promega), and 2.25  $\mu$ M of each primer in a total volume of 25 $\mu$ l. The PCR products for all samples were loaded together with dye buffer (Orange G, Sigma Aldrich, UK) onto a 1 % (w/v) agarose gel for electrophoresis (Applied Biosystems, Warrington, UK). Electrophoresis conditions were 100 V for 30-40 min. Ethidium-bromide stained gels were visualised under UV-transillumination. PCR products were confirmed by comparing the expected size of the products against a DNA ladder of known fragment

sizes. For most primer sets HyperLadder™ I (BioLine, London, UK) was used which has a DNA fragment range of 200-10037 base pairs (bp).

DNA Marker	Primers Pair	Sequence	Fragment size (bp)	References
rbcl*	rbcl-PNp-F rbcl-PNp-R	TTATTACACTTACACCGT GCTGG TTT ACCTACAACTGTTCC AGCG	135	Castelyn et al. (2009)
rbcl	DPrbcL1 DPrbcL7	AAGGAGGAADHHATGTCT AAASHDCCTTGTGTWAGTYTC	1200	Daugbjerg and Andersen (1997)
ITS (1st round PCR)*	ITS1 ITS4	GGTGAACCTGAGGAAGGAT TCCTCCGCTTATTGATATGC	872	White et al. (1990)
ITS (2nd round PCR)*	18SF-euk ITS4	CTTATCATTTAGAGGAAGGTGAAGTCG TCCTCCGCTTATTGATATGC	397	Hubbard et al. (2008) White et al. (1990)
ITS (2nd round PCR)*	PNA11F ITS4	TCTTCATTGTGAATCTGA TCCTCCGCTTATTGATATGC	825	Hubbard et al. (2008) White et al. (1990)
ITS2	Pseudo 5-F Pseudo 3-R	CGATACGTAATGCGAATTGCAA GTGGGATCCRCAGACACTCAGA	385	Penna et al. (2007)
LSU	D1R D2C	ACCCGCTGAATTTAAGCATA CCTTGGTCCGTGTTTCAAGA	700	Scholin et al. (1994)
LSU	D3Ca DIR	ACGAACGATTTGCAGTCAG ACCCGCTGAATTTAAGCATA	800	Scholin et al. (1994) McDonald et al. (2007)
LSU (2nd round PCR)	D1-186F D1-548R	GTT CCT TGG AAA AGG ACA GCT GA AGACATCAACTCTGACTG	362	McDonald et al. (2007)
SSU	EK-555F EK-1520R	AGTCTGGTGCCAGCAGCCGC CYGCAGGTTACCTAC	714	Lopez-Garcia et al. (2001)
SSU	DIN-1F EK-1269R	GTTGTTGCGGTTAAAAAGC AAGAACGGCCATGCACCAC	910	Lopez-Garcia et al. (2001)
SSU (2nd round PCR)	575F EK-1269R	GTAATTCCAGCTCCAATAGC AAGAACGGCCATGCACCAC	700	Schnetzer et al. (2011) Lopez-Garcia et al. (2001)
Sequencing Vector	M13F M13R	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	1536	Primers supplies by Invitrogen (Paisley, UK)

**Table 5.1:** Details of the primer pairs used for the PCR amplification of phytoplankton DNA in Lugol's iodine fixed archived L4 water samples. Primer pairs used in final analysis are marked by an asterisk

### 5.2.3.1 *rbcL*

PCR amplifications were achieved using the *rbcL* primers PNp-F and PNp-R (Table 5.1) (Casteleyn et al., 2009) under the following PCR conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 55 °C for 45 s, 72 °C for 60 s, and a final extension at 72 °C for 10 min. To find separate genotypes using the *rbcL* marker, PCR products were ligated into a pCR™ 2.1-TOPO® vector system using the TOPO® TA Cloning® Kit according to manufacturer's instructions (Life Technologies Corporation, Paisley, UK). The vectors containing the PCR products were then transformed into TOP10 competent *Escherichia coli* cells, according to the manufacturer's instructions using 2 µL of ligated PCR product and vector construct. These were then incubated on ice for 30 min and then heat shocked for 30 s at 42 °C and returned back to ice. This ensures that the competent *E. coli* cells take up the vectors. 250 µL of media was then added to the competent cells and they were incubated at 37 °C on an agitation table for 1 h.

Transformed cells were then spread onto Luria-Bertani (LB) agar plates containing 1 % Bacto-tryptone, 0.5 % yeast extract, 1 % NaCl, and spread with X-galactosidase sugar (20 mg mL<sup>-1</sup> in dimethylformamide) for blue-white selection of cloned products. Plates were incubated at 37 °C overnight. White colonies containing ligated constructs with *rbcL* PCR products were picked from these plates and screened to check that constructs had correct sized inserts by PCR using the vector primers M13F and M13R (Table 5.1). The PCR conditions were as follows: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 7 min. Positive PCR products with the correct sized inserts were treated with ExoSAP-IT to remove primers. ExoSAP-IT is composed of two hydrolytic enzymes, Exonuclease I and rShrimp Alkaline Phosphatase, in a buffer. For ExoSAP-IT reaction 5µL of PCR product was mixed with 2 µL of ExoSAP-IT. The reaction was then incubated at 37 °C for 15 min

to degrade remaining primers. The reaction was then incubated at 80 °C for 15 min to inactivate ExoSAP-IT. 5µL of this reaction was used for a sanger sequencing reaction (Sanger et al., 1977) using Applied Biosystems (Warrington, UK) BigDye v.3.1 kit, according to the manufacturer's instructions. Four of these reactions that related to dates of L4 samples taken in 2009 (22/6, 29/6, 17/8, and 24/8) were sent to the Source BioScience sequencing facility, Nottingham. Samples were run on a 3730 ABI capillary sequencer for visualisation of results.

#### **5.2.3.2 ITS**

For ITS, several primer combinations were used in a number of nested PCR reactions. For 1<sup>st</sup> round PCR the eukaryotic primers ITS1 and ITS4 were used under the following PCR conditions: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 50 °C for 45 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. The PCR products from this reaction then went through a 2<sup>nd</sup> round of PCR with the following *Pseudo-nitzschia* specific primers 18SFeuk and ITS4 (for sample dated 17/8) and PNA1F and ITS4 (for samples dated 22/6, 29/6, 24/8). PCR conditions for PNA1F and ITS4 were as follows: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 44 °C for 30 s, 72 °C for 90 s, and a final extension at 72 °C for 10 min. These reactions were cleaned up using ExoSAPIT and sent to the Research and Testing Laboratory, Lubbock, Texas for 454 sequencing. The PCR reactions failed to work at the Research and Testing Laboratory under the same PCR conditions. Another attempt was made to sequence the DNA from these L4 samples. This was done using the primer combinations ITS1 and ITS4 (1<sup>st</sup> round PCR) and PNA1F and ITS4 (2<sup>nd</sup> round PCR) and cloning the PCR products into competent *Escherichia coli* (as with rbcL). Positive PCR products were treated with ExoSAPIT to remove primers and 5µl of this reaction was used for a sanger sequencing reaction.

Sequenced reactions were then sent to the Source BioScience sequencing facility, Nottingham to run on a 3730 ABI capillary sequencer for visualisation of results.

#### **5.2.3.3 SSU**

For SSU amplicon testing, four genomic DNA samples were sent, 22/6, 29/6, 17/8 and 24/8 that related to dates of L4 samples taken in 2009 at concentrations of 2.5, 2.6, 2.9 and 2.6 ng  $\mu\text{L}^{-1}$  respectively. These were sent for 454 sequencing using eukaryotic primers euk516F (5' GGAGGGCAAGTCTGGT 3') and euk1055R (5' CGGCCATGCACCACC 3') at the Research and Testing Laboratory, Lubbock, Texas.

#### **5.2.4 Phylogenetic analysis**

rbcL sequences were trimmed of vector sequences and inspected for ambiguous nucleotides and aligned using Clustal W (Thompson et al., 1994) in BioEdit 7.1.3 (Hall, 1999). The final alignment was 125 nucleotides. For phylogenetic analysis, 48 sequences from known *Pseudo-nitzschia* species and more general diatom species were downloaded from Genbank and imported to the final alignment of rbcL sequences (Table 5.1). Sequences that would not align or were redundant due to sequence (Genbank) replication were excluded from the phylogenetic analysis. Neighbour-Joining (NJ), Maximum Likelihood (ML), and Bayesian Inference (BI) statistical methods were used to determine which one provides the most accurate representation of the phylogenetic relationships between all sequences (L4 rbcL sequences and known *Pseudo-nitzschia* spp. sequences). These methods were used to infer phylogenetic trees.

For Neighbour-Joining analysis, a phylogenetic tree (Saitou and Nei, 1987) was obtained according to the p-distance substitution model (Nei and Kumar, 2000), with the neighbour-joining option in MEGA 5.0 (Tamura et al., 2011). Branch statistical support was obtained with 100 bootstrap replicates (Felsenstein 1985). Percentage bootstrap supports represent the strength of support for each node/branch of the phylogenetic tree.

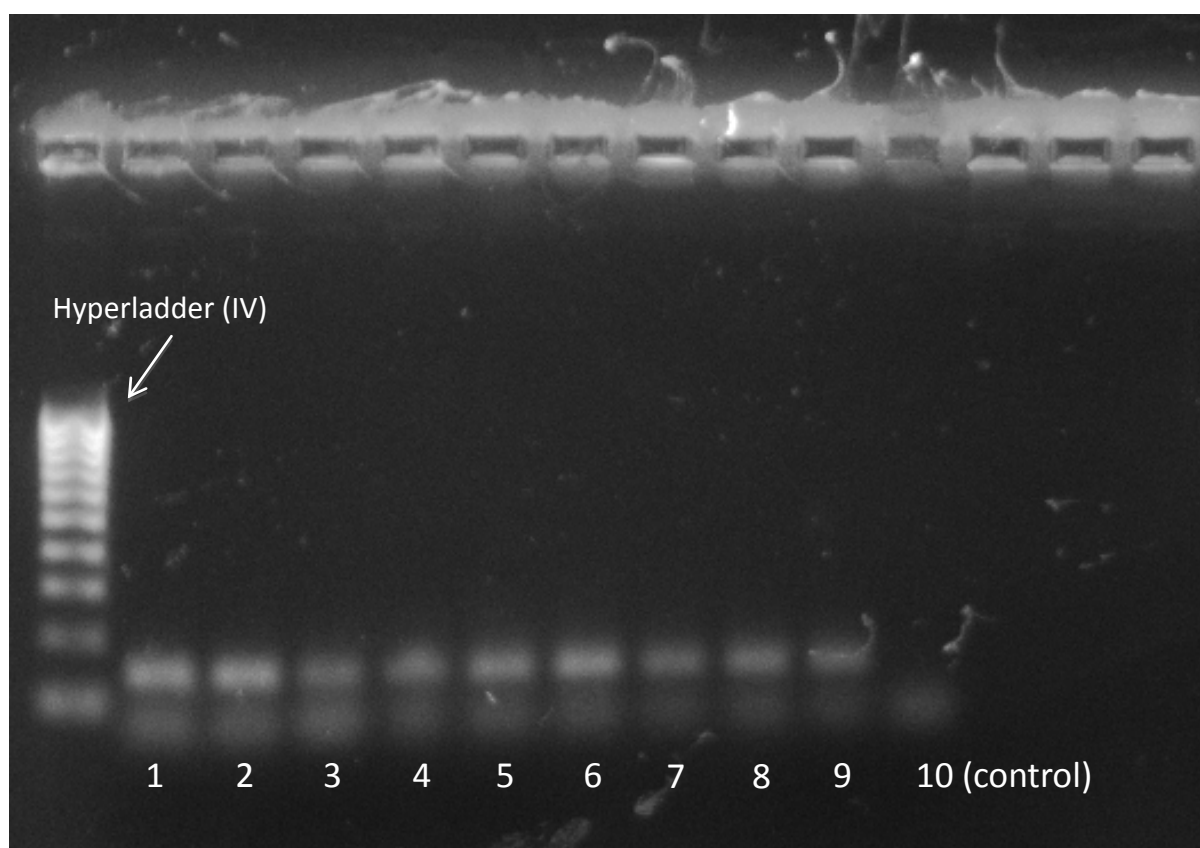
A Maximum Likelihood (ML) tree was obtained, based on the Tamura 3-parameter model (Felsenstein 1985), with settings generated by the Maximum Likelihood option in MEGA 5.0 (Tamura et al., 2011). The tree with the highest log likelihood (-743.1830) was calculated. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the Maximum Parsimony method was used; otherwise BIONJ (Neighbour Joining algorithm) method with MCL (Monte Carlo Localisation algorithm) distance matrix was used. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+ G, parameter = 0.3095)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 23.9957% sites). The ML tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 90 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 105 positions in the final dataset. Branch statistical support was obtained with 100 bootstrap replicates (Felsenstein 1985). Percentage bootstrap supports represent the strength of support for each node/branch of the phylogenetic tree.

Bayesian Inference was carried out using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) under a General Time Reversible (GTR) model (Lanave et al., 1984; Rodríguez et al., 1990) with gamma distribution and invariable sites. Four Markov chains were run for 1,000,000 generations, at a sampling frequency of 1000, from a random starting tree. The first 5,000 trees were discarded as a burn-in. In a Bayesian analysis, inferences of phylogeny are based upon the posterior probabilities of phylogenetic trees. The posterior probabilities are represented by a value between 0 and 1.

## **5.3 Results**

### **5.3.1 Primer development**

DNA was successfully extracted from 2009 preserved L4 water samples. Different primer sets were tested for their suitability in amplifying specific regions of the phytoplankton community DNA. SSU, *rbcL*, and ITS marker regions were successfully amplified. Figure 5.1 shows *rbcL* PCR products visualised by agarose gel electrophoresis. Unsuccessful primers, including those that targeted the LSU region of the genome, are listed in Table 5.1.



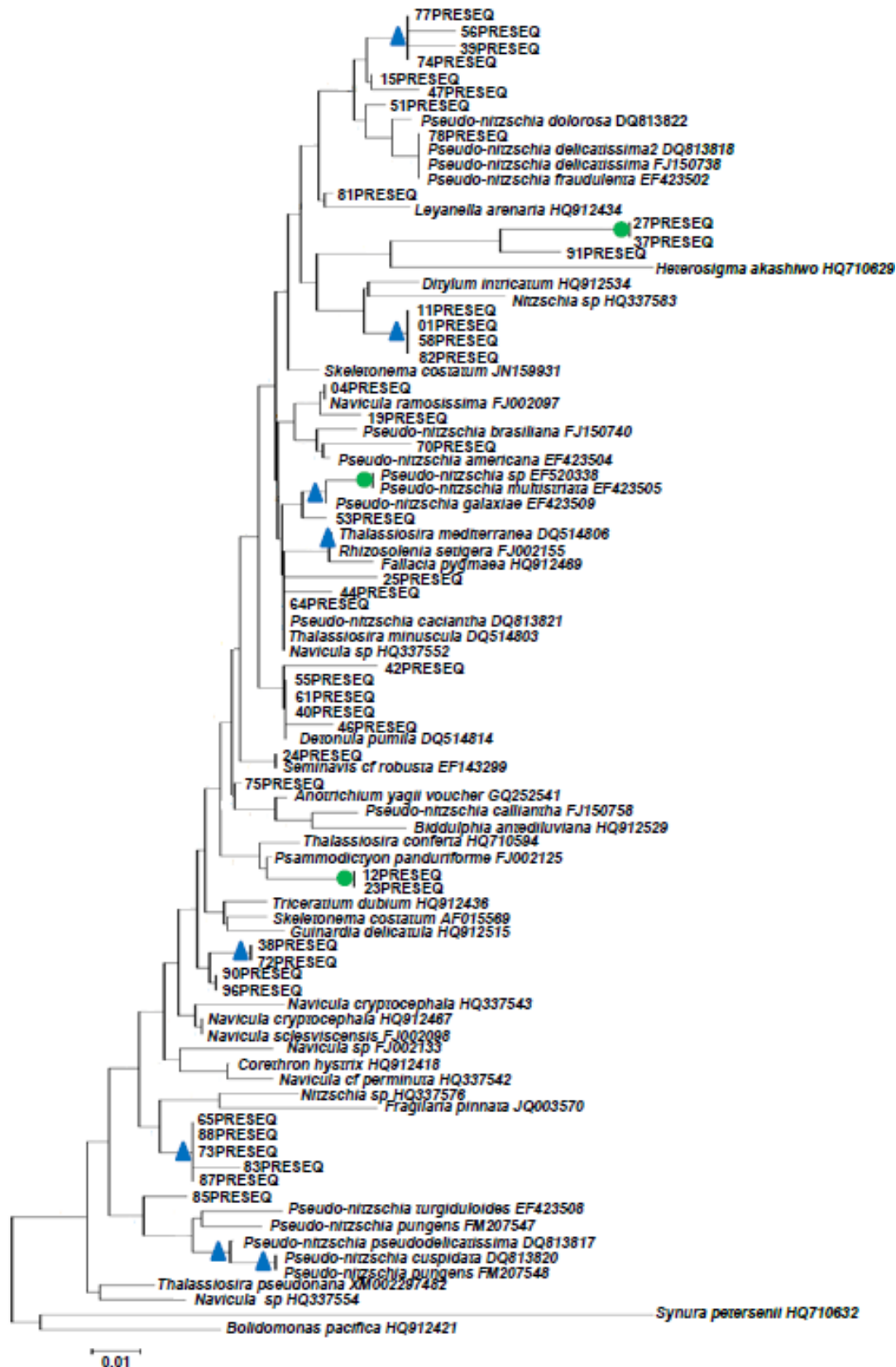
**Figure 5.1:** *rbcL* PCR products from nine L4 water samples (May-Sept) during 2009 with one control in 10<sup>th</sup> position. Hyperladder (IV) on the left hand side. Visualised by agarose gel electrophoresis using UV-transillumination.

### 5.3.2 Sequences

After cloning, the *rbcL* marker region was successfully sequenced and the results of the phylogenetic analysis are shown in figure 5.2, 5.3, and 5.4. Initial BLAST comparisons of the *rbcL* sequences showed that the closest relatives in the Genbank database were a number diatom species including *Skeletonema* spp., *Ditylum* spp., *Nitzschia* spp., and *Pseudo-nitzschia* spp.

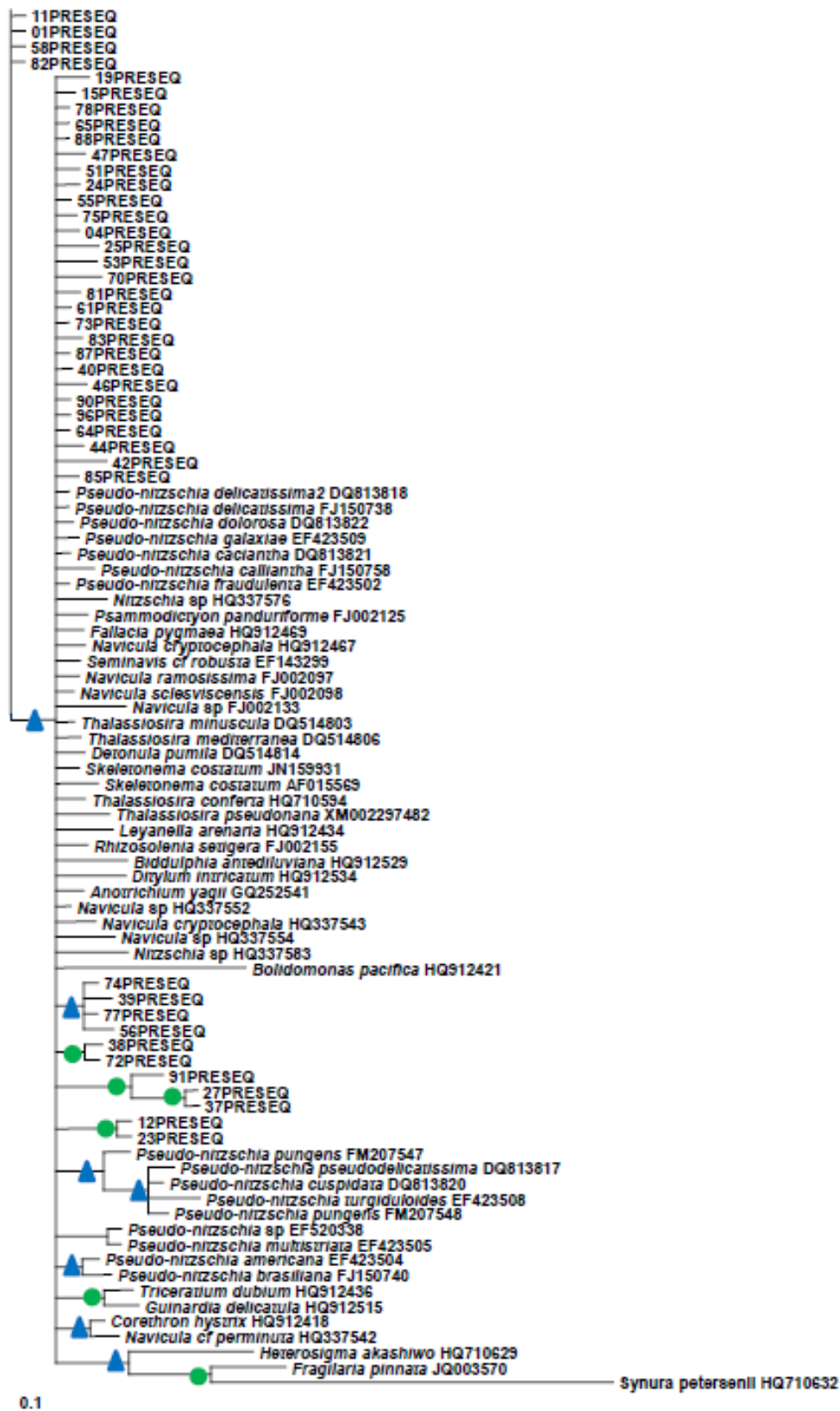
Direct sequencing of SSU PCR gel purified products and ITS PCR products, using 454, was less successful with the majority of sequences from bacteria (e.g. *Bacillus* sp) and fungi (e.g. *Alternaria* sp).





**Figure 5.2:** Maximum likelihood phylogenetic tree based on *rbcL* cloned sequences from archived Lugols-preserved L4 phytoplankton samples. Shapes present on the nodes indicate the percentages of bootstraps that were greater than 70 % (circle) and greater than 50 % (triangle). Scale bar corresponds to 0.01 nucleotide substitutions per site. Cloned L4 sequences are represented by a number +PRESEQ and Genbank sequences include species and accession number.





**Figure 5.4:** Bayesian Inference phylogenetic tree based on rbcL cloned sequences from archived Lugols-preserved L4 phytoplankton samples. Shapes present on the nodes indicate the posterior probabilities that were greater than 0.70 (circle) and greater than 0.50 (triangle). Scale bar corresponds to 0.1 nucleotide substitutions per site. Cloned L4 sequences are represented by a number +PRESEQ and Genbank sequences include species name and accession number.

### 5.3.2 Phylogenetic analysis

The topologies of the trees inferred from NJ and ML phylogenetic analyses of the rbcL dataset were similar, with ML producing the most accurate tree. Analysis showed that there was a good correspondence between the phylogenies represented in these trees and that several L4 rbcL sequences clustered with diatom species, including *Pseudo-nitzschia* spp., as well as other heterokonts. The phylogenetic tree inferred from BI was less similar with the majority of L4 rbcL sequences clustering separately to the sequences of known diatom and *Pseudo-nitzschia* species. All three phylogenetic trees are shown here with percentage bootstrap supports > 50 % and > 70 % (Fig. 5.2, 5.3) as well as BI posterior probabilities > 0.50 and > 0.70 (Figure 5.4).

Figure 5.2 shows that the rbcL sequence for 78PRESEQ clustered with the GenBank sequences for *P. dolorosa*, *P. delicatissima*, *P. delicatissima2*, and *P. fraudulenta*. Sequences for 70PRESEQ and 19PRESEQ clustered with GenBank sequences for *P. americana* and *P. brasiliiana*. The sequence for 85PRESEQ clustered with GenBank sequences for *P. pungens*, *P. turgiduloides*, *P. cuspidata*, and *P. pseudodelicatissima*. The sequence for 53PRESEQ clustered with GenBank sequences for *P. galaxiae* and *P. multistriata*. A number of sequences also clustered with *Navicula* sp., *Ditylum* sp., *Detonula* sp., *Seminavis* sp., and *Leyanella* sp. While these sequences clustered with a number of GenBank sequences the bootstrap values were low suggesting low confidences. The bootstrap value for sequences clustered with 53PRESEQ is greater than 50 %. A number of L4 rbcL sequences clustered together with bootstrap values of > 70 % (23PRESEQ, 37PRESEQ and 12PRESEQ, 13PRESEQ) and > 50 % (11PRESEQ, 01PRESEQ, 58PRESEQ, 82PRESEQ and 38PRESEQ, 72PRESEQ).

Similar clustering was evident in figure 5.3 for NJ analysis of L4 rbcL sequences. The main differences were that sequences for 04PRESEQ and 19PRESEQ clustered with *Navicula ramosissima* in this phylogenetic tree, again with low confidence. Similar clustering was evident for the L4 sequences which clustered together as in the ML tree with moderate to high bootstrap values. For Bayesian Inference analysis many of the L4 sequences were positioned separately from Genbank sequences on the phylogenetic tree with some clustering of L4 sequences as observed in ML and NJ trees. No clustering was observed between L4 rbcL sequences and Genbank sequences.

## **5.4 Discussion**

### **5.4.1 SSU and ITS markers**

One of the goals of the present study was to achieve positive amplification of DNA fragments from Lugol's iodine preserved L4 water samples. This was achieved for all PCR products using SSU, ITS and rbcL primers despite L4 samples being stored for up to 2 years before analysis. However, on sequencing these PCR products, bacteria and fungus sequences were obtained for SSU and ITS regions despite the use of *Pseudonitzschia* specific primers. These sequences accounted for approximately 100 % of the total sequences obtained. This was an issue not fully appreciated at the start of the study. On further investigation it became apparent that SSU and ITS regions are generally more difficult to amplify and sequence than the rbcL region (MacGillivray and Kaczmariska, 2011). This is largely because of the increased likelihood of amplifying heterotrophic contaminant DNA both in cultures and in natural samples using these markers compared to the photoautroph-specific rbcL marker (MacGillivray and Kaczmariska, 2011). Furthermore, the direct amplification and sequencing of the

SSU and ITS regions are not always possible and require a cloning step (Evans et al., 2007; MacGillivray and Kaczmarek, 2011). Due to these factors the practicality of using SSU and ITS markers for the resolution of *Pseudo-nitzschia* in aged preserved samples was, and is likely to be, fairly limited.

In combination with contamination issues, the fixative Lugol's iodine, which was used for the preservation and storage of samples in this study, is recognised to reduce the quality of sample DNA (Godhe et al., 2002). Lugol's iodine and formalin are among the most common fixatives used to preserve microalgal cells. While fixing cells is necessary for identification and enumeration purposes, as well as retrospect studies, many fixatives have been found to destroy and alter the DNA required as a template for molecular studies (Godhe et al., 2002; Auinger et al., 2008). The alteration of DNA structure can cause sequencing errors and mutations during PCR (Douglas and Rogers, 1998; Srinivasan et al., 2002). Of the fixatives, Lugol's iodine has been found to have a lesser effect on sequence analysis than, for example, formalin and glutaraldehyde (Auinger et al., 2008). However, it is still recognised that Lugol's iodine can bind to nucleic acids and make DNA inaccessible for amplification (Godhe et al., 2002). While some studies have not been able to obtain any amplified products from microalgal cells preserved in Lugol's iodine (Marín et al., 2001; Godhe et al., 2002), other studies have been more successful (Bowers et al., 2000; Tengs et al., 2001; Auinger et al., 2008). However, these have required a ten times higher cell concentration to achieve positive PCR results (Bowers et al., 2000). This could be problematic when analysing environmental samples that may not always contain a high enough cell concentration or amount of extracted DNA needed for amplification.

#### 5.4.2 rbcL marker and phylogenetic analysis

The amplification and sequencing of DNA from L4 preserved water samples using an rbcL marker was more successful than using SSU and ITS markers. This was mainly due to the fact that the rbcL fragment is a chloroplast marker and therefore targets the DNA of photoautotrophic taxa exclusively thus reducing the amplification of contaminant DNA (Evans et al., 2007; MacGillivray and Kaczmarska, 2011). Other reported benefits to using the rbcL marker include; the ease of amplification, sequencing and alignment across diatom taxa, as well as the lack of indels and introns (Evans et al., 2007; MacGillivray and Kaczmarska, 2011). Despite its potential suitability as a marker there are a number of drawbacks to using the rbcL fragment some of which have been identified in this study. Previous studies, have found that the rbcL marker has provided limited resolution in biologically defined and closely related species e.g. *Pseudo-nitzschia* (MacGillivray and Kaczmarska, 2011). In some instances the rbcL marker is unable to reflect species diversity fully. This relates to the shortness of the gene and the fact that the mode of chloroplast inheritance is largely unknown for the majority of diatoms (MacGillivray and Kaczmarska, 2011). This in turn effects the segregation of diatom species which is becoming more important due to the increasing number of cryptic species being identified in recent years (MacGillivray and Kaczmarska, 2011).

In the present study, the analysis of rbcL sequences revealed that some of the taxa present were closely related to a number of *Pseudo-nitzschia* spp. However, this analysis did not provide high enough resolution for the unequivocal determination of specific *Pseudo-nitzschia* species. Nevertheless, the closely related *Pseudo-nitzschia* spp. highlighted by BLAST searches and the phylogenetic analysis were in agreement with previous species identified, using transmission electron microscopy, by Klein et al.

2010 in the eastern English Channel. The ineffectiveness of the *rbcl* marker for discriminating between *Pseudo-nitzschia* species in this study was a likely consequence of the degradation of water samples due to sample fixation and storage conditions. This may have reduced the concentration of accessible DNA for amplification. Contrastingly, previous studies which have analysed fresh plankton cells have found this marker to be very effective, particularly in *Pseudo-nitzschia*, displaying a high degree of species specificity (Amato et al., 2007; McDonald et al., 2007). For this reason, the *rbcl* fragment has been identified as a promising DNA barcode for diatoms when combined with other sensitive markers.

To further explore the efficacy of the *rbcl* gene as a taxonomic marker to identify between species of *Pseudo-nitzschia* at L4, a subsequent set of experiments should be developed which focuses on the use of fresh cells. For instance, as an alternative to sample fixation and storage, DNA could be extracted straight from concentrated weekly water samples collected at L4 and then frozen until needed. Following the methods in the present study, the extracted DNA would then be amplified and sequenced using *rbcl* primers. The sequences obtained for the analysis of this fresh cellular matter could then be compared to that of preserved cellular matter. This would indicate whether or not sample fixation and storage conditions were indeed negatively impacting on the molecular analysis of L4 samples. It would also reveal how sensitive this marker was when used on fresh material. In combination with this approach, *Pseudo-nitzschia* cells could be isolated from L4 waters and cultured to form uni algal cultures. The DNA could then be extracted from these cultures and analysed. This would provide concentrated *Pseudo-nitzschia* DNA which would increase the likelihood of getting a strong signal from the *rbcl* gene.



While the effects of fixatives such as Lugol's iodine on microalgae DNA amplification have been explored in a small number of studies (Godhe et al., 2002; Auinger et al., 2008), there has been little investigation into *Pseudo-nitzschia* DNA amplification and sequencing from aged Lugol's iodine preserved samples. The development of the molecular methods in the present study for this purpose was very time consuming and many problems were encountered when attempting to find compatible primers. This process was also costly due to the different DNA kits, reagents, and primers used, as well as the cost of cloning and sequencing. In order for molecular methods such as the one used in the present study to be applied to environmental monitoring and retrospective monitoring studies many challenges need to be overcome which simplify and reduce the cost of these methods.

#### **5.4.3 An alternative molecular method**

One alternative to the method used herein is based on quantitative PCR (qPCR) coupled with melt curve analysis (Andree et al., 2011). The application of qPCR methods for the detection and quantification of toxic *Pseudo-nitzschia* species has been conducted by Dr. Evelyn Kready at the National University of Ireland, Galway. This method has been developed to be rapid so that results are available on the same day and reagent costs are kept to a minimum. The assay is commonly based on ITS1 and ITS2 gene regions, and SYBR Green dye for melt curve analysis, to differentiate between *Pseudo-nitzschia* species and strains (Andree et al., 2011). Melt curve analysis involves an incubation step (1 min at 60 °C) after qPCR amplification whereby the temperature of the DNA is slowly increased to 95 °C (1 °C per 15 s). This produces a melt curve profile of the denaturation of DNA, monitored by changes in fluorescence, which is unique for each species of *Pseudo-nitzschia* (Andree et al., 2011). However, before the application of an assay like this it is necessary to determine what species are found

locally (Andree et al., 2011). This would involve developing melt curve profiles for each of the *Pseudo-nitzschia* species found locally and these can then be compared to the melting temperatures of unknown *Pseudo-nitzschia* species in environmental samples. The use of this assay on Lugol's fixed archived samples is unknown and yet to be investigated but may provide a faster and less expensive alternative to explore *Pseudo-nitzschia* spp. in archived samples and fresh environmental samples from L4 in the future.

#### **5.4.4 Conclusions**

The present study aimed to investigate the application of molecular methods to archived Lugol's iodine preserved L4 samples to identify the *Pseudo-nitzschia* species present during 2009. Few studies have attempted to amplify and sequence phytoplankton DNA from aged fixed environmental samples and those studies that have done this have had variable success. In the present study, DNA was successfully extracted and amplified from preserved L4 samples that had been stored for up to 2 years. Sequence analysis showed that, while only bacteria and fungi sequences were detected using ITS and SSU markers, a number of diatom and *Pseudo-nitzschia* spp. sequences were detected for the *rbcL* marker. This suggests that the *rbcL* fragment may be a more promising marker, and when combined with sequence information for other sensitive markers, could provide valuable information on *Pseudo-nitzschia* species dynamics. Sequencing results from the present study suggest that Lugol's iodine may have reduced the quality of L4 water samples and either destroyed some of the DNA or made it inaccessible. Further work needs to be done to understand the effects of Lugol's Iodine on DNA extraction and amplification for these samples. These molecular methods also need to be applied to fresh L4 water samples to determine how successful they are at extracting and amplifying DNA from non-fixed microalgal cells.



## **Chapter 6: Summary, conclusions and future work**



## 6.1 Overall conclusions and discussion

### 6.1.1 Project rationale and aims

The main objective of the project was to investigate harmful algal bloom dynamics in the western English Channel utilising time series data from the monitoring station L4, data from laboratory studies, and data from toxin analysis using LC-MS as a determinant method (Fig. 6.1). Although there are a number of toxic phytoplankton species present in UK waters, a general initial review revealed that not all species are problematic in all waters around the British Isles, that a number of these toxic phytoplankton species are difficult to culture, and that toxin analysis can be complicated both by the need for licenses to handle authentic toxins and by a lack of authentic toxins of known concentration needed for analysis. On taking all these factors into consideration and after consideration of the knowledge gaps in the literature with regards to toxic phytoplankton around the British Isles, it was decided that the focal point of this project would be the genus *Pseudo-nitzschia* and a study of the factors influencing the production of the toxin domoic acid (DA). Despite a plethora of literature regarding the genus *Pseudo-nitzschia*, little was known at the inception of the study, about *Pseudo-nitzschia* dynamics in the English Channel and the environmental factors impacting on toxin production in these waters. Furthermore, of the laboratory studies examining key environmental factors, the complex relationships between multiple macronutrient and micronutrient availabilities on *Pseudo-nitzschia* growth and toxin production remained unclear.

There were therefore three overall aims to the study:

The first was to develop reliable and reproducible analytical protocols for the determination of domoic acid (DA) in seawater and in *Pseudo-nitzschia* spp. cells.

The second was to determine the important environmental factors influencing *Pseudo-nitzschia* spp. diversity, occurrence, and toxin production using field data from the western English Channel.

The third was to examine the impacts of key environmental factors on *Pseudo-nitzschia* spp. growth dynamics and DA production using laboratory based multifactorial culture experiments.

In the first instance, a critical literature review was undertaken of the extensive published research associated with *Pseudo-nitzschia*, DA production and associated analytical methods to assess the then current state of knowledge. In particular the review focused on the diversity, morphology, and identification of *Pseudo-nitzschia* species and examined the trophic impacts of DA, the ecological roles of DA, and the factors effecting its production. The analytical methods available at the time for detecting, extracting, concentrating, and quantifying DA were critically evaluated.

## **6.1.2 Main findings**

### ***6.1.2.1 Methods for DA determination***

Building on published analytical methods, new methods were established and developed, for the accurate and reproducible extraction and determination of DA concentrations, and then applied in subsequent experimental and field studies. One such method developed from previously published methods (Wang et al., 2007; De la Iglesia et al., 2008) was based on the extraction, purification, and concentration of DA from seawater and algal culture matrices using Strata-X solid phase extraction cartridges. This method combined the protocols of two different methods (Wang et al., 2007; De la Iglesia et al., 2008) and allowed optimisation of the recovery of DA and ensured good reproducibility. For detection and quantification of DA in seawater and algal culture samples a published LC-MS method was selected and developed for use on an LC-MS coupled with an ESI ion trap interface (Hummert et al., 2002). Early in the project a method suitable for analysis of DA and okadaic acid (OA) in one run was sought. As the project progressed toxin analysis focused solely on DA. Despite this change in focus, the method originally selected was deemed suitable and further modifications to improve performance were made. One such improvement was the addition of an internal standard, kainic acid (KA), which enhanced the accuracy of the LC-MS analysis. To ensure that DA determinations using LC-MS methods were quantifiable calibrations of DA and KA were performed. Calibrations were monitored over time, in ratio to each other, and at varying concentrations to confirm the reliability and precision of the revised LC-MS methods using KA internal standard. Another improvement was that one of the buffering agents, which is usually added to the mobile phase, was eliminated which enhanced chromatography. Also, analysis time was reduced allowing for a greater number of samples to be processed.



In addition to LC-MS DA determination, an alternative analytical method, based on an enzyme linked immunosorbent assay (ELISA) published for use in DA determination in bivalve molluscs, was investigated. However, using ELISA for use with algal cultures was found to be variable with significant overestimations of DA concentrations when compared with calibrated determinations using LC-MS. Considering LC-MS provides a definitive measurement of DA, ELISA was not pursued as an alternative analytical tool for future work.

#### **6.1.2.2 Field monitoring**

LC-MS measurements of DA concentrations were made alongside cell count data from the monitoring station L4 in the western English Channel during 2009. Cell count data showed that *Pseudo-nitzschia* spp. occurred throughout the year ranging from 40 cells L<sup>-1</sup> and 250 x 10<sup>3</sup> cells L<sup>-1</sup> with two peaks in abundance occurring in June/July and in August. Three distinct groups/complexes of *Pseudo-nitzschia* species, according to cell morphology and size, were enumerated; a *P. delicatissima*-group, a *P. seriata*-group, and a *P. pungens/multiseries*-group. The smaller celled *P. delicatissima*-group species formed the main background species throughout the year, followed by the occurrence of the *P. pungens/multiseries*-group during spring/summer months, and the *P. seriata*-group during summer/autumn months. The highest cell numbers were recorded for the *P. delicatissima*-group which peaked in August and accounted for 100 % of the total diatom community at that time. BEST analysis in PRIMER revealed that the three *Pseudo-nitzschia* groups were ecologically different with a number of environmental parameters influencing the abundance of the species within these groups. The *P. delicatissima*-group was significantly influenced the physical environmental factors, namely temperature, hours of light, and rainfall ( $P < 0.05$ ). In contrast the *P.*

*pungens/multiseries*-group was significantly influenced by macronutrients i.e. by chemical factors; and the *P. seriata*-group was significantly influenced by temperature and nitrate concentrations.

Particulate DA (pDA) was detected at low concentrations (maximum  $0.4 \text{ ng mL}^{-1}$ ) at L4 from May to July, persisting in the seawater for five weeks. This is the first time that DA has been detected at the long term monitoring site in the western English Channel and results suggest that presence of DA significantly correlated ( $P < 0.05$ ) with the occurrence of *P. seriata*-group and *P. pungens/multiseries*-group species during a period of low silicate and nitrate availability. Although one or more of these group species produced low pDA concentrations, results showed that there is potential for toxic blooms to occur at this site in the future. This is evidenced in the L4 time series data which shows that *Pseudo-nitzschia* can be present in high numbers exceeding the threshold cell concentration of  $150 \times 10^3 \text{ cells L}^{-1}$  set by current monitoring programmes after which toxin analysis is carried out. If blooms of toxigenic *Pseudo-nitzschia* species coincide with favourable conditions for toxin production then DA concentrations could reach higher values  $1000 \text{ pg mL}^{-1}$  (Trainer et al., 2012). To understand the variability of toxin production at L4 and the scope for the occurrence of such toxic bloom events further monitoring needs to be completed at this site. The detailed and high-resolution data for a number of biological, chemical, and physical parameters collected at this site is an essential resource in the study of toxigenic phytoplankton species. Findings from this study have been submitted for publication to the journal *Continental Shelf Research* (Downes-Tettmar et al., 2013).

A number of studies have used group level discrimination, based on cell morphology and size, when monitoring *Pseudo-nitzschia* spp. in the marine environment (Fehling et

al., 2006; Kaczmarek et al., 2007; Martin et al., 2009; Sahraoui et al., 2009). While this can provide valuable insight about *Pseudo-nitzschia* population dynamics, it is important to understand the dynamics at species level especially where toxigenic species may occur. To provide this information molecular methods are frequently combined with microscopy to further resolve the species complexes within the genus *Pseudo-nitzschia*. To elucidate the *Pseudo-nitzschia* species present at L4 during 2009 molecular methods were developed using existing primers to identify *Pseudo-nitzschia* species from Lugol's iodine preserved seawater samples. Three DNA marker regions were investigated, partial SSU, the ITS, and partial rbcL. Multiple primer sets were tested, using polymerase chain reaction, for their compatibility with L4 samples. DNA was successfully extracted from 2009 preserved L4 water samples and SSU, rbcL, and ITS marker regions were amplified.

After 454 sequencing, sequence data for SSU and ITS showed that L4 samples for the dates 22/6, 29/6, 17/8 and 24/8 in 2009 contained bacteria and fungi, which is a likely consequence of the deterioration of Lugol's iodine preserved water samples and the poor selectivity between the target marker regions. After cloning rbcL PCR products and using Sanger sequencing, sequence data indicated that *Pseudo-nitzschia* spp were present in water samples and that rbcL was a promising target marker region for sequencing diatoms from L4 preserved water samples. rbcL sequences were compared to known *Pseudo-nitzschia* sequences in Genbank and, although a low confidence was found, sequences from L4 clustered with a number of different *Pseudo-nitzschia* spp. These were *P. dolorosa*, *P. delicatissima*, *P. delicatissima2*, *P. fraudulenta*, *P. americana* and *P. brasiliana*, *P. pungens*, *P. turgiduloides*, *P. cuspidata*, and *P. pseudodelicatissima*, *P. galaxiae* and *P. multistriata*. Clustering of these sequences suggested that sequences from L4 phytoplankton species were closely related to certain *Pseudo-nitzschia* species

or groups. Despite this relatedness, we can only infer group level taxa confidently and only the successful application of alternative primers for these marker regions on live samples would allow increased resolution of the *Pseudo-nitzschia* species present. This study confirms that the application of molecular methods for the identification of *Pseudo-nitzschia* spp in Lugol's iodine preserved samples is not ideal due to the variable degradation of DNA in stored environmental samples. However, this is the first time molecular methods have been applied to L4 samples to determine *Pseudo-nitzschia* diversity, and it is one of a few studies which investigates sequencing from preserved Lugols iodine water samples.

#### **6.1.2.3 Culture studies**

Whilst many studies have addressed the effects of individual or dual macronutrients (nitrate, phosphate, and silicate) on *Pseudo-nitzschia* growth and toxin production there have been no studies of the impacts of at least three macronutrients in combination. A systematic multifactorial laboratory experiment was therefore undertaken over a 40 day time period, using concentrations of nitrate (110 and 880  $\mu\text{M}$ ), phosphate (4.5 and 36.2  $\mu\text{M}$ ), and silicate (13.3 and 106  $\mu\text{M}$ ), to assess the effect of macronutrient availability on the growth and toxin production of *Pseudo-nitzschia multiseries*. Growth rates across all cultures varied between 0.36 and 0.41  $\text{d}^{-1}$  and were not found to be significantly different across treatments. Contrastingly, differences were found between the maximum cell abundances as cultures entered the stationary phase of growth, these differences were due to phosphate availability which was found to have a significant positive ( $P = 0.0014$ ) effect. Furthermore, the decline of viable cells during the late stationary phase was significantly affected by the availability of all the nutrients ( $P <$

0.0001). Particulate DA (pDA) and dissolved DA (dDA) was detected from Day 14 and increased up until Day 40 and was found to be significantly negatively affected by phosphate ( $P = 0.005$ ) and silicate ( $P < 0.0001$ ). Where cultures were supplied with high concentrations of these nutrients, pDA was reduced. dDA concentrations were highest when nitrate ( $P = 0.0001$ ) and silicate ( $P = 0.0004$ ) concentrations were low. These findings confirm that the availability of nitrate, phosphate, and silicate all significantly affect the growth, decline, and domoic production of *P. multiseriata*, and highlight the importance of both phosphate and silicate availability for DA production.

The above work was followed up with a further laboratory experiment which investigated the impacts of both macronutrient (phosphate and silicate) and micronutrient (iron and copper) availability on *Pseudo-nitzschia* growth dynamics, photosynthetic efficiency and DA production. Whilst the effects of micronutrients on *Pseudo-nitzschia* growth and DA production have previously been addressed (Bates et al., 2000; Maldonado et al., 2002; Wells et al., 2005), the relationships between these nutrients and macronutrients are less clear, complex, and not easily investigated. Previous studies have found that the availability of macronutrients and micronutrients can impact on *Pseudo-nitzschia* spp. in different ways, causing an accumulation of cellular DA when silicate and/or phosphate are limiting, but only when iron is in excess (Maldonado et al., 2002). This could impact on the overall toxicity of *Pseudo-nitzschia* cells. A multifactorial experiment was designed, following a similar protocol to the macronutrient experiment whereby eight nutrient combinations were examined. For this experiment, to control the chemical composition of the media it was necessary first to remove trace metal contamination from all flaskware, media containers, and from the basal medium itself (modified Aquil medium). This involved extreme rigour, with several much time consuming acid washing, purification, and sterilisation steps

required. Despite this, limited growth and DA production were observed in experimental *P. multiseriis* cultures. Photosynthetic efficiency was similar between treatments, initially with  $F_V/F_M$  ratios representative of healthy cells, and decreasing during the experimental period of 22 days. Thorough investigations were made to determine the cause of the limited culture growth (a feature which has also been evident in previous trace metals studies). The results highlighted the challenges in culturing *Pseudo-nitzschia* spp. under such conditions. From the experimental work it was concluded that the method used for media purification might have been too efficient, effectively removing trace elements essential for *P. multiseriis* growth. Further optimisation of the growth media and methods for purification remain necessary for future trace metal studies.

## **6.2 Environmental implications**

### **6.2.1 Monitoring and time series data**

Findings from the L4 time series and additional monitoring data show that toxigenic *Pseudo-nitzschia* species were present and annual blooms of *Pseudo-nitzschia* spp. were recorded at this site. Although L4 is not a shellfish aquaculture site and cellular domoic acid (DA) concentrations were low, there may be potential for these blooms to become more frequent and problematic in future. Whilst there are reports of a slight negative trend in total diatom abundance over the last 20 years at L4 (Widdicombe et al., 2010), overall there has been a marked increase in diatom abundance, including *Pseudo-nitzschia* spp. in the northeast Atlantic and North Sea over the last 50 years (Hinder et al., 2012). This finding was based on data acquired by a continuous plankton recorder (CPR) which also recorded a decline in dinoflagellate abundance over the same time period (Hinder et al., 2012). The study by Hinder et al. (2012) provides compelling

evidence that toxigenic diatom species may pose an increasing threat in future years compared to toxigenic dinoflagellate taxa in waters around the British Isles. The work presented herein exemplifies the value of high resolution and high temporal long-term time series data, such as that compiled at the monitoring station L4 in the western English Channel, for characterising phytoplankton species dynamics and the variations in key biogeochemical, physical, and ecological parameters.

The reported shift in the functional groups of microalgae over the last 50 years has been suggested to result from increasing sea temperatures and windy summer conditions (Hinder et al., 2012). Combining this information with time series data, predictions might be made as to which species might become more dominant and whether these conditions favour those diatom species that are toxigenic or not. For example, the occurrence of *P. seriata*-group species have previously been linked to increases in sea surface temperature during summer months (Fehling et al., 2006), and at L4 the occurrence of this *Pseudo-nitzschia* group has been associated with toxin production. Therefore, an increase in sea surface temperatures throughout the year could favour the growth of this species and in turn increase the likelihood of toxic events. Previous findings indicate that *Pseudo-nitzschia* taxa respond to different sets of environmental conditions and only by understanding how these vary seasonally and annually over a number of years can robust conclusions be drawn.

The apparent separation between *Pseudo-nitzschia* groups in terms of their toxicity and response to environmental conditions could help inform and improve harmful phytoplankton monitoring programmes. In a recent study, Fehling et al. (2012) suggested that due to the different affinity of *Pseudo-nitzschia* groups to different water masses and environmental conditions, as well as a difference in toxicity between

groups, the current practice of enumerating *Pseudo-nitzschia* as a single group is insufficient. Monitoring programmes currently use the threshold of  $150 \times 10^3$  cells L<sup>-1</sup> as a guideline above which toxin analysis is undertaken on shellfish samples. However, this number could predominantly be made up of *P. delicatissima*-group species which have not been shown to be associated with toxin production around the British Isles. To make this distinction when identifying and enumerating *Pseudo-nitzschia* species could save time and better inform monitoring programmes as to the toxic nature of future blooms.

### 6.2.2 Forecasting

To further understand *Pseudo-nitzschia* occurrences and predict future bloom events, long-term time series data, such as those from L4, could provide an advantageous platform on which to develop or inform existing ecosystem models (Trainer et al., 2012). Due to the increasing frequency and intensity of harmful algal blooms (HABs), and the deleterious consequences of these blooms on industry and human/ecosystem health there has been an emphasis on developing forecasting tools for the predictive monitoring of HABs (Ryan et al., 2011; Lelong et al., 2012). These forecasting tools have mainly been developed for toxic dinoflagellate species with relatively few having been developed for toxigenic *Pseudo-nitzschia* species (Anderson et al., 2009; Lane et al., 2009; Palma et al., 2010). Much of the data available for *Pseudo-nitzschia* bloom occurrences is episodic and the role of key environmental factors is based on empirical relationships. This has led to the requirement of a mechanistic approach where long term monitoring (biological, physical, and chemical parameters) and experimental eco-physiological data are central to improving forecasting accuracy (Trainer et al., 2012). Using this approach the abundance of *Pseudo-nitzschia* spp should be monitored over a

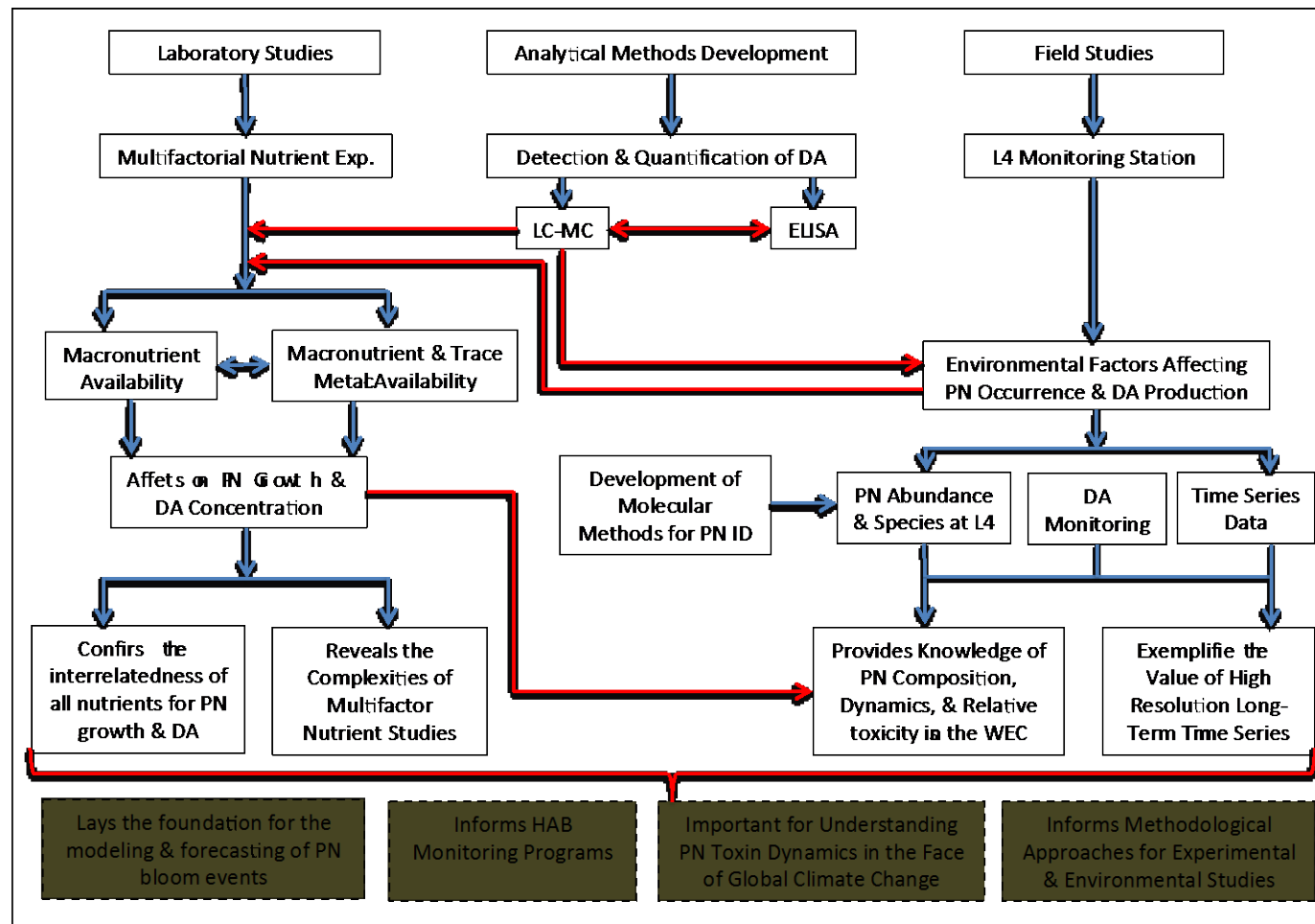


number of years and the environmental parameters that correspond to genus proliferations and toxicity investigated. Identification of *Pseudo-nitzschia* spp. using transmission electron microscopy and molecular methods is required, and these strains then need to be isolated and cultured in order to define their biological and physiological characteristics (e.g. growth rates, optimal growth conditions, toxin production). Perhaps only once all such detailed information is collated for each *Pseudo-nitzschia* strain in a specific region can model simulations best represent the complexities of toxic bloom events in the natural environment.

The research herein lays the foundations from which such detailed information could be gathered and used for forecasting (Fig. 6.2). The development of a robust chromatographic method for the analysis of DA combined with environmental monitoring and analysis of *Pseudo-nitzschia* species and at L4 has enabled us to start to build up a profile of *Pseudo-nitzschia* dynamics at L4. Experiments on cultures confirmed previously published results that the interrelationship of macronutrient factors is important for both PN growth and DA production. Further micronutrient availability culture experiments, which set out to examine the role of domoic acid in iron and copper acquisition, equally revealed the complexity and interrelationship of factors.

There are many interactions between environmental variables and climate change factors that can either be synergistic or antagonistic in their control over phytoplankton dynamics (Boyd et al., 2010). Furthermore, the interaction of various environmental factors on toxin production are of increasing significance in our changing global oceans and are affected not only by temperature changes but by anthropogenic nutrient inputs and the progressive acidification of the oceans caused by CO<sub>2</sub> emissions (Boyd et al.,

2010). However, there has been little exploration into how these complex interactions could affect toxin production. An example of one such study has shown that there is a synergism between increasing CO<sub>2</sub> concentrations in seawater, changing pH, and silicate limitation which all act to greatly magnify cellular DA concentrations in *P. fraudulenta* when compared with pre-industrial CO<sub>2</sub> concentrations (Tatters et al., 2012). Similar findings have been found for *P. multiseriis* cultures that have been exposed to high CO<sub>2</sub> concentrations and phosphate limitation (Sun et al., 2011). Such studies emphasise the significance of multifactor studies that address these confounding interactions and can help to improve the resolution of predictive tools.



**Figure 6.1:** A diagram representing the three approaches used in this project, the cross over between these approaches (red lines), the main findings, and the outcomes of this work (yellow boxes).

## **6.3 Recommendation for future work**

### **6.3.1 Continuation of monitoring**

To understand harmful algal species dynamics we need to increase understanding of the seasonal and annual variations of species, toxicity, and the important environmental factors influencing occurrence and toxin production. Long term data monitoring such as that undertaken at L4 is of central importance to this. Considering the shifts that have been observed in the phytoplankton functional groups, it is important to maintain these investigations to monitor long-term changes and to inform forecasting tools. The molecular identification of HAB species is a key component of this and the knowledge gained can be used to compare species diversity and relatedness across the rest of the British Isles. At a more local level, results could be compared to continuous plankton recorder data to investigate how representative L4 is in terms of the biology compared to other sites in the English Channel. Additionally, the continuation of field investigations would allow for the identification of new and existing HAB species which, due to increases in sea surface temperature, may become a threat in this region and the British Isles in future years.

### **6.3.2 Measuring other toxins in the western English Channel**

The chromatographic methods used herein for toxin detection and quantification could be expanded to monitor suites of toxins. At the start of this project LC-MS methods were developed to measure both DA and OA from seawater samples in one LC-MS run. OA is a toxin produced by the dinoflagellates *Dinophysis* sp. and *Prorocentrum* sp. and causes diarrheal shellfish poisoning. Due to time constraints and the final objectives of the project, the simultaneous analysis of these toxins was not taken forward. However,

LC-MS methods could be developed to assess the toxicity of other potential HAB species present at L4. This would allow further characterisation of the phytoplankton dynamics at this site and add value to the use of the monitoring station as a platform from which to study HABs.

### **6.3.3 Additional tools for the determination of toxins at L4**

As an alternative to collecting, filtering and extracting DA from seawater more recent methods have been developed whereby toxins are extracted from a porous resin which has been suspended in the water column (Lane et al., 2010; MacKenzie, 2010). An example of this is solid phase absorption tracking (SPATT) (MacKenzie, 2010) which is used to quantify the concentrations of dissolved toxins in seawater. SPATT allows for the analysis of many toxins including azaspiracids and yessotoxins and was originally proposed for use in monitoring programmes where sensitive methods were sought that could be used as an early warning system for shellfish contamination (MacKenzie, 2010). Low concentrations of toxins can be detected even when the abundance of toxic algal species are low (Lane et al., 2010). A lot of work is still being undertaken to validate SPATT methods and the application of these bags at L4 might provide valuable feedback on the reliability and precision of using this approach and how SPATT data corresponds to the occurrence of causative phytoplankton species. In addition to this, the information gained from using SPATT could enhance our understanding of toxin production and release at L4.

#### 6.3.4 Improving *Pseudo-nitzschia* cell isolation and cultivation

During the course of this project several attempts were made to isolate and culture *Pseudo-nitzschia* spp. from L4. A number of species were successfully isolated but following introduction to growth media some of these cultures failed to thrive. Of those that did, six *Pseudo-nitzschia* spp. strains were established in culture. These cultures were later identified as either *P. fraudulenta* or *P. delicatissima* using electron microscopy and neither was found to produce DA. To ascertain which species of *Pseudo-nitzschia* are responsible for producing DA at L4, more species need to be isolated, cultured, and analysed for DA production. To achieve this, one option could be to use a flow cytometer such as a FACS Aria high-speed digital cell sorter which, depending on the compatibility of this technology with sorting *Pseudo-nitzschia* cells, could isolate single cells from mixed phytoplankton populations. There appears to be no evidence in the literature of the use of this method for isolating harmful algal species to date. Development of a reliable method for sorting *Pseudo-nitzschia* cells, would reduce the time it takes to isolate single cells from natural samples and would potentially increase the number of strains that could be acquired from any one sample, thus improving culture success.

Due to complications isolating and culturing toxic *Pseudo-nitzschia* species from L4, and the requirement to use a confirmed toxin producer for LC-MS method development, a toxic strain of *P. multiseriata* was acquired which had been donated by Dr. E. Keady at the Marine Institute, Galway. This strain was used in LC-MS method development and laboratory experimental work. However, out of the fourteen confirmed DA producing *Pseudo-nitzschia* spp., *P. multiseriata* has been studied the most extensively and as a result of this more information is still needed on the toxicity and growth dynamics of other potentially toxic species. It is well documented in the literature that there are

substantial variations in the toxicity of *Pseudo-nitzschia* strains, both of the same species and between species. Therefore, future experimental work should include other potentially toxic *Pseudo-nitzschia* species and, in order to extend our knowledge of DA production in the western English Channel, these species would preferably be isolated from this region.

### **6.3.5 Culture studies and growth conditions**

It is clear from the experimental work in this project, which investigated macronutrient and trace metal availability on *P. multiseriis* growth and toxin production, that further work is needed to optimise the growth media to which cultures are exposed. The use of different media recipes or alternative media adaptations needs to be explored to ensure maximal growth under controlled conditions. This includes examining the different methods of media purification to limit the negative impacts of this process on the chemical composition of the artificial seawater. An alternative method could be to use natural seawater collected at a time when nutrient and trace metal concentrations were at low to trace concentrations. Despite the experimental complexities encountered in this project surrounding the study of trace metals, the interrelated effects of macronutrient and micronutrient availability still pose important questions regarding the mechanisms for DA production. In the natural environment and in laboratory cultures there appears to be a close coupling between the availability of these nutrients and DA production. It is not known whether there is an interaction between these nutrients when either one is limiting that increases the production and release of DA. Furthermore, it is unclear as to whether DA production is a direct or indirect consequence of macronutrient and micronutrient limitation in the external environment.

It has been argued that it is the coupling of these nutrients that could account for the regional variations in DA production (Maldonado et al., 2002).

Like many other marine toxins, the fate of DA remains rather ambiguous yet it is clear that toxins are released into the seawater and in the case of *Pseudo-nitzschia* this release has been found to be an active process (Bates et al., 2003). Under experimental conditions of iron limitation as much as 95 % of intracellular DA was observed to be released into surrounding media. While it has been hypothesised that extracellular DA is involved in iron chelation little is known about the breakdown products of DA and how long these may persist in the water column. It has been suggested that there are two main pathways for the degradation of DA in the water column, photodegraded and degradation by bacteria (Stewart et al., 1998; Hagström et al., 2007). To date, there are only a few studies that have explored the photodegradation of DA and the breakdown products yielded from this photochemical process. Systematic laboratory studies are required to more thoroughly understand the fate of DA and in particular the fate during photodegradation.

The few photodegradation studies so far conducted have advanced an understanding of the fate of dissolved domoic acid in the water column and have demonstrated that simulated sunlight can potentially modify the chemical structure of DA and produce a suite of isomers and decarboxylated derivatives. Studies in this area have so far mainly investigated the production of the photoisomers isodomoic D, E, and F over a 24 hour period and have shown that the photochemical dynamics of dissolved DA are very complex (Wright et al., 1990; Bates et al., 2003; Campbell et al., 2005; Bouillon et al., 2006; Bouillon et al., 2008). It is apparent that there is a need for greater understanding of DA photoproducts, other than the three geometric isomers, that are derived from



photodegradation. To fully understand the fate and impact of dissolved DA in seawater a comprehensive study into the additional photoproducts that arise upon exposure to sunlight needs to be conducted. This work should take into account photoproducts produced over a time period greater than 24 hours and sampling of irradiated solutions should be conducted at regular time intervals.

## 6.4 Summary

While there have been many advances in our understanding of *Pseudo-nitzschia* bloom dynamics and toxin production in the last two decades, including the ones reported as part of this thesis, there are still many scientific questions that remain unanswered. The development of new methods for species identification, toxin quantification, and sampling are adding to knowledge. As a result of this, the number of identified *Pseudo-nitzschia* species, those confirmed to produce DA, and the environmental triggers of DA production are ever increasing. However, there are still fundamental questions that need elucidating, such as the role of DA, the environmental conditions triggering *Pseudo-nitzschia* bloom development, and the effects of DA contamination in the marine ecosystem. Overall, the importance of this species lies in the impacts it may have on marine wildlife and on human health. Our understanding of this genus allows us to examine which species are likely to be a potential threat, informs management for susceptible bloom areas, and may provide information allowing the establishment of an accurate early warning system of future HAB events.

## References

- Almandoz, G.O., Ferrario, M.E., Ferreyra, G.A., Schloss, I.R., Esteves, J.L. and Paparazzo, F.E., 2007. The genus *Pseudo-nitzschia* (Bacillariophyceae) in continental shelf waters of Argentina (Southwestern Atlantic Ocean, 38-55°S). Harmful Algae. 6, 93-103.
- Amato, A., Kooistra, W.H.C.F., Levialdi Ghiron, J.H., Mann, D.G., Pröschold, T. and Montresor, M., 2007. Reproductive isolation among sympatric cryptic species in marine diatoms. Protist. 158, 193-207.
- Anderson, C.R., Siegel, D.A., Kudela, R.M. and Brzezinski, M.A., 2009. Empirical models of toxigenic *Pseudo-nitzschia* blooms: Potential use as a remote detection tool in the Santa Barbara Channel. Harmful Algae. 8, 478-492.
- Andree, K.B., Fernandez-Tejedor, M., Elandaloussi, L.M., Quijano-Scheggia, S., Sampedro, N., Garces, E., Camp, J. and Diogene, J., 2011. Quantitative PCR coupled with melt curve analysis for detection of selected *Pseudo-nitzschia* spp. (Bacillariophyceae) from the Northwestern Mediterranean sea. Applied Environ. Microb. 77, 1651-9.
- Auinger, B.M., Pfandl, K. and Boenigk, J., 2008. Improved methodology for identification of protists and microalgae from plankton samples preserved in Lugol's iodine solution: Combining microscopic analysis with single-cell PCR. Appl. Environ. Microbiol. 74, 2505-2510.

- Bargu, S., Goldstein, T., Roberts, K., Li, C. and Gulland, F., 2011. *Pseudo-nitzschia* blooms, domoic acid, and related California sea lion strandings in Monterey Bay, California. *Marine Mammal Science*. 28, 237-253.
- Bargu, S. and Silver, M.W., 2003. Field evidence of krill grazing on the toxic diatom genus *Pseudo-nitzschia* in Monterey Bay, California. *Bull. Mar. Sci.* 72, 629-638.
- Bates, S.S., 1998. Ecophysiology and metabolism of ASP toxin production. In: Anderson, D.M., Cembella, A. and Hallegraeff, G.M. (Eds.), *Physiological Ecology of Harmful Algal Blooms*. Springer-Verlag, Heidelberg, pp. 405-426
- Bates, S.S., 2000. Domoic-acid-producing diatoms: another genus added! *J. Phycol.* 36, 978-983.
- Bates, S.S., de Freitas, A.S.W., Milley, J.E., Pockington, R., Quilliam, M.A., Smith, J.C. and Worms, J., 1991. Controls on domoic acid production by the diatom *Nitzschia pungens* f. *multiseries* in culture: Nutrients and Irradiance. *Can. J. Fish. Aquat. Sci.* 48, 1136-1144.
- Bates, S.S., Douglas, D.J., Doucette, G.J. and Leger, C., 1995. Enhancement of domoic acid production by reintroducing bacteria to axenic cultures of the diatom *Pseudo-nitzschia multiseries*. *Nat. Toxins*. 3, 428-435.
- Bates, S.S., Gaudet, J., Kaczmarek, I. and Ehrman, J.M., 2004. Interaction between bacteria and the domoic-acid-producing diatom *Pseudo-nitzschia multiseries*

(Hasle) Hasle; can bacteria produce domoic acid autonomously? Harmful Algae. 3, 11-20.

Bates, S.S., Hiltz, M.F. and Leger, C., 1999. Domoic acid toxicity of large new cells of *Pseudo-nitzschia multiseria* resulting from sexual reproduction In: Martin, J.L. and Haya, K. (Eds.), Proceedings of the sixth canadian workshop on harmful algae. Can. Tech. Rep. Fish. Aquat. Sci., pp. 21-26

Bates, S.S., Leger, C., Satchwell, M. and Boyer, G.L., 2000. The effects of iron and domoic acid production by *Pseudo-nitzschia multiseria*. In: Hallegraeff, G.M., Blackburn, S.I., Bolch, C. and Lewis, R.J. (Eds.), Proceedings of the 9th International conference on harmful algal blooms, Hobart, Tasmania. Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 320-323

Bates, S.S., Leger, C. and Smith, K.M., 1996. Domoic acid production by the diatom *Pseudo-nitzschia multiseria* as a function of division rate in silicate limited chemostat culture. In: Yasumoto, T., Oshima, Y. and Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms, Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 163-166

Bates, S.S., Leger, C., Wells, M.L. and Hardy, K., 2003. Photodegradation of domoic acid. In: Bates, S.S. (Eds.), Proceedings of the Eighth Canadian Workshop on Harmful Algae, Fisheries, Oceans Canada, pp. 42-47

- Bates, S.S., Worms, J. and Smith, J.C., 1993. Effects of ammonium and nitrate on growth and domoic acid production by *Nitzschia pungens* in batch culture. Can. J. Fish. Aquat. Sci. 50, 1248-1254.
- Baugh, K.A., Bush, J.M., Bill, B.D., Lefebvre, K.A. and Trainer, V.L., 2006. Estimates of specific toxicity in several *Pseudo-nitzschia* species from the Washington coast, based on culture and field studies. African J. Mar. Sci. 28, 403-407.
- Besiktepe, S., Ryabushko, L., Ediger, D., Yilmaz, D., Zenginer, A., Ryabushko, V. and Lee, R., 2008. Domoic acid production by *Pseudo-nitzschia calliantha* Lundholm, Moestrup et Hasle (bacillariophyta) isolated from the Black Sea. Harmful Algae. 7, 438-442.
- Blanco, J., Acosta, C.P., Bermúdez de la Puente, M. and Salgado, C., 2002. Depuration and anatomical distribution of the amnesic shellfish poisoning (ASP) toxin domoic acid in the king scallop *Pecten maximus*. Aquat. Toxicol. 60, 111-121.
- Blanco, J., Livramento, F. and Rangel, I.M., 2010. Amnesic shellfish poisoning (ASP) toxins in plankton and molluscs from Luanda Bay, Angola. Toxicon. 55, 541-546.
- Bouillon, R.-C., Kieber, R.J., Skrabal, S.A. and Wright, J.L.C., 2008. Photochemistry and identification of photodegradation products of the marine toxin domoic acid. Mar. Chem. 110, 18-27.

- Bouillon, R.C., Knierim, T.L., Kieber, R.J., Skrabal, S.A. and Wright, J.L.C., 2006. Photodegradation of the algal toxin domoic acid in natural water matrices. *Limnol. Oceanogr.* 51, 321-330.
- Bowers, H.A., Tengs, T., Glasgow, H.B., Burkholder, J.M., Rublee, P.A. and Oldach, D.W., 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. *Appl. Environ. Microbiol.* 66, 4641-4648.
- Boyd, P.W., Strzepek, R., Fu, F. and Hutchins, D.A., 2010. Environmental control of open-ocean phytoplankton groups: Now and in the future. *Limnol. Oceanogr.* 55, 1353-1376.
- Bresnan, E., Davidson, K., Gowen, R., Higman, W., Lawton, L., Lewis, J., Percy, L., McKinney, A., Milligan, S., Shammon, T. and Swan, S., 2007. Relating harmful phytoplankton to shellfish poisoning and human health. Workshop 15th and 16th October 2007 at Dunstaffnage Marine Laboratory, Oban, Scotland.
- Bresnan, E., Fraser, S. and Moffat, C., 2003. Monitoring programme for toxin producing phytoplankton in scottish coastal waters 1st April 2002 - 31st March 2003. Fisheries Research Services Contract Report. 14/03, 36.
- Brewer, P. and Riley, J., 1965. The automatic determination of nitrate in seawater. *Deep Sea Res.* 12, 765-772.

- Brzezinski, M.A., Olson, R.J. and Chisholm, S.W., 1990. Silicon availability and cell-cycle progression in marine diatoms. *Mar. Ecol. Prog. Ser.* 67, 83-96.
- Busse, L.B., Venrick, E.L., Antrobus, R., Miller, P.E., Vigilant, V., Silver, M.W., Mengelt, C., Mydlarz, L. and Prezelin, B.B., 2006. Domoic acid in phytoplankton and fish in San Diego, CA, USA. *Harmful Algae*. 5, 91-101.
- Campbell, A., McKinnon, K., Whitehead, R., Skrabal, S., Keiber, R. and Wright, J., 2005. Transformation and photodegradation of domoic acid in seawater. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R. and Vargo, G.A. (Eds.), *Harmful Algae 2002*. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 150-152
- Campbell, D., Kelly, M., Busman, M., Bolch, C., Wiggins, E., Moeller, P., Morton, S., Hess, P. and Shumway, S., 2001. Amnesic shellfish poisoning in the king scallop, *Pecten maximus*, from the west coast of Scotland. *J. Shellfish Res.* 20, 75-84.
- Campbell, D.A., Kelly, M.S., Busman, M., Wiggins, E. and Fernandes, T.F., 2003. Impact of preparation method on gonad domoic acid levels in the scallop, *Pecten maximus* (L.). *Harmful Algae*. 2, 215-222.
- Casteleyn, G., Adams, N.G., Vanormelingen, P., Debeer, A., Sabbe, K. and Vyverman, W., 2009. Natural hybrids in the marine diatom *Pseudo-nitzschia pungens* (Bacillariophyceae): Genetic and morphological evidence. *Protist*. 160, 343-354.

- Casteleyn, G., Leliaert, F., Backeljau, T., Debeer, A.-E., Kotaki, Y., Rhodes, L., Lundholm, N., Sabbe, K. and Vyverman, W., 2010. Limits to gene flow in a cosmopolitan marine planktonic diatom. PNAS. 107, 12952-12957.
- Cembella, A.D., Antia, N.J., Harrison, P.J. and Rhee, G.Y., 1984. The utilization of inorganic and organic phosphorous compounds as nutrients by eukaryotic microalgae: A multidisciplinary perspective: Part 2. Crit. Rev. Microbiol. 11, 13-81.
- Churro, C.I., Carreira, C.C., Rodrigues, F.J., Craveiro, S.C., Calado, A.J., Casteleyn, G. and Lundholm, N., 2009. Diversity and abundance of potentially toxic *Pseudo-nitzschia* peragallo in Aveiro Coastal Lagoon, Portugal and description of a new variety, *P. pungens* var. *aveirensis* var. nov. Diatom Res. 24, 35-62.
- Ciminiello, P., Dell'Aversano, C., Fattorusso, E., Forino, M., Magno, G.S., Tartaglione, L., Quilliam, M.A., Tubaro, A. and Poletti, R., 2005. Hydrophilic interaction liquid chromatography/mass spectrometry for determination of domoic acid in Adriatic shellfish. Rapid Commun. Mass Spectrom. 19, 2030-2038.
- Clarke, K.R. and Ainsworth, M., 1993. A method of linking multivariate community structure to environmental variables. Mar. Ecol. Prog. Ser. 92, 205-219.
- Clarke, K.R. and Gorley, R., 2006. PRIMER v6: User manual/Tutorial. PRIMER-E, Plymouth.



- Clayden, J., Read, B. and Hebditch, K.R., 2005. Chemistry of domoic acid, isodomoic acids, and their analogues. *Tetrahedron*. 61, 5713-5724.
- Cochlan, W.P., Herndon, J. and Kudela, R.M., 2008. Inorganic and organic nitrogen uptake by the toxigenic diatom *Pseudo-nitzschia australis* (Bacillariophyceae). *Harmful Algae*. 8, 111-118.
- Costa, P.R., Rosa, R., Duarte-Silva, A., Brotas, V. and Sampayo, M.A.M., 2005. Accumulation, transformation and tissue distribution of domoic acid, the amnesic shellfish poisoning toxin, in the common cuttlefish, *Sepia officinalis*. *Aquat. Toxicol.* 74, 82-91.
- Croteau, R., Kuchan, T. and Lewis, N., 2000. Natural Products (Secondary Metabolites). In: Buchanan, B., Gruissem, W. and Jones, R. (Eds.), *Biochemistry and Molecular Biology of Plants*. American Society of Plant Physiologist, pp. 1250-1251
- Cusack, C., Bates, S.S., Quilliam, M.A., Patching, J. and Raine, R., 2002. Confirmation of domoic acid production by *Pseudo-nitzschia australis* (Bacillariophyceae) isolated from Irish waters. *J. Phycol.* 38, 1106-1112.
- D'Alelio, D., Ribera d'Alcala, M., Dubroca, L., Sarno, D., Zingone, A. and Montresor, M., 2010. The time for sex: A biennial life cycle in a marine planktonic diatom. *Limnol. Oceanogr.* 55, 106-114.

- Daugbjerg, N. and Andersen, R.A., 1997. A molecular phylogeny of the heterokont algae based on analyses of chloroplast-encoded *rbcL* sequence data. *J Phycol.* 33, 1031-1041.
- Davidovich, N.A. and Bates, S.S., 1998. Sexual reproduction in the pennate diatoms *Pseudo-nitzschia multiseriata* and *P.pseudodelicatissima* (Bacillariophyceae). *J. Phycology.* 34, 126-137.
- Davidson, K., Gowen, R.J., Tett, P., Bresnan, E., Harrison, P.J., McKinney, A., Milligan, S., Mills, D.K., Silke, J. and Crooks, A.-M., 2012. Harmful algal blooms: How strong is the evidence that nutrient ratios and forms influence their occurrence? *Estuar. Coast. Shelf S.* 115, 399-413.
- De la Iglesia, P., Giménez, G. and Diogène, J., 2008. Determination of dissolved domoic acid in seawater with reversed-phase extraction disks and rapid resolution liquid chromatography tandem mass spectrometry with head-column trapping. *J. Chromatogr. A.* 1215, 116-124.
- Doucette, G.J., King, K.L., Thessen, A.E. and Dortch, Q., 2008. The effect of salinity on domoic acid production by the diatom *Pseudo-nitzschia multiseriata*. *Nova Hedwigia.* 31-46.
- Doucette, G.J., Mikulski, C.M., Jones, K.L., King, K.L., Greenfield, D.I., Marin Iii, R., Jensen, S., Roman, B., Elliott, C.T. and Scholin, C.A., 2009. Remote, subsurface detection of the algal toxin domoic acid onboard the Environmental Sample Processor: Assay development and field trials. *Harmful Algae.* 8, 880-888.

- Douglas, D.J., Bates, S.S., Bourque, L.A. and Selvin, R.C., 1993. Domoic acid production by axenic and non-axenic cultures of the pennate diatom *Nitzschia pungens* f. *multiseriis*. Toxic phytoplankton blooms in the sea. 595-600.
- Douglas, D.J., Ramsey, U.P., Walter, J.A. and Wright, J.L.C., 1992. Biosynthesis of the neurotoxin domoic acid by the marine diatom *Nitzschia pungens* forma *multiseriis*, determined with [13C]-labelled precursors and nuclear magnetic resonance. J. Chem. Soc. Chem. Commun. 714-716.
- Douglas, M.P. and Rogers, S.O., 1998. DNA damage caused by common cytological fixatives. Mut. Res. 401, 77-88.
- Downes-Tettmar, N., Rowland, S., Widdicombe, C., Woodward, E.M.S. and Llewellyn, C.A., 2013. Seasonal variation in *Pseudo-nitzschia* spp. and domoic acid in the western English Channel. Cont. Shelf Res. 53, 40-59.
- Ehrman, J.M. and Kaczmarek, I., 2004. A virtual instrument metaphor for object identification: An example application using toxic diatoms. Limnol. Oceanogr. Bulletin. 13, 1-4.
- El-Sabaawi, R. and Harrison, P., 2006. Interactive effects of irradiance and temperature on the photosynthetic physiology of the pennate diatom *Pseudo-nitzschia granii* (Bacillariophyceae) from the northwest subarctic pacific. J. Phycol. 42, 778-785.

- Evans, K.M., Wortley, A.H. and Mann, D.G., 2007. An assessment of potential diatom "barcode" genes (cox1, rbcL, 18S and ITS rDNA) and their effectiveness in determining relationships in *Sellaphora* (Bacillariophyta). *Protist*. 158, 349-364.
- Feely, R.A., Sabine, C.L., Hernandez-Ayon, J.M., Ianson, D. and Hales, B., 2008. Evidence for Upwelling of Corrosive "Acidified" Water onto the Continental Shelf. *Science*. 320, 1490-1492.
- Fehling, J., Davidson, K. and Bates, S.S., 2005. Growth dynamics of non-toxic *Pseudo-nitzschia delicatissima* and toxic *P. seriata* (Bacillariophyceae) under simulated spring and summer photoperiods. *Harmful Algae*. 4, 763-769.
- Fehling, J., Davidson, K., Bolch, C.J.S. and Bates, S.S., 2004a. Growth and domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) under phosphate and silicate limitation. *J. Phycol.* 40, 674-683.
- Fehling, J., Davidson, K., Bolch, C.J.S., Brand, T.D. and Narayanaswamy, B.E., 2012. The relationship between phytoplankton distribution and water column characteristics in North West European shelf sea waters. *PLoS ONE*. 7, 1-16.
- Fehling, J., Davidson, K., Bolch, C.J.S. and Tett, P., 2006. Seasonality of *Pseudo-nitzschia* spp. (Bacillariophyceae) in Western Scottish waters. *Mar. Ecol. Prog. Ser.* 323, 91-105.

- Fehling, J., Green, D.H., Davidson, K., Bolch, C.J.S. and Bates, S.S., 2004b. Domoic acid production by *Pseudo-nitzschia seriata* (Bacillariophyceae) in Scottish waters. J. Phycol. 40, 622-630.
- Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evol. 39, 783-791.
- Flynn, K.J., 2010. Do external resource ratios matter? Implications for modelling eutrophication events and controlling harmful algal blooms. J Mar. Syst. 83, 170-180.
- Fritz, L., Quilliam, M.A., Wright, J.L.C., Beale, A.M. and Work, T.M., 1992. An outbreak of domoic acid poisoning attributed to the pennate diatom *Pseudo-nitzschia australis*. J. Phycol. 28, 439-442.
- Furey, A., Lehane, M., Gillman, M., Fernandez-Puente, P. and James, K.J., 2001. Determination of domoic acid in shellfish by liquid chromatography with electrospray ionization and multiple tandem mass spectrometry. J. Chromatogr. A. 938, 167-174.
- Garet, E., González-Fernández, Á., Lago, J., Vieites, J.M. and Cabado, A.G., 2010. Comparative Evaluation of Enzyme-Linked Immunoassay and Reference Methods for the Detection of Shellfish Hydrophilic Toxins in Several Presentations of Seafood. Journal of Agricultural and Food Chemistry. 58, 1410-1415.

- Garrison, D.L., Conrad, S.M., Eilers, P.P. and Waldron, E., 1992. Confirmation of domoic acid production by *Pseudo-nitzschia australis* (Bacillariophyceae) cultures. J. Phycol. 28, 604-607.
- Garthwaite, I., 2000. Keeping shellfish safe to eat: a brief review of shellfish toxins, and methods for their detection. Trends in Food Science & Technology. 11, 235-244.
- Garthwaite, I., Ross, K.M., Miles, C.O., Hansen, R.P., Foster, D., Wilkins, A.L. and Towers, N.R., 1998. Polyclonal antibodies to domoic acid, and their use in immunoassays for domoic acid in sea water and shellfish. Nat. Toxins. 6, 93-104.
- Geider, R.J. and La Roche, J., 2002. Redfield revisited: Variability of C:N:P in marine microalgae and its biochemical basis. Eur. J. Phycol. 37, 1-17.
- Godhe, A., Anderson, D.M. and Rehnstam-Holm, A.-S., 2002. PCR amplification of microalgal DNA for sequencing and species identification: Studies on fixatives and algal growth stages. Harmful Algae. 1, 375-382.
- Goldstein, T., Mazet, J.A.K., Zabka, T.S., Langlois, G., Colegrove, K.M., Silver, M., Bargu, S., Van Dolah, F., Leighfield, T., Conrad, P.A., Barakos, J., Williams, D.C., Dennison, S., Haulena, M. and Gulland, F.M.D., 2008. Novel symptomatology and changing epidemiology of domoic acid toxicosis in California sea lions (*Zalophus californianus*): an increasing risk to marine mammal health. Proceedings of the Royal Society B: Biological Sciences. 275, 267-276.

Grasshoff, K., 1976. Methods of seawater analysis. In: chemie, V. (Eds.), Weiheim, pp. pp. 317

Guillard, R., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W. and Chanley, M. (Eds.), Culture of Marine Invertebrate Animals. Plenum Publishing Co., New York, pp. 29-60

Gulland, E.M.D., Haulena, M., Fauquier, D., Lander, M.E., Zabka, T., Duerr, R. and Langlois, G., 2002. Domoic acid toxicity in Californian sea lions (*Zalophus californianus*): clinical signs, treatment and survival. Veterinary Record. 150, 475-480.

Haché, S., 2000. Determination of a signal (pheromone) produced by the toxic diatom *Pseudo-nitzschia multiseriis* and having a role in sexual communication. Honours thesis, Université of Moncton, Moncton, NB, 29 pp

Hagström, J.A., Granéli, E., Maneiro, I., Barreiro, A., Petermann, A. and Svensen, C., 2007. Release and degradation of amnesic shellfish poison from decaying *Pseudo-nitzschia multiseriis* in presence of bacteria and organic matter. Harmful Algae. 6, 175-188.

Hagström, J.A., Granéli, E., Moreira, M.O.P. and Odebrecht, C., 2011. Domoic acid production and elemental composition of two *Pseudo-nitzschia multiseriis* strains, from the NW and SW Atlantic Ocean, growing in phosphorus- or nitrogen-limited chemostat cultures. J. Plankton Res. 33, 297-308.

- Hall, T.A., 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41, 95-98.
- Hallegraeff, G.M., 2003. Harmful algal blooms: a global review. In: Hallegraeff, G.M., Anderson, D. and Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO Publishing, France, pp. 32
- Hasle, G.R., 1965. *Nitzschia* and *Fragilariopsis* species studied in the light and electron microscopes. II. The group *Pseudonitzschia*. Ny Serie. 18, 1-45.
- Hasle, G.R., 1995. *Pseudo-nitzschia pungens* and *P. multiseries* (Bacillariophyceae): Nomenclatural history, morphology, and distribution. J. Phycol. 31, 428-435.
- Hasle, G.R., 2002. Are most of the domoic acid-producing species of the diatom genus *Pseudo-nitzschia* cosmopolites? Harmful Algae. 1, 137–146.
- Hasle, G.R. and Lundholm, N., 2005. *Pseudo-nitzschia seriata* f. *obtusa* (Bacillariophyceae) raised in rank based on morphological, phylogenetic and distributional data. Phycologia. 44, 608-619.
- Hasle, G.R. and Syvertsen, E.E., 1997. Marine Diatoms. In: Tomas, C.R. (Eds.), Identifying marine phytoplankton. Academic Press, San Diego, CA, pp. 5-385



- Hays, G.C., Richardson, A.J. and Robinson, C., 2005. Climate change and marine plankton. *Trends Ecol. Evol.* 20, 337-344.
- Heino, J., 2008. Influence of taxonomic resolution and data transformation on biotic matrix concordance and assemblage-environment relationships in stream macro-invertebrates. *Boreal Environ. Res.* 13, 359-369.
- Hiltz, M., Bates, S.S. and Kaczmarek, I., 2000. Effect of light: dark cycles and cell apical length on the sexual reproduction of the pennate diatom *Pseudo-nitzschia multiseries* (Bacillariophyceae) in culture. *Phycologia*. 39, 59-66.
- Hinder, S., Hays, G., Brooks, C., Davies, A., Edwards, M., Walne, A. and Gravenor, M., 2011. Toxic marine microalgae and shellfish poisoning in the British Isles: history, review of epidemiology, and future implications. *Environ. Health Persp.* 10, 54.
- Hinder, S.L., Hays, G.C., Edwards, M., Roberts, E.C., Walne, A.W. and Gravenor, M.B., 2012. Changes in marine dinoflagellate and diatom abundance under climate change. *Nature Clim. Change*. 2, 271-275.
- Holligan, P.M. and Harbour, D.S., 1977. The vertical distribution and succession of phytoplankton in the Western English Channel in 1975-1976. *J. Mar. Biol. Ass. UK*. 57, 1075-1093.
- Horner, R.A. and Postel, J.R., 1993. Toxic diatoms in western Washington waters (U.S. west coast). *Hydrobiologia*. 269-270, 197-205.

- Howard, M.D.A., Cochlan, W.P., Ladizinsky, N. and Kudela, R.M., 2007. Nitrogenous preference of toxigenic *Pseudo-nitzschia australis* (Bacillariophyceae) from field and laboratory experiments. *Harmful Algae*. 6, 206-217.
- Hubbard, K.A., Rocap, G. and Armbrust, E.V., 2008. Inter- and intraspecific community structure within the diatom genus *Pseudo-nitzschia* (Bacillariophyceae). *J. Phycol.* 44, 637-649.
- Huelsenbeck, J.P. and Ronquist, F., 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*. 17, 754-755.
- Hummert, C., Rühl, A., Reinhardt, K., Gerdt, G. and Luckas, B., 2002. Simultaneous analysis of different algal toxins by LC-MS. *Chromatographia*. 55, 673-680.
- Jackson, A.E., Ayer, S.W. and Laycock, M.V., 1992. The effect of salinity on growth and amino acid composition in the marine diatom *Nitzschia pungens*. *Can. J. Bot.* 70, 2198-2201.
- James, K.J., Gillman, M., Lehane, M. and Gago-Martinez, A., 2000. New fluorimetric method of liquid chromatography for the determination of the neurotoxin domoic acid in seafood and marine phytoplankton. *J. Chromatogr. A*. 871, 1-6.
- Kaczmarek, I., Martin, J.L., Ehrman, J.M. and LeGresley, M.M., 2007. *Pseudo-nitzschia* species population dynamics in the Quoddy Region, Bay of Fundy. *Harmful Algae*. 6, 861-874.

- Kawatsu, K., Hamano, Y. and Noguchi, T., 1999. Production and characterisation of a monoclonal antibody against domoic acid and its application to enzyme immunoassay. *Toxicon*. 37, 1579-1589.
- Keller, M.D., Bellows, W.K. and Guillard, R.R.L., 1988. Microwave treatment for sterilization of phytoplankton culture media. *J. Exp. Mar. Biol. Ecol.* 117, 279-283.
- Kirkwood, D., ICES/CM, 1989. Simultaneous determination of selected nutrients in sea water
- Klein, C., Claquin, P., Bouchart, V., Le Roy, B. and Véron, B., 2010. Dynamics of *Pseudo-nitzschia* spp. and domoic acid production in a macrotidal ecosystem of the Eastern English Channel (Normandy, France). *Harmful Algae*. 9, 218-226.
- Kleivdal, H., Kristiansen, S.I., Nilsen, M.V. and Briggs, L., 2007a. Single-laboratory validation of the biosense direct competitive enzyme linked immunosorbent assay (ELISA) for determination of domoic acid toxins in shellfish. *J. AOAC int.* 90, 1000-1010.
- Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., Goksøyr, A., Briggs, L., Holland, P. and McNabb, P., 2007b. Determination of domoic acid toxins in shellfish by biosense ASP ELISA - a direct competitive enzyme-linked immunosorbent assay: collaborative study. *J. AOAC Int.* 90, 1011-27.

- Kotaki, Y., Furio, E.F., Satake, M., Lundholm, N., Katayama, T., Koike, K., Fulgueras, V.P., Bajarias, F.A., Takata, Y., Kobayashi, K., Sato, S., Fukuyo, Y. and Kodama, M., 2005. Production of isodomoic acids A and B as major toxin components of a pennate diatom *Nitzschia navis-varingica*. *Toxicon*. 46, 946-953.
- Kreuder, C., Miller, M., Jessup, D., Lowenstine, L., Harris, M., Ames, J., Carpenter, T., Conrad, P. and Mazet, J., 2003. Patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998-2001. *J. Wildlife Diseases*. 39, 495-509.
- Kudela, R.M., Roberts, A. and Armstrong, M., 2004. Laboratory analysis of nutrient stress and toxin production in *Pseudo-nitzschia* spp. from Monterey bay, California. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R. and Vargo, G.A. (Eds.), *Harmful Algal Blooms*. Florida and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, Paris, pp. 136-138
- Lanave, C., Preparata, G., Saccone, C. and Serio, G., 1984. A new method for calculating evolutionary substitution rates. *J. Mol. Evol.* 20, 86-93.
- Lane, J.Q., Raimondi, P. and Kudela, R.M., 2009. Development of a logistic regression model for the prediction of toxigenic *Pseudo-nitzschia* blooms in Monterey Bay, California. *Mar. Ecol. Prog. Ser.* 383, 37-51.
- Lane, J.S., Roddam, M., Langlois, G.W. and Kudela, R.M., 2010. Application of Solid Phase Adsorption Toxin Tracking (SPATT) for field detection of the hydrophilic

phycotoxins domoic acid and saxitoxin in coastal California Limnol. Oceanogr. Methods. 8, 645-660.

Lawrence, J.F., Charbonneau, C.F. and Menard, C., 1991. Liquid chromatographic determination of domoic acid in mussels, using AOAC paralytic shellfish poison procedure: collaborative study. J. Assoc. Off. Anal. Chem. 74, 68-72.

Lawrence, J.F., Charbonneau, C.F., Menard, C., Quilliam, M.A. and Sim, P.G., 1989. Liquid chromatographic determination of domoic acid in shellfish products using the AOAC paralytic shellfish poison extraction procedure. J. Chromatogr. A. 462, 349-356.

Lawrence, J.F., Lau, B.P.Y., Cleroux, C. and Lewis, D., 1994. Comparison of UV absorption and electrospray mass spectrometry for the high-performance liquid chromatographic determination of domoic acid in shellfish and biological samples. J. Chromat. A. 659, 119-126.

Laycock, M., de Freitas, A. and Wright, J., 1989. Glutamate agonists from marine algae. J. Applied Phycol. 1, 113-122.

Leahey, R.J.G., Burkill, P.H. and Sleight, M.A., 1994. A comparison of fixatives for the estimation of abundance and biovolume of marine planktonic ciliate populations. J. Plankton Res. 16, 375-389.

Lefebvre, K.A., Bargu, S., Kieckhefer, T. and Silver, M.W., 2002a. From sanddabs to blue whales: the pervasiveness of domoic acid. Toxicon. 40, 971-977.

- Lefebvre, K.L., Silver, M.S., Coale, S.C. and Tjeerdema, R.T., 2002b. Domoic acid in planktivorous fish in relation to toxic *Pseudo-nitzschia* cell densities. Mar. Biol. 140, 625-631.
- Lelong, A., Hégaret, H., Soudant, P. and Bates, S.S., 2012. *Pseudo-nitzschia* (Bacillariophyceae) species, domoic acid and amnesic shellfish poisoning: revisiting previous paradigms. Phycologia. 51, 168-216.
- Lewis, N.I., Bates, S.S., McLachlan, J.L. and Smith, J.C., 1993. Temperature effect on growth, domoic acid production, and morphology of the diatom *Nitzschia pungens* f. *multiseries*. In: Smayda, T.J. and Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, Amsterdam, pp. 601-606
- Lincoln, J.A., Turner, J.T., Bates, S.S., Léger, C. and Gauthier, D.A., 2001. Feeding, egg production, and egg hatching success of the copepods *Acartia tonsa* and *Temora longicornis* on diets of the toxic diatom *Pseudo-nitzschia multiseries* and the non-toxic diatom *Pseudo-nitzschia pungens*. Hydrobiologia. 453-454, 107-120.
- Lindstrom, S.C. and Fredericq, S., 2003. rbcL gene sequences reveal relationships among north-east Pacific species of *Porphyra* (Bangiales, Rhodophyta) and a new species, *P. aestivalis*. Phycological Res. 51, 211-211.
- Litaker, R.W., Stewart, T.N., Eberhart, B.-T.L., Wekell, J.C., Trainer, V.L., Kudela, R.M., Miller, P.E., Roberts, A., Hertz, C., Johnson, T.A., Frankfurter, G., Smith,

G.J., Schnetzer, A., Schumacker, J., Bastian, J.L., Odell, A., Gentien, P., Le Gal, D., Hardison, D.R. and Tester, P.A., 2008. Rapid Enzyme-linked Immunosorbent Assay for Detection of the Algal Toxin Domoic Acid. J. Shellfish Res. 27, 1301-1310.

Llewellyn, C.A., Fishwick, J.R. and Blackford, J.C., 2005. Phytoplankton community assemblage in the English Channel: a comparison using chlorophyll a derived from HPLC-CHEMTAX and carbon derived from microscopy cell counts. J. Plankton Res. 27, 103-119.

Lombardi, A.T. and Wangersky, P.J., 1991. Influence of phosphorus and silicon on lipid class production by the marine diatom *Chaetoceros gracilis* grown in turbidostat cage cultures. Mar. Ecol. Prog. Ser. 77, 39-47.

López-García, P., Rodríguez-Valera, F., Pedrós-Alió, C. and Moreira, D., 2001. Unexpected diversity of small eukaryotes in deep-sea Antarctic plankton. Nature. 409, 603-607.

Loureiro, S., Garcés, E., Fernandez-Tejedor, M., Vaqué, D. and Camp, J., 2009a. *Pseudo-nitzschia* spp. (Bacillariophyceae) and dissolved organic matter (DOM) dynamics in the Ebro Delta (Alfacs Bay, NW Mediterranean Sea). Estuar. Coast. Shelf S. 83, 539-549.

Loureiro, S., Jauzein, C., Garcés, E., Collos, Y., Camp, J. and Vaqué, D., 2009b. The significance of organic nutrients in the nutrition of *Pseudo-nitzschia delicatissima* (Bacillariophyceae). J. Plankton Res. 31, 399-410.

- Lundholm, N., Bates, S.S., Baugh, K.A., Bill, B.D., Connell, L.B., Léger, C. and Trainer, V.L., 2012. Cryptic and pseudo-cryptic diversity in diatoms-with descriptions of *Pseudo-nitzschia hasleana* sp. nov. and *P. fryxelliana* sp. nov. J. Phycol. 48, 436-454.
- Lundholm, N., Clarke, A. and Ellegaard, M., 2010. A 100-year record of changing *Pseudo-nitzschia* species in a sill-fjord in Denmark related to nitrogen loading and temperature. Harmful Algae. 9, 449-457.
- Lundholm, N., Hansen, J.P. and Kotaki, Y., 2004. Effect of pH on growth and domoic acid production by potentially toxic diatoms of the genera *Pseudo-nitzschia* and *Nitzschia*. Mar. Ecol. Prog. Ser. 273, 1-15.
- Lundholm, N., Hansen, P. and Kotaki, Y., 2005. Lack of allelopathic effects of the domoic acid-producing marine diatom *Pseudo-nitzschia multiseries*. Mar. Ecol. Prog. Ser. 288, 21-33.
- Lundholm, N., Moestrup, Ø., Hasle, G.R. and Hoef-Emden, K., 2003. A study of the *Pseudo-nitzschia pseudodelicatissima/cuspidata* complex (Bacillariophyceae): What is *P. pseudodelicatissima*? J. Phycol. 39, 797-813.
- Lundholm, N., Moestrup, Ø., Kotaki, Y., Hoef-Emden, K., Scholin, C. and Miller, P., 2006. Inter- and intraspecific variation of the *Pseudo-nitzschia delicatissima* complex (Bacillariophyceae) illustrated by rRNA probes, morphological data and phylogenetic analyses. J. Phycol. 42, 464-481.



- Lundholm, N., Skov, J., Pocklington, R. and Moestrup, Ø., 1994. Domoic acid, the toxic amino acid responsible for amnesic shellfish poisoning, now in *Pseudo-nitzschia seriata* (Bacillariophyceae) in Europe. *Phycologia*. 33, 475-478.
- MacGillivray, M.L. and Kaczmarek, I., 2011. Survey of the efficacy of a short fragment of the *rbcL* gene as a supplemental DNA barcode for diatoms. *J Eukaryot. Microbiol.* 58, 529-536.
- Macintyre, H.L., Stutes, A.L., Smith, W.L., Dorsey, C.P., Abraham, A. and Dickey, R.W., 2011. Environmental correlates of community composition and toxicity during a bloom of *Pseudo-nitzschia* spp. in the Northern Gulf of Mexico. *J. Plankton Res.* 33, 273-295.
- MacKenzie, L.A., 2010. In situ passive solid-phase adsorption of micro-algal biotoxins as a monitoring tool. *Current Opinion in Biotechnology*. 21, 326-331.
- Mafra, L.L., Léger, C., Bates, S.S. and Quilliam, M.A., 2009. Analysis of trace levels of domoic acid in seawater and plankton by liquid chromatography without derivatization, using UV or mass spectrometry detection. *J. Chromatogr. A*. 1216, 6003-6011.
- Maldonado, M.T., Hughes, M.P., Rue, E.L. and Wells, M.L., 2002. The Effect of Fe and Cu on Growth and Domoic Acid Production by *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia australis*. *Limnol. Oceanogr.* 47, 515-526.

- Manhart, J.R., Fryxell, G.A., Villac, M.C. and Segura, L.Y., 1995. *Pseudo-nitzschia pungens* and *P. multiseriis* (Bacillariophyceae): Nuclear ribosomal DNAs and species differences. J. Phycol. 31, 421-427.
- Mantoura, R.F.C. and Woodward, E.M.S., 1983. Optimization of the indophenol blue method for the automated determination of ammonia in estuarine waters. Estuar. Coast. Shelf S. 17, 219-224.
- Marchetti, A., Maldonado, M.T., Lane, E.S. and Harrison, P.J., 2006. Iron requirements of the pennate diatom *Pseudo-nitzschia*: Comparison of oceanic (high-nitrate, low chlorophyll waters) and coastal species Limnol. Oceanogr. 51, 2092-2101.
- Marín, I., Aguilera, A., Reguera, B. and Abad, J.P., 2001. Preparation of DNA suitable for PCR amplification from fresh or fixed single dinoflagellate cells. Biotechniques. 30, 88-93.
- Maroulis, M., Monemvasios, I., Vardaka, E. and Rigas, P., 2008. Determination of domoic acid in mussels by HPLC with post-column derivatization using 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) and fluorescence detection. J. Chromat. B. 876, 245-251.
- Marshall, H.G., Burchardt, L. and Lacouture, R., 2005. A review of phytoplankton composition within Chesapeake Bay and its tidal estuaries. J. Plankton Res. 27, 1083-1102.

- Martin, J.L., Hanke, A.R. and LeGresley, M.M., 2009. Long term phytoplankton monitoring, including harmful algal blooms, in the Bay of Fundy, Eastern Canada. *J. Sea Res.* 61, 76-83.
- Maucher, J.M. and Ramsdell, J.S., 2005. Ultrasensitive detection of domoic acid in mouse blood by competitive ELISA using blood collection cards. *Toxicon.* 45, 607-613.
- McCarron, P. and Hess, P., 2006. Tissue distribution and effects of heat treatments on the content of domoic acid in blue mussels, *Mytilus edulis*. *Toxicon.* 47, 473-479.
- McDonald, S.M., Sarno, D. and Zingone, A., 2007. Identifying *Pseudo-nitzschia* species in natural samples using genus-specific PCR primers and clone libraries. *Harmful Algae.* 6, 849-860.
- McKay, R., Geider, R.J. and La Roche, J., 1997. Physiological and biochemical response of the photosynthetic apparatus of two marine diatoms to nutrient stress. *Plant Physiol.* 114, 615-622.
- Medlin, L.K. and Kooistra, W.H.C.F., 2010. Methods to estimate the diversity in the marine photosynthetic protist community with illustrations from case studies: A review. *Diversity.* 2, 973-1014.

- Menden-Deuer, S., Lessard, E.J. and Satterberg, J., 2001. Effect of preservation on dinoflagellate and diatom cell volume and consequences for carbon biomass predictions. *Mar. Ecol. Prog. Ser.* 222, 41-50.
- Miguez, A., Fernandez, M. and Fraga, S., 1996. First detection of domoic acid in Galicia (NW of Spain) In: Yasumoto, T. (Eds.), *Intergovernmental Oceanographic Commission of UNESCO*. Paris, pp. 143-145
- Miller, P.E. and Scholin, C.A., 1998. Identification and enumeration of cultured and wild *Pseudo-nitzschia* (Bacillariophyceae) using species-specific LSU rRNA-targeted fluorescent probes and filter-based whole cell hybridization. *J. Phycol.* 34, 371-382.
- Moncheva, S. and Parr, B., 2010. *Manual for phytoplankton sampling and analysis in the Black sea*. 68 pp.
- Mos, L., 2001. Domoic acid: a fascinating marine toxin. *Environ. Toxicol. Pharmacol.* 9, 79-85.
- Moschandreou, K.K., Papaefthimiou, D., Katikou, P., Kalopesa, E., Panou, A. and Nikolaidis, G., 2010. Morphology, phylogeny and toxin analysis of *Pseudo-nitzschia pseudodelicatissima* (Bacillariophyceae) isolated from the Thermaikos Gulf, Greece. *Phycologia*. 49, 260-273.
- Nei, M. and Kumar, S., 2000. *Molecular Evolution and Phylogenetics*. In: (Eds.), Oxford University Press, New York, pp. 333

- Newsome, H., Truelove, J., Hierlithy, L. and Collins, P., 1994. Determination of domoic acid in serum and urine by immunological analysis. *Bulletin Environ. Contam. Toxicol.* 47,
- Nguyen, A.-L., Luong, J.H.T. and Masson, C., 1990. Capillary Electrophoresis for Detection and Quantitation of Domoic Acid in Mussels. *Analytical Letters.* 23, 1621-1634.
- Olson, M.B., Lessard, E.J., Cochlan, W.P. and Trainer, V.L., 2008. Intrinsic growth and microzooplankton grazing on toxigenic *Pseudo-nitzschia* spp. diatoms from the coastal northeast Pacific. *Limnol. Oceanogr.* 53, 1352-1368.
- Orive, E., Laza-Martinez, A., Seoane, S., Alonso, A., Andrade, R. and Miguel, I., 2010. Diversity of *Pseudo-nitzschia* in the Southeastern Bay of Biscay. *Diatom Res.* 25, 125-145.
- Orsini, L., Procaccini, G., Sarno, D. and Montresor, M., 2004. Multiple rDNA ITS-types within the diatom *Pseudo-nitzschia delicatissima* (Bacillariophyceae) and their relative abundances across a spring bloom in the Gulf of Naples. *Mar. Ecol. Prog. Ser.* 271, 87-98.
- Orsini, L., Sarno, D., Procaccini, G., Poletti, R., Dahlmann, J. and Montresor, M., 2002. Toxic *Pseudo-nitzschia multistriata* (Bacillariophyceae) from the Gulf of Naples: morphology, toxin analysis and phylogenetic relationships with other *Pseudo-nitzschia* species. *European J. Phycol.* 37, 247-257.

- Osada, M., Marks, L.J. and Stewart, J.E., 1995. Determination of domoic acid by two different versions of a competitive enzyme-linked immunosorbent assay (ELISA). *Bulletin Environ. Contam. Toxicol.* 54, 797-804.
- Osada, M. and Stewart, J.E., 1997. Gluconic acid gluconolactone: Physiological influences on domoic acid production by bacteria associated with *Pseudo-nitzschia multiseries*. *Aquat. Microb. Ecol.* 12, 203-209.
- Palma, S., Mouriño, H., Silva, A., Barão, M.I. and Moita, M.T., 2010. Can *Pseudo-nitzschia* blooms be modeled by coastal upwelling in Lisbon Bay? *Harmful Algae*. 9, 294-303.
- Pan, Y., Bates, S.S. and Cembella, A.D., 1998. Environmental stress and domoic acid production by *Pseudo-nitzschia*: A physiological perspective. *Nat. Toxins*. 6, 127-135.
- Pan, Y., Subba Rao, D.V. and Mann, K.H., 1996a. Changes in domoic acid production and cellular chemical composition of the toxigenic diatom *Pseudo-nitzschia multiseries* under phosphate limitation. *J. Phycol.* 32, 371-381.
- Pan, Y., Subba Rao, D.V., Mann, K.H., Brown, R.G. and Pocklington, R., 1996b. Effects of silicate limitation on production of domoic acid, a neurotoxin, by the diatom *Pseudo-nitzschia multiseries*. I. Batch culture studies. *Mar. Ecol. Prog. Ser.* 131, 225-233.

- Pan, Y.L., Parsons, M.L., Busman, M., Moeller, P.D.R., Dortch, Q., Powell, C.L. and Doucette, G.J., 2001. *Pseudo-nitzschia* sp. cf. *pseudodelicatissima*- a confirmed producer of domoic acid from the northern gulf of mexico. Mar. Ecol. Prog. Ser. 220, 83-92.
- Pan, Y.L., Rao, D.V.S. and Mann, K.H., 1996c. Acclimation to low light intensity in photosynthesis and growth of *Pseudo-nitzschia multiseries* Hasle, a neurotoxic diatom. J. Plankton Res. 18, 1427-1438.
- Parsons, M.L., Scholin, C.A., Miller, P.E., Doucette, G.J., Powell, C.L., Fryxell, G.A., Dortch, Q. and Soniat, T.M., 1999. *Pseudo-nitzschia species* (Bacillariophyceae) in louisiana coastal waters: Molecular probe field trials, genetic variability, and domoic acid analyses. J. Phycology. 35, 1368-1378.
- Penna, A., Bertozzini, E., Battocchi, C., Galluzzi, L., Giacobbe, M.G., Vila, M., Garces, E., Lugliè, A. and Magnani, M., 2007. Monitoring of HAB species in the Mediterranean Sea through molecular methods. J. Plankton Res. 29, 19-38.
- Piñeiro, N., Leão, J.M., Gago Martínez, A. and Rodríguez Vázquez, J.A., 1999. Capillary electrophoresis with diode array detection as an alternative analytical method for paralytic and amnesic shellfish toxins. J. Chromatogr. A. 847, 223-232.
- Pingree, R.D. and Griffiths, D.K., 1978. Tidal fronts on the shelf seas around the British Isles. J. Geophys. Res.-Oc Atm. 83, 4615-4622.

- Plumley, F.G., 1997. Marine algal toxins: Biochemistry, genetics, and molecular biology. *Limnol. Oceanogr.* 42, 1252-1264.
- Pocklington, R., Milley, J.E., Bates, S.S., Bird, C.J., De Freitas, A.S.W. and Quilliam, M.A., 1990. Trace determination of domoic acid in seawater and phytoplankton by high performance liquid chromatography of the fluorenylmethoxycarbonyl (FMOC) derivative. *Intern. J. Environ. Anal. Chem.* 38, 351-368.
- Quijano-Scheggia, S., Garcés, E., Andree, K.B., de la Iglesia, P., Diogène, J., Fortuño, J.-M. and Camp, J., 2010. *Pseudo-nitzschia* species on the Catalan coast: characterization and contribution to the current knowledge of the distribution of this genus in the Mediterranean Sea. *Scientia Marina*. 72, 395-410.
- Quijano-Scheggia, S., Garcés, E., Sampedro, N., van Lenning, K., Flo, E., Andree, K., Fortuño, J.-M. and Camp, J., 2008. Identification and characterisation of the dominant *Pseudo-nitzschia* species (Bacillariophyceae) along the NE Spanish coast (Catalonia, NW Mediterranean). *Sci. Mar.* 72, 343-359.
- Quijano-Scheggia, S.I., Garcés, E., Lundholm, N., Moestrup, Ø., Andree, K. and Camp, J., 2009. Morphology, physiology, molecular phylogeny and sexual compatibility of the cryptic *Pseudo-nitzschia delicatissima* complex (Bacillariophyta), including the description of *P. arenysensis* sp. nov. *Phycologia*. 48, 492-509.
- Quilliam, M.A., 2003. Chemical methods for domoic acid, the amnesic shellfish poisoning (ASP) toxin. In: Hallegraeff, G.M., Anderson, D.M. and Cembella,



A.D. (Eds.), Manual on harmful marine microalgae. Intergovernmental Oceanographic Commission (UNESCO), Paris, pp. 247

Quilliam, M.A., Thomas, K. and Wright, J.L.C., 1998. Analysis of domoic acid in shellfish by thin-layer chromatography. *Natural Toxins*. 6, 147-152.

Raine, R., Edwards, M., Reid, C., Bresnan, E. and Fernand, L., 2008. Harmful algal blooms (HABs). MCCIP Annual Report Card 2007-2008. 1-8.

Raven, J.A., 1998. Small is beautiful: the picophytoplankton. *Funct. Ecol.* 12, 503-513.

Rees, A.P., Hope, S.B., Widdicombe, C.E., Dixon, J.L., Woodward, E.M.S. and Fitzsimons, M.F., 2009. Alkaline phosphatase activity in the Western English Channel: Elevations induced by high summertime rainfall. *Estuar. Coast. Shelf S.* 81, 569-574.

Rhodes, L., Scholin, C. and Garthwaite, I., 1998. *Pseudo-nitzschia* in New Zealand and the role of DNA probes and immunoassays in refining marine biotoxin monitoring programmes. *Nat. Toxins*. 6, 105-111.

Rines, J.E.B., Donaghay, P.L., Dekshenieks, M.M., Sullivan, J.M. and Twardowski, M.S., 2002. Thin layers and camouflage: hidden *Pseudo-nitzschia* spp. (Bacillariophyceae) populations in a fjord in the San Juan Islands, Washington, USA. *Mar. Ecol. Prog. Ser.* 225, 123-137.

- Rodríguez, F., Oliver, J.L., Marín, A. and R, M.J., 1990. The general stochastic model of nucleotide substitution. *J. Theor. Biol.* 142, 485-501.
- Romero, M., Kotaki, Y., Relox, J., Lundholm, N., Takata, Y., Kodama, M. and Fukuyo, Y., 2012. Two new ASP toxin production types in strains of *Nitzschia navis-varingia* from the Philippines. *Coast. Mar. Sci.* 35, 67-69.
- Rue, E. and Bruland, K., 2001. Domoic acid binds iron and copper: a possible role for the toxin produced by the marine diatom *Pseudo-nitzschia*. *Mar. Chem.* 76, 127-134.
- Ryan, J., Greenfield, D., Marin, R., Preston, C., Roman, B., Jensen, S., Pargett, J., Birch, C., Mikulski, G., Doucette, G. and Scholin, C., 2011. Harmful phytoplankton ecology studies using an autonomous molecular analytical and ocean observing network. *Limnol. Oceanogr.* 56, 1255-1272.
- Sahraoui, I., Hlaili, A.S., Mabrouk, H.H., Léger, C. and Bates, S.S., 2009. Blooms of the diatom genus *Pseudo-nitzschia* H. Peragallo in Bizerte lagoon (Tunisia, SW Mediterranean). *Diatom Res.* 24, 175-190.
- Saitou, N. and Nei, M., 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4, 406-425.
- Sanger, F., Nicklen, S. and Coulson, A.R., 1977. DNA sequencing with chain-terminating inhibitors. *PNAS.* 74, 5463-5467.

- Schnetzler, A., Miller, P.E., Schaffner, R.A., Stauffer, B.A., Jones, B.H., Weisberg, S.B., DiGiacomo, P.M., Berelson, W.M. and Caron, D.A., 2007. Blooms of *Pseudo-nitzschia* and domoic acid in the San Pedro Channel and Los Angeles harbor areas of the Southern California Bight, 2003-2004. *Harmful Algae*. 6, 372-387.
- Schnetzler, A., Moorthi, S.D., Countway, P.D., Gast, R.J., Gilg, I.C. and Caron, D.A., 2011. Depth matters: Microbial eukaryote diversity and community structure in the eastern North Pacific revealed through environmental gene libraries. *Deep-Sea Res. PT I*. 58, 16-26.
- Scholin, C., Miller, P.E., Buck, K., Chavez, F.P., Harris, P., Haydock, P., Howard, J. and Cangelosi, G., 1997. Detection and quantification of *Pseudo-nitzschia australis* in cultured and natural populations using LSU rRNA-targeted probes. *Limnol. Oceanog.* 42, 1265-1272.
- Scholin, C.A., Gulland, F., Doucette, G.J., Benson, S., Busman, M., Chavez, F.P., Cordaro, J., DeLong, R., De Vogelaere, A., Harvey, J., Haulena, M., Lefebvre, K., Lipscomb, T., Loscutoff, S., Lowenstine, L.J., Marin, R., Miller, P.E., McLellan, W.A., Moeller, P.D.R., Powell, C.L., Rowles, T., Silvagni, P., Silver, M., Spraker, T., Trainer, V. and Van Dolah, F.M., 2000. Mortality of sea lions along the central California coast linked to a toxic diatom bloom. *Nature*. 403, 80-84.
- Scholin, C.A., Herzog, M., Sogin, M. and Anderson, D.M., 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium*

(Dinophyceae).II. sequence analysis of a fragment of the LSU rRNA gene. J. Phycol. 30, 999-1011.

Sekula-Wood, E., Schnetzer, A., Benitez-Nelson, C.R., Anderson, C., Berelson, W.M., Brzezinski, M.A., Burns, J.M., Caron, D.A., Cetinic, I., Ferry, J.L., Fitzpatrick, E., Jones, B.H., Miller, P.E., Morton, S.L., Schaffner, R.A., Siegel, D.A. and Thunell, R., 2009. Rapid downward transport of the neurotoxin domoic acid in coastal waters. *Nature Geosci.* 2, 272-275.

Sierra-Beltrán, A., Palafox-Urbe, M., Grajales-Montiel, J., Cruz-Villacorta, A. and Ochoa, J.L., 1997. Sea bird mortality at Cabo San Lucas, Mexico: Evidence that toxic diatom blooms are spreading. *Toxicon.* 35, 447-453.

Sierra-Beltrán, A.P., Cruz, A., Núñez, E., Del Villar, L.M., Cerecero, J. and Ochoa, J.L., 1998. An overview of the marine food poisoning in Mexico. *Toxicon.* 36, 1493-1502.

Skoog, D.A., Holler, F.J. and Crouch, S.R., 2007. Principles of Instrumental Analysis. In: (Eds.), Thomson Brooks/Cole, Belmont, CA,

Smayda, T.J., 2006. Harmful algal bloom communities in Scottish coastal waters: Relationship to fish farming and regional comparisons - A review Scottish Executive Environment Group Paper 2006/3. 219.

Smith, D.S. and Kitts, D.D., 1995. Enzyme immunoassay for the determination of domoic acid in mussel extracts. *J. Agric. Food Chem.* 43, 367-371.

- Smith, E.A., Papapanagiotou, E.P., Brown, N.A., Stobo, L.A., Gallacher, S. and Shanks, A.M., 2006. Effect of storage on amnesic shellfish poisoning (ASP) toxins in king scallops (*Pecten maximus*). Harmful Algae. 5, 9-19.
- Smyth, T.J., Fishwick, J.R., Al-Moosawi, L., Cummings, D., Harris, C., Kitidis, V., Rees, A.P., Martinez-Vicente, V. and Woodward, E.M.S., 2010. A broad spatio-temporal view of the Western English Channel observatory. J. Plankton Res. 32, 585-601.
- Sournia, A., Chrétiennot-Dinet, M.-J. and Ricard, M., 1991. Marine phytoplankton: How many species in the world ocean? J. Plankton Res. 13, 1093-1099.
- Southward, A.J., Langmead, O., Hardman-Mountford, N.J., Aiken, J., Boalch, G.T., Dando, P., Genner, M.J., Joint, I., Kendall, M., Halliday, N.C., Harris, R.P., Leaper, R., Mieszkowska, N., Pingree, R.D., Richardson, A.J., Sims, D.W., Smith, T., Walne, A.W. and Hawkins, S.J., 2005. Long-term oceanographic and ecological research in the Western English Channel. Adv. Mar. Biol. 47, 105.
- Srinivasan, M., Sedmak, D. and Jewell, S., 2002. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am. J. Pathol. 161, 1961-1971.
- Stewart, J.E., 2008. Bacterial involvement in determining domoic acid levels in *Pseudo-nitzschia multiseries* cultures. Aquat. Microb. Ecol. 50, 135-144.

- Stewart, J.E., Marks, L.J., Gilgan, M., Pfeiffer, E. and BM, Z., 1998. Microbial utilization of the neurotoxin domoic acid: blue mussels (*Mytilus edulis*) and soft shell clams (*Mya arenaria*) as sources of the microorganisms. Can. J. Microbiol. 44, 456-464.
- Stewart, J.E., Marks, L.J., Wood, C.R., Risser, S.M. and Gray, S., 1997. Symbiotic relations between bacteria and the domoic acid producing diatom *Pseudo-nitzschia multiseriata* and the capacity of these bacteria for gluconic acid/gluconolactone formation. Aquat. Microb. Ecol. 12, 211-221.
- Stubbs, B., Coates, L., Milligan, S., Morris, S., Higman, W. and Algoet, M., 2008. Biotxin monitoring programme for England and Wales-1st April 2007 to 31st March 2008. Shellfish News. 26, 35-39.
- Stubbs, B., Milligan, S. and Lees, D., 2005. The marine biotoxin monitoring programme for England and Wales: 2004 –2005. Shellfish News 19, 26-29.
- Stubbs, B., Milligan, S., Morris, S., Higman, W. and Algoet, M., 2007a. Biotxin monitoring programme for England and Wales 1st April 2005 to 31st May 2006. Shellfish News. 23, 48-51.
- Stubbs, B., Milligan, S., Morris, S., Higman, W. and Algoet, M., 2007b. Biotxin Monitoring Programme for England and Wales 1st June 2006 to 31st March 2007. Shellfish News. 24, 48-51.

- Subba Rao, D.V., Pan, Y. and Mukhida, K., 1998. Production of domoic Acid by *Pseudo-nitzschia multiseriis* Hasle, affected by lithium. Mar. Ecol. 19, 31-36.
- Sun, J., Hutchins, D.A., Feng, Y., Seuber, E.L., Caron, D.A. and Fu, F., 2011. Effects of changing pCO<sub>2</sub> and phosphate availability on domoic acid production and physiology of the marine harmful bloom diatom *Pseudo-nitzschia multiseriis*. Limnol. Oceanogr. 56, 829-840.
- Sun, T. and Wong, W.H., 1999. Determination of domoic acid in phytoplankton by high-performance liquid chromatography of the 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate derivative. J. Agric. Food Chem. 47, 4678-4681.
- Sunda, W.G. and Huntsman, S.A., 1995. Iron uptake and growth limitation in oceanic and coastal phytoplankton. Mar. Chem. 50, 189-206.
- Sunda, W.G., Price, N.M. and Morel, F.M.M., 2005. Trace metal ion buffers and their use in culture studies. In: Andersen, R.A. (Eds.), Algal Culturing Techniques. Academic Press, pp. 35-63
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S., 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731-2739.

- Tatters, A.O., Fu, F. and Hutchins, D.A., 2012. High CO<sub>2</sub> and silicate limitation synergistically increase the toxicity of *Pseudo-nitzschia fraudulenta*. PLoS ONE. 7, 1-7.
- Tengs, T., Bowers, H.A., Ziman, A.P., Stoecker, D.K. and Oldach, D.W., 2001. Genetic polymorphism in *Gymnodinium galatheanum* chloroplast DNA sequences and development of a molecular detection assay. Mol. Ecol. 10, 515-523.
- Ter Braak, C.J.F. and Šmilauer, P., 2002. CANOCO reference manual and CanoDraw for windows user's guide: software for canonical community ordination (version 4.5). Microcomputer Power, Ithaca, 500 pp. <http://www.canoco.com>.
- Thessen, A.E., Bowers, H.A. and Stoecker, D.K., 2009. Intra- and interspecies differences in growth and toxicity of *Pseudo-nitzschia* while using different nitrogen sources. Harmful Algae. 8, 792-810.
- Thessen, A.E., Dortch, Q., Parsons, M.L. and Morrison, W., 2005. Effect of salinity on *Pseudo-nitzschia* species (Bacillariophyceae) growth and distribution. J. Phycol. 41, 21-29.
- Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research. 22, 4673-4680.



- Trainer, V., Adams, N., Bill, B.D., Stehr, C., Wekell, J.C., Moeller, P., Busman, M. and Woodruff, D., 2000. Domoic acid production near California coastal upwelling zones, June 1998. *Limnol. Oceanogr.* 45, 1818-1833.
- Trainer, V., Wells, M.L., Cochlan, W.P., Trick, C.G., Bill, B.D., Baugh, K.A., Beall, B.F., Herndon, J. and Lundholm, N., 2009. An ecological study of a massive bloom of toxigenic *Pseudo-nitzschia cuspidata* off the Washington State coast. *Limnol. Oceanogr.* 54, 1461-1474.
- Trainer, V.L., Adams, N.G., Bill, B.D., Anulacion, B.F. and Wekell, J.C., 1998. Concentration and dispersal of a *Pseudo-nitzschia* bloom in Penn Cove, Washington, USA. *Nat. Toxins.* 6, 113-125.
- Trainer, V.L., Bates, S.S., Lundholm, N., Thessen, A.E., Cochlan, W.P., Adams, N.G. and Trick, C.G., 2012. *Pseudo-nitzschia* physiological ecology, phylogeny, toxicity, monitoring and impacts on ecosystem health. *Harmful Algae.* 14, 271-300.
- Turner, J.T. and Tester, P.A., 1997. Toxic marine phytoplankton, zooplankton grazers, and pelagic food webs. *Limnol. Oceanogr.* 42, 1203-1214.
- Uronen, P., 2007. Harmful algae in the planktonic food web of the Baltic Sea. *Monogr. Boreal Environ. Res.* 28, 44.
- Utermöhl, H., 1958. Zur vervollkommnung der quantitativen phytoplankton methodik. *Mitteil. Int. Verein. Theor. Angew. Limnol.* 9, 1-38.

- Vale, P. and Sampayo, M.A.M., 2001. Domoic acid in Portuguese shellfish and fish. *Toxicon*. 39, 893-904.
- Van Dolah, F.M., 2000. Marine algal toxins: Origins, health effects, and their increased occurrence. *Environ. Health Perspectives*. 108, 133-141.
- Vigilant, V. and Silver, M., 2007. Domoic acid in benthic flatfish on the continental shelf of Monterey Bay, California, USA. *Mar. Biol.* 151, 2053-2062.
- Villac, M.C. and Fryxell, G.A., 1998. *Pseudo-nitzschia pungens* var. *cingulata* var. nov. (Bacillariophyceae) based on field and culture observations. *Phycologia*. 37, 269-274.
- Vrieling, E.G., Koeman, R.P.T., Scholin, C.A., Scheerman, P., Peperzak, L., Veenhuis, M. and Gieskes, W.W.C., 1996. Identification of a domoic acid-producing *Pseudo-nitzschia* species (Bacillariophyceae) in the Dutch Wadden Sea with electron microscopy and molecular probes. *European J. Phycol.* 31, 333-340.
- Wang, Z., King, K.L., Ramsdell, J.S. and Doucette, G.J., 2007. Determination of domoic acid in seawater and phytoplankton by liquid chromatography-tandem mass spectrometry. *J. Chromatogr.A*. 1163, 169-176.
- Wang, Z., Maucher-Fuquay, J., Fire, S.E., Mikulski, C.M., Haynes, B., Doucette, G.J. and Ramsdell, J.S., 2012. Optimization of solid-phase extraction and liquid chromatography/ tandem mass spectrometry for the determination of domoic

acid in seawater, phytoplankton, and mammalian fluids and tissues. *Analytica Chimica Acta*. 715, 71-79.

Wekell, J.C., Gauglitz, E.J., Bamett, H.J., Hatfield, C.L., Simons, D. and Ayres, D., 1994. Occurrence of domoic acid in washington state razor clams (*Siliqua patula*) during 1991-1993. *Nat. Toxins*. 2, 197-205.

Wells, M.L., 2003. The level of iron enrichment required to initiate diatom blooms in HNLC. *Mar. Chem.* 82, 101-114.

Wells, M.L., Trick, C.G., Cochlan, W.P., Hughes, M.P. and Trainer, V.L., 2005. Domoic acid: The synergy of iron, copper, and the toxicity of diatoms. *Limnol. Oceanogr.* 50, 1908-1917.

White, T.J., Bruns, T., Lee, S. and Taylor, J., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: (Eds.), *PCR Protocols, A Guide to Methods and Applications*. Academic Press, pp. 315-322

Widdicombe, C.E., Eloire, D., Harbour, D., Harris, R.P. and Somerfield, P.J., 2010. Long-term phytoplankton community dynamics in the Western English Channel. *J. Plankton Res.* 32, 643-655.

Windust, A., 1992. The response of bacteria , microalgae and zooplankton to the diatom *Nitzschia pungens* forma multiseriis, and its toxic metabolite domoic acid. MSc thesis, Dalhousie University, Halifax, Nova Scotia, pp

- Woodward, E.M.S. and Rees, A.P., 2001. Nutrient distributions in an anticyclonic eddy in the Northeast Atlantic Ocean, with reference to nanomolar ammonium concentrations. *Deep Sea Res. II.* 48, 775-793.
- Work, T.M., Barr, B., Beale, A.M., Fritz, L., Quilliam, M.A. and Wright, J.L.C., 1993. Epidemiology of domoic acid poisoning in brown pelicans (*Pelecanus occidentalis*) and brandt cormorants (*Phalacrocorax penicillatus*) in California. *J. Zoo Wildlife Med.* 24, 54-62.
- Wright, J., Falk, M., McInnes, G. and JA, W., 1990. Identification of Isodomoic acid D and two new geometrical isomers of domoic acid in toxic mussels. *Can. J. Chem.* 68, 22-25.
- Wright, J.L.C., Boyd, R.K., Freitas, A.S.W.d., Falk, M., Foxall, R.A., Jamieson, W.D., Laycock, M.V., McCulloch, A.W., McInnes, A.G., Odense, P., Pathak, V.P., Quilliam, M.A., Ragan, M.A., Sim, P.G., Thibault, P., Walter, J.A., Gilgan, M., Richard, D.J.A. and Dewar, D., 1989. Identification of domoic acid, a neuroexcitatory amino acid, in toxic mussels from eastern Prince Edward Island. *Can. J. Chem.* 67, 481-490.
- Wyatt, N.J., Kitidis, V., Woodward, E.M.S., Rees, A.P., Widdicombe, S. and Lohan, M., 2010. Effects of high CO<sub>2</sub> on the fixed nitrogen inventory of the Western English Channel. *J. Plankton Res.* 32, 631-641.

- Zaman, L., Arakawa, O., Shimosu, A., Onoue, Y., Nishio, S., Shida, Y. and Noguchi, T., 1997. Two new isomers of domoic acid from a red alga, *Chondria armata*. *Toxicon*. 35, 205-212.
- Zang, J. and Chi, J., 2002. Automated analysis of nanomolar concentrations of phosphate in natural seawaters, with liquid waveguide. *Environ. Sci. Technol.* 36, 1048-1053.
- Zhao, J.-Y., Thibault, P. and Quilliam, M.A., 1997. Analysis of domoic acid isomers in seafood by capillary electrophoresis. *Electrophoresis*. 18, 268-276.
- Zhiming, Y. and Subba Rao, D., 1998. Impact of germanium on the growth of *Psuedonitzschia pungens f. multiseries* and the production of algal toxin. *Chinese Science Bulletin*. 43, 2091-2095.