ANTIBACTERIAL FREE FATTY ACIDS FROM THE MARINE DIATOM, PHAEODACTYLUM TRICORNUTUM

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Antibacterial free fatty acids from the marine diatom, *Phaeodactylum tricornutum*

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A thesis submitted for the degree of Doctor of Philosophy

University of St Andrews

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Abstract

The aim of this thesis was to isolate the compounds responsible for the antibacterial activity of cell extracts of the marine diatom, *Phaeodactylum tricornutum*. Marine microalgae are not only important primary producers but, due to their phylogenetic diversity, they are also a potential source of novel bioactive compounds. The marine diatom, *P. tricornutum*, was selected for study because its cell extracts are known to be antibacterial but the compounds responsible have not been isolated. In this thesis, the compounds responsible for the antibacterial activity are isolated from aqueous methanol *P. tricornutum* cell extracts by column chromatography and reverse phase high-performance liquid chromatography using a bioassay-guided approach. The compounds in three active fractions were identified by mass spectrometry and nuclear magnetic resonance spectroscopy as the unsaturated fatty acids (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid, (9*Z*)-hexadecenoic acid and (6*Z*, 9*Z*, 12*Z*)-hexadecatrienoic acid. The fatty acids were found to be antibacterial against *Staphylococcus aureus* at micromolar concentrations. *P. tricornutum* exists in different cell morphs and, interestingly, extracts prepared from cultures in the fusiform morph were found to have greater antibacterial activity than extracts from oval cultures. This is explained by greater levels of the three antibacterial fatty acids in the fusiform cell extracts. The antibacterial fatty acids are proposed to be released by enzyme action when the diatom cells lose their integrity. The release of free fatty acids by diatoms is suggested to be a simple, very low cost population-level activated defence mechanism against potential pathogenic bacteria triggered when the cell loses its integrity. Further, this pathway may act against multiple threats to the microalga, including grazers, as fatty acids exhibit activity in diverse

biological assays. Finally, whilst two of the fatty acids, (9*Z*)-hexadecenoic acid and (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid, inhibited the growth of MRSA their usefulness as therapeutic compounds may be limited due to their instability and their broad biological activity.

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Abbreviations

Cultures, media and chemical solutions

ATCC: American Type Culture Collection, Manassas, Virginia, U.S.A.
CCAP: Culture Collection of Algae and Protozoa, Dunstaffnage, Argyll, U.K.
ESAW: enriched seawater, artificial water
HEPES: hydroxyethylpiperazineethanesulphonic acid
MT: Mary Tatner collection
NaCl: sodium chloride
NCIMB: United Kingdom National Collections of Industrial, Food and Marine Bacteria, Aberdeen, Aberdeenshire, U.K.
SAG (or EPSAG): Sammlung von Algenkulturen der Universität Göttingen [Culture Collection of Algae at the University of Göttingen], Germany
TOM: modified Bold's basal medium for heterotrophs
UTEX: Culture Collection of Algae at the University of Texas at Austin, Texas, U.S.A.

Units of measurement

º: degree
ºC: degrees Centigrade
AU: absorbance unit
cfu: colony forming unit
cm: centimetre
Da: Dalton
fg: femtogram
ft: foot
g: gram
g: times the force of gravity
h: hour
kV: kilo Volt
L: litre
mg: milligram
MHz: megahertz
min: minute
mL: millilitre
mm: millimetre
mM: millimolar
nm: nanometre
ppm: parts per million
psi: pounds per square inch
rpm: revolutions per minute
µL: microlitre
µm: micrometre

µM: micromolar
$\mu mols^{-1}$ m^{-2}: micromols per second per square metre
v/v: volume per volume
v/v/min: volume per volume per minute

Miscellaneous

-ve: negative
+ve: positive
Ø: diameter
A_{570}: absorbance at 570 nm
A_{750}: absorbance at 750 nm
ANOVA: analysis of variance
CI-MS: chemical ionisation mass spectrometry
DMS: dimethyl sulphide
DMSP: dimethyl-sulphoniopropionate
EI-MS: electron impact mass spectrometry
EPA: eicosapentaenoic acid
FAME: fatty acid methyl esters
GLC: gas-liquid chromatography
HMBC: heteronuclear multiple bond correlation
HSQC: heteronuclear multiple quantum coherence
L:D: light:dark ratio (in hours)
MGDG: monogalactosyldiacylglycerol
MRSA: multi-resistance *Staphylococcus aureus*
NMR: nuclear magnetic resonance
PCA: principle components analysis
PUA: polyunsaturated aldehyde
pers. comm.: personal communication
ppm: parts per million
PTFE: polytetrafluoroethylene
RDA: radial diffusion assay
RP-HPLC: reversed phase high performance liquid chromatography
SD: standard deviation of the mean
SE: standard error of the mean
SEM: scanning electron microscopy
TAG: triacylglycerides
TFA: trifluoroacetic acid
U.K.: United Kingdom
w x h x d: width by height by depth

Chapter 1: Introduction

1.0 Global crisis of antibiotic resistance

The mass production and misuse of antibiotics over the last 60 years has lead to an increased occurrence of multi-antibiotic-resistant bacteria or 'superbugs'. Presently, many antibiotics are unable to cure certain infections and bacterial resistance poses a serious problem for global healthcare in the 21st century. There are now significant difficulties in successfully treating a multitude of infectious diseases.

Initially, the discovery of new antibacterial medications proved to be highly successful and so concern regarding resistant bacterial strains was limited (Rolinson, 1961; Cohen, 1992). However, bacteria continued to acquire resistance to the newly introduced therapeutics whilst becoming increasingly less susceptible to the established drugs (Jevons, 1961; Neu, 1992). Methicillin-resistant Staphylococci strains were reported six months after its introduction in 1960 (Jevons, 1961; Knox, 1961), and the 1960s saw the emergence of methicillin-resistant *S. aureus* strains with resistance to multiple classes of antibiotics (MRSA) (Benner and Kayser, 1968). Subsequently, bacterial antibiotic resistance has been disseminating not only geographically but also between species (Neu, 1992; Alanis, 2005; Grundmann *et al*., 2006). Whilst MRSA remains the most common drug-resistant pathogen (Grundmann *et al*., 2006), numerous bacterial pathogens with multiple drug resistances have been reported and further resistant species continue to emerge (Table 1.1). Worryingly, bacteria have acquired resistance mechanisms for every class of medicinal antibiotic (Alanis, 2005) and MRSA strains resistant to even the 'last resort antibiotic', vancomycin, have been reported (Srinivasan *et al*., 2002).

Table 1.1 – Selected species of human pathogenic bacteria with antibiotic-resistant strains that are causing increased concern (reviewed by Neu (1992), Alanis (2005) and Thomson and Bonomo (2005)).

Gram positive	Gram negative
Clostridium difficile	*Acinetobacter baumannii*
Enterococcus spp.	*Campylobacter jejuni*
Mycobacterium tuberculosis[a]	*Escherichia coli*
Staphylococcus aureus	*Haemophilus influenzae*
Streptococcus pneumoniae	*Klebsiella pneumoniae*
Streptococcus pyogenes	*Neisseria gonorrhoeae*
	Pseudomonas aeruginosa
	Salmonella typhi
	Vibrio cholerae

[a] These are not conventional Gram positive (as they do not take up the stain) but are widely considered to be so due to the structure of their cell wall (Trifiro *et al*., 1990).

Accordingly, the occurrence of antibiotic-resistant infections is increasing steadily (Payne, 2004; Office for National Statistics, 2007) and between 1994-2004 the percentage of *S. aureus* infections reportedly caused by MRSA in the UK increased from <2 % to >40 % (National Audit Office, 2004). Indeed, MRSA was recorded as cause of death in more than 1600 cases in England and Wales for 2005 (Office for National Statistics, 2007). Further, antibiotic-resistant infections are more expensive to treat because they demand higher antibiotic use or more expensive therapies, longer hospital stays and precautions to prevent dissemination (Gould, 2006). To solve such a huge problem requires a multitude of approaches (Table 1.2) though one potential solution is the discovery of novel antibiotics. However, despite the worsening problem of antibiotic resistance, the discovery, development and approval of novel antibacterial drugs is in serious decline, mainly because the pharmaceutical industry has re-channelled resources into the more profitable disease areas of chronic illnesses and lifestyle disorders, for example obesity (Payne, 2004; Alanis, 2005). Furthermore, new anti-infective markets have been identified, for example anti-HIV and other antiviral treatments, and this has meant reduced investment in antibacterial research (Alanis, 2005). There remains an urgent need for the discovery of new therapeutic antibacterial agents, particularly compounds that affect bacteria by mechanisms that differ from existing drugs (Neu, 1992; Demain, 2006).

1.1 Exploitation of natural products

Natural products are compounds produced by a biological source and their exploitation has proved to be the most consistent and successful strategy for the discovery of new pharmaceuticals (Harvey, 2000; Demain, 2006). They have proved particularly successful as a source of new anti-infective drugs and between 1983-1994

Table 1.2 – A plethora of steps can be taken to address the worsening problem of antibiotic resistance. Further suggestions in Neu (1992), Kunin (1993), Srinivasan *et al*. (2002), National Audit Office (2004), Gould (2006) and Grundmann *et al*. (2006).

Steps that may combat antibiotic-resistant bacteria
Increased surveillance to identify patients carrying antibiotic-resistant infections.
Isolation of patients with antibiotic-resistant infections.
Improved epidemiological investigations to pinpoint sources of infection.
Design and implementation of more effective and faster detection assays.
Improved hand hygiene for health workers.
Improved cleanliness in medical facilities.
Discovery and development of novel therapeutic antibiotics.
Prescription of more selective drugs in place of broad-spectrum medications.
Enforcement of full-term therapies for all infections.
Stricter controls on the availability of pharmaceuticals, especially in the developing world.

63 % of newly approved anti-infective drugs and 78 % of antibacterial drugs were from natural origin (Cragg *et al*., 1997). Drugs from natural origin include the unaltered natural product, chemically altered analogues or compounds modelled on a natural product parent (Cragg *et al*., 1997). Chemical modifications can improve efficacy or eliminate deleterious side effects (Munro *et al*., 1999; Rouhi 2003a). However, despite playing such a pivotal role in the drug discovery process, natural product discovery programs in the larger pharmaceutical companies were terminated in the 1990s mainly because they were considered to be relatively slow, labour intensive, expensive and yielding few compounds (Rouhi, 2003b; Alanis, 2005; Battershill *et al*., 2005). Biodiversity ownership issues have also contributed (Battershill *et al*., 2005). Large pharmaceutical companies have embraced the new technologies of combinatorial chemistry and rational drug design (Battershill *et al*., 2005), but thanks in part to the poor performance of these new approaches, there is now a resurgence in exploring natural products as sources of novel compounds (Rouhi, 2003b). Such new research into natural products has become more attractive due to developments in separation technology, high-speed approaches for sample de-replication, advances in structure elucidation and new methods for titre improvement (Munro *et al*., 1999; Harvey, 2000; Rouhi, 2003b; Demain, 2006). Continuing improvements in natural products chemistry is also providing alternatives to natural synthesis (Burkart, 2003; Demain, 2006) and issues surrounding organism ownership have been clarified with the implementation of the United Nations Convention on Biological Diversity (United Nations, 1992; Battershill *et al*., 2005).

Natural products offer plenty of potential for the discovery of novel compounds because more than 90 % of the world's biodiversity remains to be tested for biological

activity (Harvey, 2000; Demain, 2006). Whilst natural products cannot compete in terms of the number of compounds generated by other technologies, these programs do provide high quality lead compounds with greater structural diversity than can be offered by standard combinatorial chemistry (Harvey, 2000; Rouhi, 2003b; Battershill *et al*., 2005).

1.2 Marine natural products

As many accessible terrestrial plants and microbes have already been explored for their antibiotic potential, the oceans have been identified as a great source of new organisms that can be exploited for their metabolites (Bèrdy, 1989; Donia and Hamann, 2003; Battershill *et al*., 2005). This is due to the rich, unexplored biodiversity found within the oceans due to the greater variety of habitats and environmental conditions that exist in the seas compared with terrestrial environments (Ruggieri, 1976; Jensen and Fenical, 1994; Patrzykat and Douglas, 2003; Battershill *et al*., 2005). Marine organisms are known to synthesise wide-ranging novel chemical structures with unusual chemistry not seen in terrestrial organisms (Ruggieri, 1976; Rinehart *et al*., 1981; Scheuer, 1990; Jensen and Fenical, 1994; Faulkner, 2002; Paul *et al*., 2006), often as a result of their diverse and novel biosynthetic pathways (Harper *et al*., 2001; Moore, 2005). Moreover, the number of novel compounds isolated from terrestrial microbial cultures has inevitably decreased, and indeed, more than 90% of bioactive cultures discovered produce previously known agents (Fenical, 1993). Encouragingly though, the proportion of marine microbes producing antibiotic compounds compares well with terrestrial isolates (Sponga *et al*., 1999).

In short, marine microbes have been neglected by drug screening programs and so represent a major untapped resource for the discovery of novel pharmaceuticals (Rinehart *et al*., 1981 Paul, 1988; Jensen and Fenical, 1994).

1.3 The Algae

The term 'algae' essentially describes all photosynthetic organisms that are not considered higher plants (Hinde, 1995). The algae are crucial for correct functioning of the oceanic ecosystem where they form an important base for the food chain (Sze, 1998). They also play a major role in maintaining the stability of the biosphere as they contribute approximately 50 % of the world's fixed carbon (Lips and Avissar, 1986; Field *et al*., 1998; Shurin *et al*., 2006). The algae are a very diverse group which reflects their polyphyletic origins (Radmer, 1996; Sze, 1998; Falkowski *et al*., 2004; Figure 1.1). Only the blue-green algae are prokaryotes (now more commonly referred to as the Cyanobacteria). The eukaryotic algae are composed of both multicellular species (the seaweeds or macroalgae) and single-celled species (the microalgae), though, the microalgae includes species that form colonies or filaments. The work in this thesis considers only the marine eukaryotic microalgae but examples from other algal groups are included where appropriate.

The genetic diversity of the eukaryotic microalgae derives from the broad variety of habitats that these organisms inhabit. For example species can be planktonic or benthic or exist in mutualistic symbioses with invertebrate organisms (Radmer, 1996; Sze, 1998). Thanks to their polyphyletic origins, the eukaryotic microalgae express a broad array of metabolites with rich chemical diversity (Lincoln *et al*., 1991; Borowitzka, 1995; Shimizu, 1996; Faulkner, 2002; Moore, 2005). Estimates for the

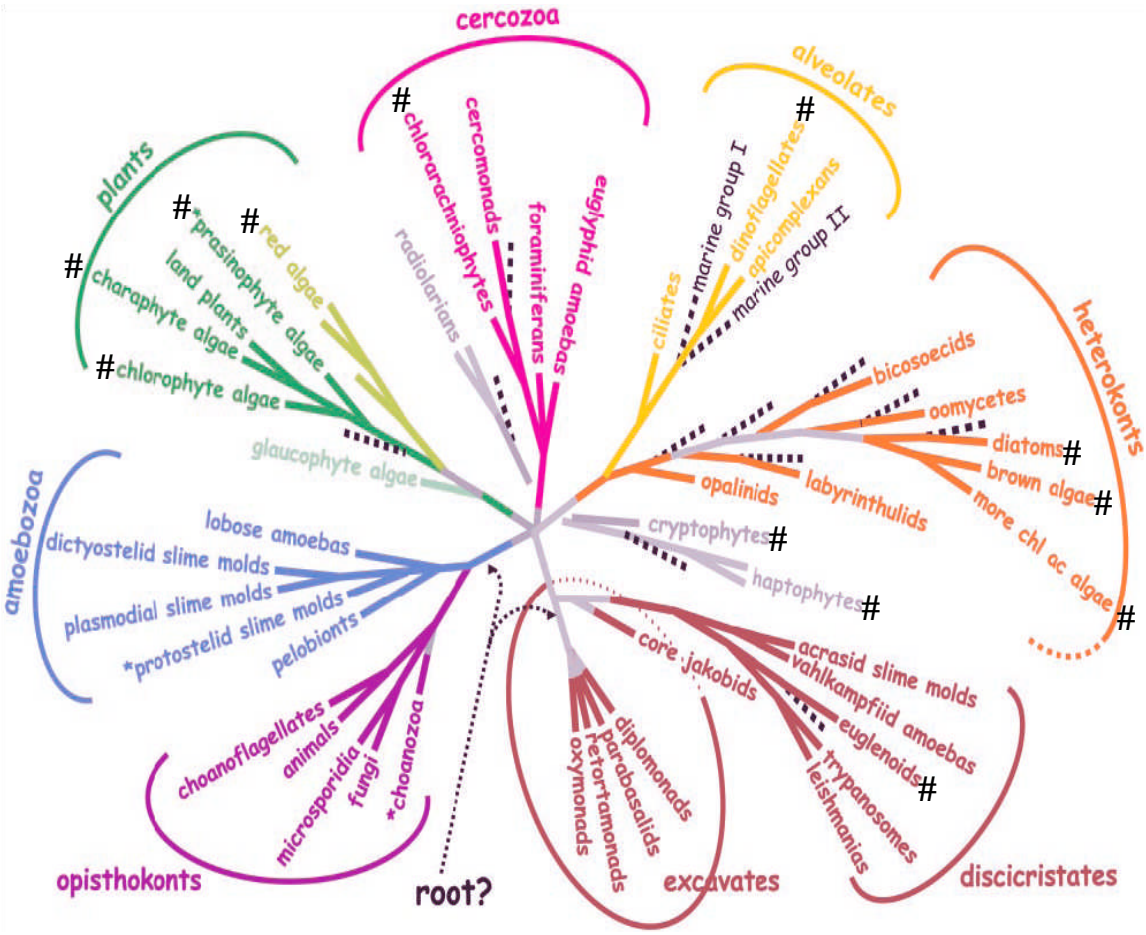


Figure 1.1 – The phylogenetic origins of eukaryotes showing that algae have diverse phylogeny and are found in 5 of the 8 major branches (Baldauf, 2003). Algal groups signified by '#'.

number of eukaryotic microalgal species vary but it is commonly recognised that there are between 100,000 and one million species, including at least 100,000 species of diatom alone (Norton *et al*., 1996; Falkowski *et al*., 2004). Despite the exploitation of the eukaryotic microalgae in various industries (Table 1.3) relatively few workers have attempted to exploit the microalgae for the discovery of novel bioactive compounds and there have been no very large-scale screening programs. Some eukaryotic microalgae have been shown to produce compounds with antibacterial activity (reviewed by Aubert *et al*., 1979; Metting and Pyne, 1986; Lincoln *et al*., 1990; Pesando, 1990) but, importantly, only a small percentage of the described eukaryotic microalgal species have been examined for the discovery of novel antibacterial compounds (Kellam and Walker, 1989; Harvey, 2000). This was perhaps due to difficulties associated with their collection, identification, storage and culture but, happily, many of these problems have now been overcome. As many microalgae can be cultured under laboratory conditions a sustainable supply is possible (Borowitzka, 1999b; Day *et al*., 1999; Culture Collection of Algae and Protozoa, 2007). Improvements in molecular biology aid microalgal identification (Olmos *et al*., 2000; Bolch, 2001) whilst steps are being taken to ensure stable strain storage (Day *et al*., 1999; Taylor and Fletcher, 1999). Algae can be successfully grown on very cheap media in outdoor ponds or raceways (Raymont and Adams, 1958; Goldman and Ryther, 1976; Mann and Ryther, 1977; Aaronson and Dubinsky, 1982; Richmond, 1999) but the controlled culture of microalgae is presently relatively inefficient and thus expensive (Borowitzka, 1999c; Chen and Chen, 2006). During culture microalgae can suffer light limitation due to self-shading meaning heterotrophy is highly desirable (Wen and Chen, 2003; Lebeau and Robert, 2003b; Chen and Chen, 2006). To this end, Zaslavskaia *et al*. (2001) has transformed an

Table 1.3 – Common industrial uses of eukaryotic microalgae. For general reviews see de la Noüe and de Pauw (1988), Cannell (1990), Richmond (1990), Radmer (1996) and Pulz and Gross (2004).

Application	Important Genera	Selected reviews
Human health food	*Chlorella*	Jensen (1993), Yamaguchi (1997)
Aquacultural feeds (for *Artemia*, rotifers, bivalve molluscs and fish larvae)	*Chaetoceros*, *Cyclotella*, *Isochrysis*, *Skeletonema*	Becker (1994), Duerr *et al*. (1998)
Agricultural feeds	*Chlorella*	Becker (1994), Spolaore *et al*. (2006)
Wastewater treatment and bioremediation	Various	Becker (1994), Semple *et al*. (1999)
Glycerol and lipids	*Dunaliella*, *Phaeodactylum*	Cohen (1986)
EPA, DHA and other fatty acids	*Crypthecodinium*, *Nannochloropsis*, *Nitzschia*, *Phaeodactylum*	Borowitzka (1988a); Molina Grima *et al*. (1999a); Lebeau and Robert (2003a)
Polysaccharides	*Porphyridium*	Cohen (1986)
Biofuels (alcohols, hydrogen, methane gas)	Various	Cohen (1986), Yamaguchi (1997)
β-carotene and other pigments	*Dunaliella*	Spolaore *et al*. (2006)
Vitamins	Various	Borowitzka (1988b)
Bioactive compounds	Various	Metting and Pyne (1986), Lincoln *et al*. (1990), Borowitzka (1999a)
Isotope-labelled compounds	*Phaeodactylum*	Apt and Behrens (1999)
Nanotechnology	*Thalassiosira*	Parkinson and Gordon (1999), Wee *et al*. (2005)

obligate autotrophic diatom to grow entirely heterotrophically, though certain species may already be cultured heterotrophically (Gladue and Maxey, 1994; Chu *et al*., 1996) or mixotrophically (Xu *et al*., 2004; Fernández Sevilla *et al*., 2004; Cerón García *et al*., 2005). Further improvements have also been made in the design and efficiency of bioreactors meaning that the microalgae can be exploited using existing biotechnology (Borowitzka, 1997; Borowitzka, 1999b; Richmond, 1999).

Although feasible, it is very unlikely that any bioactive compound would be produced as a final product by culturing the microalga itself. It is much more desirable to chemically synthesise a pharmaceutical compound for commercial, regulatory and safety reasons (Hutchinson, 1994; Burkart, 2003; Rouhi, 2003a). Alternatively, the gene or pathway producing the compound of interest could be transferred into viral or bacterial vector species (Pfeifer *et al*., 2001; Burkart, 2003; Wenzel and Müller, 2005). Only if these other avenues fail is it likely that the microalga would be cultured, although microorganisms can produce structurally complex molecules which may be difficult or impossible to manufacture by chemical synthesis (Hutchinson, 1994; Borowitzka, 1995; Wenzel and Müller, 2005).

In short, the eukaryotic microalgae exhibit huge genetic and chemical diversity but have been completely under explored for the production of bioactive compounds despite being shown to produce such compounds using present day biotechnology (Harvey, 2000). Indeed, the microalgae can be considered to be in the unique position of sharing the advantages of huge chemical diversity, at least as great as that of the higher plants, with the benefits of a microorganism, in so much that their growth and metabolite expression can be altered in a controlled way using conventional culture

methods (Cannell, 1993). For the reasons detailed above the eukaryotic microalgae, and especially the diatoms, are becoming increasingly attractive as a potential exploitable source of novel antibacterial molecules.

1.4 Microalgal chemical defence

From an ecological viewpoint, microalgae are able to survive and even flourish in an environment that is richly populated with competitors, pathogens, parasites and predators. Indeed, one mL of coastal seawater typically contains ~10^3 microalgae, ~10^6 bacteria and ~10^7 viruses (Cole, 1982; Børsheim *et al*., 1990; Selph *et al*., 2001) of which some will be competitors whilst others potential pathogens and parasites. To be successful in such a densely populated milieu, microalgae must possess strategies that provide defence or competitive advantages and the production of antibacterial compounds may partially explain their success (Steemann-Nielsen, 1955; Jørgensen, 1956; Sieburth, 1960).

Many studies have described the antibacterial nature of microalgal cell extracts and cell-free culture supernatant but in only very few instances have the compounds responsible for the activity been isolated and a full structural characterisation performed (e.g., Findlay and Patil, 1984; Ohta *et al*., 1994). In most of these studies little attention was paid to whether the expression of antibacterial compounds is constitutive or inducible, the environmental factors that control their expression, if pathogen challenge affects titre or what happens in their absence. Many of these parameters are difficult to investigate but it does mean that an evaluation of their ecological relevance is rather difficult and even unclear (Engel *et al*., 2002). Furthermore, the antibacterial compounds are often found only at very low levels or

may be too unstable in seawater to have any relevance to surrounding bacteria (Aubert *et al*., 1979; Glombitza, 1979). Moreover, antibacterial activity is often absent in preparations from many ecologically successful species and this may be because these algae use different strategies to outcompete or defend against bacteria (Duff *et al*., 1966; Reichelt and Borowitzka, 1984; Cannell *et el*., 1988; Kellam and Walker, 1989; Kjelleberg and Steinberg, 2001). These different strategies may involve compounds that prevent bacterial colonisation of the algal surface or may encourage a bacterial flora that prevents the settlement of undesirable epiphytes (Engel *et al*., 2002). Certainly, it is known that microalgae often harbour characteristic bacterial flora (Grossart *et al*., 2005), but the role of antibacterial compounds in the defence of microalgae against bacteria has yet to be conclusively proven (Paul, 1988; Engel *et al*., 2002). Despite doubts as to why microalgae have antibacterial compounds the widespread nature of these compounds warrants further investigation because these compounds provide one of the more simple explanations for how microalgae can compete with and defend against bacteria.

Antibacterial compounds have been considered to function as allelochemicals to provide a competitive advantage against surrounding microbes or defend against bacterial parasites but much less attention has been paid to the potential role against bacterial pathogens (Hay, 1996) despite bacteria potentially playing a major role in reducing microalgal numbers (Mitchell, 1971; Mayali and Azam, 2004; Kim *et al*., 2007). Often defence pathways are evaluated in terms of the protection that they provide against grazers, perhaps due to the importance of this selection pressure on microalgal evolution (Hay, 1996; Smetacek, 2001). However, it is entirely reasonable that microalgae may have defence pathways that are capable of combating bacterial

pathogens. It seems most likely that some of the defence pathways already described for the microalgae are multifunctional (Wolfe, 2000) and thus defend the cell against a variety of threats. Of course, other pathways described in microalgae, not thought to act in defence, may indeed do so.

Defence strategies described in the microalgal literature are often classified as either constitutive, which is to say they are always present, or inducible, meaning that they are formed in response to a biotic elicitor, or termed 'activated' when the defence is only triggered after cell death (Métraux, 1994; Harvell and Tollrian, 1999; Pohnert *et al*., 2007). Defence strategies may be further considered as either physical (mechanical, structural and behavioural) or chemical. For physical defences, the protection provided by the diatom shell (Hamm *et al*., 2003) is a good example of a constitutive defence. The shell can thicken in response to the presence of grazers, which may be considered an induced, physical defence (Pondaven *et al*., 2007).

Constitutive chemical defences include not only those that are always present during normal cell functioning but also those that may be affected by the cell's nutrient status. The major advantage of constitutive defences is that they are maintained whenever nutritionally feasible, and such defences are desirable where a threat is frequently encountered (Tollrian and Harvell, 1999). The toxins of *Alexandrium lusitanicum*, or feeding deterrent compounds produced by *P. tricornutum*, are examples of constitutive defences and, though their levels do change during culture, this is due to altered nutrient conditions and not a specific biological elicitor (Mascarenhas *et al*., 1995; Shaw *et al*., 1995a; Shaw *et al*., 1995b). The disadvantages of constitutive pathways are that they may be considered metabolically

‘costly’ to maintain, and this is especially so in an environment with shifting threats or changing predatory pressure (Tollrian and Harvell, 1999; Wolfe, 2000). A potentially less ‘costly’ strategy is an inducible one where defence metabolites are only produced or upregulated once a potential threat is perceived. For example, the presence of feeding copepods can cause an upregulation of paralytic shellfish toxin production in marine dinoflagellates (Tollrian and Harvell, 1999; Selander *et al*., 2006). Induced defences reduce the opportunity for self-toxicity and may be considered less wasteful as they are only produced when required (Tollrian and Harvell, 1999; Wolfe, 2000). A potentially more efficient option is an activated defence whereby the bioactive metabolites are freed from essential cell components only after death (Pohnert, 2004; Pohnert *et al*., 2007). Activated defences have the advantages of little or no self-toxicity and a negligible or zero ‘cost’ of maintenance because extra protein synthesis is thought not to be required other than is necessary for normal cell functioning (Wolfe *et al*., 1997; Pohnert, 2000; Wolfe, 2000; Pohnert, 2004; Pohnert *et al*., 2007). The obvious disadvantage of activated defences is that they can act only after death and are therefore unable to protect the individual cell. However, they may be able to act effectively for a population of closely genetically related surrounding cells (kin selection) (Wolfe, 2000; Pohnert *et al*., 2007).

1.4.1 Microalgal activated defence pathways

The two best-characterised activated defence pathways in the microalgae are the β-dimethyl-sulphoniopropionate (DMSP) and oxylipin pathways. One further activated pathway that may be considered to act in defence is the generation of reactive oxygen species, such as superoxide (Marshall *et al*., 2005a). This has been shown to occur in diverse microalgal species upon cellular disintegration, though this has not been

investigated in diatoms (Marshall *et al*., 2005b). This potential defence pathway is certainly worthy of consideration as a protective strategy against pathogens and grazers (Marshall *et al*., 2005a; Ross *et al*., 2005). Furthermore, the generation of reactive oxygen species may act in wound repair for larger microalgae (Ross *et al*., 2005).

The DMSP pathway is normally triggered by attack from a grazing predator when the non-toxic substrate, DMSP, which normally functions as an antioxidant, osmolyte or cryoprotectant (Kirst *et al*., 1991), is cleaved by the enzyme DMSP lyase to yield dimethyl sulphide (DMS) and acrylic acid (Wolfe and Steinke, 1996; Wolfe *et al*., 1997). The acrylic acid may reach toxic concentrations inside a grazer (Wolfe *et al*., 1997; Wolfe, 2000) whilst the DMS may act as a diffusible warning signal of digestion (Wolfe and Steinke, 1996; Wolfe *et al*., 1997). Normally DMSP and DMSP lyase are kept apart in a healthy cell but these mix when the cell is broken by mechanical disruption which occurs during ingestion by a grazer (Wolfe and Steinke, 1996; Strom *et al*., 2003). The DMSP pathway can also be triggered during lysis by viral pathogens (Malin *et al*., 1998).

Recently the oxylipin pathway has been described for diatoms and this defence mechanism is thought to reduce or prevent predation by herbivore grazers like copepods (Pohnert, 2005). When a diatom cell loses it's integrity, for example during predator attack, lipases immediately act on membrane lipids to yield various free mono- and polyunsaturated fatty acids (Jüttner, 2001; Pohnert, 2002; Pohnert *et al*., 2004). The fatty acids, 16:3n4 and 20:5n3, are freed from the chloroplast-derived glycolipids, especially monogalactosyldiacylglycerol (MGDG), whilst the 20:5n3

may also be freed from phospholipids (d'Ippolito *et al*., 2004; Cutignano *et al*., 2006; d'Ippolito *et al*., 2006; Figure 1.2; see Appendix I for an explanation of fatty acid nomenclature). Most fatty acids are released during the first few minutes (Jüttner, 2001; Pohnert 2002; Pohnert *et al*., 2004) and lipase activity is concentrated around the extruding cytoplasm of damaged cells (Pohnert, 2002). Subsequently, other enzymes, the lipoxygenases and hydroperoxide lyases, rapidly act to cleave and convert the free fatty acids to oxylipins, including C7-C11 polyunsaturated aldehydes (PUA) that have biological activity (Pohnert, 2000; Pohnert, 2002; Pohnert *et al*., 2002; d'Ippolito *et al*., 2005; Barofsky and Pohnert, 2007; Fontana *et al*., 2007). The suite of PUA are species- and even strain-specific (Wichard *et al*., 2005) and many PUA and oxylipins have been identified that have all been produced from the same few fatty acid precursors (Pohnert, 2004). PUA can detrimentally affect grazers by direct toxicity, exhibiting antiproliferative properties, reducing grazer fecundity, reducing hatching success in copepods or reducing larval survival (Miralto *et al*., 1999; Caldwell *et al*., 2002; Pohnert *et al*., 2002; Adolph *et al*., 2003; Romano *et al*., 2003; Ianora *et al*., 2004; Adolph *et al*., 2004; Caldwell *et al*., 2005; Taylor *et al*., 2007). However PUA pathways have been detected in less than 40 % diatom species (Wichard *et al*., 2005) which has prompted some workers to speculate that other compounds must be involved in the deleterious effects seen on grazing copepods and invertebrates by species that do not produce PUA (Wichard *et al*., 2005; Fontana *et al*., 2007). However, Jüttner (2001) reports that the precursor, 20:5n3, can itself be toxic for grazers.

The DMSP and oxylipin pathways are highly metabolically efficient because essential cell constituents are rapidly converted into compounds that are highly toxic for

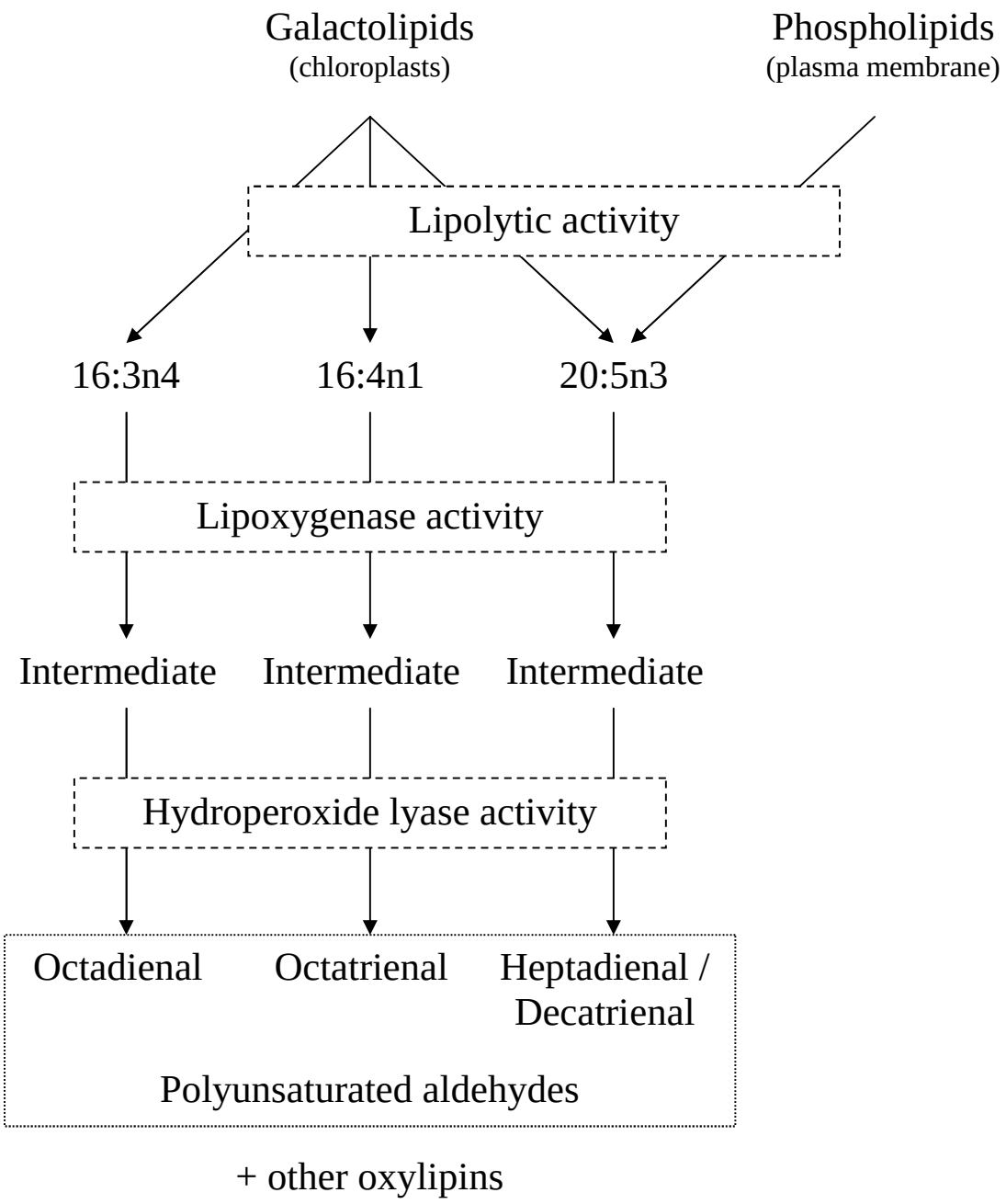


Figure 1.2 – Biosynthetic pathways for the production of polyunsaturated aldehydes by diatoms (adapted from d'Ippolito *et al*., 2004 and Fontana *et al*., 2007). Lipases (lipolytic activity) act on phospholipids or glycolipids to yield free fatty acids. The free fatty acids are altered by lipoxygenases, hydroperoxide lyases and lipolytic acyl hydrolases to form a suite of oxylipin compounds including polyunsaturated aldeydes such as octadienal and decatrienal. These pathways have largely been characterised in *Skeletonema costatum* and *Thalassiosira rotula*.

grazers without additional costs associated with maintaining constitutive toxins (Wolfe *et al*., 1997; Pohnert, 2000; Jüttner, 2001; Pohnert, 2002; Pohnert *et al*., 2007). DMSP may be slightly more 'costly' to maintain because there is evidence to suggest that microalgal cells signal the presence of the damaging DMSP pathway to potential predators (Wolfe *et al*., 1997; Strom *et al*., 2003). This is concluded because protist grazers selectively ingest low DMSP-pathway-capable strains of *Emiliana huxley* whilst avoiding more potentially toxic isolates (Wolfe and Steinke, 1996; Wolfe *et al*., 1997; Wolfe, 2000; Strom *et al*., 2003).

Although the DMSP and oxylipin defensive reactions occur upon death and thus seem to make little sense for an individual cell, it could benefit a population of genetically similar or identical individuals (Wolfe, 2000; Pohnert and Boland, 2002; Pohnert *et al*., 2007). Microalgal defences must be metabolically inexpensive due to the short lifecycle. Thus cellular resources can be allocated to growth and division (Pohnert, 2000; Jüttner, 2001; Pohnert, 2005). It is highly likely that to reduce the metabolic costs of maintaining defence strategies, a single multifunctional strategy that is able to act against various threats will be under positive selection pressure (Wolfe, 2000). There is evidence to suggest that this may be the case for the DMSP and oxylipin pathways because end products of these pathways are not only toxic to grazers but are also antimicrobial (Bisignano *et al*., 2001; Adolph *et al*., 2004; Prost *et al*., 2005). It could be the case that many antibacterial compounds, which have been found, and occasionally identified, previously in microalgal cultures are the products of multifaceted defence pathways that may act against a variety of threats, including bacterial pathogens and grazers.

1.5 Broad aims of this thesis

The broad aim of this thesis is to study the antibacterial compounds from a model microalgal species, paying particular attention to their isolation and identification. Further, the ecological significance of these compounds will be considered as will their production and whether or not they function to defend the microalga against bacteria. Finally, the potential for commercial exploitation of microalgal-derived antibacterial compound(s) will be explored.

1.6 *P. tricornutum*: species of choice

P. tricornutum is an unusual pennate marine diatom that was first isolated and described by Bohlin (1897). Some early publications incorrectly refer to the organism as *Nitzschia closterium* forma *minutissima* (Lewin, 1958). It is an obligate photoautotroph (Hayward, 1968a; Hayward, 1968b) that has been isolated from coastal and estuarine environments (Hayward, 1968b; Craggs, 1994; Li *et al*., 2006) though strains have been isolated from an inland lake (Rushforth *et al*., 1988). It is an unusual diatom in so much that it does not have a conventional silica shell (Lewin *et al*., 1958; Lewin, 1958; Borowitzka and Volcani, 1978) and thus shows little or no requirement for silicon (D'Elia *et al*., 1979).

P. tricornutum was selected for the isolation and characterisation of antibacterial compounds in this thesis for numerous reasons. First, previous workers have shown that cell extracts from this species are antibacterial although the molecules responsible for this activity have never been isolated and identified (Table 1.4). Diatoms have proved to generate a high hit ratio of antibacterial activity in cell extracts (Duff *et al*., 1966; Aubert *et al*., 1979; Aaronson and Dubinsky, 1982; Viso *et al*., 1987; Kellam

Table 1.4 – Previous reports in the literature for antibacterial activity of *P. tricornutum*. Often antibacterial activity has been found in non-polar extracts.

Reference	Antibacterial extract(s)	Spectrum of activity	Notes
Duff *et al*. (1966)	Antibacterial activity in late exponential phase cultures sequentially extracted with acetone, chloroform, chloroform:methanol (1:1) and methanol:water (4:1).	Gram positive: *Brevibacterium* spp., *Corynebacterium* spp., *Micrococcus* spp. *Streptococcus faecalis*; Gram negative: *Flavobacterium* sp.	Similar activity found in each extract.
Brown *et al*. (1977)	Cell-free culture filtrates antibacterial.	Gram negative: *Escherichia coli*	
Cooper *et al*. (1983)	Cultures in early- or late exponential phase or stationary phase extracted with chloroform:methanol:water mixes to yield antibacterial organic and aqueous extracts.	Gram positive: *Bacillus subtilis* (spores), *Staphylococcus aureus*; Gram negative: *Alcaligenes cupidus*, *Alteromonas communis*, *Alteromonas haloplanktis*, *Escherichia coli*, *Vibrio fischeri*, *Vibrio parahaemolyticus.*	Greatest activity in late exponential phase but aqueous and organic extracts equally active.
Cooper *et al*. (1983)	Antibacterial component (>10 kDa) detected in culture supernatant.	Gram negative: *Pseudomonas aeruginosa*	
Cooper *et al*. (1985)	Antibacterial fraction containing six fatty acids isolated (14:0, 16:2, 16:3, 16:4, 18:4, 20:5)[a].	Gram positive: *Bacillus subtilis*; Gram negative: *Vibrio parahaemolyticus.*	
Kellam and Walker (1989)	Stationary phase cultures extracted with methanol then hexane.	Gram positive: *Bacillus subtilis*, *Staphylococcus aureus.*	Hexane fraction more active.
Bickerdike (2002)	Cultures extracted with numerous solvents. Greatest activity (against an unidentified Gram positive bacterium) in 100 % methanol extracts. A fraction was subsequently isolated with antibacterial activity.	Gram positive: *Planococcus citreus*, MRSA; Gram negative: *Escherichia coli*, *Psychrobacter immobilis*.	Compound in antibacterial fraction not fully identified.

[a] see Appendix I for a brief explanation of fatty acid nomenclature

and Walker, 1989; Pesando, 1990; Lincoln *et al*., 1990) but only in a limited number of studies have the authors succeeded in purifying and identifying the compounds responsible for the antibacterial activity (Aubert *et al*., 1970; Pesando, 1972; Findlay and Patil, 1984).

Second, this diatom can be grown rapidly in inexpensive culture media (Hayward, 1968b; Ansell *et al*., 1964; D'Elia *et al*., 1979) and is amenable to large-scale culture (Raymont and Adams, 1958; Ansell *et al*., 1964; Goldman and Ryther, 1976). It is robust and can survive in highly variable conditions of salinity (Hayward, 1968b; Styron *et al*., 1976), nutrient status (Hayward, 1965), pH (Raymont and Adams, 1958; Hayward, 1968b; Goldman *et al*., 1982), temperature (Hayward, 1968b; Styron *et al*., 1976; Véron *et al*., 1996), irradiance and light quality (Hayward, 1968b; Terry *et al*., 1983; Geider *et al*., 1985; Véron *et al*., 1996), light regime (Nelson *et al*., 1979) or turbulence (Brindley Alias *et al*., 2004). As a result, it often dominates in man-made habitats, such as aquaculture ponds (Raymont and Adams, 1958; Goldman and Ryther, 1976; Goldman *et al*., 1982). However, relatively little is known with respect to the pathways, if any, that defend this diatom against potential pathogens, such as bacteria or viruses. This diatom is not thought to produce bioactive polyunsaturated aldehydes (Wichard *et al*., 2005) though other products of the lipoxygenase/hydroperoxide lyase pathways (polar oxo-acids) have been discovered in *P. tricornutum* cultures (Pohnert *et al*., 2002). It is not clear whether this diatom has the DMSP pathway but DMS has been detected in cultures upon cellular disintegration (Ackman *et al*., 1966).

Third, this diatom has been widely studied and it is considered the 'model' pennate diatom. Indeed, there have been approximately 1000 peer-reviewed publications on this organism (Web of Knowledge, 2007). The genome has been sequenced and is undergoing annotation (Lopez *et al*., 2005; J. A. Berges, University of Wisconsin, pers. comm.), which makes it amenable to follow up studies, especially of a genomic nature. Genetic manipulation has been demonstrated for this species (Zaslavskaia *et al*., 2000) and a strain has been produced that grows heterotrophically (Zaslavskaia *et al*., 2001).

1.6.1 *P. tricornutum*: cell morphology

This species is curious because it exhibits phenotypic plasticity meaning it exists in a number of different morphs: oval, fusiform (crescent-shaped) and triradiate (Figure 1.3) though there are numerous intermediary forms (Barker, 1935; Wilson, 1946; Lewin *et al*., 1958; Hayward, 1968b; Borowitzka and Volcani, 1978; Figure 1.3). It is unclear whether the triradiate morph is found in nature or is an artefact of culture (Lewin *et al*., 1958). The cells typically divide clonally and give rise to daughter cells of the same shape though variations in this life cycle have been reported (Wilson, 1946; Lewin *et al*., 1958; Coughlan, 1962; Borowitzka and Volcani, 1978).

Little is known with respect to the conditions that govern the expression of these different morphs though it is likely to change in response to environmental conditions. Many authors have reported that on successive culture transfer on to solid media the oval morph becomes more plentiful but in liquid media the fusiform morph becomes more numerous (Barker, 1935; Lewin *et al*., 1958; Gutenbrunner *et al*., 1994). Nevertheless, ovals can be maintained in liquid culture media for many generations

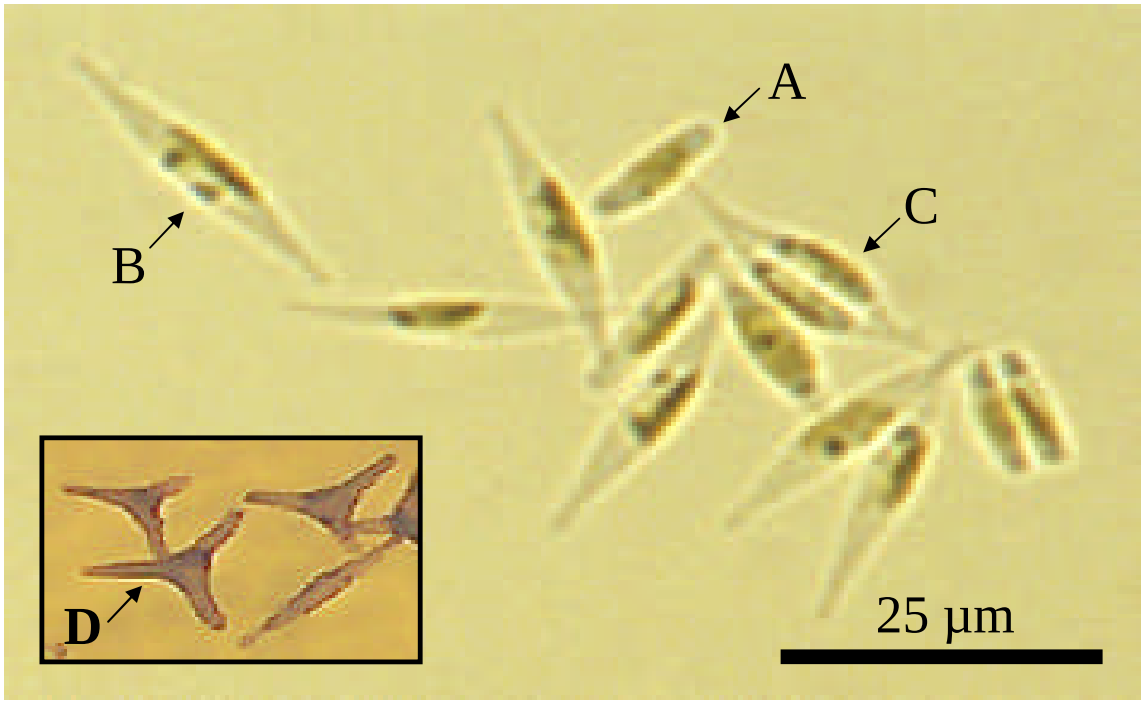


Figure 1.3 – Photograph showing the different morphology of cells in cultures of the marine diatom, *P. tricornutum*: (A) oval, (B) fusiform, (C) intermediate and (D; inset) triradiate.

(Lewin *et al*., 1958; Cooksey and Cooksey, 1974; Borowitzka *et al*., 1977). In liquid medium levels of calcium or copper may affect morphology (Cooksey and Cooksey, 1974; Markina and Aizdaicher, 2006) but osmolarity does not (Gutenbrunner *et al*., 1994).

Such phenotypic plasticity may be an important mechanism for the survival and adaptation of a population until rarer genetic mutations can occur (Morales *et al*., 2002). The expression of different cell morphs probably allows greater opportunity for success in the changing and highly competitive environment of coastal and estuarine areas where this diatom is often found. In these habitats, natural selection favours the evolution of plastic genotypes as an unpredictable environment tends to prevent the production of an optimal phenotype (Morales *et al*., 2002). One obvious difference between the morphs that may provide an advantage in the field is the greater buoyancy of fusiform cells meaning they are better adapted to a planktonic existence (Lewin *et al*., 1958). However, well-adapted organisms must be able to sense environmental change and respond by producing suitable phenotypes for a variety of environmental conditions (Morales *et al*., 2002). *P. tricornutum* can sense external signals, such as those produced by members of the same species (Iwasa and Shimizu, 1972), other species (Vardi *et al*., 2006), or changes in water movement, osmotic stress or iron concentration (Falciatore *et al*., 2000; Scala and Bowler, 2002) though none of these have yet been shown to affect cell morphology.

Whilst previous studies have shown that oval and fusiform cells differ physiologically and chemically (Table 1.5) no workers have investigated whether or not the different morphs differ with respect to levels of antibacterial activity. Due to greater

Table 1.5 – Comparison of cellular characteristics for oval and fusiform cells of *P. tricornutum*.

Characteristic	Oval	Fusiform	References
Cell size (length)	8 µm	25-35 µm	Lewin (1958)
Growth in liquid media	Tend to clump	Found as single cells	Lewin (1958)
Generation time	No difference		Darley (1968)
Buoyancy	Low	High	Lewin *et al*. (1958)
DNA content	No difference		Darley (1968)
Cell walls	Siliceous (1 pennate valve)	Non-siliceous	Lewin *et al*. (1958) Borowitzka *et al*. (1977)
Mucilagenous capsule	Yes (16 % dry weight)	No	Lewin *et al*. (1958)
Toluidine blue stain	Red	Slightly blue	Gutenbrunner *et al*. (1994)
Packed cell volume (2.2 $x10^{10}$ cells)	5.8 mL	2.7 mL	Lewin *et al*. (1958)
Cell mass (mg)	2.00 $x10^{-8}$	2.02 $x10^{-8}$	Lewin *et al*. (1958)
Cell lipid by dry weight (%)	24	34	Lewin *et al*. (1958)

Cell protein by dry weight (%)	34	41	Lewin *et al*. (1958)
Cell carbohydrate by dry weight (%)	2	3	Lewin *et al*. (1958)
Cell silica by dry weight (%)	0.4-0.5	0.4-0.5	Lewin *et al*. (1958)
12, 50, 116, 120 kDa proteins	Present	Absent	Gutenbrunner *et al*. (1994)
A 21 kDa protein	Absent	Present	Gutenbrunner *et al*. (1994)
66 and 90 kDa proteins	More	Less	Gutenbrunner *et al*. (1994)
Form of colonies on agar	Light brown, spreading with irregular margin	Dark brown, rounded with entire margin	Lewin (1958); Lewin *et al*. (1958)
Motility	Motile (1 to 2.9 µm min^{-1})	Non-motile	Lewin (1958); Iwasa and Shimizu (1972)
Immunogenicity	Same level of response		Gutenbrunner *et al*., 1994

competition for space and nutrients, benthic organisms are thought to produce more bioactive compounds compared to planktonic species (Cembella, 2003), thus it is predicted in this thesis that the benthic-preferring oval morph will have greater levels of antibacterial activity.

1.7 Specific aims

1. To identify the compounds responsible for the antibacterial activity of cell extracts prepared from *P. tricornutum* cultures.

2. To investigate whether or not the different cell morphs have different quantities of antibacterial compounds and explore why this may be so.

3. To evaluate the commercial and ecological significance of isolated antibacterial compounds.

Chapter 2: Growth of *Phaeodactylum tricornutum* in a custom-designed small-scale culture system

2.1.0 Introduction

Before investigating the nature and production of antibacterial compounds by *P. tricornutum* it is important to study the alga's growth under specific laboratory conditions. Culture parameters, such as irradiance, temperature, mixing rate and nutrient availability, can all affect algal growth by increasing lag phase, hastening entry into stationary phase or decreasing maximum cell concentration (Cole, 1982; Fawley, 1984; Yongmanitchai and Ward, 1991; Ohta *et al*., 1995; Wen and Chen, 2001; Brindley Alias *et al*., 2004; Jiang and Gao, 2004; Xu *et al*., 2004; Schapira *et al*., 2006). These parameters can ultimately affect the production of algal metabolites, such as antibacterial compounds (Trick *et al*., 1984; Cannell *et al*., 1988; Ohta *et al*., 1995). Therefore the growth conditions will be checked to ensure that these produce unialgal and axenic *P. tricornutum* cultures that, in turn, yield cell extracts with antibacterial activity.

This chapter describes the characterisation of axenic *P. tricornutum* growth under the controlled conditions experienced in a custom designed small-scale batch culture system that will permit further studies on the antibacterial activity of cell extracts from this organism. A description of the culture system and growth conditions is given and the effect of bottle position in the system on algal growth is investigated. Procedures employed throughout this study are detailed for the harvest of diatom cells and for the subsequent preparation of cell extracts. An outline is given for the radial diffusion assay, used to screen extracts for antibacterial activity, and the measures

taken to standardise this assay, with particular attention paid to characterising and quantifying the growth of the assay bacteria.

2.2.0 Materials and methods

2.2.1 General methods used throughout this thesis

All chemicals, unless otherwise stated, were purchased from Sigma Aldrich Ltd. A comprehensive list of manufacturer and supplier details can be found in Appendix II. All solutions were made using deionised water (Option 3 Water Purifier; Elga). Filtered seawater was prepared by passing through a 0.45 µm cellulose nitrate membrane filter (Whatman International Ltd.) under vacuum. The orbital mixer (OM501; Denley Instruments Ltd.) was always operated at room temperature, 170 rpm with a 1-inch throw. All vessels used for culturing algae were acid washed for 4 days with 10% (v/v) hydrochloric acid (VWR International) and extensively rinsed with deionised water (Probert and Klaas, 1999). The speed vac consisted of a centrifuge chamber (GL11 Gyrovap; Philip Harris Scientific) connected to a condenser (CT02-50; Christ GmbH) and a vacuum pump (MZ2C; Vacuubrand GmbH). All equipment, solutions and media that could be autoclaved were sterilised by autoclaving at 121 ºC at 15 psi for 15 min (ASA270; Astell Scientific Ltd.). Other liquids were sterilised by filtration through sterile 0.2 µm cellulose acetate syringe filters (Nalgene). Operations requiring sterile conditions were performed in a laminar flow hood (M51424/2; Microflow Ltd.). Statistical analyses were performed using the SPSS package version 12.0 for Microsoft Windows. Data were tested for normality typically with the Shapiro-Wilk test (Shapiro and Wilk, 1965), as this test is most suitable for smaller sample sizes (Shapiro and Wilk, 1965; Gaten, 2000; Mendes and Pala, 2003; Garson, 2007), and for homogeneity of variance with Levene's test

(Levene, 1960). Only where data deviated from normality or equal variance are test results given. Where data violated the assumptions of normal distribution and equal variance, which are required for ANOVA, ANOVA was still performed because it is robust enough to cope with such deviations (Field, 2000; Pallant, 2005). Standard deviation (SD) and standard error (SE) values are always given of the mean.

2.2.2 Small-scale batch culture system

The small-scale batch culture system consisted of 12 bottles positioned on a wooden rack supplied with air in a temperature-controlled lightbox (Figure 2.1). It was operated as a closed system to reduce the risk of microbial contamination.

2.2.2.1 The lightbox

The lightbox was composed of reflective white sides, white roof and lightly coloured floor and had internal dimensions of 90 x 60 x 44 cm (w x h x d). Four 61 cm cool white fluorescent tubes (General Electric F18W/33; Lightbulbs Direct Ltd.) provided illumination from the top and rear. Irradiance at bottle-height was measured with a digital quantum light meter (LI-189; LI-COR Biosciences UK Ltd.) and this ranged from 25 µmol s^{-1} m^{-2} at positions 1 and 12 to 45 µmol s^{-1} m^{-2} at positions 6 and 7. A 14:10 h light:dark regime operated and the temperature was controlled at 20 ºC.

2.2.2.2 Culture bottles

Culture bottles consisted 470 mL transparent polycarbonate centrifuge bottles (VWR International) with rubber stoppers (Ø 40.5 mm, 2-hole; Fisher Scientific) (Figure 2.2). One stopper hole was fitted with 6 cm and the other 19 cm of 316L stainless steel tubing (external Ø 6.35 mm; RS Components Ltd.). Silicone rubber tubing (bore

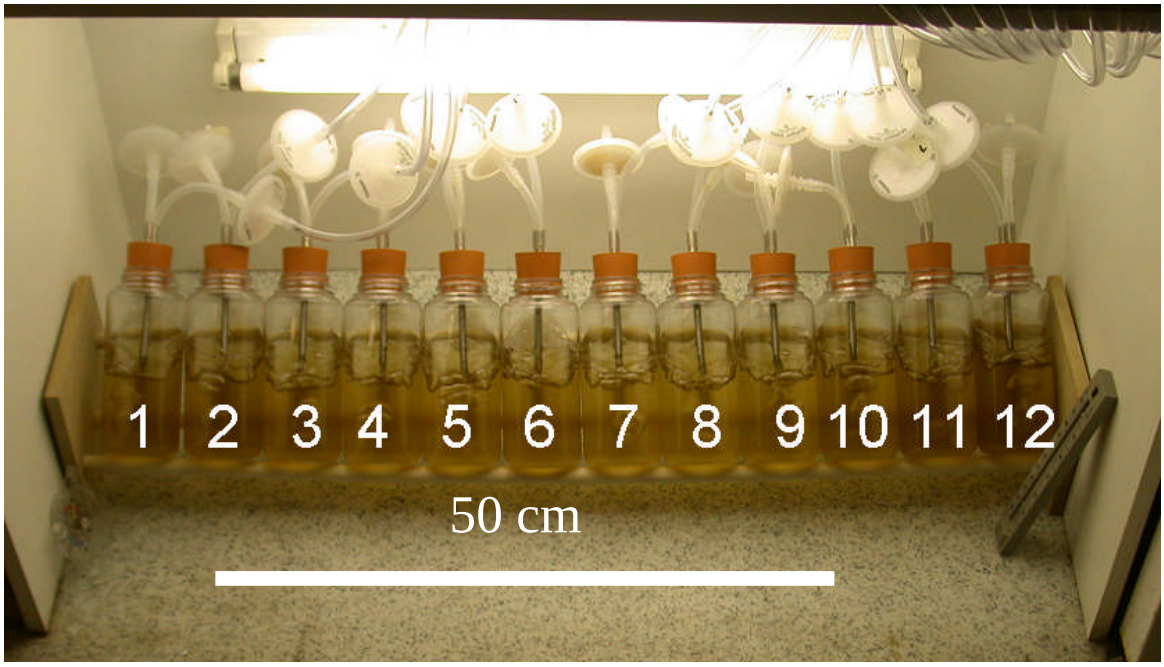


Figure 2.1 – The custom small-scale batch culture system used to axenically culture *P. tricornutum* showing bottle positions numbered 1 to 12.

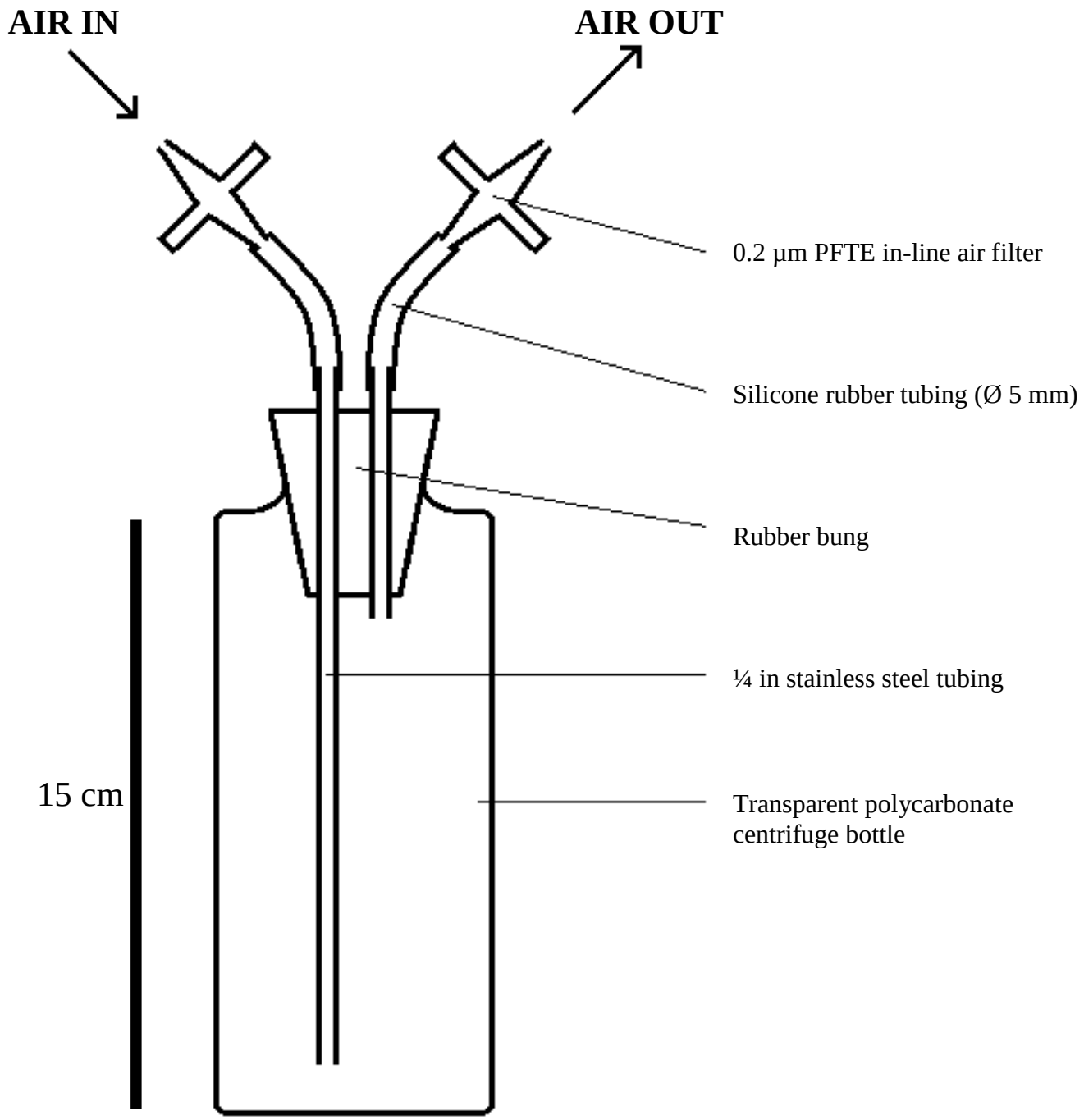


Figure 2.2 – Schematic diagram of the 470 mL transparent polycarbonate centrifuge bottles used in the custom batch culture system. Filter sterilised air flowing into the bottle is directed to the bottom of the vessel for gaseous exchange and culture mixing. Diagram is not to scale.

Ø 5 mm; Fisher Scientific) was attached to external ends of the stainless steel tubing and to this were connected 0.2 µm PTFE air filters (Midisart 2000; Sartorius AG). The rack consisted of two square ends (15 cm x 15 cm; medium density fibre board) connected by 3 x 87 cm length of wooden doweling (Ø 15 mm) that angled the bottles at 60° to the floor.

2.2.2.3 *Phaeodactylum tricornutum*

A slope of axenic *P. tricornutum* Bohlin SAG 1090-6 was purchased from the Experimental Phycology and Culture Collection of Algae (EPSAG), University of Göttingen. This strain was isolated in 1951 by Dr. M. R. Droop from a rock pool on the island of Segelskär, Finland (EPSAG, 2007a). The strain is held elsewhere as CCAP 1052/6 and UTEX 646. A volumetric flask containing 300 mL sterile modified half-strength Enriched Seawater, Artificial Water medium (modified ESAW; Appendices III, IV and V) was aseptically inoculated with a loopful of *P. tricornutum* from the original slope and cultured in the lightbox. The culture was swirled daily by hand and every 17-20 days, 15 mL culture was used to aseptically inoculate a fresh flask. This process of sub-culture continued throughout the study. Oval and fusiform morphs dominated cultures whilst the triradiate morph was only seen very rarely. The initial stock culture was confirmed as axenic (Appendix VI), however, throughout the study sub-cultures were checked for contamination by streaking the culture across sterile 2216E agar plates (Difco) that were monitored for non-*P. tricornutum* growth. Very occasionally, colonies other than *P. tricornutum* developed that indicated contamination and so these cultures were not used.

2.2.2.4 Inoculum

Sterile bottles were filled with 300 mL sterile modified ESAW medium. Each bottle was inoculated with 3.15 x10^7 cells from a 17-20 day-old volumetric flask culture to give a final concentration 1 x10^5 cells mL^{-1}. Inoculum volume was kept constant between batches by topping up to 15 mL with sterile modified ESAW medium. Air was supplied by a pump (XX5522050; Millipore) at a rate of 2.4 L min^{-1} $bottle^{-1}$ (7.6 v/v/min). To counter the effects of variable irradiance across the lightbox, every 48 h the bottles occupying positions 6 and 7 were moved to positions 1 and 12, respectively (Figure 2.1); bottles in the other positions moved one position towards to the middle. At the same time, each bottle was topped up to 315 mL with sterile deionised water to counter small evaporative losses.

2.2.3 *P. tricornutum* growth in small-scale batch culture system

To assess the growth of *P. tricornutum* in the small-scale batch culture system 6 cultures were grown in bottles (as Section 2.2.2) for 12 days and aseptically sampled every 24 h by removing 1 mL of culture. Each culture had the absorbance at 750 nm (A_{750}) determined using a spectrophotometer (Ultraspec K; LKB Biochrom) with modified ESAW medium as reference. This experiment was performed in triplicate (three batches of six bottles). To confirm that culture A_{750} could be used to assess, quickly and reliably, algal growth the relationship with algal cell number per mL was investigated. This was performed by determining total algae per mL each day for four cultures of the first batch using a New Improved Neubauer haemocytometer (Weber Scientific International Ltd.) under 25x objective lens in bright field (Leitz Diaplan; Leitz Wetzlar). A mean of four counts was performed for each sample.

2.2.4 Preparation of algal cell extracts

Algal cells were harvested by centrifugation at 3580 *g* for 11 min at 4 ºC (Beckman J2-21M/E; Beckman Coulter Ltd.). The supernatant was discarded and the cell pellet resuspended in 50 mL sterile 3.2% aqueous NaCl. The cell suspension was transferred to a sterile 50 mL falcon tube and centrifuged at 3000 *g* for 15 minutes at 4 ºC. After discarding the supernatant, the cell pellets were stored at –80 ºC. For extraction, each cell pellet was thawed and resuspended in 1 mL sterile 3.2% NaCl solution and re-centrifuged at 12000 *g* for 2 min at ~20 ºC before discarding the supernatant. The cell pellet was completely resuspended in 0.6 mL methanol:water (5:1) and ultrasonicated (Status US 200; Philip Harris Scientific) on ice using a Ø 2 mm probe (MS72; Philip Harris Scientific) for 2 min in 30 s bursts with 30 s breaks to reduce sample warming. The probe was set to an active:passive interval of 0.6:0.4 s and was wiped clean between samples. Lysate was kept on ice, agitated on the orbital mixer for 1 h and then cellular debris removed by centrifuging at 12000 *g* for 1 h at 4 ºC. The supernatant was transferred to a sterile 1.5 mL Eppendorf tube and dried to completion using the speed vac at 30 ºC. Dried extracts were massed and reconstituted to a concentration of 60 mg mL^{-1} with sterile 50 mM HEPES aqueous solution pH 7.8 (Acros Organics). The algal extracts were stored at –80 ºC until use. To assess the extraction efficiency, four cell pellets were re-extracted twice more and tested for antibacterial activity (see Section 2.2.7). Total antibacterial activity was calculated as the activity in each tested 4 µL sample multiplied by total volume of extract.

2.2.5 Testing for antibacterial activity by radial diffusion assay

The radial diffusion assay (RDA) was used to test cell extracts for antibacterial activity and a broad range of bacterial species was selected for use in this assay including Gram positive, Gram negative, marine and terrestrial strains. This increased the possibility of identifying numerous antibacterial molecules that may act on different targets. The bacterial species used for the RDA are listed in Table 2.1. *Staphylococcus aureus* SH1000 was gifted by Dr Kate Cosgrove (University of Sheffield, UK), *Pseudomonas aeruginosa* HW was gifted by Dr Andrew Mearns-Spragg (Aquapharm Bio-Discovery Ltd.), *Pseudomonas aeruginosa* 10775 was purchased (NCIMB Ltd.) and all other bacterial strains are held in the Comparative Immunology and Marine Microbiology Group collection, University of St. Andrews. Each bacterial strain was kept on appropriate agar plates (Table 2.1) at 4 ºC until required and sub-cultured onto fresh plates every 4 weeks. For long-term storage, glycerol stocks were made by adding 0.3 mL sterile glycerol to 1.7 mL of stationary phase bacterial culture and kept at –80 ºC.

2.2.6 Standardisation of the radial diffusion assay

To ensure that the RDA could be performed in a standard and reproducible way it was necessary to have an accurate and rapid method for enumerating bacterial colony forming units (cfu). For each bacterial species the relationship between cfu mL^{-1} and culture absorbance at 570 nm (A_{570}) was tested to ensure that the latter could be used as a reliable measure for cfu. A universal bottle containing 15 mL appropriate medium (Table 2.1) was inoculated with a single bacterial colony and cultured on the orbital mixer until late exponential phase. Cells were harvested by centrifugation at 2060 *g* for 10 minutes at 4 ºC. The cell pellet was washed by discarding the

Table 2.1 – Bacterial strains used throughout experimentation giving details on their Gram stain, the growth medium used, the salt concentration of the wash solution, the incubation temperature and the positive control used in the RDA.

Bacterium	Strain number	Gram stain	Growth medium[a]	NaCl wash solution (%)	Incubation temperature (ºC)[b]	Positive control for RDA (µg mL^{-1})
Escherichia coli	B Strain Type	-ve	LB	0.9	37	Ampicillin (40)
Listonella (= *Vibrio*) *anguillarum*	MT 1637	-ve	2216E	3.2	25	Cecropin P1 (100)
Micrococcus luteus	NCIMB 9278	+ve	2216E	3.2	25	Melittin (100)
Planococcus citreus	NCIMB 1493	+ve	2216E	3.2	25	Melittin (100)
Pseudomonas aeruginosa	NCIMB 10775	-ve	LB	0.9	37	Melittin (100)
Pseudomonas aeruginosa	HW	-ve	LB	0.9	37	Melittin (100)
Staphylococcus aureus	SH1000	+ve	LB	0.9	37	Ampicillin (5)
Staphylococcus epidermidis	ATCC 10145	+ve	Nutrient[c]	n/a[d]	37	n/a[d]
Staphylococcus epidermidis	CIG	+ve	LB	0.9	37	Melittin (100)

[a] This medium was used for liquid cultures and modified appropriately for agar plates or for the RDA.

[b] Incubation temperature used for static growth on agar plates.

[c] Nutrient agar (Sigma Aldrich Ltd.).

[d] Not applicable as this bacterium was not used for RDA.

supernatant and making the volume up to 10 mL using appropriate sterile NaCl solution (Table 2.1). The cell pellet was resuspended using a vortex (Whirlimixer; Jencons Scientific Ltd.) and centrifuged as before. This procedure was repeated to complete the washing. This initial cell suspension (100 %) was diluted with appropriate sterile NaCl solution (Table 2.1) to give cell suspensions of 75 %, 50 %, 25 %, 10 % or 1 %. The A_{570} of cell suspensions was determined using the spectrophotometer with medium as reference. In turn, 1, 10, 25, 50, 75 and 100 % cell suspensions were serially diluted in NaCl solution and 100 µL of each dilution spread across triplicate agar plates. These were incubated at appropriate temperature (Table 2.1) until colonies could be counted. This experiment was performed twice for each bacterial species.

Data were collected to determine exponential growth phase. This is important, as bacteria are more susceptible to antibiotics when they are actively growing (Ganz *et al*., 1985). Every 4 h, until 24 h post-inoculation, at least three cultures for each bacterium were harvested, washed and the A_{570} determined as above. *M. luteus* cultures were also sampled after 26, 30, 36, 42, 48 or 54 h. For *Ps. aeruginosa* 10775 cultures were harvested every 6 h until 30 h.

To check that there was a significant relationship between the concentration of an antibiotic and clear zone area on a RDA plate, standard solutions of melittin (Sigma Aldrich Ltd.) in water at concentrations of 50, 100, 200, 400 and 600 µg mL^{-1} were tested as positive control against *P. citreus* (as Section 2.2.7). Melittin is an antibacterial peptide with activity against *P. citreus*.

2.2.7 Performing the radial diffusion assay

The RDA method was a modified version of the two-layer radial diffusion assay technique as described by Lehrer *et al*. (1991). A 15 mL 1/10-nutrient strength lower agar appropriate for the target bacterium (Table 2.1; prepared as Appendix III) was melted by heating to 100 ºC and cooled to ~40 ºC. Then 1 x10^7 cfu of exponentially growing bacteria was added (prepared as above) and the agar mixed gently by hand. It was immediately poured into a sterile square Petri dish (120 x 120 mm; Greiner Bio-One) on a level surface. Once set, the plate was briefly chilled (20 min at 4 ºC) and wells of Ø 3 mm were bored using a sterile plastic Pasteur pipette. Four µL of sample was added to each well and a suitable positive control (Table 2.1) and appropriate negative control(s) added to each plate. Samples were allowed to diffuse for 4 h at 4 ºC. For the top layer, 15 mL full-nutrient strength upper agar appropriate for the target bacterium (Table 2.1; prepared as Appendix III) was melted by heating to 100 ºC and cooled to ~40 ºC. This was poured over the lower agar, allowed to set, and the plate incubated at the appropriate temperature for the bacterial species (Table 2.1). After 24 h, ~20 mL stain was poured on to the agar surface. This stain consisted of 20 mg Coomassie brilliant blue reagent (G-250; Pierce), 210 mL methanol (VWR International), 630 mL deionised water and 150 mL 37% formaldehyde. The plate was gently agitated on a rocker (A600; Denley) for 16 h. The stain was poured away and clear zones measured with a rule to the nearest half millimetre. Area of bacterial growth inhibition was calculated as total area of clear zone minus the area of the well.

2.2.8 Statistical analyses

The data collected for each day to test the effect of culture bottle position on *P. tricornutum* growth was shown to be normally distributed by Shapiro-Wilk test and

show equal variances by Levene's test except day 12 where the variances were not equal ($F_{5,12} = 3.271$, $p < 0.05$). One-way ANOVA were performed for each day on the A_{750} data generated for the three batches. For all analyses $p \leq 0.05$ was considered significant.

2.3.0 Results

2.3.1 *P. tricornutum* growth in small-scale batch culture system

Algal growth was monitored for 12 days to assess how *P. tricornutum* grows under the conditions encountered in the small-scale batch culture system. The growth curve showed that, after inoculation, the culture reached late exponential phase between days 9 – 11 with onset of stationary phase at day 11 (Figure 2.3). The relationship between culture A_{750} and algal cell number per mL was investigated and, as expected, this was very highly significant ($p < 0.001$) confirming that A_{750} can be used to measure algal cell number (Figure 2.4). Culture A_{750} was favoured over other wavelengths, such as those that rely on levels of chlorophyll *a*, because cell pigment contents have been shown to change during algal growth (Borowitzka, 1988b; Fernández Sevilla *et al*., 2004).

One-way ANOVAs confirmed that there was no significant difference ($p > 0.05$) between bottles each day for the growth of *P. tricornutum* therefore confirming that algal growth is consistent for each bottle in the small-scale culture system.

2.3.2 Preparation of algal extracts

Extraction efficiency for antibacterial molecules was estimated by repeated re-extraction of cell pellets. The initial extraction removed a mean total clear zone area

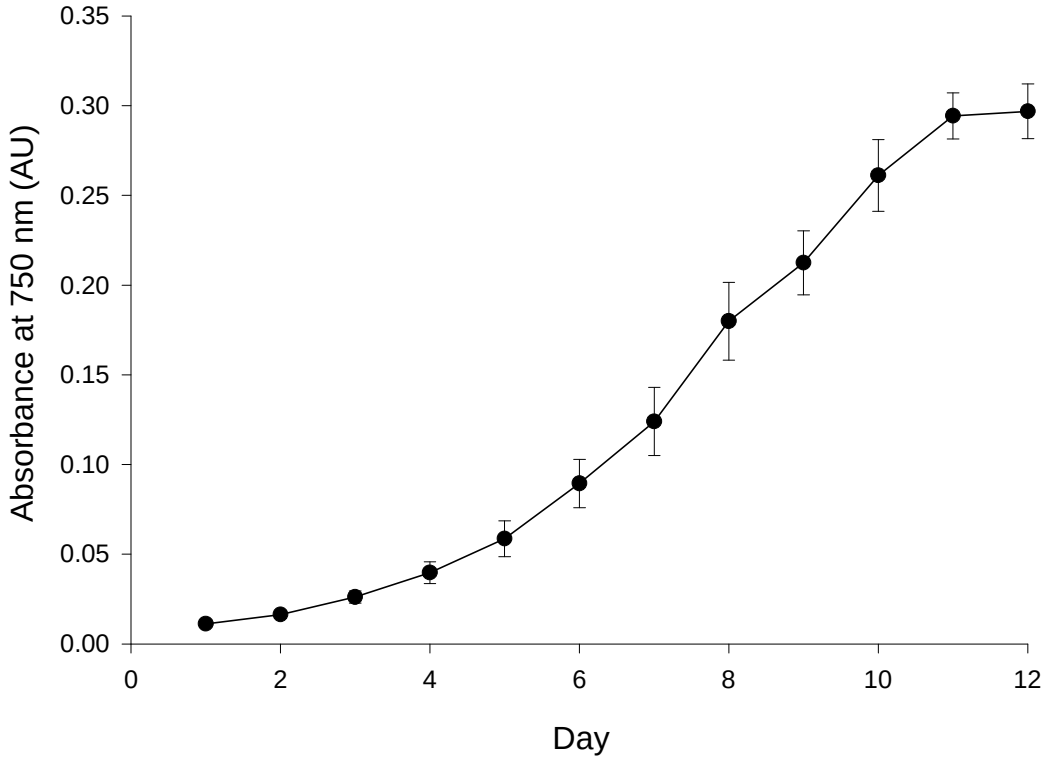


Figure 2.3 - Growth of *P. tricornutum* in modified ESAW medium in the custom small-scale batch culture system for 12 days at 20 ºC. n = 18; error bars ± 1 SD.

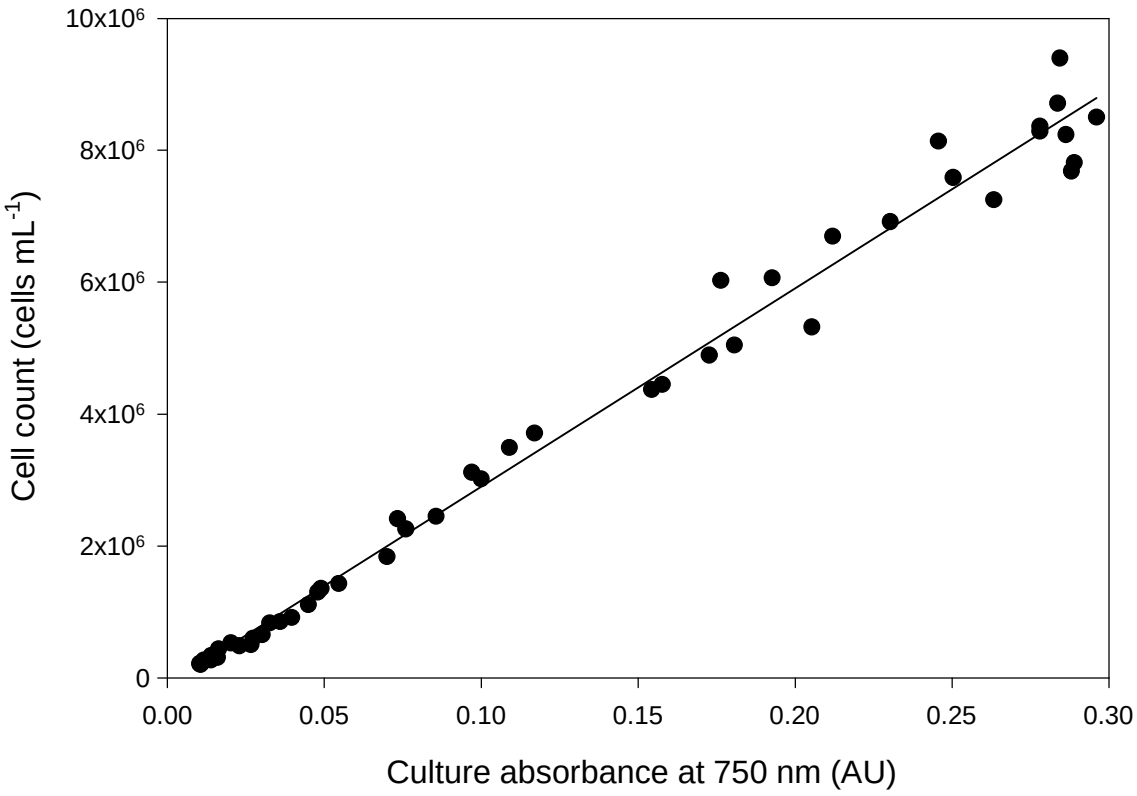


Figure 2.4 – Growth of *P. tricornutum* in modified ESAW medium in the custom small-scale batch culture system showing the relationship between culture absorbance at 750 nm and number of algal cells per mL. Regression: cells $mL^{-1} = 3.01 \times 10^{7} (A_{750}) - 1.09 \times 10^{5}$. Correlation is very highly significant ($F_{1,47} = 3514$, $r^2 = 0.987$; $p < 0.001$).

on the RDA (antibacterial activity) of 3,566 mm^2 (Figure 2.5). After first re-extraction a further area of 1,020 mm^2 antibacterial activity was extracted whilst the second re-extraction contained 431 mm^2 antibacterial activity (Figure 2.5). This meant that the initial extraction, first and second re- extractions contained 71 %, 20 % and 9 % respectively of the total antibacterial activity removed by the three extractions. Small quantities of methanol-soluble antibacterial compounds may have still been present after the second re-extraction but the initial extraction is efficient as it removes a large proportion of the antibacterial activity present in *P. tricornutum* cells. Methanol was selected as the extraction solvent because it has been used previously to obtain antibacterial extracts from *P. tricornutum* (Duff *et al*., 1966; Cooper *et al*., 1983; Kellam and Walker, 1989; Bickerdike, 2002).

2.3.3 Standardisation of the radial diffusion assay

Experiments were designed to show that culture A_{570} could be used to estimate the number of bacterial cfu in a washed bacterial suspension and for each bacterium highly significant relationships ($p < 0.01$) were found to exist between these parameters (Figure 2.6). This enabled the construction of growth curves for each bacterial species (Figure 2.7). These data mean that the lower layer of agar, used in the RDA, can be seeded consistently with an accurate number of exponentially growing bacterial cfu (1×10^7).

Different concentrations of the antibacterial peptide, melittin, were tested as positive control against *P. citreus* to confirm that a very highly significant relationship ($p < 0.001$) existed between the concentration of antibacterial compound and the area of bacterial growth inhibition that developed on the RDA plate (Figure 2.8). This

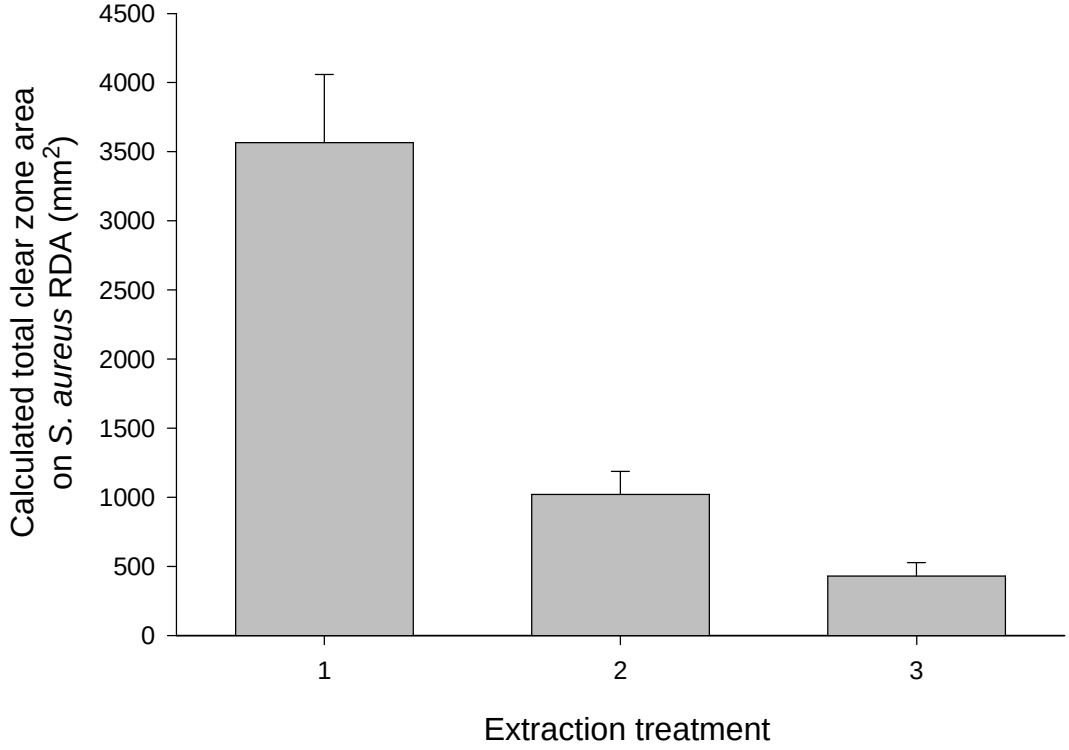


Figure 2.5 – Quantity of antibacterial activity against *S. aureus* in aqueous methanol cell extracts from sequential extraction of a *P. tricornutum* cell pellet: (1) the initial extraction; (2) re-extraction of the same cell pellet; and (3) a second re-extraction of the cell pellet. The data is presented as the total clear zone area calculated to be in the total volume of the extract. This was determined from the clear zone area caused by the 4 µL of extract tested. n = 4; error bars are ± 1 SE.

Figure 2.6 – Relationships between cfu mL^{-1} and cell suspension absorbance at 570 nm for (A) *E. coli* (regression: cfu mL^{-1} = 3.86 x10^8 [A_{570}] – 4.56 x10^7; $F_{1,10}$ = 14.0, r^2 = 0.542; $p < 0.01$); (B) *L. anguillarum* (regression: cfu mL^{-1} = 3.77 x10^8 [A_{570}] – 9.29 x10^6; $F_{1,10}$ = 141, r^2 = 0.927; $p < 0.001$); (C) *M. luteus* (regression: cfu mL^{-1} = 2.22 x10^7 [A_{570}] – 1.82 x10^6; $F_{1,10}$ = 42.1, r^2 = 0.789; $p < 0.001$); (D) *P. citreus* (regression: cfu mL^{-1} = 2.67 x10^8 [A_{570}] – 2.29 x10^7; $F_{1,10}$ = 177, r^2 = 0.941; $p < 0.001$); (E) *Ps. aeroginosa* 10775 (regression: cfu mL^{-1} = 4.07 x10^8 [A_{570}] – 9.09 x10^6; $F_{1,10}$ = 350, r^2 = 0.969; $p < 0.001$); (F) *Ps. aeruginosa* HW (regression: cfu mL^{-1} = 2.31 x10^8 [A_{570}] + 5.17 x10^6; $F_{1,4}$ = 31.0, r^2 = 0.857; $p < 0.01$); (G) *S. aureus* (regression: cfu mL^{-1} = 3.48 x10^8 [A_{570}] – 2.17 x10^5; $F_{1,10}$ = 330, r^2 = 0.968; $p < 0.001$); and (H) *S. epidermidis* CIG (regression: cfu mL^{-1} = 1.84 x10^8 [A_{570}] – 9.35 x10^5; $F_{1,10}$ = 240, r^2 = 0.956; $p < 0.001$).

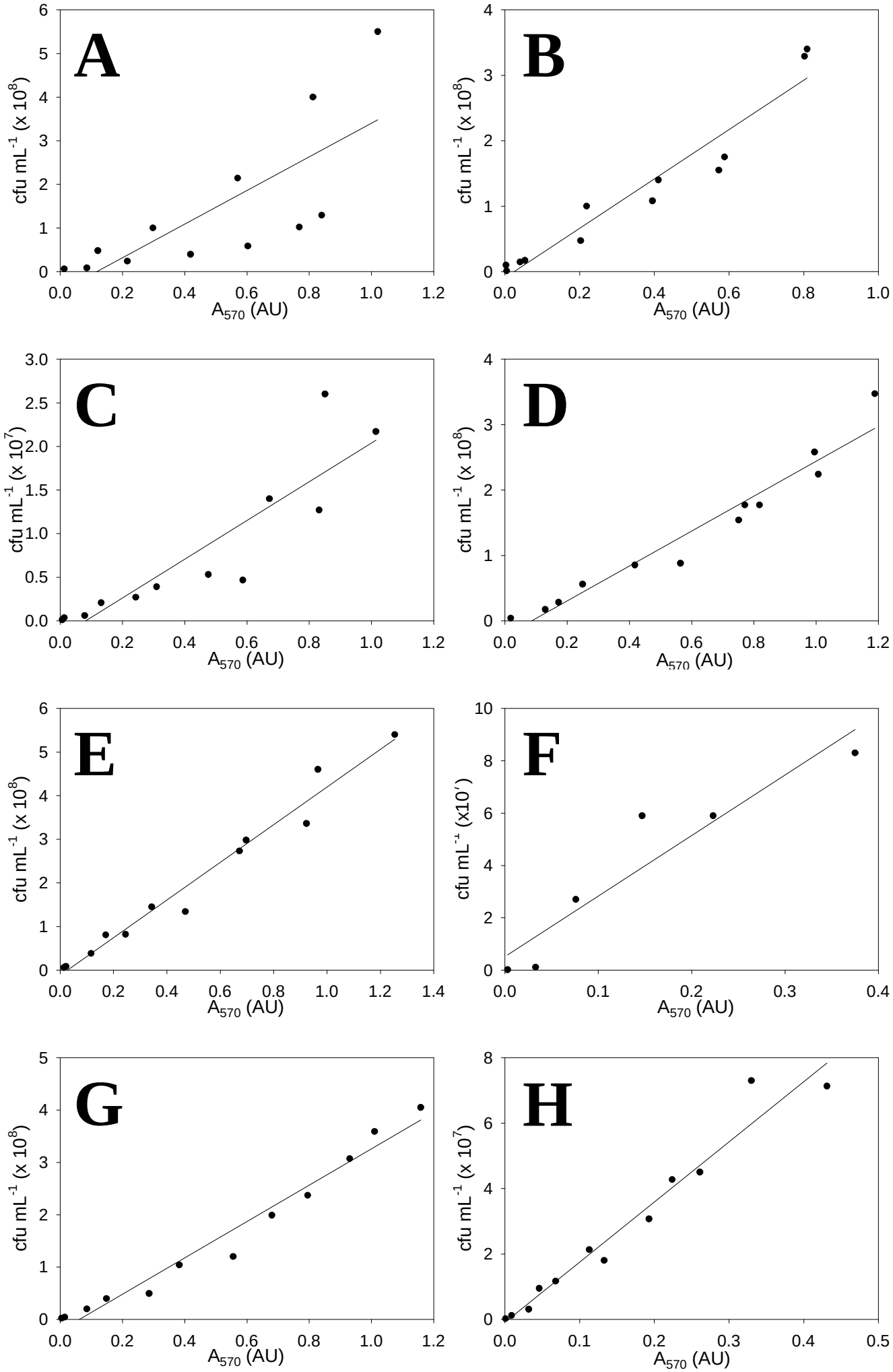
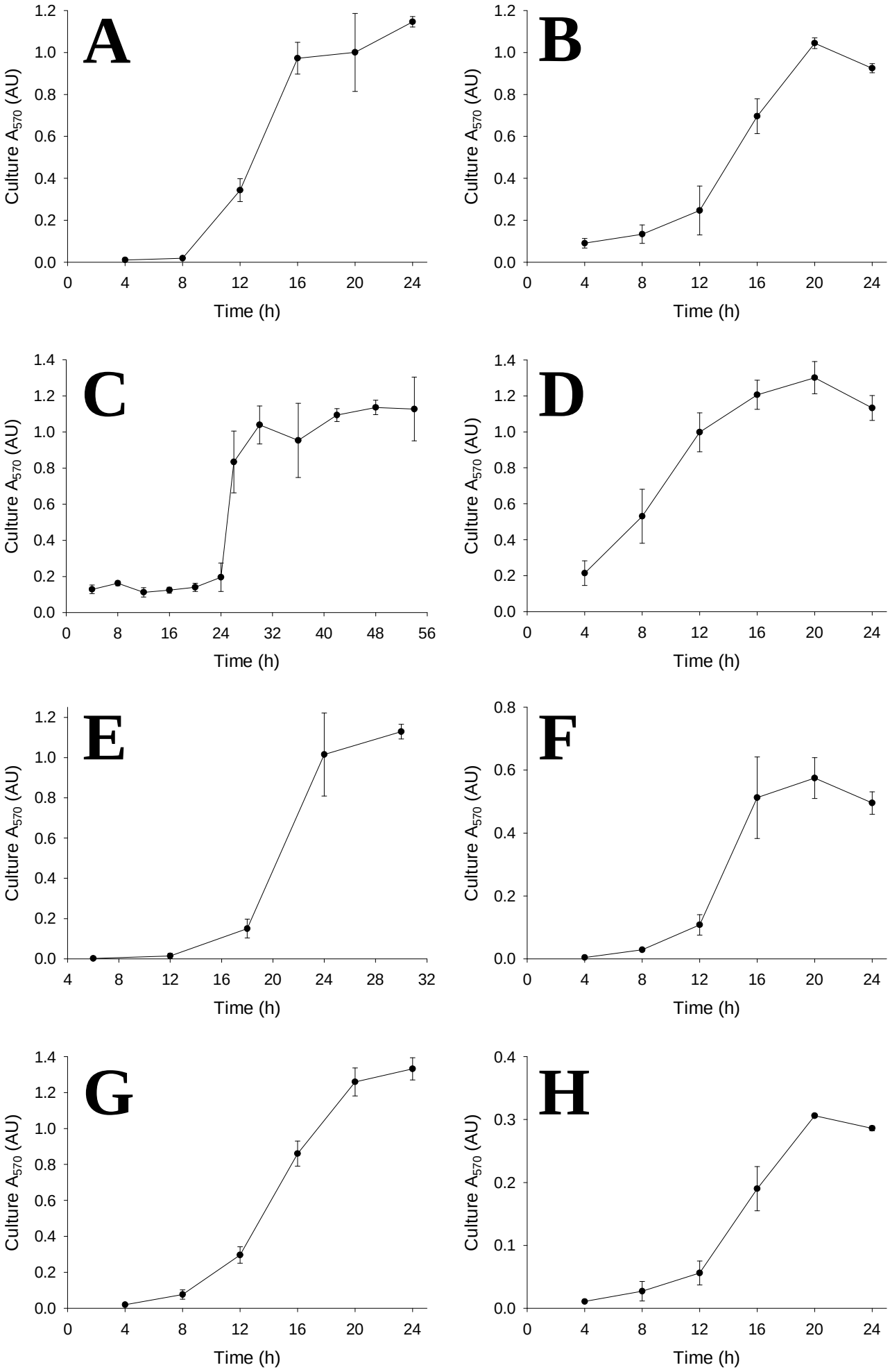

A
B
C
D
E
F
G
H
cfu mL-1 (x 10^8)
cfu mL-1 (x 10^7)
cfu mL-1 (x10')
A570 (AU)

Figure 2.7 – Growth of (A) *E. coli* , (B) *L. anguillarum*, (C) *M. luteus*, (D) *P. citreus*, (E) *Ps. aeroginosa* 10775, (F) *Ps. aeruginosa* HW, (G) *S. aureus* and (H) *S. epidermidis* CIG on the orbital shaker in universal bottles containing either sterile LB (A, E, F, G and H) or sterile 2216E medium (B, C and D). In all cases n = 3 (except B at 4 and 8 h where n = 6, at 12 and 16 h where n = 7; C at 24, 48 and 54 h where n = 6; and H at 12 and 16 h where n = 6); error bars are ± 1 SD (except B and F ± 1 SE).


A
B
C
D
E
F
G
H
Culture A_{570} (AU)
Time (h)

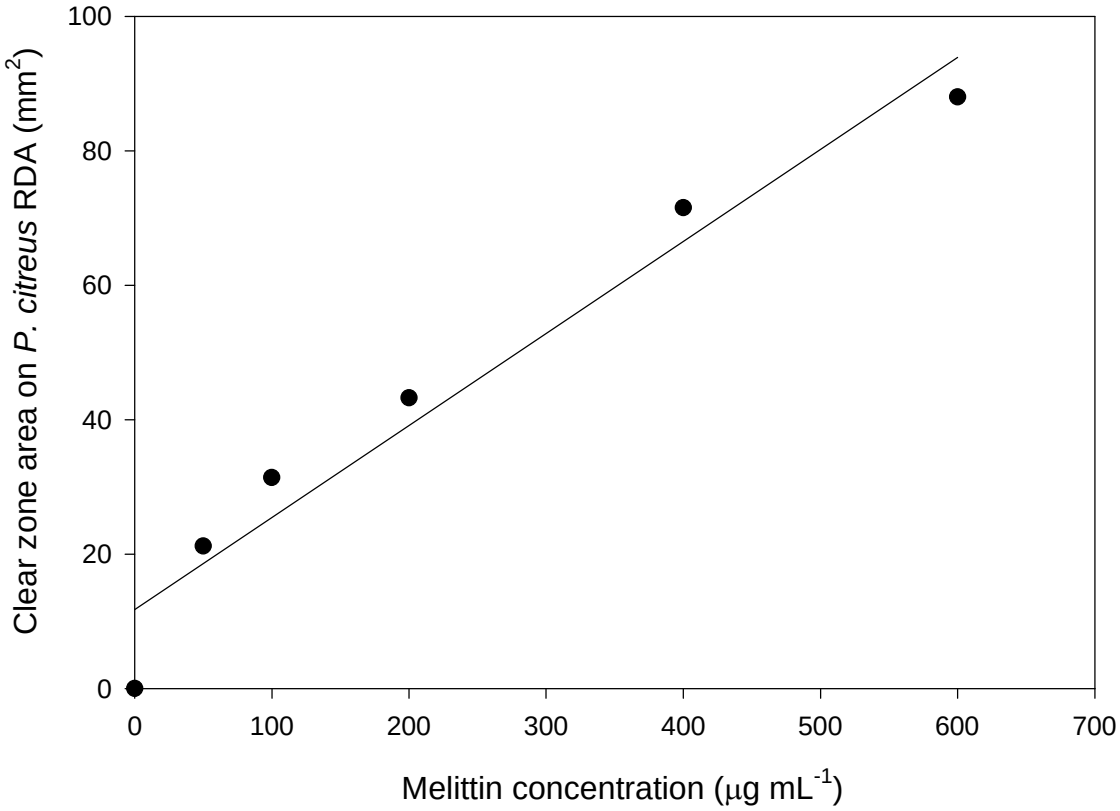


Figure 2.8 – Antibacterial activity of melittin against *P. citreus* as measured by clear zone area on RDA plate showing that the concentration of an antibacterial compound is proportional to the area of bacterial growth inhibition. The correlation is very highly significant ($F_{1,17} = 314$, $r^2 = 0.949$; $p < 0.001$); error bars not visible.

confirmed that the RDA could be used to quantify antibacterial activity and detect differences in the concentration of antibacterial compounds.

2.4.0 Discussion

The growth of axenic *P. tricornutum* under the specific growth conditions of the small-scale batch culture system has been characterised and, importantly, it was found that under these conditions culture bottle position had no significant effect on the growth of the alga. These cultures yielded cell extracts with antibacterial activity. The RDA method was standardised so that it can be performed in a reproducible manner thus minimising inter-assay variability.

A *P. tricornutum* batch culture system that permits reproducible and predictable growth was designed and implemented. The RDA method was standardised with particular attention paid to ensuring that the bacterial inoculum for the lower layer of agar would be seeded with the same number of cells at the same stage of the growth curve and therefore in the same physiological condition. The RDA can provide a measure of the level of antibacterial activity of *P. tricornutum* cell extracts but it cannot provide any insight into the qualitative make up of the extract at this stage. Greater inhibition zones could simply result from greater quantities of the same antibacterial compound(s), however, this could also arise due to qualitative changes in the compounds present in the extract, for example greater quantities of different antibacterial compound(s) with the same potency, or a different compound(s) in a lower quantity that has greater potency. Alternatively there could be quantitative or qualitative changes in the levels of compounds that mask or antagonise antibacterial activity.

Having characterised the growth of *P. tricornutum* in a model culture system and standardised the methods for testing for antibacterial activity, further studies can be performed to investigate the temporal production of antibacterial compounds by this alga. The qualitative nature of the antibacterial compounds present in cell extracts of *P. tricornutum* can also now be considered.

Chapter 3: Production of antibacterial compounds by *P. tricornutum* and preliminary studies on their chemical properties

3.1.0 Introduction

Before isolating antibacterial compounds from *P. tricornutum* it is necessary to determine if these are released from or are confined to the algal cells, if their production changes during growth and if the cell's morphology affects the level of antibacterial activity. Such studies enable the optimal production of material for the subsequent isolation of the active compounds. Further, to permit successful isolation by bioassay-guided fractionation, it is necessary to select susceptible bacterial species and make an assessment for the stability and chemical nature of the active compounds.

This chapter describes preliminary studies to characterise the antibacterial activity from cultures of *P. tricornutum* using the small-scale batch culture system. The effect of bottle position in the batch culture system is tested to ascertain if this has a significant effect on the level of antibacterial activity in cell extracts prepared from the cultures, thus confirming whether the system is suitable for such studies. Localisation of antibacterial compounds are investigated because active compounds can be released by the microalgae into the culture medium (Berland *et al*., 1972; Cooper *et al*., 1983; Trick *et al*., 1984; Cannell *et al*., 1988) or can be found within the cells and require solvent extraction (Duff *et al*., 1966; Debro and Ward, 1979; Viso *et al*., 1987; Cannell *et al*., 1988; Kellam and Walker, 1989; Chu *et al*., 2004). The spectrum of antibacterial activity of cell extracts is also determined to enable the selection of susceptible bacteria for bioassay-guided isolation of active compounds.

To allow the determination of optimal harvest time for greatest yield of antibacterial compounds the production of antibacterial activity is monitored over time because antibacterial activity of cell extracts can change during growth (Debro and Ward, 1979; Cooper *et al*., 1983). In addition, the stability of the compounds responsible for the antibacterial activity of cell extracts is investigated with respect to changes in heat, pH and salinity. Similarly, the qualitative nature of the antibacterial compounds is studied by digesting cell extracts with broad-spectrum proteinases, to assess the contribution of proteinaceous species with measurements of protein and fatty acid concentration in antibacterial extracts providing further insight. Finally, the affect of cell morphology on the level of antibacterial activity in cell extracts is analysed.

3.2.0 Materials and methods

3.2.1 Testing *P. tricornutum* culture supernatant for antibacterial activity

Algal cells were removed from three 10-day-old cultures (cultured as Section 2.2.2) by centrifuging at 3580 *g* for 11 min at room temperature. The supernatant was collected and filtered through a sterile 0.2 µm cellulose acetate syringe filter. This was tested for its ability to inhibit bacterial growth (*E. coli*, *L. anguillarum*, *M. luteus*, *P. citreus S. aureus* or *S. epidermidis* CIG), first by RDA (as Section 2.2.7) and second by turbimetric assay. For the turbimetric assay, into each well of a sterile 24-well plate (flat-bottomed; Corning Inc.) was dispensed 0.4 mL sterile LB medium solution (100 g L^{-1}; this gave full-strength LB medium in the final well volume). To each experimental well was added 1.6 mL sterile culture supernatant. To each control well was added 1.6 mL modified ESAW. Each well was then inoculated with 5 $x10^5$ cfu *S. aureus* (exponential phase) and incubated on an orbital mixer (operating as Section 2.2.1). At 6, 12, 22 and 28 h bacterial growth was determined for three

experimental wells and the three corresponding control wells by removing 1.5 mL culture and measuring A_{570} with a spectrophotometer (using as references LB plus culture supernatant or modified ESAW). This experiment was repeated for the other five bacterial species above, except that 0.4 mL sterile 2216E medium (187 g L^{-1}) was used for marine species.

3.2.2 Production of antibacterial compounds by *P. tricornutum* during culture

To measure antibacterial activity in *P. tricornutum* cell extracts during the growth curve the small-scale batch culture system was set up and inoculated as Section 2.2.2. At days 4, 6, 8, 10, 12 and 14 post-inoculation, two algal cultures were selected at random for harvest as in Section 2.2.4 (using Microsoft Excel random number generator) and the A_{750} determined using modified ESAW medium as reference. To maintain consistent conditions of light and airflow in the remaining flasks, harvested bottles were replaced with water-filled bottles. The experiment was performed twice to give quadruplicate replicates for each day.

Cell pellets were extracted (Section 2.2.4), resuspended to 60 mg mL^{-1} and tested for antibacterial activity against *S. aureus* by RDA (Section 2.2.7). Antibacterial activity per cell was calculated by dividing the total number of cells extracted by the total quantity of antibacterial activity in the extract (this is calculated by dividing the quantity of antibacterial activity in 4 µL of sample by four then multiplying by total extract volume). Yield of antibacterial activity was calculated as the product of the antibacterial activity of each cell and the number of cells at harvest.

3.2.3 Antibacterial activity of cell extracts between culture bottles

The effect of culture bottle position in the small-scale batch culture system was tested to see whether this affected the level of antibacterial activity in extracts by inoculating the culture system, harvesting at day 10, extracting and testing for activity versus *S. aureus* (as Sections 2.2.2, 2.2.4 and 2.2.7). This experiment was performed in triplicate.

3.2.4 Spectrum of antibacterial activity in *P. tricornutum* cell extracts

The spectrum of antibacterial activity for 30 cell extracts that came from cultures grown, harvested at day 10 and prepared as Sections 2.2.2 and 2.2.4. These were tested for antibacterial activity by RDA (Section 2.2.7) against Gram positive and Gram negative marine and terrestrial bacteria: *E. coli*, *L. anguillarum*, *M. luteus*, *P. citreus*, *Pseudomonas aeruginosa* 10775, *Pseudomonas aeruginosa* HW, *S. aureus* and *S. epidermidis* CIG (n = 30; except *Ps. aeruginosa* 10775 where n = 8, and *Ps. aeruginosa* HW where n = 5).

3.2.5 Stability of antibacterial activity in cell extracts

Antibacterial activity in cell extracts (prepared as Sections 2.2.2 and 2.2.4) were investigated for stability under various physicochemical conditions. After experimental treatments, in each case samples were tested for antibacterial activity by RDA against *S. aureus* (Section 2.2.7).

To examine the effect of temperature on cell extracts, previously prepared cell extract (60 mg mL^{-1} in sterile 50 mM HEPES solution; pH = 7.8) was defrosted from –80 ºC and divided into seven aliquots of 8 µL. One was the control treatment (returned to

–80 ºC), whilst the others were kept at –20, 4, 25, 37 or 55 ºC for 4 h. Also, one aliquot was autoclaved at 121 ºC for 15 min. After treatment, extracts were returned to –80 ºC before being defrosted and assayed for antibacterial activity. Treatments were performed for three cell extracts.

To assess the stability of cell extract to different pH, five aliquots of dried extract were resuspended in sterile buffers at pH 3, 5, 7, 9 or 11 to 20 mg mL^{-1}. McIlvaine's sodium phosphate/citric acid buffer (Hale, 1966) was used to obtain pH values between 3 – 7, whilst Sørensen-Walbum glycine/NaCl/sodium hydroxide buffer (Hale, 1966) was used for pH 9 and 11. The buffer pH values were confirmed post-autoclaving. Extracts were stored for 24 h at 4 ºC before being assayed for antibacterial activity. Each sterile buffer acted as negative controls in the RDA. The experiment was repeated for a further two cell extracts.

Third, to test the stability of antibacterial activity in cell extracts at different salinities dried extracts were resuspended to 20 mg mL^{-1} in sterile deionised water or a sterile solution of 1, 2, 3, 4 or 5 % NaCl. The samples were stored for 24 h at 4 ºC before being assayed for antibacterial activity. Each salinity solution was a negative control. The experiment was repeated for a further two cell extracts.

Finally, two cell extracts (prepared as Sections 2.2.2 and 2.2.4) were subjected to digestion by broad-spectrum proteinase either proteinase K or trypsin to evaluate the role of proteins in the antibacterial activity. For experimental group A, 10 μL of 1 mg mL^{-1} proteinase K solution was added to 10 μL extract and incubated for 4 h at 37 ºC; for experimental group B, 10 μL of 1 mg mL^{-1} trypsin solution was added to 10 μL

extract and incubated for 4 h at 25 ºC. Two negative controls consisted of proteinase K or trypsin (10 µL) and 10 µL sterile 50 mM HEPES only. An additional negative control consisted of 10 µL cell extract with 10 µL water that was incubated at 25 or 37 ºC.

To ensure that the proteinase K and trypsin were active at the concentrations used, additional controls consisting of 10 µL of the commercially available antibacterial peptide, melittin (100 µg mL^{-1} in water), with 10 µL of proteinase K or trypsin solutions were incubated at the appropriate temperature for 4 h. To ensure that the melittin was antibacterial on the RDA, a further treatment of 10 µL melittin solution and 10 µL water was included. These treatments were performed in duplicate. The melittin samples (and associated positive and negative controls) were tested versus *P. citreus* and not *S. aureus* because the latter is not susceptible to killing by melittin at the concentration tested.

3.2.6 Total mass of protein in *P. tricornutum* cell extracts

The total mass of protein in the 23 antibacterial cell extracts produced in Section 3.2.2 was investigated using the Bradford assay (Bradford, 1976). Briefly, 492 µL double-deionised water and 500 µL Coomassie Protein Assay Reagent (Pierce) was added to 8 µL cell extract and mixed by inversion before determining the absorbance at 595 nm (Ultrospec 3300 pro; Biochrom Ltd.). A calibration curve (plotting protein concentration against absorbance at 595 nm) was produced for a series of albumin standard solutions ranging 0.05 – 2 mg mL^{-1} (Sigma Aldrich Ltd.).

3.2.7 Total mass of fatty acids in *P. tricornutum* cell extracts

The total mass of fatty acids was investigated for 50 µL of each of the 23 *P. tricornutum* cell extracts found to be antibacterial (produced in Section 3.2.2). This was kindly performed by Dr. Mike Walton (Sea Mammal Research Unit, University of St Andrews). Briefly, 50 µg of an internal standard (saturated fatty acid 23:0) was added to each cell extract and methyl esters of fatty acids (FAME) prepared by dissolving each extract in 1 mL toluene and adding 2 mL 1 % sulphuric acid in methanol. The mixture was left overnight in a stoppered tube flushed with nitrogen at 50 ºC. Then 2 mL of 2 % potassium bicarbonate in water was added and the esters extracted twice with 3 mL hexane:diethyl ether 1:1 using Pasteur pipettes to collect the upper layers. The combined fractions were washed with 4 mL water and any residual water was removed by mixing with anhydrous sodium sulphate. The solution was filtered through non-absorbent cotton wool (pre-washed with diethyl ether) and the solvent removed with gentle warming under a stream of nitrogen. The resultant FAME were analysed by gas-liquid chromatography (GLC) on a gas chromatograph (Trace GC-2000; ThermoQuest Ltd.) equipped with a flame-ionisation detector and fitted with a DB23 fused silica capillary column (25 m x 0.25 mm internal diameter; SGE Ltd.). Hydrogen was used as the carrier gas and sample application was by split injection. Oven temperature was programmed to start at 60 ºC and held at 60 ºC for 2 min, then rise to 150 ºC at 20 ºC min^{-1}, held for 2 min and then rise to 205 ºC at 1.8 ºC min^{-1} and finally rise to 230 ºC at 5 ºC min^{-1}. Reference standard FAME mixtures (Sigma Aldrich Ltd.) were also analysed and separated FAME identified by reference to these authentic samples.

3.2.8 Test for muramidase activity in cell extracts

Though the presence of muramidases has not been reported in microalgae, *P. tricornutum* cell extracts were tested for muramidase activity because they are very active against *M. luteus* (see below), a bacterium that is highly sensitive to the action of this enzyme (Fleming, 1922). Three antibacterial cell extracts (prepared as Sections 2.2.2 and 2.2.4) were tested using a modified RDA (based on Section 2.2.7). For this modification the LB or 2216E media in the lower agar was replaced with 0.0055 g *Micrococcus lysodeikticus* (*M. luteus*) cell walls (Sigma Aldrich Ltd.). It was autoclaved, poured and allowed to set. The agar was not inoculated with bacteria and an upper agar was not required. Once set, wells were punched into the agar and 4 μL of each sample loaded into individual wells. The positive control consisted 50 mg mL^{-1} lysozyme (Sigma Aldrich Ltd.) in 50 mM HEPES buffer. The plate was incubated overnight at room temperature and then examined. Clear zones indicate muramidase action.

3.2.9 Antibacterial activity of different cell morphs

To investigate whether different algal morphs exhibited different levels of antibacterial activity, 51 *P. tricornutum* cultures were grown, harvested, extracted and tested for antibacterial activity against *S. aureus* by RDA (as Sections 2.2.2, 2.2.4 and 2.2.7), except that the morphology of the culture just before harvest was determined from at least triplicate cell counts under the microscope (as Section 2.2.3). The antibacterial activity attributable to each cell in the culture was calculated and plotted against the culture's morphology. To further investigate whether or not the morphs had different levels of antibacterial activity, cultures enriched for each morph are required. However, the small-scale batch culture system proved unsuitable for the

production of such cultures (Appendix VII). Hence, *P. tricornutum* was cultured in two batches of ten shake-flasks containing 150 – 300 mL sterile modified ESAW medium to give cultures enriched in either oval or fusiform cells. In the first batch the fusiform-enriched inoculum contained 61 % fusiforms, 36 % ovals and 3 % intermediates whilst in the second batch this inoculum contained 74 % fusiforms, 21 % ovals and 4 % intermediates whilst in both batches the oval-enriched inoculums contained 100 % ovals (calculated from at least triplicate counts under the microscope). In the first batch the initial cell concentration was 1 x10^5 cells mL^{-1} but in the second batch it was double this. Inoculum volumes were kept the same within each batch with sterile modified ESAW medium. Flasks were kept in the lightbox (Section 2.2.2.1) and shaken once daily by hand. The presence of algal growth on the sides of the flasks was monitored but this was not seen. Prior to cell harvest 1 mL of culture was taken for A_{750} determination and cell morphology of the culture determined from at least duplicate cell counts (as Section 2.2.3). The volume of culture harvested was noted. Diatom cells were harvested at day 15 in batch one and at day 13 in batch two by centrifuging at 3000 *g* for 15 min at 4 ºC. The supernatants were discarded whilst cell pellets were stored at –80 ºC until extraction with aqueous methanol (Section 2.2.4). Dried cell extracts were resuspended in sterile HEPES so that 1 µL contained extract from 7.5 x10^6 cells. Extracts were stored at –80 ºC until each was tested once for antibacterial activity against *S. aureus* by RDA (Section 2.2.7).

3.2.10 Statistical analyses

Data sets for the growth of six bacterial species in the presence or absence of algal supernatant were shown to be normally distributed and have equal variances by

Shapiro-Wilk and Levene's tests. Data obtained at 28 h for each bacterium was tested for significant differences between growth of bacteria in the presence or absence of algal supernatant by student's t-test.

Data collected for determination of significant differences in the antibacterial activity between bottles were shown to be non-normally distributed by Shapiro-Wilk test ($W_{36} = 0.887$, $p < 0.01$) and have unequal variance by Levene's test ($F_{11,24} = 3.357$, $p < 0.01$). One-way ANOVA was performed on these data with bottle number as the only factor.

Temperature stability data were shown to deviate from normality by Shapiro-Wilk test ($W_{21} = 0.870$, $p < 0.01$) but show equal variances by Levene's. Treatment groups were tested for significant differences by one-way ANOVA.

pH stability data were shown to deviate from normality by Shapiro-Wilk test ($W_{15} = 0.854$, $p < 0.05$) but show equal variances by Levene's. Treatment groups were tested for significant differences by one-way ANOVA.

Salinity stability data were shown to deviate from normality by Shapiro-Wilk test ($W_{18} = 0.856$, $p < 0.05$) but show equal variances by Levene's. Treatment groups were tested for significant differences by one-way ANOVA.

The clear zone area on RDA data obtained to test for a significant difference between the antibacterial activity attributable to each cell in morph-enriched cultures was shown to be non-normally distributed by Shapiro-Wilk test ($D_{20} = 0.763$, $p < 0.001$)

and have unequal variances by Levene's test ($F_{3,16} = 26.08$, $p < 0.001$). Two-way ANOVA (with batch and morphology as factors) was performed to test these data for significant difference between the fusiform- or oval-enriched cultures.

Shapiro-Wilk and Levene's tests showed that the data collected for A_{750} of morph-enriched cultures at harvest was normally distributed and have equal variances. Two-way ANOVA (with batch and morphology as factors) was also performed on these data to determine whether there was a significant difference between the fusiform- or oval-enriched cultures.

Where one-way ANOVA showed significant differences existed between treatment groups the *post hoc* Tukey's HSD was used to identify the treatment groups that significantly differed. For all analyses $p \leq 0.05$ was considered significant.

3.3.0 Results

3.3.1 Testing *P. tricornutum* culture supernatant for antibacterial activity

The presence of antibacterial factors in *P. tricornutum* sterile culture supernatant was examined, first by RDA, but no activity was found against any bacterial species tested. Second, bacterial growth in the presence or absence of sterile algal culture supernatant showed no negative effects on growth of any of the six bacterial species tested (Figure 3.1). In fact, for five species bacterial growth was faster (seen as a shift left in the growth curve; Figure 3.1) and reached significantly greater A_{570} values at 28 h in the presence of culture supernatant ($p < 0.01$; Table 3.1). The effect of increased bacterial growth rate and greater final A_{570} in the presence of algal supernatant was most pronounced with *E. coli* and *L. anguillarum* and only with *S.*

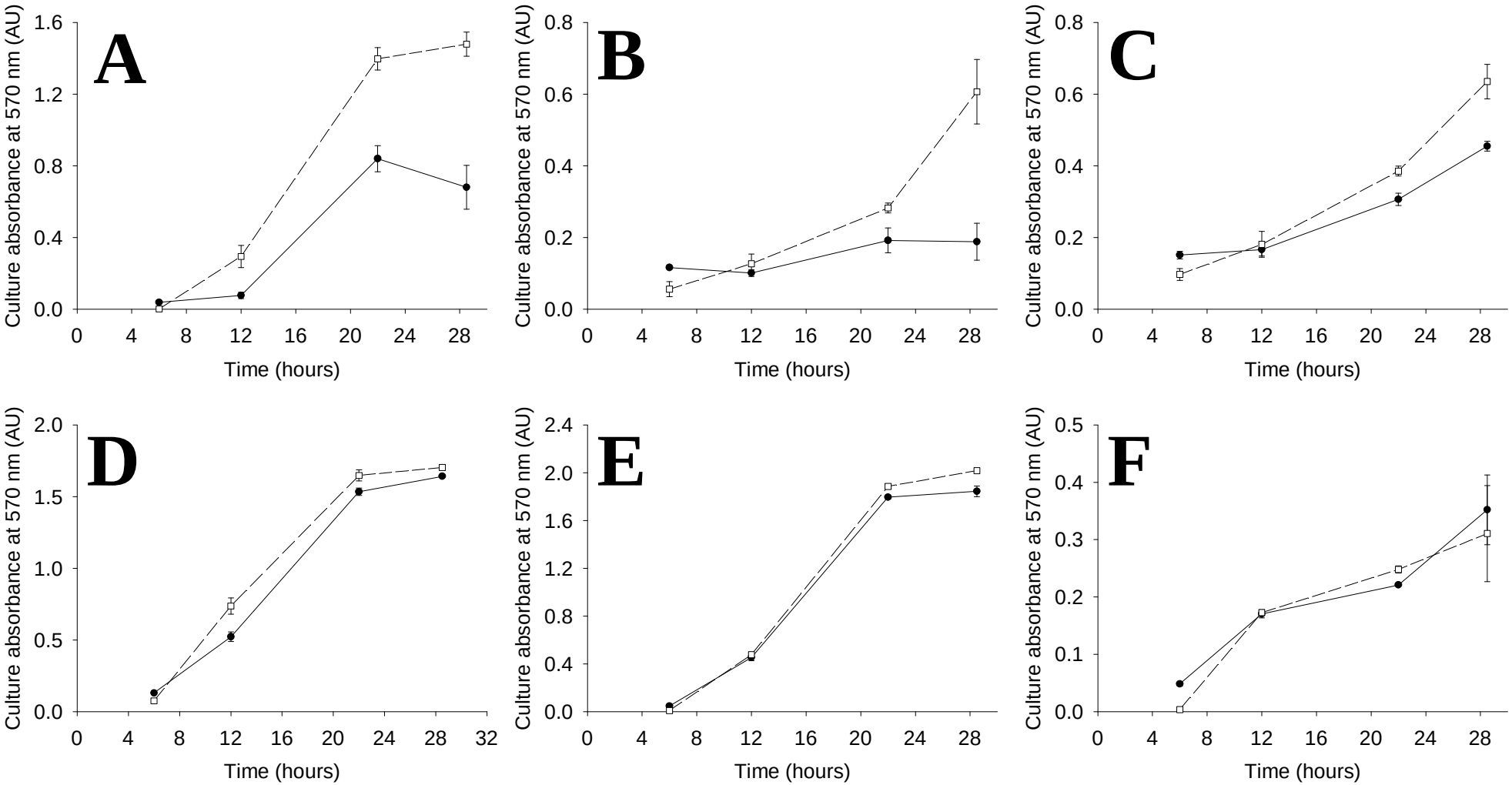


Figure 3.1 – Growth of (A) *E. coli*, (B) *L. anguillarum*, (C) *M. luteus*, (D) *P. citreus*, (E) *S. aureus* and (F) *S. epidermidis* in the absence (●) or presence (□) of sterile-filtered *P. tricornutum* culture supernatant showing that growth was significantly improved at 28 h ($p < 0.05$) in the presence of supernatant for all bacterial species except *S. epidermidis* where no significant difference exists between the treatment groups. n = 3; error bars are ± 1 SD.

Table 3.1 – T-test results for the growth of six bacterial species in the presence or absence of sterile algal culture supernatant. In five cases the bacteria reached a significantly greater A_{570} value at 28 h in the presence of sterile algal culture supernatant. For only one bacterial species (*S. epidermidis*) was no significant difference found between bacterial growth in the presence or absence of sterile algal culture supernatant. $p \leq 0.05$ was considered significant.

Bacterium species	T-test result
E. coli	$t_4 = -9.887$, $p < 0.001$
L. anguillarum	$t_4 = -6.999$, $p < 0.01$
M. luteus	$t_4 = -6.218$, $p < 0.01$
P. citreus	$t_4 = -6.118$, $p < 0.01$
S. aureus	$t_4 = -6.451$, $p < 0.01$
S. epidermidis	$t_4 = 0.693$, $p > 0.05$

epidermidis was growth in the algal supernatant not significantly different from the control ($p > 0.05$). Thus it seems likely that most, if not all, antibacterial compounds are retained within the algal cells and not released into the surrounding milieu.

3.3.2 Production of antibacterial compounds by *P. tricornutum* during culture

Cell extracts were found to be antibacterial against *S. aureus* for each day tested, but the amount of antibacterial activity changed during the growth curve (Figure 3.2). Activity was low in cell extract at day 4 but this increased steadily up to day 10, which is mid-exponential phase (Figure 3.2). Extracts from late-exponential phase cultures (day 12) showed slightly reduced levels of activity and this decreased to the lowest amount for those extracts prepared from cultures harvested in early stationary phase at day 14 (Figure 3.2). When antibacterial activity was calculated on a per cell basis, the quantity of antibacterial activity tended to decrease gradually with culture age (Figure 3.3). At day 4, the clear zone area on a RDA plate attributable to a single cell was calculated to be 4.2×10^{-6} mm^2 but at day 12 this had reduced five-fold to 0.82×10^{-6} mm^2. The yield of antibacterial activity was low at day 4 but increased steadily up to day 10 (Figure 3.3). At day 10 (mid-exponential phase), the yield of antibacterial activity from the whole culture, as calculated by total clear zone area on a RDA, was 6.7×10^3 mm^2 but reached a high of 7.1×10^3 mm^2 at day 12 (late-exponential phase) (Figure 3.3). When the culture entered early-stationary phase (day 14) antibacterial yield quickly diminished to a level lower than that for day 8 (Figure 3.3).

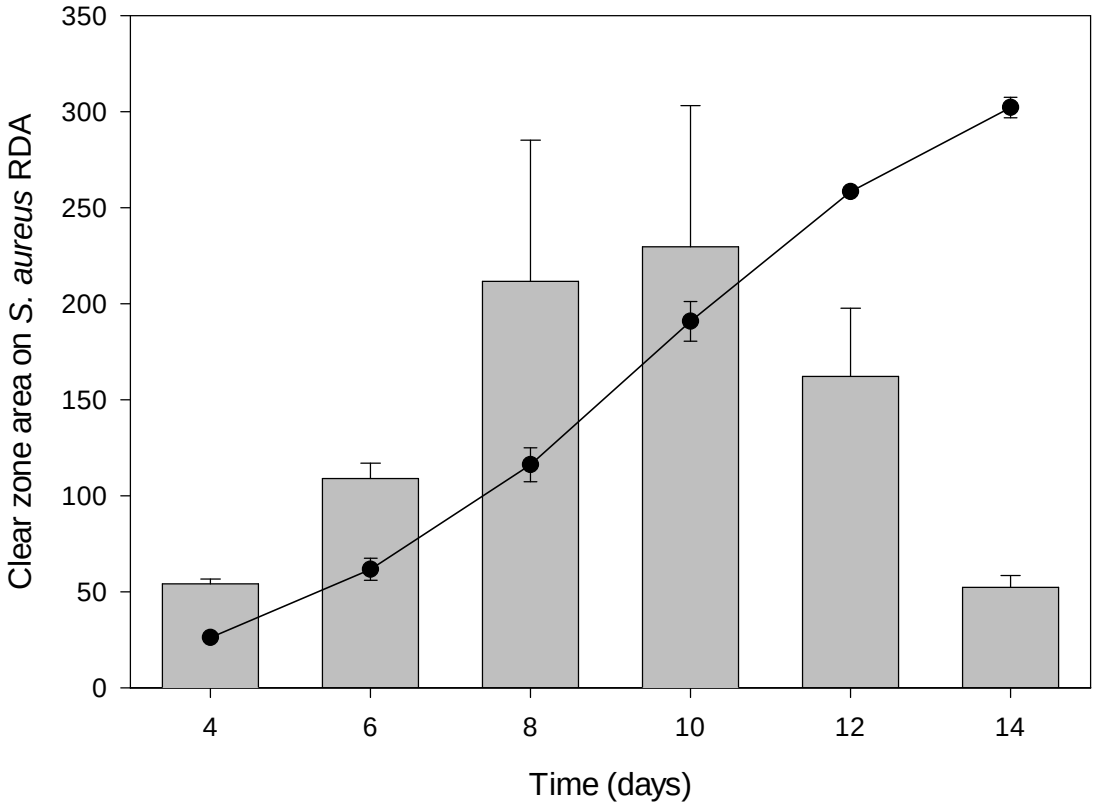


Figure 3.2 – Growth of *P. tricornutum* in the custom culture system for 14 days (●; values plotted are A_{750} x 700; units AU) and the antibacterial activity of aqueous methanol cell extracts against *S. aureus* (vertical bars; units mm^2). Extracts tested at 60 mg mL^{-1} in sterile 50 mM HEPES solution (pH 7.8). The antibacterial activity of cell extracts changes during culture with extracts prepared from cultures harvested at day 10 giving greatest antibacterial activity. Data for days 4–12: n = 4 (data for day 14: n = 3); all error bars are ± 1 SE.

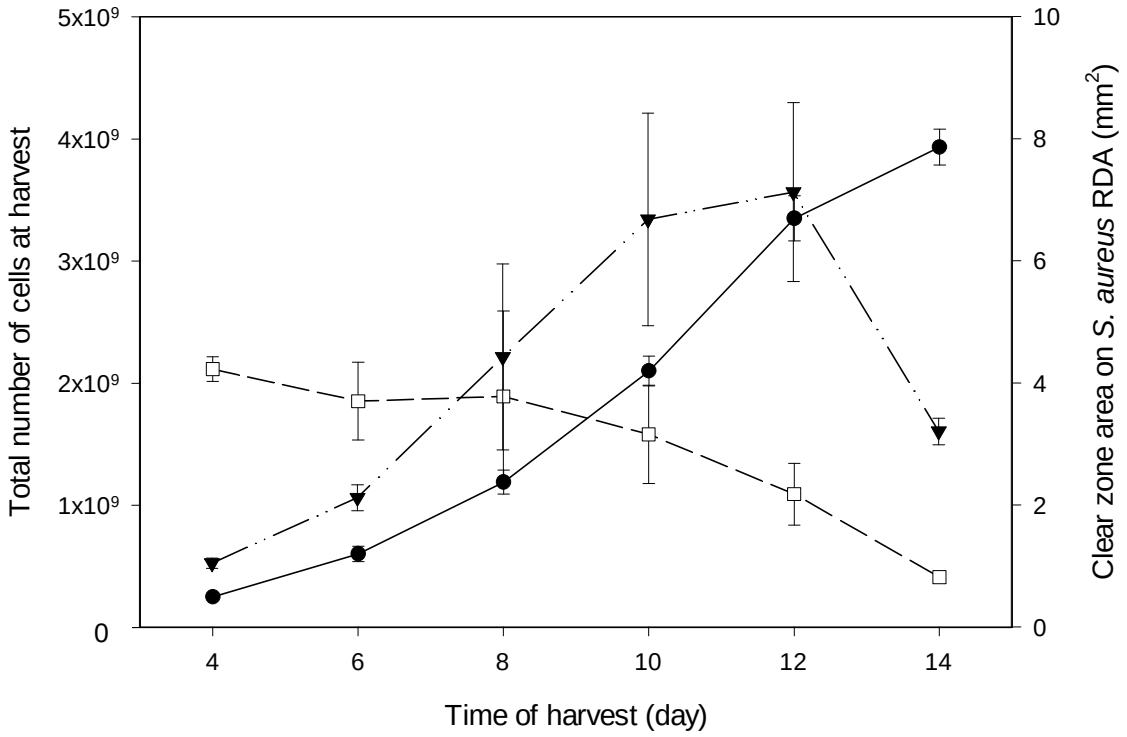


Figure 3.3 – Growth of *P. tricornutum* in the custom batch culture system for 14 days (●), amount antibacterial activity calculated for each cell as measured by clear zone area on a *S. aureus* RDA (scale x 10^{-6}) (□), and yield of antibacterial activity calculated as the total clear zone area on a *S. aureus* RDA that would be expected in the whole culture (scale x 10^{3}) (▼). The graph shows that the antibacterial activity calculated as an estimated quantity for each cell reduces during culture. However, yield of antibacterial activity is greatest at mid- to late-exponential phase,due to the greater total number of cells in the culture. Data for days 4–12: n = 4 (data for day 14: n = 3); all error bars are ± 1 SE.

3.3.3 Antibacterial activity of cell extracts between culture bottles

No significant difference was found between bottles in the level of the antibacterial activity in extracts (one-way ANOVA: $F_{11,24} = 290.5$, $p > 0.05$).

3.3.4 Spectrum of antibacterial activity in *P. tricornutum* cell extracts

Terrestrial and marine bacterial species were found to vary in their sensitivity to growth inhibition by *P. tricornutum* cell extracts (Figure 3.4). The cell extracts tested against *M. luteus* consistently gave the largest clear zone area on the RDA plates with a mean clear zone area of 180 ± 34 mm^2 (mean ± 1 SE) followed by *S. epidermidis* (154 ± 24 mm^2), *S. aureus* (138 ± 16 mm^2), and to a lesser extent *P. citreus* (97.1 ± 17mm^2) (Figure 3.4). *L. anguillarum* was only slightly sensitive to cell extracts (10.1 ± 3.0 mm^2) but *E. coli* and the two *Ps. aeruginosa* strains were not inhibited by cell extracts (Figure 3.4). The four most susceptible species were Gram positive whilst, of the four Gram negative species tested, only the marine pathogen, *L. anguillarum*, was susceptible (Figure 3.4).

3.3.5 Stability of antibacterial activity in cell extracts

Exposure of *P. tricornutum* cell extracts to different temperatures affected the ability to inhibit the growth of *S. aureus* with a significantly reduced ($p < 0.05$) amount of antibacterial activity in extracts exposed to higher temperatures (55 and 121 ºC) though no significant reduction ($p > 0.05$) in activity was seen with extracts exposed to temperatures up to 37 ºC (Figure 3.5). Significantly greater ($p < 0.05$) antibacterial activity was found in cell extracts that had been resuspended in buffers at pH 3, 9 and 11 compared to reduced amount of activity in extracts resuspended in buffers at pH 5 and 7 (Figure 3.6). The antibacterial activity of cell extracts was not significantly

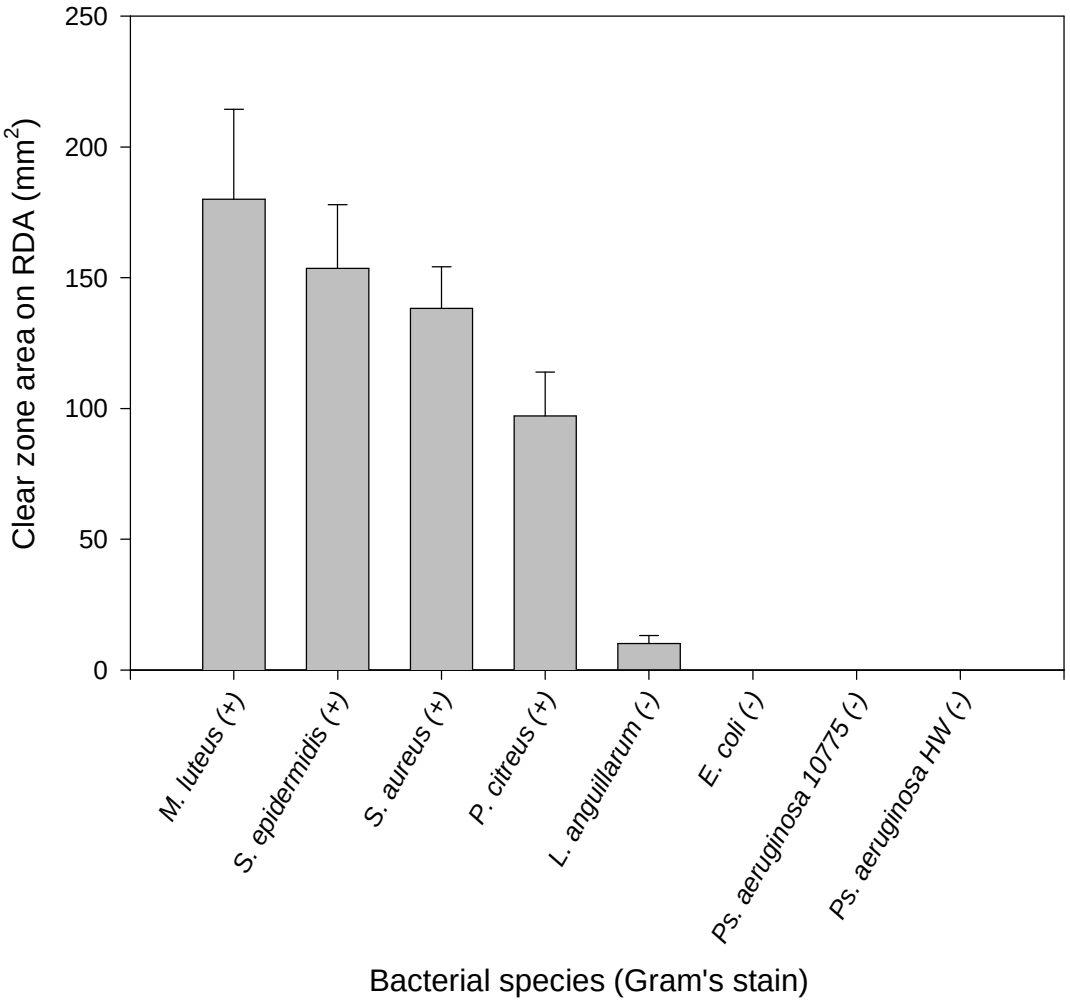


Figure 3.4 – Antibacterial activity of *P. tricornutum* aqueous methanol cell extracts as measured by clear zone area on RDA showing that the Gram positive species are more susceptible than Gram negative species. n = 30 (except *Ps. aeruginosa* 10775, n = 8; *Ps. aeruginosa* HW, n = 5); error bars are ± 1 SE.

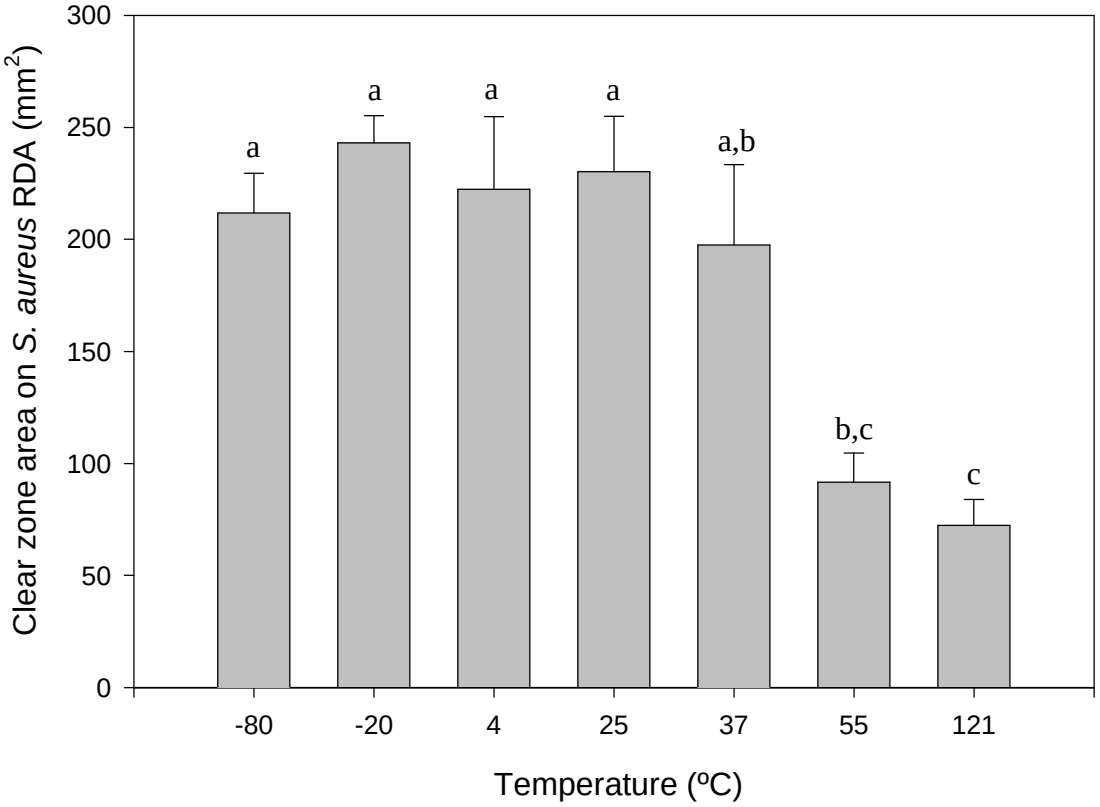


Figure 3.5 – Antibacterial activity of *P. tricornutum* aqueous methanol cell extracts against *S. aureus* (expressed as clear zone area on RDA) exposed to temperatures between –80 and 55 ºC for 4 h with an additional treatment group being autoclaved at 121 ºC for 15 min. One-way ANOVA confirmed that significant differences existed between the treatment groups (one-way ANOVA: $F_{6,14} = 9.088$, $p < 0.001$) with bars having different letters signifying those groups that differed significantly from each other by Tukey's HSD ($p \leq 0.05$). The sample at –80 ºC is the control but antibacterial activity of extracts did not reduce significantly until exposed to 55 ºC. Antibacterial activity significantly reduced further after autoclaving but some activity remained even after this harsh treatment. n = 3; error bars are ± 1 SE.

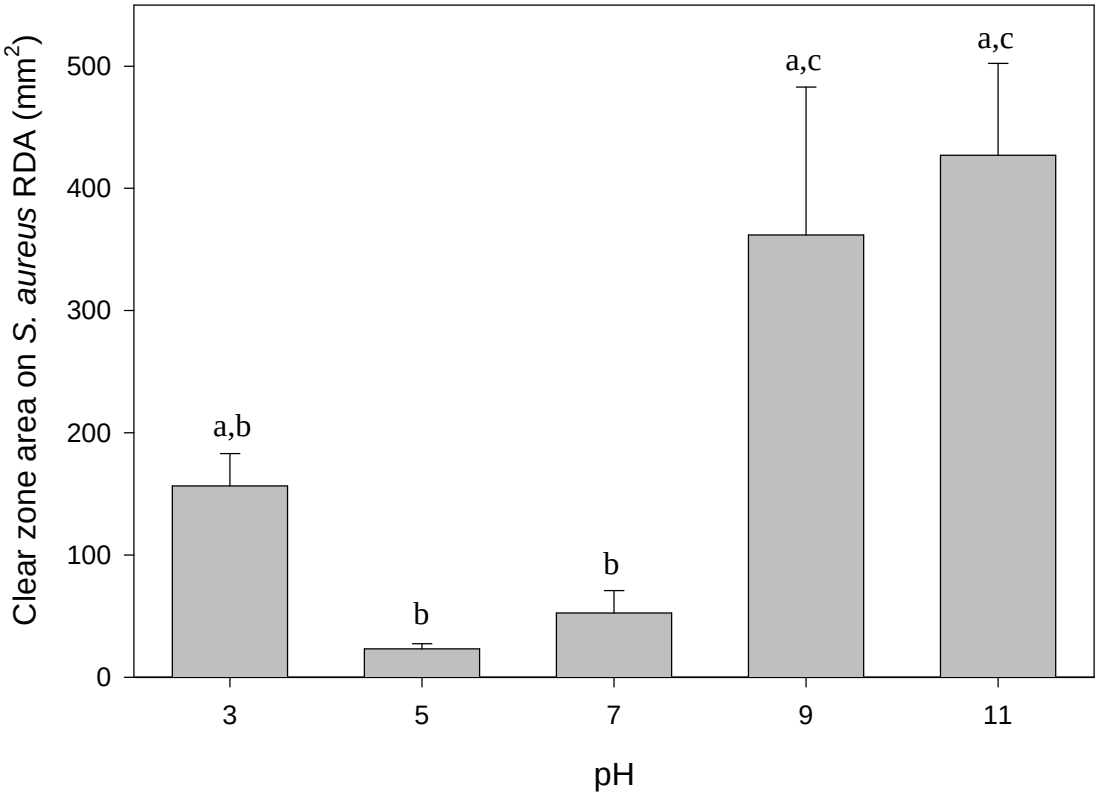


Figure 3.6 – Antibacterial activity against *S. aureus* of *P. tricornutum* aqueous methanol cell extracts resuspended in buffers at pH 3 to 11 measured as clear zone area on RDA. One-way ANOVA confirmed that significant differences existed between the treatment groups (one-way ANOVA: $F_{4,10} = 7.769$, $p < 0.01$) with bars having different letters signifying those groups that differed significantly from each other by Tukey's HSD ($p \leq 0.05$). Significantly greater antibacterial activity was found in extracts resuspended in low or high pH buffers (pH 3, 9 and 11). n = 3; error bars are ± 1 SE.

affected by salinity ($p > 0.05$) with extracts at salinity ranging 0 to 5 % showing similar levels of activity (Figure 3.7). Negative control solutions did not give any clear zones on the RDA.

Antibacterial activity in cell extracts was not affected by proteinase digestion, as activity remained similar in samples incubated in the presence or absence of trypsin and proteinase K (Figure 3.8). The trypsin and proteinase K showed no antibacterial effect but their ability to digest antibacterial compounds of a peptide nature was confirmed by the diminished level of activity in melittin samples incubated with either enzyme (Figure 3.9).

3.3.6 Total mass of protein in *P. tricornutum* cell extracts

The total mass of protein in extracts tested for activity against *S. aureus* changed depending on which day the culture was harvested (Figure 3.10). Total protein in tested cell extracts increased steadily up to a high of 3.87 ± 0.66 µg (mean ± 1 SD) at day 10 then reduced to 1.91 ± 0.52 µg (day 12) and 0.65 ± 0.28 µg at day 14 (Figure 3.10). A very highly significant relationship ($p < 0.001$) existed between the total mass of proteins in the cell extract and anti-*S. aureus* activity meaning greater quantities of proteins in extracts showing higher antibacterial activity (Figure 3.11). When assessed as 'protein per cell', greatest mass of protein was found in cells from cultures harvested at day 6 then, generally, mass of protein per cell reduced with each later harvest time (Figure 3.12).

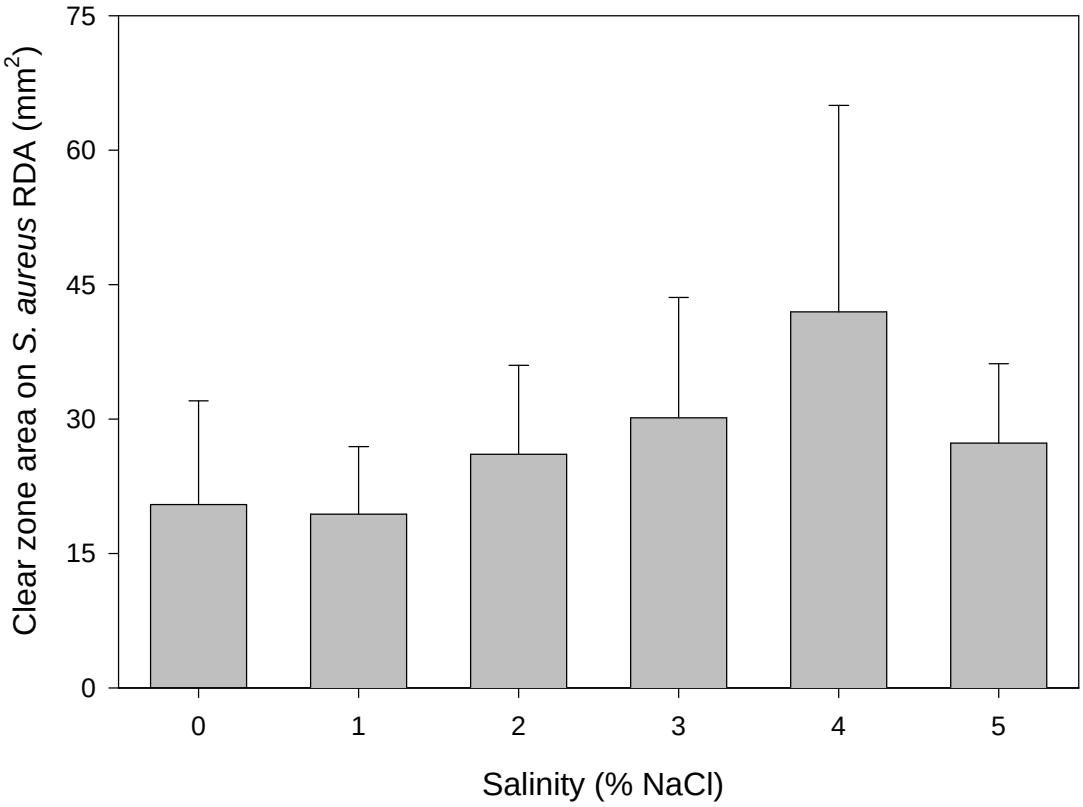


Figure 3.7 – Antibacterial activity against *S. aureus* of *P. tricornutum* aqueous methanol cell extracts resuspended in NaCl solutions ranging from 0-5 % (measured as clear zone area on RDA). One-way ANOVA confirmed that there were no significant differences between the treatment groups (one-way ANOVA: $F_{5,12} = 0.371$, $p > 0.05$). Salinity does not affect the antibacterial activity of cell extracts. n = 3; error bars are ± 1 SE.

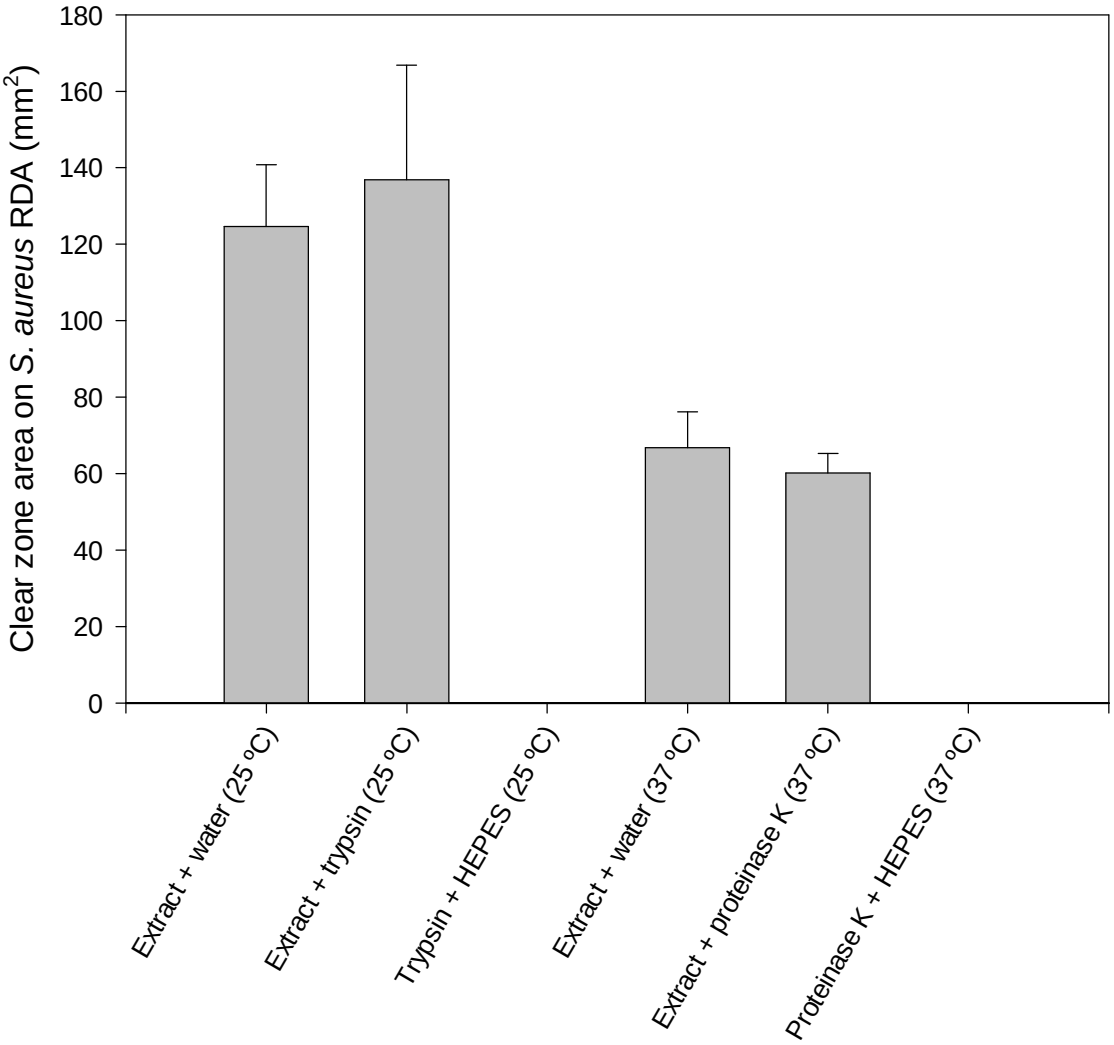


Figure 3.8 – Antibacterial activity against *S. aureus* of *P. tricornutum* aqueous methanol cell extracts (measured as clear zone area on RDA) showing similar levels of activity for cell extracts incubated in either the presence or absence of the proteinases, trypsin or proteinase K. Antibacterial activity in the cell extracts does not appear to be affected by proteinase digestion. Trypsin and proteinase K alone possessed no antibacterial activity. n = 2; error bars are ± 1 SD.

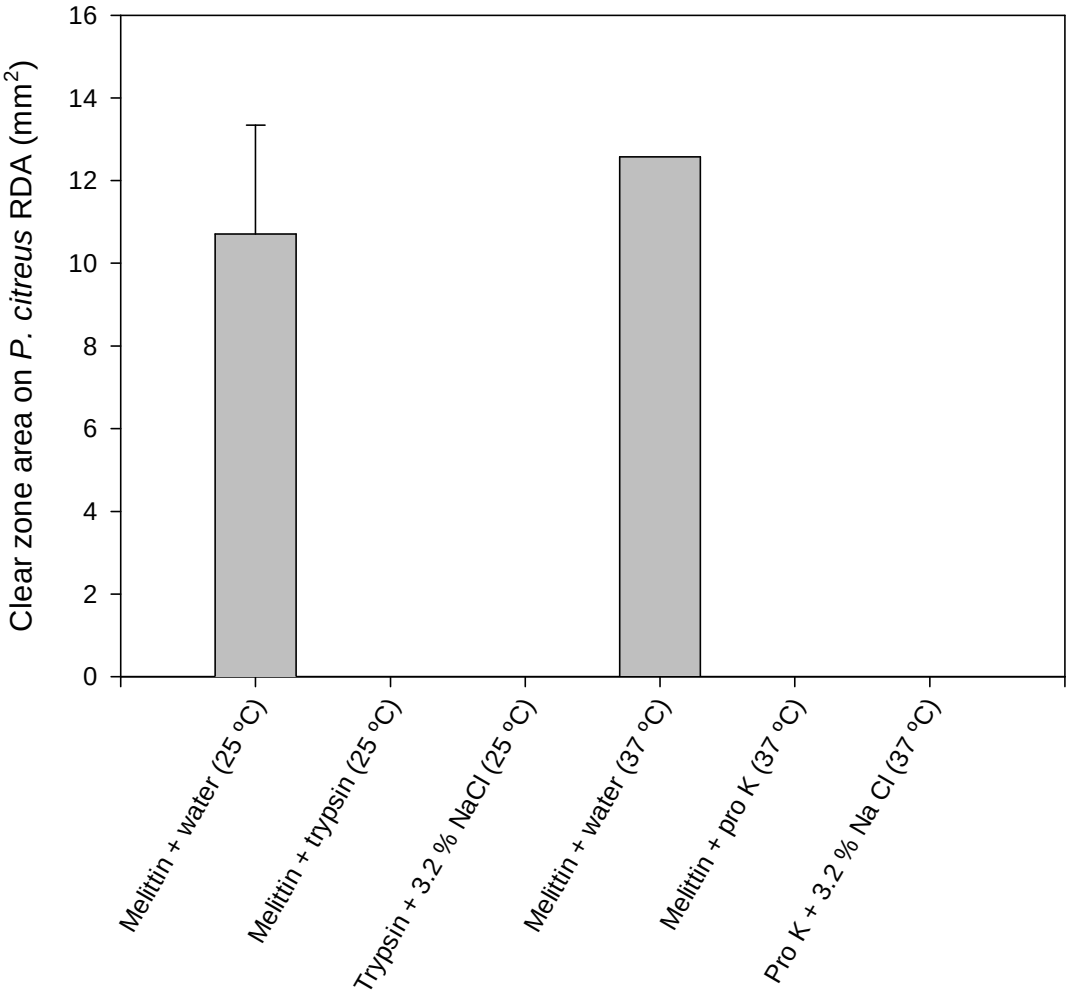


Figure 3.9 – Antibacterial activity against *P. citreus* of melittin in solution at 100 µg mL^{-1} (measured as clear zone area on RDA) showing that activity was completely abolished by incubating with proteinases, trypsin or proteinase K. Therefore, trypsin and proteinase K were both able to digest small peptides such as melittin. Trypsin and proteinase K alone showed no antibacterial activity on the RDA. n = 2; error bars are ± 1 SD (not all error bars visible).

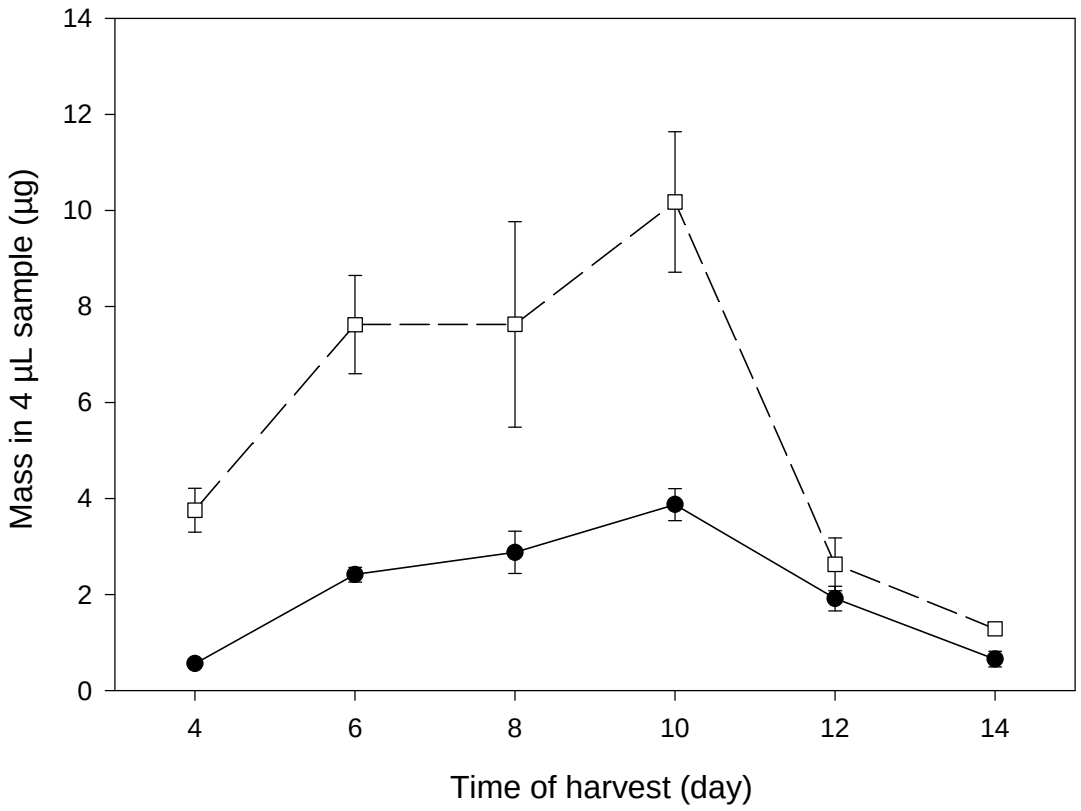


Figure 3.10 – Total mass of proteins (●) and FAME (□) in *P. tricornutum* aqueous methanol cell extracts resuspended at 60 mg mL^{-1} in sterile 50 mM HEPES (pH 7.8) and tested for antibacterial activity (prepared from algal cultures grown in the custom culture system and harvested 4 – 14 days). Values given as the total masses of protein or FAME calculated for a 4 µL sample. n = 4 (except day 14 where n = 3), error bars are ± 1 SE.

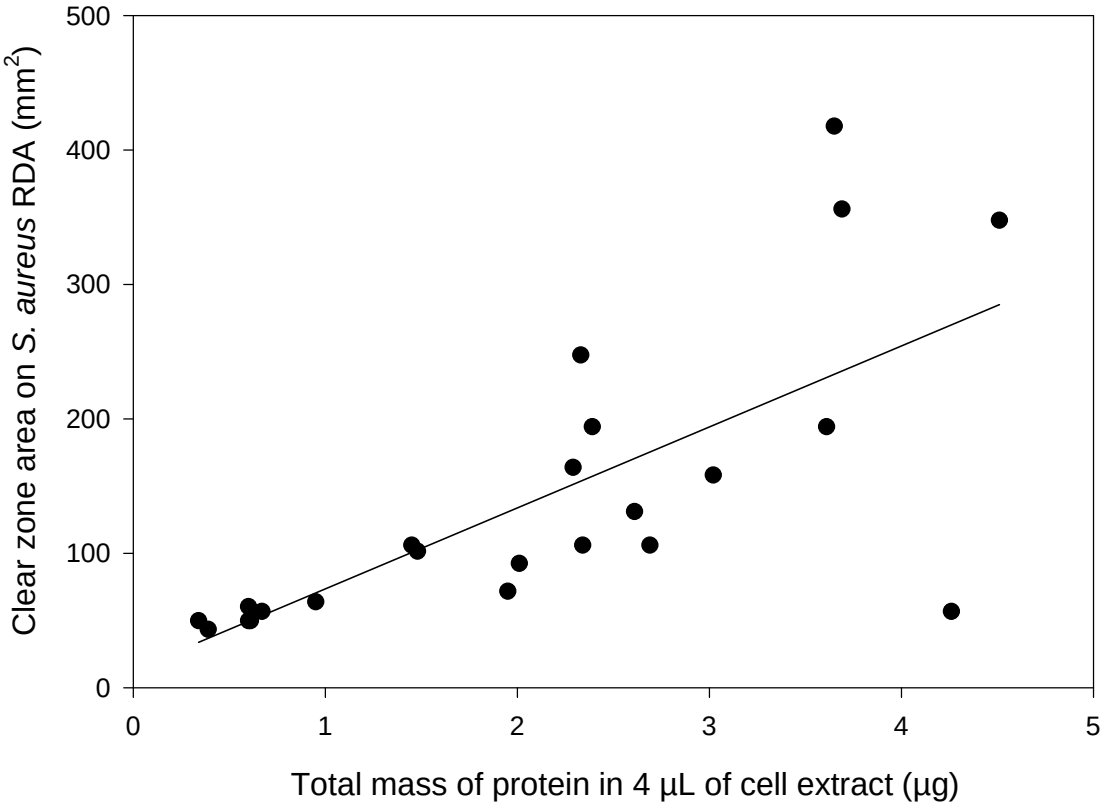


Figure 3.11 – Total mass of protein in *P. tricornutum* cell extracts tested for antibacterial activity (prepared from algal cultures grown in the custom batch culture system and harvested between days 4 – 14) showing that extracts containing a greater mass of proteins had higher levels of antibacterial activity. Relationship is very highly significant ($F_{1,22} = 22.0$, $r^2 = 0.512$; $p < 0.001$).

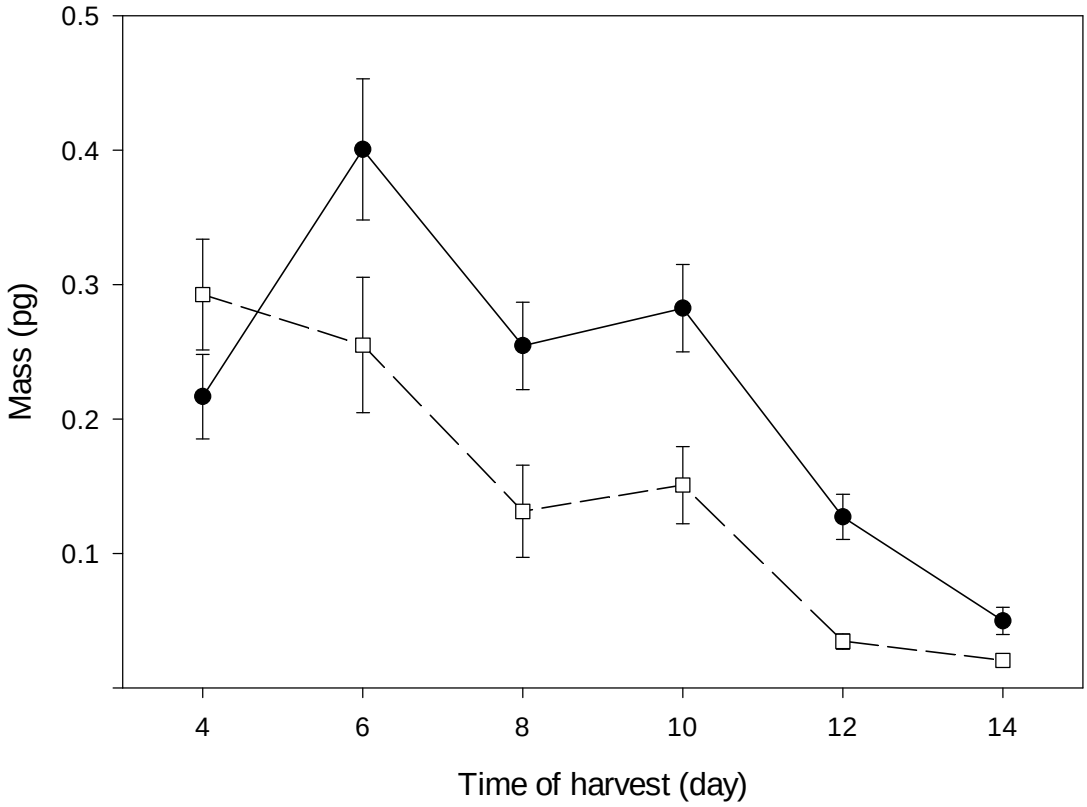


Figure 3.12 – Mass of protein (●; values plotted are mass x 5) and FAME (□) in cell extracts calculated on a per algal cell basis from cultures grown in the custom culture system and harvested between days 4 – 14 showing that levels of both cellular constituents reduce during culture. Data for days 4 – 12: n = 4 (data for day 14: n = 3); all error bars are ± 1 SE.

3.3.7 Total mass of fatty acids in *P. tricornutum* cell extracts

The total mass of FAME in extracts tested for antibacterial activity against *S. aureus* depended on which day the culture was harvested but generally there was approximately five times more FAME than protein. Total mass of FAME in extracts tested for activity increased up to a total of 10.2 ± 2.9 µg (mean ± 1 SD) at day 10 then rapidly reduced at days 12 and 14 (2.6 ± 1.1 µg and 1.28 ± 0.08 µg respectively), essentially following the same trend as protein level (Figure 3.10). A significant relationship ($p < 0.05$) existed between mass of FAME in cell extract and antibacterial activity meaning greater quantities of FAME were present in extracts showing higher antibacterial activity (Figure 3.13). When calculated as 'FAME per cell', greatest quantity of FAME were in cells from cultures harvested at day 4 with FAME mass generally reducing with each later harvest time until day 14 when FAME per cell was 14 times less than at day 4 (Figure 3.12).

3.3.8 Test for muramidase activity in cell extracts

No muramidase activity was found in any of the *P. tricornutum* cell extracts, though a clear zone was found around the lysozyme positive control.

3.3.9 Antibacterial activity of different cell morphs

When the antibacterial activity attributable to each cell in mixed morphology cultures is plotted against the relative morphology of each culture it is clear that the cultures with a greater proportion of fusiform cells produce extracts with greater antibacterial activity against *S. aureus* (Figure 3.14). Conversely, cell extracts from cultures with higher proportions of oval cells show lower levels of antibacterial activity attributable to each cell (Figure 3.14).

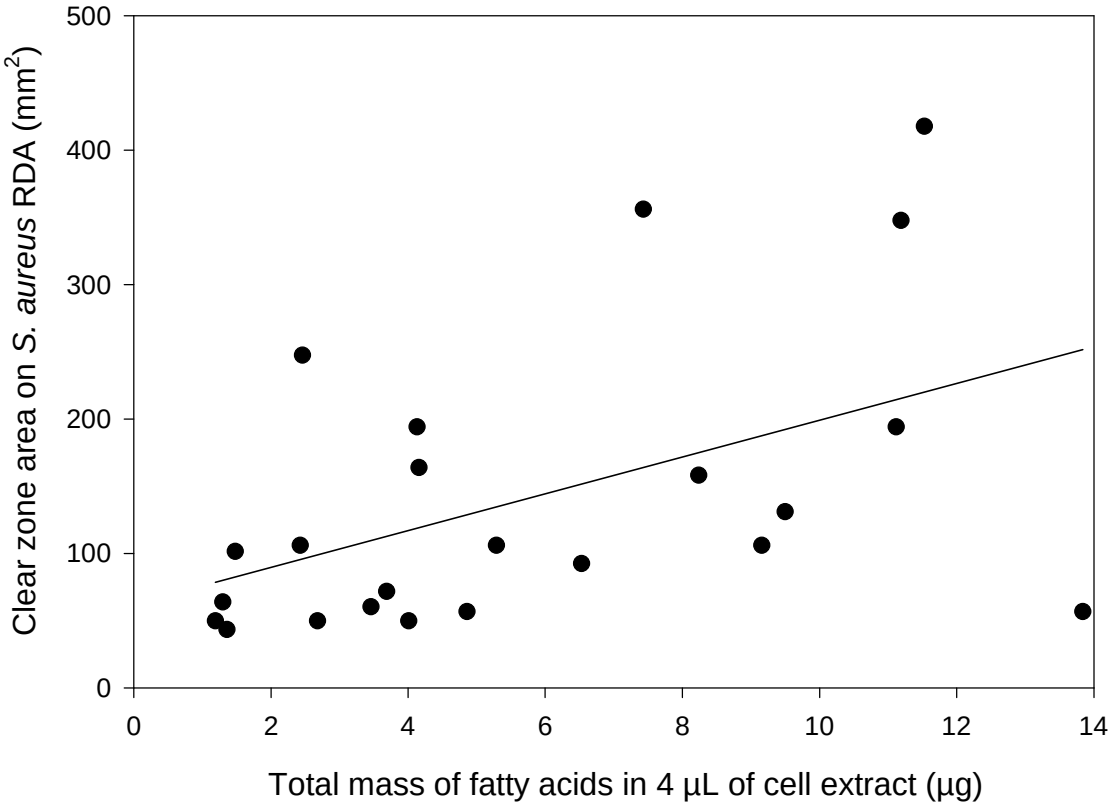


Figure 3.13 – Total mass of fatty acids (as FAME) in *P. tricornutum* cell extracts tested for antibacterial activity (prepared from algal cultures grown in the custom batch culture system and harvested between days 4–14) showing that extracts containing greater levels of fatty acids had higher levels of antibacterial activity. Relationship is significant ($F_{1,22} = 6.42$, $r^2 = 0.234$; $p < 0.05$).

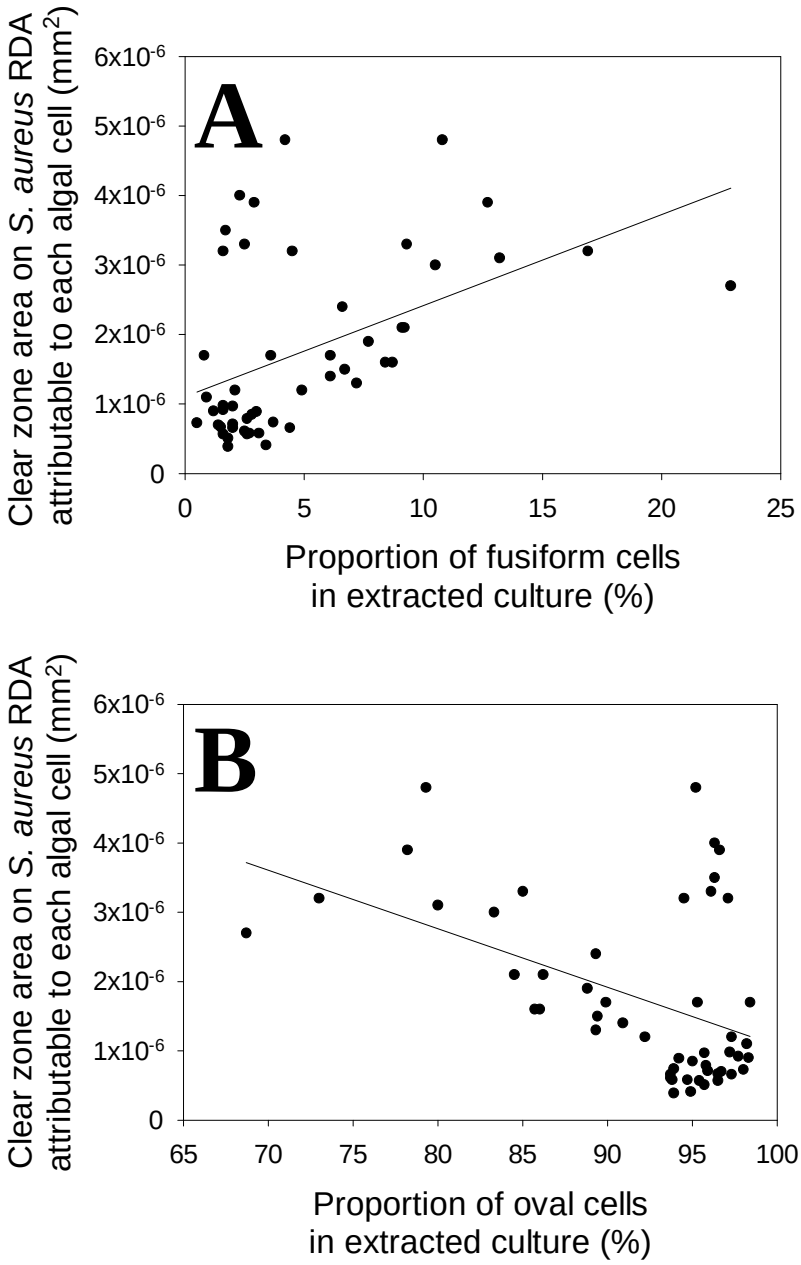


Figure 3.14 – Antibacterial activity against *S. aureus* of cell extracts from mixed morphology cultures calculated for each cell showing that a greater proportion of fusiform cells in the extracted cultures produced a greater level of antibacterial activity attributable to each cell (A); regression: $F_{1,49} = 14.05$, $r^2 = 0.472$; $p < 0.001$. Conversely, a greater proportion of oval cells in the extracted cultures produced a lower level of antibacterial activity attributable to each cell (B); regression: $F_{1,49} = 12.95$, $r^2 = 0.457$; $p < 0.001$.

At harvest, the morph-specific enriched cultures the oval-enriched cultures contained 100 % oval cells, whilst the fusiform-enriched cultures contained a range of 71 – 83 % fusiform cells (mean = 76 %). When the enriched cultures were extracted and tested for antibacterial activity against *S. aureus* by RDA the antibacterial activity attributable to each cell in fusiform-enriched cultures was significantly greater ($p < 0.05$) than for oval-enriched cultures (Figure 3.15). Interestingly, the fusiform-enriched flasks had significantly greater ($p < 0.001$) A_{750} values at harvest suggesting that these cultures grew faster than the ovals (Figure 3.16). This finding was later confirmed in other culture vessels (Appendix VIII) and has never previously been reported.

3.4.0 Discussion

Level of antibacterial activity in *P. tricornutum* cell extracts peaked at mid-exponential phase (day 10) and largely remained confined within the algal cells. Antibacterial activity per cell reduced gradually through the growth curve and the optimum time to harvest the culture for greatest yield of antibacterial activity was determined to be late-exponential phase. Fusiform cells have greater antibacterial activity than oval cells. Extracts were shown to be most active against Gram positive species and this activity was found to remain stable at high and low temperatures and at different values of salinity. However, pH affects the antibacterial activity with greatest activity found in extracts resuspended in low (pH 3) or high (pH 9 or 11) values. Activity does not appear to be primarily due to proteins, as proteinases had no effect on the RDA clear zone areas of antibacterial cell extracts.

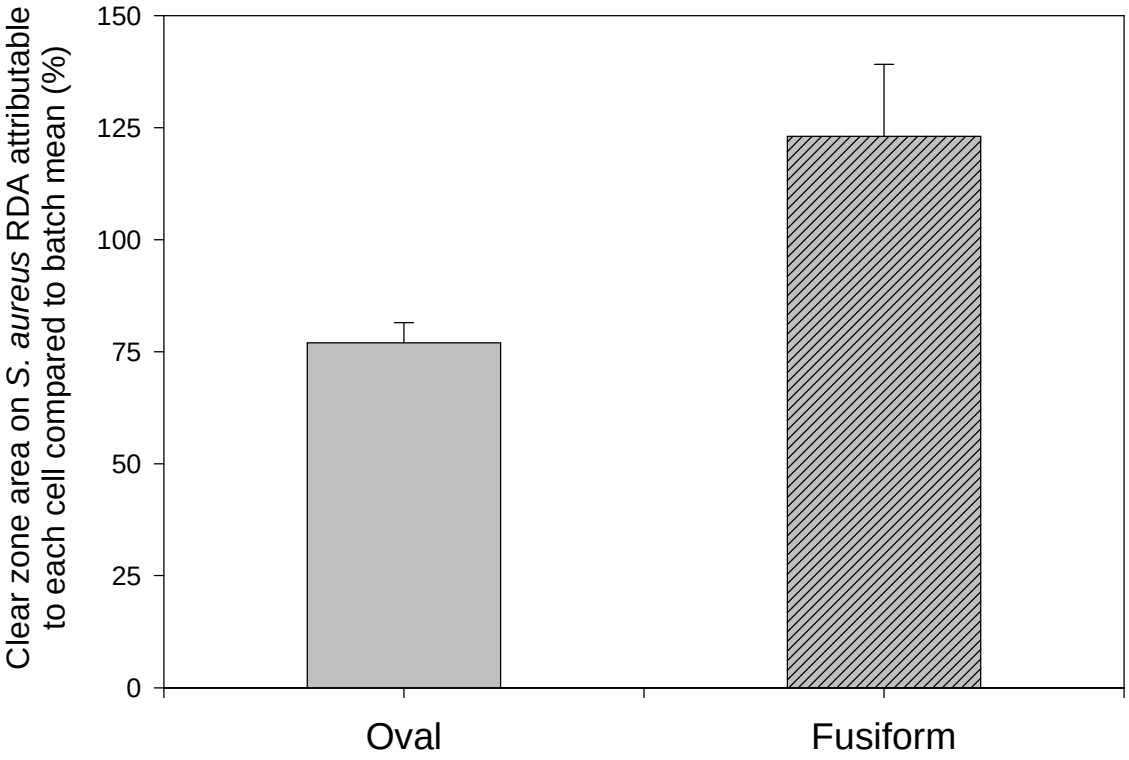


Figure 3.15 – Antibacterial activity against *S. aureus* attributable to each cell (calculated from clear zone area on RDA) in cultures enriched in either the oval or fusiform cells showing that cells in the fusiform morphology have significantly greater antibacterial activity than oval cells (two-way ANOVA: $F_{3,16} = 4.66$, $p < 0.05$). Due to inter-batch variability data is expressed as values relative to batch mean (batch mean = 100 %). n = 10; error bars are ± 1 SE.

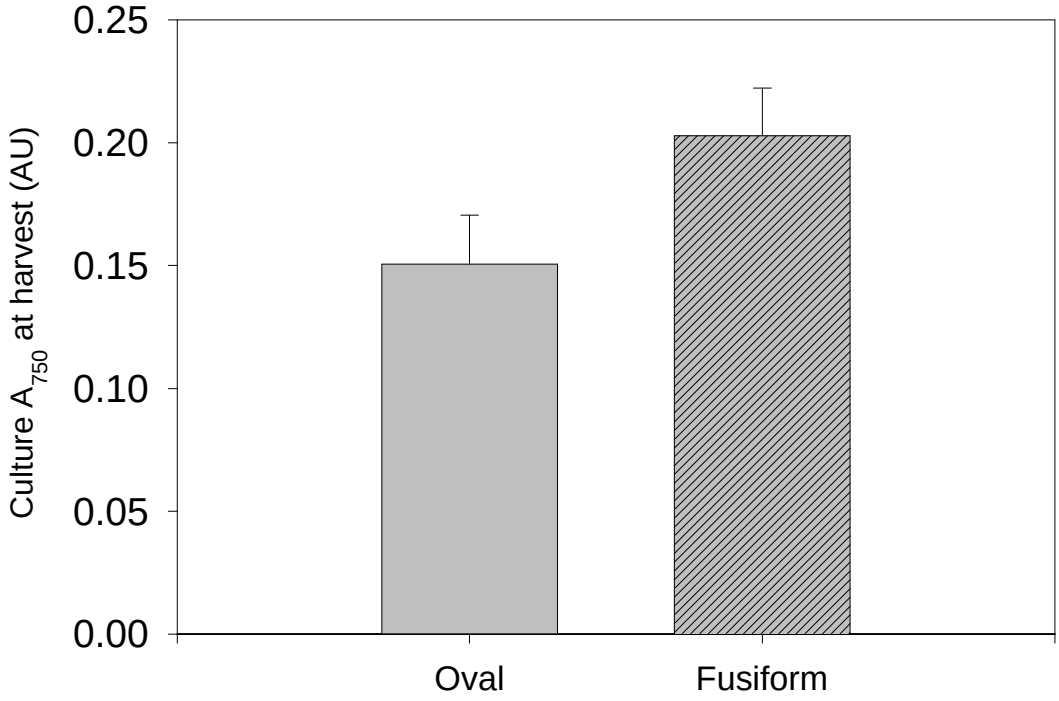


Figure 3.16 – Culture A_{750} at harvest of fusiform- and oval-enriched shakeflasks showing that the fusiform-enriched flasks had significantly greater A_{750} values than the oval-enriched flasks (two-way ANOVA: $F_{3,16} = 19.6$, $p < 0.001$). This suggests that the fusiform-enriched cultures grew faster than the oval-enriched cultures. n = 10; error bars are ± 1 SE.

In the present study, the amount of antibacterial activity in *P. tricornutum* cell extracts was shown to change during growth, with greatest antibacterial activity in extracts prepared from cultures harvested in exponential phase and lowest activity in extracts from stationary phase. Cooper *et al*. (1983) found that cell extracts prepared from late-exponential phase *P. tricornutum* show greater spectrum of antibacterial activity compared to extracts from stationary phase cultures though this was not investigated in the present work. However, in contrast to Cooper e*t al*. (1983), who found only cell extracts prepared from stationary phase cultures show anti-*S. aureus* activity, the present study found activity against this bacterium throughout the growth curve, including early- and late-exponential phases. This could reflect the use of different algal and bacterial strains, culture conditions, extraction and antibacterial assays. Previous workers have often monitored antibacterial activity at a limited number of points in the growth curve, e.g. 3 points (Debro and Ward, 1979; Kogure *et al*., 1979; Cooper *et al*., 1983). Importantly, the assessment of antibacterial activity in *P. tricornutum* cell extracts has only ever been qualitative (Cooper *et al*., 1983). Thus, the present study is the first to assess quantitatively antibacterial activity of *P. tricornutum* extracts throughout the entire growth curve.

In the present study, *P. tricornutum* culture supernatant was found to enhance the growth of most of the bacterial species tested, which confirms previous observations (Berland *et al*., 1972). Faster and greater total bacterial growth is likely to be due to the presence of growth factors or the utilisation of bio-molecules as energy sources. Other studies with *P. tricornutum* culture supernatants reported anti-*E. coli* activity, which was attributed to acrylic acid (Brown *et al*., 1977), and anti-*Ps. aeruginosa* activity attributed to a molecule with mass >10 kDa (Cooper *et al*., 1983). However,

Brown *et al*. (1977) provided few details with respect to algal culture and the physiological condition of the alga at harvest, whilst Cooper *et al*. (1983) used stationary phase cultures. In the present study, however, the culture supernatants came from exponential phase cultures, which may be important, as algal cultures can release greater quantities of compounds in stationary or death phase (Borowitzka, 1988b; Shaw *et al*., 1995b). Further, in the present study different algal and bacterial strains were used compared to those used by Brown *et al*. (1977) and Cooper *et al*. (1983). If antibacterial compounds were present in the sterile culture supernatants here they may not have been present in sufficient concentrations to have a detrimental effect on bacterial growth, or the advantageous effects of other excreted compounds may have masked activity of antibacterial compounds.

To enable an assessment of optimal time for harvest of *P. tricornutum* culture to obtain maximum antibacterial activity, the antibacterial activity of individual microalgal cells was considered over the growth curve. It was found that antibacterial activity of individual cells gradually reduced with time. This could be because the active compounds are being partitioned between dividing cells, perhaps for either metabolism or growth. Alternatively, the extraction may have become increasingly inefficient as the number of cells in the extract increased. Despite the reduction in the antibacterial activity of individual cells, the increased numbers of algal cells in the culture resulted in day 12 being determined as the day with maximum yield of activity. This may signify that the actual peak yield occurs between days 10 and 14. To be cautious, cultures should be harvested earlier rather than later as antibacterial yield decreases dramatically at day 14. This is the first study to consider how antibacterial activity of individual microalgal cells changes with time.

The faster growth of fusiform cultures may be because this cell morph tends to be dominant in liquid culture, and thus, is considered to be adapted for growth in a liquid medium (Barker, 1935; Lewin *et al*., 1958; Hayward, 1968a; Gutenbrunner *et al*., 1995). The reduced A_{750} seen in oval-dominated flasks is not due to growth on the vessel walls as this was absent. Using a different strain of *P. tricornutum*, Borowitzka and Volcani (1978) have reported that there was no difference in the growth rates of the three common morphs. In the present study, cells in the fusiform morph were shown to have greater antibacterial activity than oval cells, and as no such previous investigations have been reported, this is a novel finding. This cannot be explained by harvest of cultures in different growth phases because whilst the fusiform cultures did grow faster, the data presented above shows that the further through the growth curve a culture is harvested, the lower the antibacterial activity of the extract on a per cell basis. A possible explanation is that the antibacterial compounds are more easily extracted from the fusiform cells compared with the ovals and this could be due to the differences in cellular structure. Oval cells have a siliceous shell whereas the shell of a fusiform cell is, at best, only partially silified (Lewin *et al*., 1958; Lewin, 1958; Borowitzka and Volcani, 1978) and may therefore be more fragile and thus more susceptible to breaking during sonication.

Previous workers have reported that Gram positive bacteria are often more susceptible to killing by *P. tricornutum* cell extracts than Gram negative bacteria (Duff *et al*., 1966; Cooper *et al*., 1983; Table 1.4). Gram positive bacteria susceptible to *P. tricornutum* extracts include *Micrococcus* species (Duff *et al*., 1966) and *S. aureus* (Cooper *et al*., 1983; Kellam and Walker, 1989). As there are no other published accounts, the present study is the first to report *P. tricornutum* cell extracts showing

antibacterial activity against *S. epidermidis* and *P. citreus*. However, in contrast to Brown *et al*. (1977) and Cooper *et al*. (1983) no activity was found against *E. coli* or *Ps. aeruginosa*. It is noteworthy that the only susceptible Gram negative species (*L. anguillarum*) is a marine pathogen of fish (Toranzo *et al*., 2005). These results enable the selection of susceptible bacterial species for use for bioassay-guided fractionation of cell extracts to isolate the antibacterial compounds.

To enable the implementation of suitable protocols for future work aimed at isolating active molecules, an assessment for the stability of the antibacterial activity was necessary. Duff *et al*. (1966) reported that antibacterial activity of various *P. tricornutum* extracts remain stable in the dark at 4 ºC for at least 1 month. However, activity is lost progressively when left at room temperature in the light (Duff *et al*., 1966). In the present study, antibacterial activity in the cell extract was found to be stable over a wide range of temperatures and even the samples exposed to autoclaving retained some activity. These findings are similar to results found with the antibacterial activity in cell extracts from *Chlorococcum* that remain active, despite incubating for 1 h at 100 ºC (Ohta *et al*., 1993). Temperature stability does not help to identify the compounds responsible but it does mean that strict temperature controls will not be needed during isolation. It is noteworthy that the extracts used for temperature stability in the present study had been dried at 30 ºC for ~3 h and so the possibility exists that temperature sensitive compounds may have already been inactivated by this procedure. In the present work cell extracts were shown to be antibacterial at salinity values up to 5 % NaCl showing that the compounds responsible are active at salt concentrations of the alga's natural habitat. Moreover, greater activity was found in extracts resuspended in buffers at high or low pH with

reduced activity at pH 5 and 7. As seawater has a pH of 8.2 (Palmer and Pearson, 2003) this indicates that the compounds responsible might be soluble and active at environmental pH. This is the first study to specifically address the stability of antibacterial activity in cell extracts from *P. tricornutum* to temperature changes, different pH and salinity conditions. The antibacterial activity of cell extracts remained despite incubating with broad-acting proteinases suggesting that the major compound(s) responsible for the antibacterial activity is not a protein. However, that proteins contribute to the antibacterial activity of the extract cannot be completely ruled out because, whilst unlikely, it is possible that an antibacterial protein could resist proteinase inactivation.

In the present study, levels of fatty acids (derivatised as FAME) and proteins were assessed to provide further insight into the nature of the antibacterial compounds present. There was a large decrease in the concentrations of proteins and fatty acids in extracts from cultures harvested in late-exponential and early-stationary compared to early-exponential phase. This was because the mass of dried cell extracts were much larger for later harvest times (Figure 3.17) which suggests that other molecules were making up a larger proportion of the extract than at previous points of the growth curve. This additional mass may have been due to debris from dead or damaged algal cells. As extracts were resuspended in HEPES buffer at the same concentration each time (60 mg mL^{-1}) the concentration of fatty acids, proteins or other antibacterial molecules in these samples would be reduced, assuming that the material providing this extra mass was inactive. This explains not only why extracts from the later harvested cultures show lower concentration of protein and fatty acids in their extracts but also why these have lower levels of antibacterial activity.

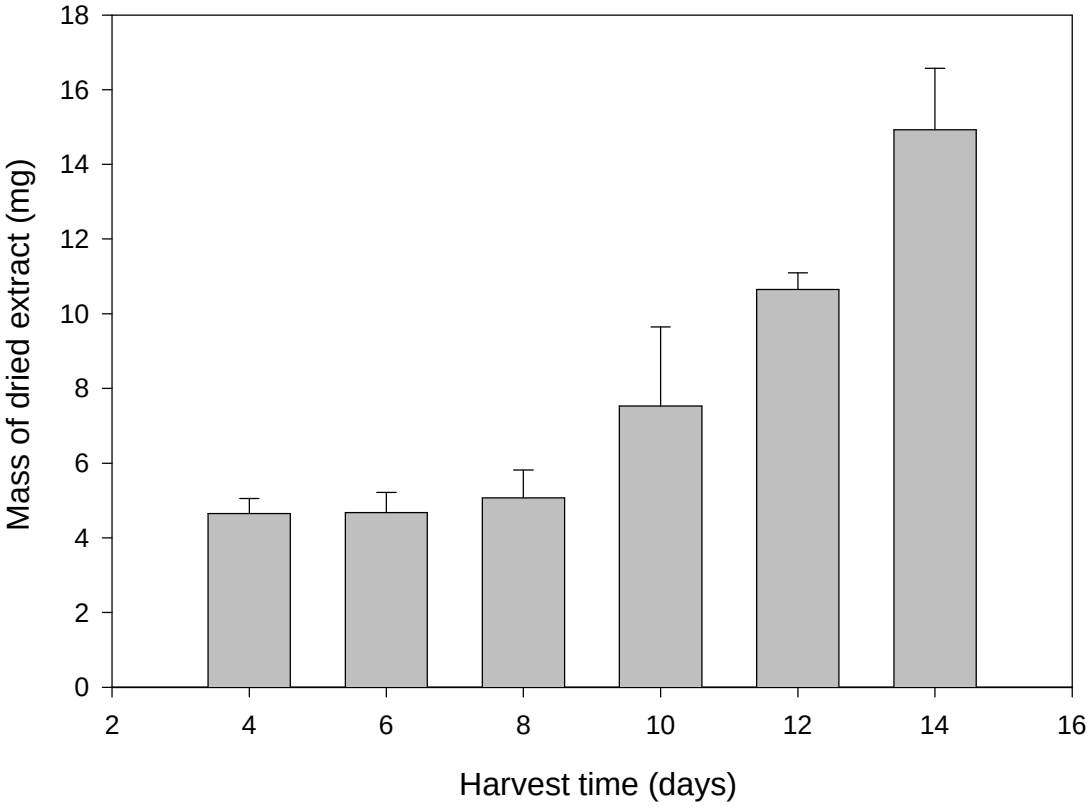


Figure 3.17 – Mass of dried cell extracts from *P. tricornutum* cultures harvested and extracted at different times during culture showing that the mass of the extracts increased with later harvest time. n = 4 (except for day 14, where n = 3); error bars are ± 1 SD.

Significant relationships were found between antibacterial activity of cell extracts and the mass of proteins and FAME found therein. However, as the mass of proteins and FAME in the cell extracts also correlate significantly with each other ($p < 0.001$; Figure 3.18), and the fact that protein concentration is often used as a general measure of organic molecules (Becker, 1994; Barbarino and Lourenço, 2005), this information does not aid with identifying the nature of the active compound(s). At this stage activity cannot be attributed conclusively to either proteins or fatty acids. The amounts of protein and FAME, when calculated on a 'per cell' basis, generally reduced with culture age and this could be because the stores of such molecules reduced as they could have been used for energy. Alternatively, the extraction may have become more inefficient as the number of cells in the extract increased. This reduction is unlikely to be due to the cells reducing in size because, even though diatoms generally reduce in size with subsequent generations (van den Hoek *et al*., 1995; Sze, 1998), this is often not observed with *P. tricornutum* (Wilson, 1946). In the present study, mass of fatty acids decreased five-fold during culture on a 'per cell' quota calculation. By contrast, Liang *et al*. (2006) found that total lipid increased with *P. tricornutum* culture age whilst Siron *et al*. (1989) report cellular fatty acid content increased three-fold during *P. tricornutum* culture. Contradictory findings probably reflect the use of different extraction protocols. Further, Liang *et al*. (2006) found that protein 'per cell' increased and peaked early in the culture (day 2) then gradually reduced with culture age and this was also found in the present study.

To summarise, there is evidence to suggest that multiple factors are responsible for the antibacterial activity of *P. tricornutum* cell extracts. First, extracts showed variable antibacterial activity against Gram positive and negative bacteria possibly

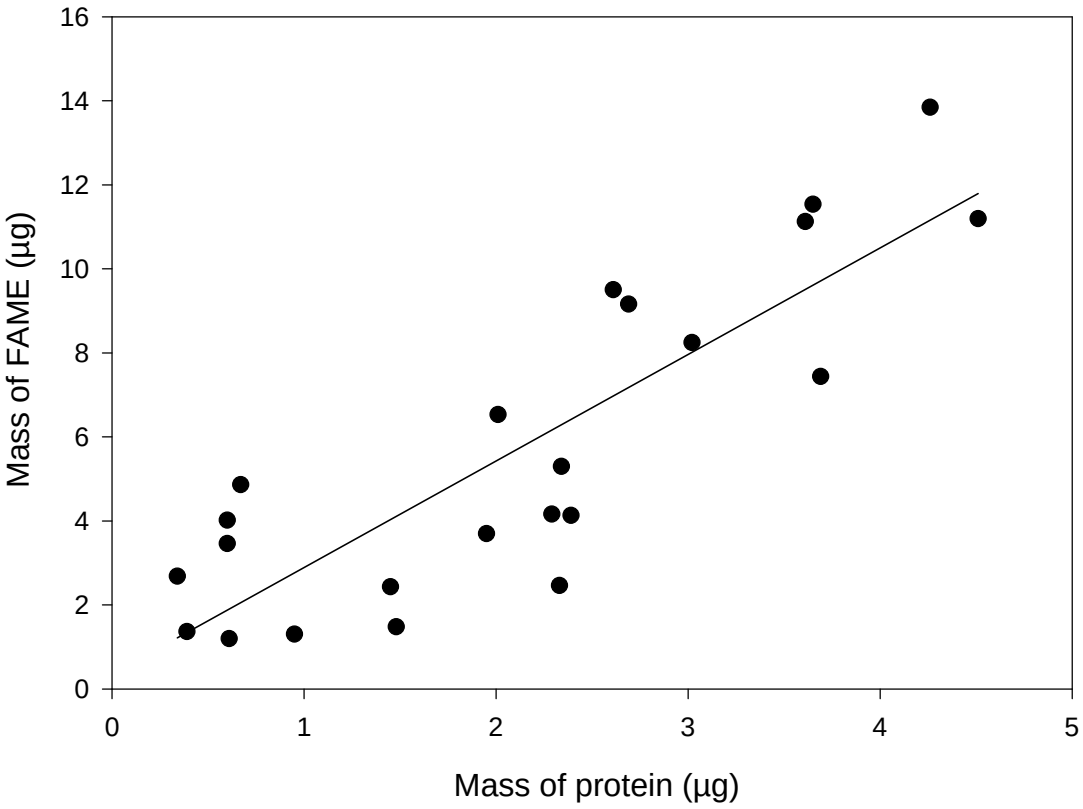


Figure 3.18 – Mass of protein and FAME in 4 µL aqueous methanol cell extracts of *P. tricornutum* resuspended at 60 mg mL^{-1} in sterile 50 mM HEPES (pH 7.8) prepared from algal cultures grown in the custom batch culture system and harvested 4 – 14 days. This shows that the mass of protein and fatty acids in the extracts correlate with each other and this correlation is very highly significant ($F_{1,21}$ = 56.33, r^2 = 0.728; p < 0.001).

indicative of two mechanisms of action. Further, Cooper *et al*. (1983) showed that spectrum of antibacterial activity changed according to the growth curve suggesting qualitative changes in the expression of antibacterial molecules. Second, antibacterial activity has been reported in lipophilic and aqueous fractions of cell extracts (Cooper *et al*., 1983). Third, evidence from the pH work reported above shows that acid and alkali soluble compounds may be present.

Now that the kinetics of production have been characterised, the optimal yield of antibacterial activity has been determined and stability of antibacterial compounds in the extract assessed, the thesis continues with the isolation of antibacterial molecules from *P. tricornutum* cell extracts by bioassay-guided fractionation.

Chapter 4: Isolation of (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid, an antibacterial fatty acid, from aqueous methanol cell extracts of the marine diatom, *Phaeodactylum tricornutum*

4.1.0 Introduction

Microalgae have been shown to produce antibacterial molecules of varied chemical species including fatty acids (Pesando, 1972; Findlay and Patil, 1984; Ohta *et al*., 1994), nucleosides (Aubert *et al*., 1970), peptides (Berland *et al*., 1972) and pigment derivatives (Blaauw-Jansen, 1954; Jørgensen, 1962; Bruce *et al*., 1967; Hansen, 1973; Trick *et al*., 1984). As any of these bio-molecules could be responsible for the activity in *P. tricornutum* cell extracts, a broad approach to isolating the antibacterial molecules is required. A much larger quantity of *P. tricornutum* biomass is also required and this can only be achieved by scaling up culture.

For ease of preparation, an alternative, cheaper medium, Miquel seawater (Allen and Nelson, 1910), was selected and tested for reliable *P. tricornutum* growth and production of antibacterial compounds because medium composition is known to affect the production of antibacterial metabolites (Cannell *et al*., 1988; Ohta *et al*., 1995). Large-scale production and extraction of algal biomass is performed from which the antibacterial compounds can be separated by chromatographic methods.

4.2.0 Materials and methods

All solvents were HPLC-grade and all water used was sterile ultra pure de-ionised water (Option 3 Water Purifier; Elga). Seawater that had been coarse-filtered through sand was obtained from the Gatty Marine Laboratory aquarium.

4.2.1 Large-scale *P. tricornutum* culture

4.2.1.1 Lightbox

An enlarged lightbox of internal dimensions of 190 cm x 70 cm x 55 cm (w x h x d) was constructed. Illumination was provided from five 6 ft white light fluorescent tubes (TLD70W/35; Philips) positioned at the rear of the box. Irradiance ranged from 200 - 450 µmol s^{-1} m^{-2} with a 14:10 h light:dark regime and lightbox temperature ranged from 15-25 ºC. These light and temperature conditions are suitable for *P. tricornutum* culture (Hayward, 1968b; Styron *et al*., 1976; Fawley, 1984; Chrismadha and Borowitzka, 1994).

4.2.1.2 Culture vessels

Nine separate culture vessels were positioned in the lightbox. They comprised 4x 18 L, 2x 22 L, 1x 23 L, 1x 10 L polypropylene carboys (Nalgene) and 1x 10 L flat-bottom glass jar (Pyrex; Corning Ltd.) (Figure 4.1). Together they provide batches of 158 L of culture. The upper opening of each vessel had a non-absorbent cotton wool bung with rigid plastic tubing passed through. The tubing enabled air, sterilised through an in-line 0.3 µm PTFE filter (Hepavent; Whatman International Ltd.) filter, to be supplied to the bottom of each vessel at 0.3 v/v/min.

4.2.1.3 Culture medium

The culture vessels were filled with seawater and sterilised with 0.25 mL 4 % sodium hypochlorite solution per litre of seawater (final concentration 10 ppm free chlorine). Air, supplied from an aquarium air line, was used to aerate the seawater for 30 min. The vessels were left in the dark for 12 h before 1 mL 12 g L^{-1} sodium thiosulphate solution per litre seawater was added to deactivate the residual chlorine. To ensure no

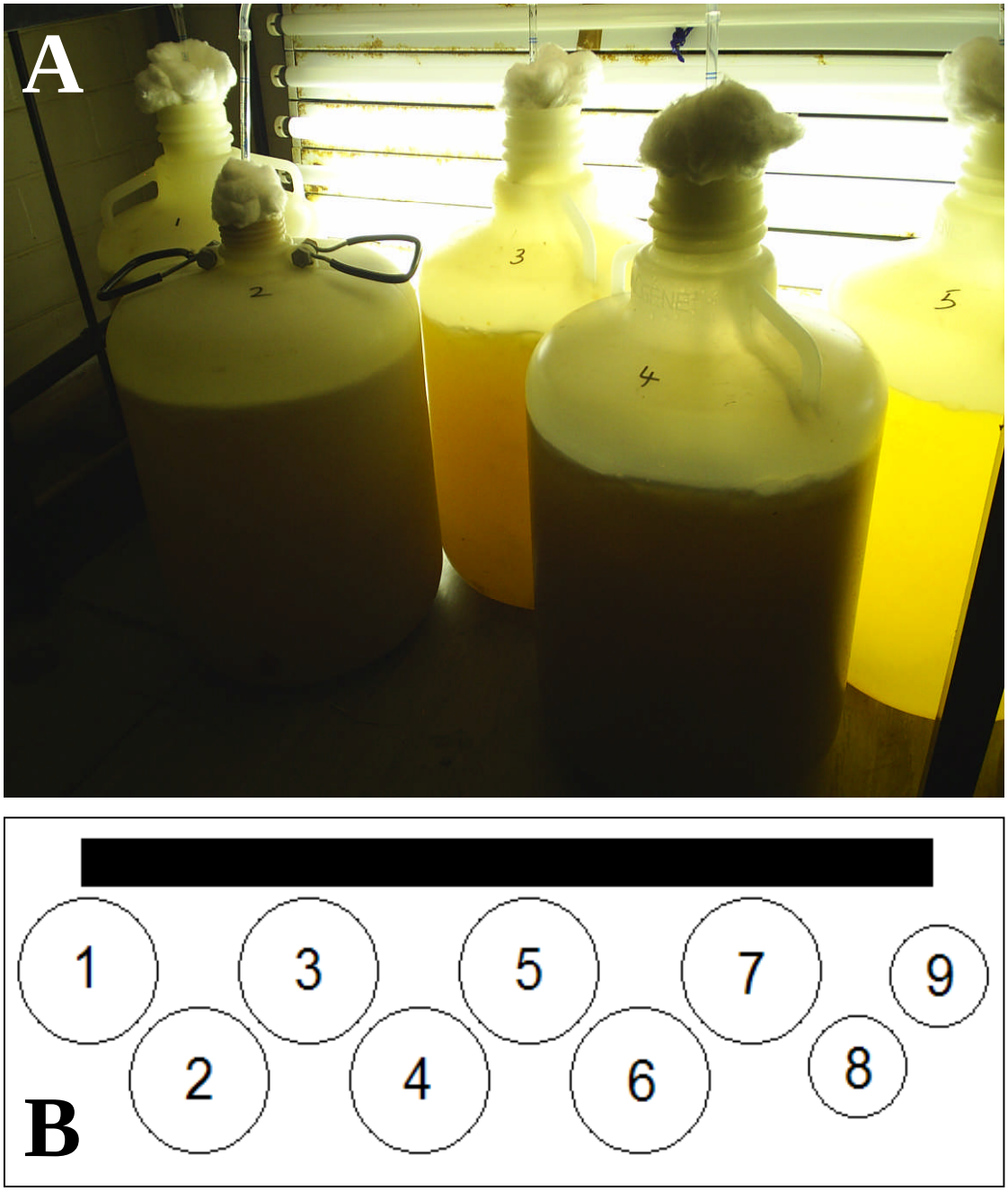


Figure 4.1 – (A) Photograph of the large-scale system for culturing 158 L batches of *P. tricornutum* in Miquel seawater medium in a lightbox supplied with sterile air and (B) the positions of the nine culture vessels in relation to the lights.

free chlorine remained, its concentration was assessed before inoculation (Free Chlorine Detection Kit; Hanna Instruments). Nutrients in sterile solution were aseptically added to give Miquel seawater medium (Appendix III).

4.2.1.4 Inoculum, growth and cell harvest

Each vessel was inoculated with a 8 to 9 day-old exponential phase mixed-morphology axenic *P. tricornutum* culture (5 % v/v) that had been cultured in 5 L glass jars (as Appendix VIII). Growth was monitored in each vessel by determining the A_{750} of 1 mL aliquots removed at intervals. Sterile medium was used as reference. The cultures were harvested at mid- to late-exponential phase by centrifugation in 400 mL batches at 1387 *g* for 5 minutes at 18 ºC (Beckman J2-21M/E). The supernatants were discarded and the cell pellets were resuspended in sterile 3.2 % NaCl solution, combined together before transferring to sterile 50 mL falcon tubes. The resuspended cell pellets were centrifuged at 3000 *g* for 15 minutes at 4 ºC (4K15; Sigma Aldrich) and the supernatant discarded. Each culture generated 1 cell pellet that was stored at –80 ºC. A total of 632 L of *P. tricornutum* was cultured in 36 vessels.

4.2.2 Comparison of growth and yield of antibacterial activity in modified ESAW and Miquel seawater media

To ensure that the new culture conditions had no significant effect on *P. tricornutum* growth or production of antibacterial compounds, the small-scale batch culture system was inoculated and run (as Section 2.2.2) for 10 days, except that six culture bottles contained Miquel seawater medium and six contained modified ESAW medium. At 24 h intervals, 1 mL aliquots were aseptically removed from each culture and the A_{750} determined. The respective sterile medium was used as reference. After 10 days, the

algae in each bottle was harvested and the cells extracted as Section 2.2.4. Extracts at 60 mg mL^{-1} were tested for antibacterial activity against *S. aureus* by RDA as Section 2.2.7.

4.2.3 Extraction of scale up biomass

Each cell pellet (15 – 35 mL) was resuspended to 50 mL with methanol:water (5:1) and extracted on ice by agitating on the orbital shaker for 16 h. The extracts were centrifuged at 5525 *g* for 1 h at 4 ºC to remove cellular debris and the supernatants stored at –80 ºC. To test that each extract contained antibacterial activity, 100 µL of each extract was dried at 30 °C with a speed vac and resuspended in 50 µL sterile 50 mM HEPES buffer (pH 7.8) before testing for antibacterial activity against *S. aureus* by RDA (as Section 2.2.7). Active extracts (from 533 L of *P. tricornutum* culture) were dried on the speed vac at 30 ºC before storage at –80 ºC. The yield was 18.98 g.

4.2.4 Separation of methanol:water (5:1) cell extracts

4.2.4.1 Sep Pak separation

To 1.2 g of the dried cell extract 60 mL of 70% methanol was added and the extract resuspended. Of this, 20 mL was loaded on to a Sep Pak cartridge (Sep Pak Vac 35cc tC18 – 10 g; Waters Ltd.) and the cartridge eluted with 20 mL of 70 % aqueous methanol followed by 20 mL each of methanol in increasing 5 % steps until 100 %. Eluates were collected as pigmented bands and 1 % by volume of each dried on the speed vac at 30 ºC, resuspended in methanol to 40 mg mL^{-1} and antibacterial activity of each tested against *S. aureus* by RDA (as Section 2.2.7). The remainder of each fraction was dried using the speed vac and stored at –20 ºC.

4.2.4.2 RP-HPLC of Sep Pak fractions 8 and 10

As fraction 8 was highly antibacterial, it was resuspended in 1.3 mL buffer A (99.93 % HPLC-grade water, 0.07 % TFA) and centrifuged at 13000 g for 20 min at room temperature. The supernatant was filtered through a 0.2 µm nylon filter (Acrodisc; Gelman Sciences Ltd.) and 1 mL was injected on to a C_{18} column (Gemini 5 µm, 250 x 10 mm, 110 Å; Phenomenex) of the HPLC (Finnegan Surveyor; Thermo Fisher Scientific Inc.). The column was eluted at 2 mL min^{-1} on a gradient of 0 – 100 min, 0 – 100 % v/v B; 100 – 120 min, 100 % v/v B (buffer B was 99.93 % HPLC-grade methanol, 0.07 % TFA). The eluate was monitored at 214 nm and 258 nm with a UV-VIS detector and 2 mL fractions collected in borosilicate glass tubes (Foxy Jr; Teledyne Isco Inc.). The same process was performed for active fraction 10. Fractions were tested for antibacterial activity against *S. aureus* by disc diffusion as described below (Section 4.2.5).

4.2.4.3 RP-HPLC of Sep Pak fraction 7

As fraction 7 was also antibacterial, it was resuspended in 2 mL buffer A (5 % acetonitrile, 0.07 % trifluoracetic acid (TFA), 94.93 % water) and prepared for RP-HPLC by filtering through a 0.2 µm nylon membrane syringe filter. One mL was injected on to the C_{18} column and eluted at 2 mL min^{-1} by a gradient of 0 – 100 min, 0 – 100 % v/v B; 100 – 120 min, 100 % v/v B (buffer B was 99.93 % HPLC-grade acetonitrile, 0.07 % TFA). The eluate was monitored for 0 – 100 min at 214 nm and 278 nm with a UV-VIS detector and 2 mL fractions collected and tested for antibacterial activity against *S. epidermidis* ATCC 10145 as described in Section 4.2.5 below.

4.2.5 Disc diffusion assay for antibacterial activity

A disc diffusion assay was used in preference to the RDA because a drying step was not required. For this assay, 25 µL of each fraction was pipetted on to sterile paper discs (Ø 6 mm AA; Whatman) and allowed to dry to completion at room temperature. This was repeated twice more for each disc to give 75 µL of each fraction per disc. A sterile swab was used to pick bacterial colonies from a stock agar plate and these were spread evenly across the surface of a sterile nutrient agar plate. The extract-impregnated discs were placed on the agar surface and the plates were incubated at 37 ºC for 18 h. Clear zones appearing around discs were interpreted as an indication of antibacterial activity. The clear zone was measured with a ruler to the nearest half millimetre and area of bacterial growth inhibition calculated as total area of clear zone minus the area of the disc.

4.2.6 Mass spectrometry and NMR spectroscopy

As fraction 96 from the RP-HPLC separation of Sep Pak fraction 7 exhibited greatest antibacterial activity (see Section 4.3.3 below) it was dried to completion on the speed vac at 30 ºC (dried mass = 1.7 mg) and resuspended in ~1.3 mL methanol. Chemical ionisation mass spectrometry (CI-MS) was performed for this fraction by Dr. Catherine Botting (School of Biology, University of St Andrews) using a 10 µL injection in negative ionisation mode at 20 kV with peak detection between 50 to 1500 Da. The remainder of fraction 96 was re-dried on the speed vac at 30 ºC and resuspended in ~1.5 mL methanol-d_4. The sample was filtered through lens tissue in a glass Pasteur pipette before the ^{1}H-NMR spectrum was recorded at 300 MHz (Avance 300; Bruker BioSpin GmbH). The NMR experimentation was kindly performed by Dr. Tomas Lebl (School of Chemistry, University of St Andrews). Chemical shifts

(δ) are given in parts per million (ppm) with respect to tetramethylsilane (δ ^{1}H for CD_3OD = 3.31 ppm).

4.2.7 Statistical analyses

The data collected on the growth and the level of antibacterial activity in methanol:water (5:1) cell extracts of *P. tricornutum* cultures grown for 10 days in either Miquel seawater or modified ESAW media were tested for normality by Shapiro-Wilk test and for homogeneity of variance by Levene's test. Neither the antibacterial activity nor A_{750} data were normally distributed ($W_{12} = 0.830$, $p < 0.05$ and $W_{12} = 0.828$, $p < 0.05$, respectively) but Levene's test showed that both sets of data had equal variance. T-tests were used to test for significant differences between growth and antibacterial activity of extracts of cultures in the two media. For all analyses $p \leq 0.05$ was considered significant.

4.3.0 Results

4.3.1 Large-scale *P. tricornutum* culture

Growth of *P. tricornutum* tended to vary between culture vessels (Figure 4.2) probably because there were small differences in irradiance, temperature, airflow or agitation. Most cultures were harvested in late exponential stage, with only three of 36 vessels failing to show good growth. These three were not used in subsequent experimentation.

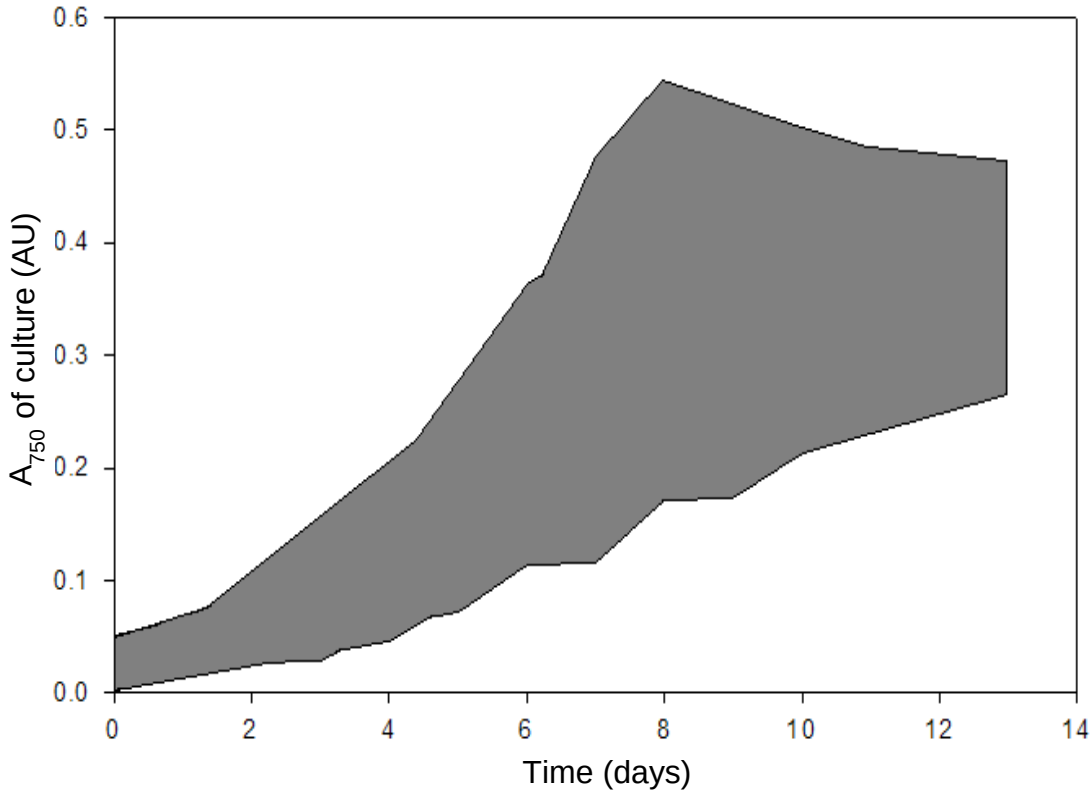


Figure 4.2 – Range of *P. tricornutum* growth (shaded area) in large-scale cultures of Miquel seawater medium showing that culture growth varied between vessels. Three cultures (not shown) were not harvested for further work due to poor growth.

4.3.2 Comparison of growth and yield of antibacterial activity in modified ESAW and Miquel seawater media

P. tricornutum cultured in Miquel seawater medium had a longer lag phase compared to those grown in modified ESAW medium (Figure 4.3), perhaps because the inoculum was also cultured in modified ESAW. The modified ESAW medium cultures showed significantly more growth at day 10 compared with Miquel seawater medium cultures ($t_{10} = 11.78$, $p < 0.001$) (Figure 4.3). However, no significant differences were found between the quantity of antibacterial activity in cell extracts prepared from those cultures grown in modified ESAW and those grown in Miquel seawater medium ($t_{10} = 1.073$, $p > 0.05$) (Figure 4.4).

4.3.3 Separation of methanol:water (5:1) cell extracts

The antibacterial activities of Sep Pak fractions against *S. aureus* are shown in Figure 4.5. Greatest activity was found in fractions 8 and 10, but no activity was found in fractions 1, 2, 13 and 14 (Figure 4.5). No activity was found in any fraction from the RP-HPLC separation of Sep Pak fractions 8 and 10 against *S. aureus*. However, two fractions (nos. 95 and 96) from the RP-HPLC separation of Sep Pak fraction 7 were antibacterial against *S. epidermidis* (Figure 4.6). Fraction 96 had greater antibacterial activity (45.9 mm^2) than fraction 95 (23.3 mm^2). This activity coincided with a major peak on the 214 nm spectrum at 94.168 min (Figure 4.7).

4.3.4 Mass spectrometry and NMR spectroscopy

Mass spectrometry of antibacterial fraction 96 from the RP-HPLC separation of Sep Pak fraction 7 revealed a prominent *m/z* ion at 301.26 Da corresponding to $[M]^-$ (Figure 4.8). The minor *m/z* ion at 603.58 Da probably corresponds to a dimer

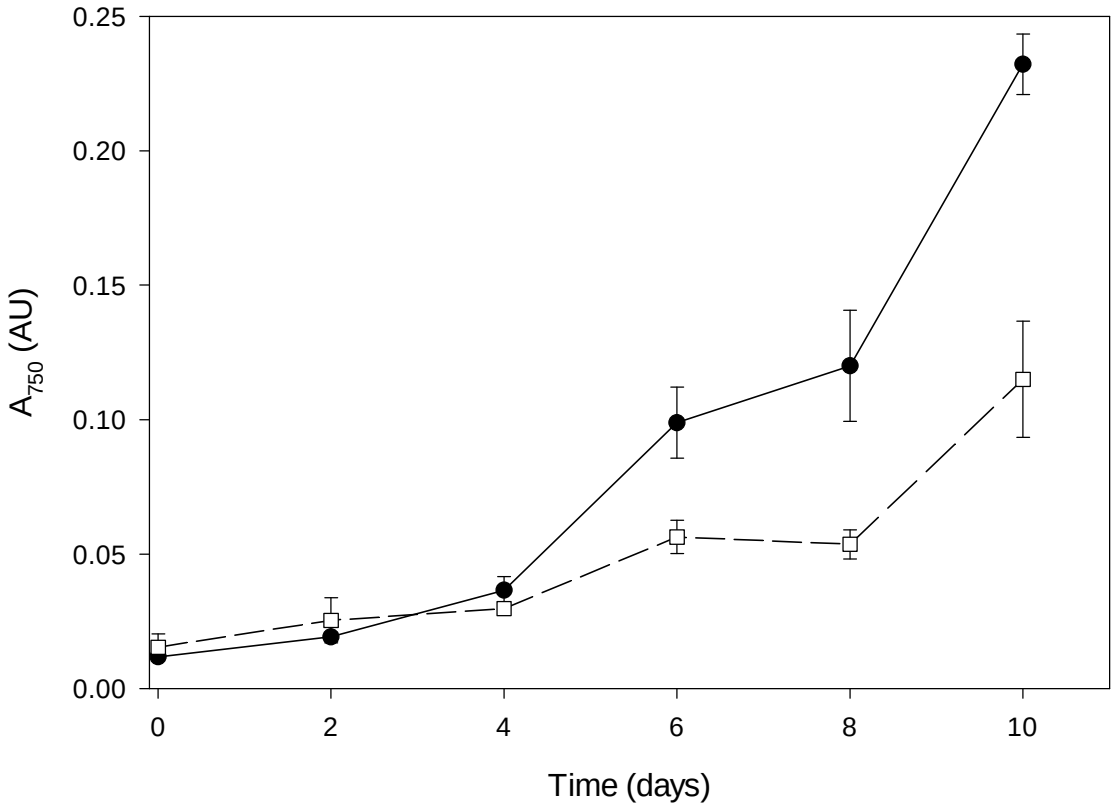


Figure 4.3 – Growth of *P. tricornutum* for 10 days in modified ESAW medium (●) or Miquel seawater medium (□) using the small-scale batch culture system showing that the modified ESAW cultures grow significantly better after 10 days (t_{10} = 11.78, p < 0.001). The other days not tested for significant differences. n = 6; error bars ± 1 SD.

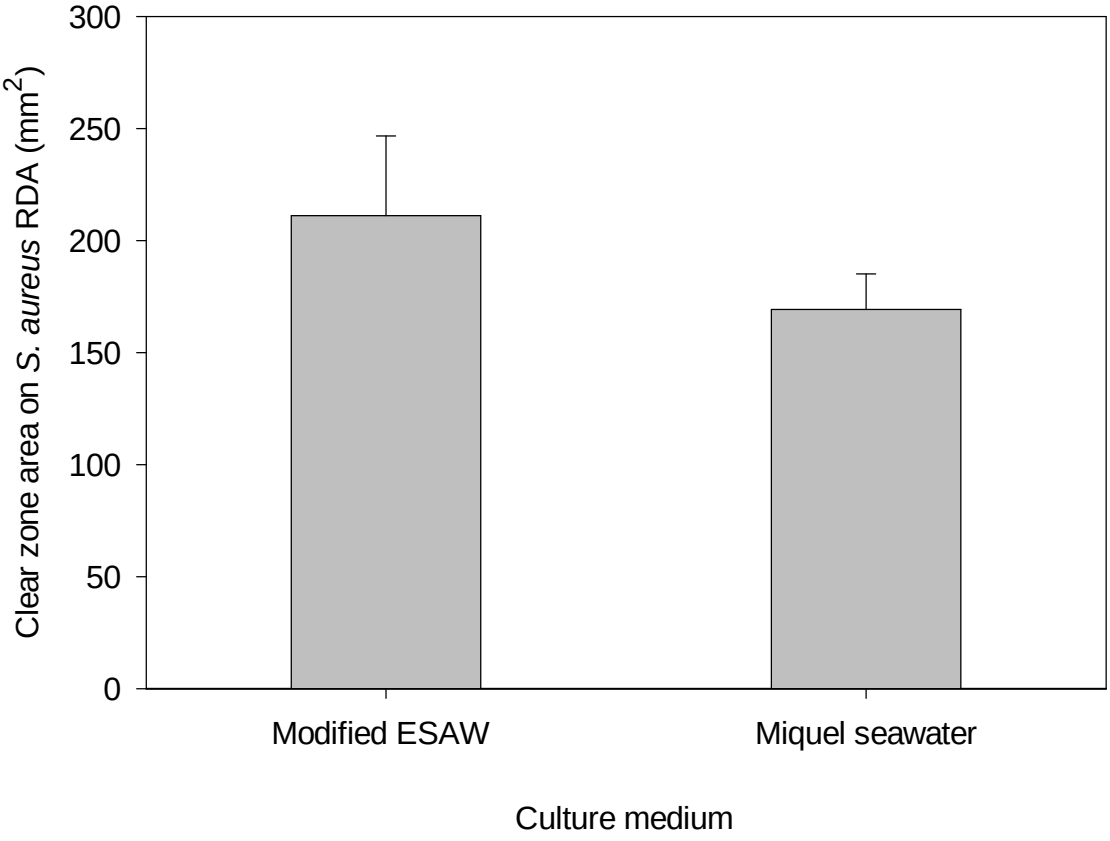


Figure 4.4 – Antibacterial activity of *P. tricornutum* aqueous methanol cell extracts against *S. aureus* prepared from cultures grown in modified ESAW or Miquel seawater medium showing that extracts from cultures grown in these media did not significantly differ in their levels of antibacterial activity. n = 6; error bars are ± 1 SE.

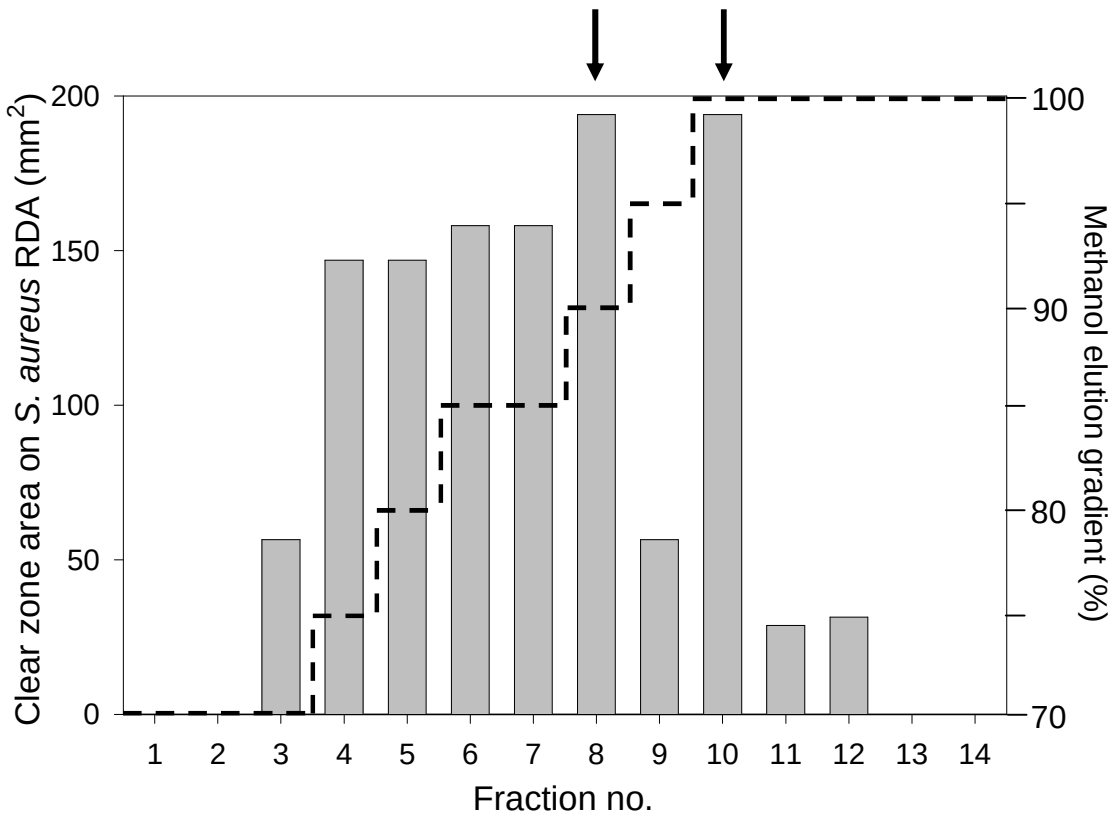


Figure 4.5 – Antibacterial activity against *S. aureus* of 14 fractions collected from C_{18} Sep Pak cartridge separation of *P. tricornutum* aqueous methanol cell extracts. At least two peaks of activity are present (marked with arrows) suggesting the presence of multiple antibacterial compounds. For antibacterial activity testing, 1 % by volume of each fraction was resuspended to 40 mg mL^{-1}. The dashed line shows the elution gradient (from 70–100 % methanol) used on the Sep Pak column.

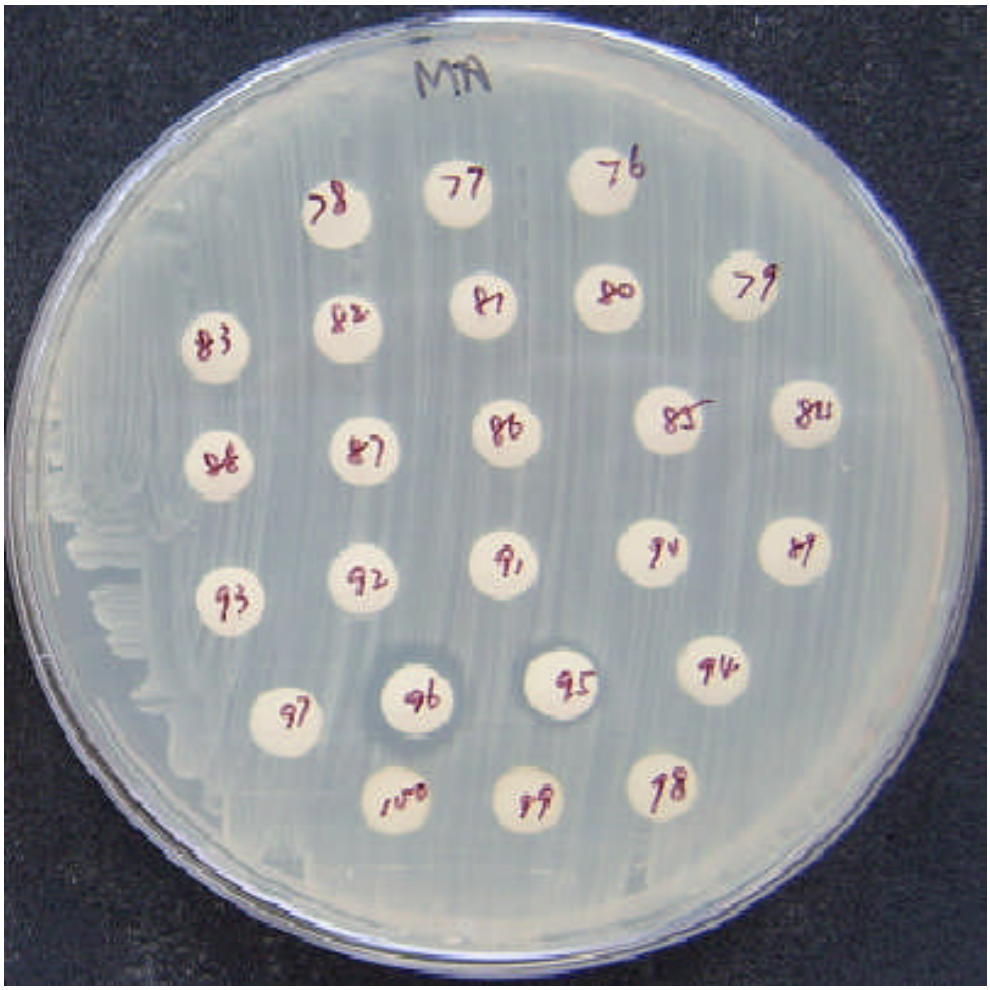


Figure 4.6 – Antibacterial activity of fractions 76-100 from RP-HPLC separation of Sep Pak fraction 7 against *S. epidermidis* showing that only fractions 95 and 96 were active (fractions 1-74 not shown).

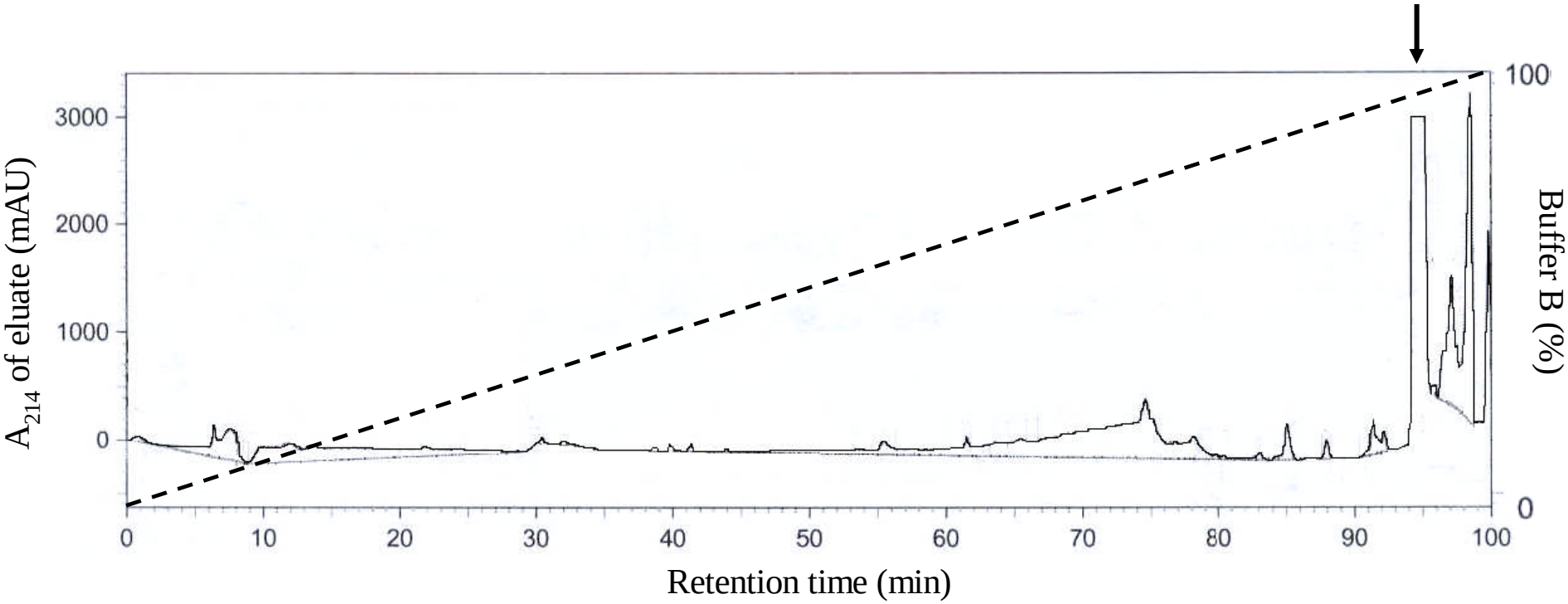


Figure 4.7 – A_{214} of eluate (0 – 100 min) during RP-HPLC C_{18} separation of Sep Pak fraction 7 showing the peak at 94.168 min corresponding to antibacterial fractions 95 and 96 (marked with arrow). Dashed line shows the elution gradient (buffer A: 5 % acetonitrile, 0.07 % TFA, 94.93 % water; buffer B: 99.93 % acetonitrile, 0.07 % TFA).

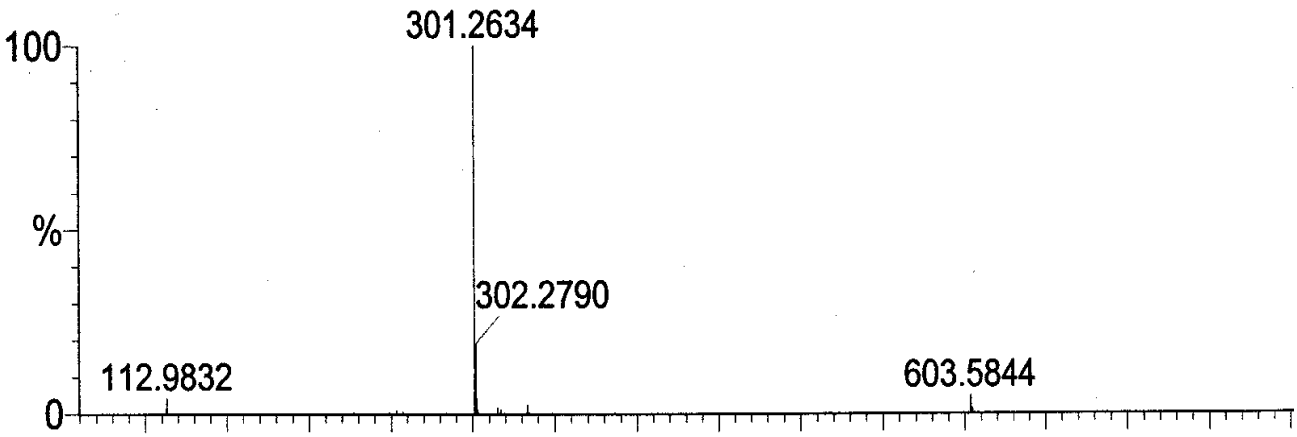


Figure 4.8 – Mass spectrum of antibacterial fraction 96 from the RP-HPLC separation of Sep Pak fraction 7 showing predominant peak at 301.26 Da corresponding to $[M]^-$ (only data 50 – 800 Da shown because no peaks found 800 – 1500 Da).

$[M+M]^-$ (Figure 4.8). The ^{1}H-NMR spectrum (Figure 4.9) showed a signal at δ 0.98 (3H) due to the terminal methyl group (Table 4.1). The signal at δ 2.02-2.18 (4H) indicates the presence of a methylene group adjacent to the terminal methyl group, and this signal also represents the methylene group at C_4 (Table 4.1). The degree of unsaturation in the carbon chain was determined from the integration of the olefinic protons at δ 5.25-5.45 ppm (Table 4.1). The presence of 10 olefinic protons indicates 5 double bonds. The signal at δ 2.78-2.90 (8H) is the protons in the methylene groups between the double bonds (Table 4.1) whilst the signals at δ 2.29 and δ 1.67 are attributable to the methylene groups adjacent to the carbonyl group (C_2) and C_3, respectively (Table 4.1). The ^{1}H-NMR spectrum is characteristic of an unsaturated fatty acid. These data, in addition to the CI-MS results, enable the identification of the molecule present in fraction 96 from the RP-HPLC separation of Sep Pak fraction 7 as (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid (Figure 4.10). This fatty acid will be referred to henceforth as 20:5n3 (see Appendix I). The ^{1}H-NMR spectrum compares very well to previously published ^{1}H-NMR spectra for 20:5n3 (Guil-Guerrero *et al*., 2001; Fu *et al*., 2004). Yield of 20:5n3 was calculated to be ~0.2 mg L^{-1} culture.

4.4.0 Discussion

In this chapter, the long-chain unsaturated fatty acid, 20:5n3, was isolated from aqueous methanol cell extracts of *P. tricornutum* and shown to have antibacterial activity against *S. epidermidis*. Sep Pak separation revealed that additional antibacterial factors were present in the aqueous methanol cell extracts, although their activities were lost during subsequent RP-HPLC fractionation.

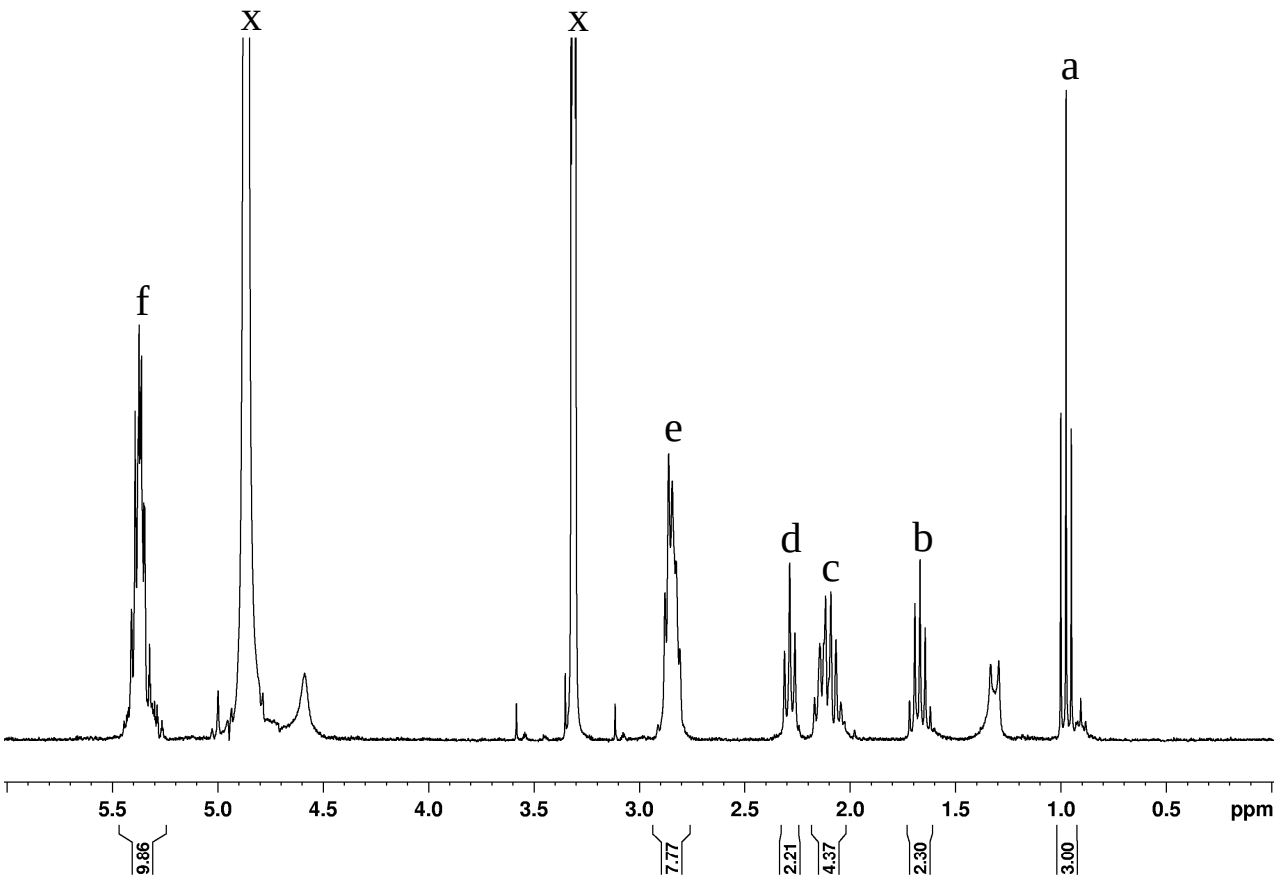


Figure 4.9 – ^{1}H-NMR spectrum of antibacterial fraction 96 from the RP-HPLC separation of Sep Pak fraction 7 in methanol-d_4 at 300 MHz showing the peaks attributable to the H atoms in: the terminal CH_3 group (a), the CH_2 at C_3 (b), the CH_2 group adjacent to the terminal CH_3 group and the CH_2 group at C_4 (c), the CH_2 group adjacent to the carbonyl group (d), the CH_2 groups between the C=C groups (e) and the five C=C double bonds (f). The other peaks are residual solvent (x).

Table 4.1 – ^{1}H-NMR data obtained in methanol-d_4 at 300 MHz for antibacterial fraction 96 from the RP-HPLC separation of Sep Pak fraction 7.

	^{1}H[a]	Peak label on ^{1}H-NMR spectrum
C1	-	-
C2	2.29, t, 2H, 7.4	d
C3	1.67, q, 2H, 7.5	b
C4	2.02-2.18, m, 2H	c
C5	5.25-5.45, m, 1H	f
C6	5.25-5.45, m, 1H	f
C7	2.78-2.90, m, 2H	e
C8	5.25-5.45, m, 1H	f
C9	5.25-5.45, m, 1H	f
C10	2.78-2.90, m, 2H	e
C11	5.25-5.45, m, 1H	f
C12	5.25-5.45, m, 1H	f
C13	2.78-2.90, m, 2H	e
C14	5.25-5.45, m, 1H	f
C15	5.25-5.45, m, 1H	f
C16	2.78-2.90, m, 2H	e
C17	5.25-5.45, m, 1H	f
C18	5.25-5.45, m, 1H	f
C19	2.02-2.18, m, 2H	c
C20	0.98, t, 3H, 7.6	a

[a] Data given as: chemical shift (ppm), multiplicity, number of protons, coupling constant (Hz) (where given).

Figure 4.10 – Structure of (5*Z*, 8*Z*, 11*Z*, 14*Z*, 17*Z*)-eicosapentaenoic acid, the compound in antibacterial fraction 96 from the RP-HPLC separation of Sep Pak fraction 7.

It is well established that free fatty acids have antibacterial properties (Kodicek and Worden, 1945; Nieman, 1954; Kabara *et al*., 1972; Butcher *et al*., 1976; Ko *et al*., 1978; Kanai and Kondo, 1979; Bergsson *et al*., 1998). The exact mechanism(s) of antibacterial activity by fatty acids remains unknown but it is likely that they act on cellular membranes affecting their structure and function (Galbraith and Miller, 1973a; Galbraith and Miller, 1973b; Miller *et al*., 1977; Freese, 1978; Thormar *et al*., 1987; McGaw *et al*., 2002). Fatty acids may cause leakage of molecules from the cell (Galbraith and Miller, 1973b; Thormar *et al*., 1987), act on numerous proteins located in the membrane (Miller *et al*., 1977), affect nutrient uptake (Sheu and Freese, 1972; Galbraith and Miller, 1973c), interfere with cellular respiration (Borst *et al*., 1962), inhibit bacterial fatty acid synthesis (Zheng *et al*., 2005) or mediate their effects by a peroxidative process involving hydrogen peroxide (Knapp and Melly, 1986; Wang and Johnson, 1992). It is also highly likely that unsaturated fatty acids act upon multiple cellular targets to inhibit bacterial growth (Miller *et al*., 1977). Antibacterial free fatty acids have been isolated from marine microalgae in bioassay-guided separations and they include: linolenic acid from *Chlorococcum* HS-101 (Ohta *et al*., 1994), hexadecatetraenoic acid from *Navicula delognei* (Findlay and Patil, 1984), 6, 9, 12-hexadecatrienoic acid and 9, 12-hexadecadienoic acid from *Chaetoceros* spp. (Wang, 1999).

Over 20 years ago, Cooper *et al*. (1985) isolated an antibacterial fraction from *P. tricornutum* cell extracts that contained a mixture of six fatty acids, including 20:5n3. These authors then tested the closest commercially available structural homologue, arachidonic acid (20:4n6), and found it to be antibacterial. Thus these authors concluded that EPA was responsible for some of the antibacterial activity in the

isolated fraction (Cooper *et al*., 1985). Other studies have confirmed EPA is antibacterial (Saito *et al*., 1984; Knapp and Melly, 1986; Ohta *et al*., 1995; Benkendorff *et al*., 2005; Shin *et al*., 2007). Interestingly, Pesando (1972) isolated a fraction containing 20:5n3 with antibacterial activity from chloroform:methanol (2:1) cell extracts of the marine diatom, *Asterionella japonica*, but attributed the activity to a photo-oxidation product of EPA and not EPA itself. Hence the present study is the first to isolate EPA from microalgal cell extracts and confirm it to be responsible for antibacterial activity.

As the Sep Pak separation produced two major peaks with antibacterial activity it is likely that more than one antibacterial compound is present in the aqueous methanol cell extracts (Figure 4.5). The presence of multiple active factors in *P. tricornutum* is quite plausible, especially as multiple antibacterial compounds have been isolated from a single microalga species (Bruce *et al*., 1967; Aubert *et al*., 1970; Findlay and Patil, 1984). However, it was not possible to isolate further antibacterial compounds from Sep Pak fractions 8 or 10 as the activity was lost during fractionation by RP-HPLC. The activity in these fractions could have been missed because they were not tested at a sufficiently high concentration.

This is the first time that 20:5n3, isolated and identified from cell extracts of a marine microalga, has been conclusively shown to be responsible for some of the antibacterial activity of the extracts. The work described in this chapter also strongly suggests the presence of further antibacterial compounds in aqueous methanol cell extracts of *P. tricornutum*. The isolation and identification of these further antibacterial compounds will form the focus of the next chapter.

Chapter 5: Isolation of two antibacterial fatty acids, (9*Z*)-hexadecenoic acid and (6*Z*, 9*Z*, 12*Z*)-hexadecatrienoic acid, from aqueous methanol cell extracts of the marine diatom, *Phaeodactylum tricornutum*

5.1.0 Introduction

In the previous chapter, eicosapentaenoic acid was isolated from aqueous methanol cell extracts of *P. tricornutum* and shown to be antibacterial. During its isolation, it became apparent that further antibacterial compounds of unknown chemical species were present. These compounds could not be purified using the techniques employed in the previous chapter; hence an alternative separation strategy was selected for isolating these further compounds. The aim of this chapter is to isolate these antibacterial compounds from cell extracts using silica gel chromatography and RP-HPLC. As such the compounds of interest will be separated initially according to polarity and then by their hydrophobicity. Isolated antibacterial compounds will undergo structural studies using mass spectrometry, ^{1}H- and ^{13}C-NMR spectroscopy.

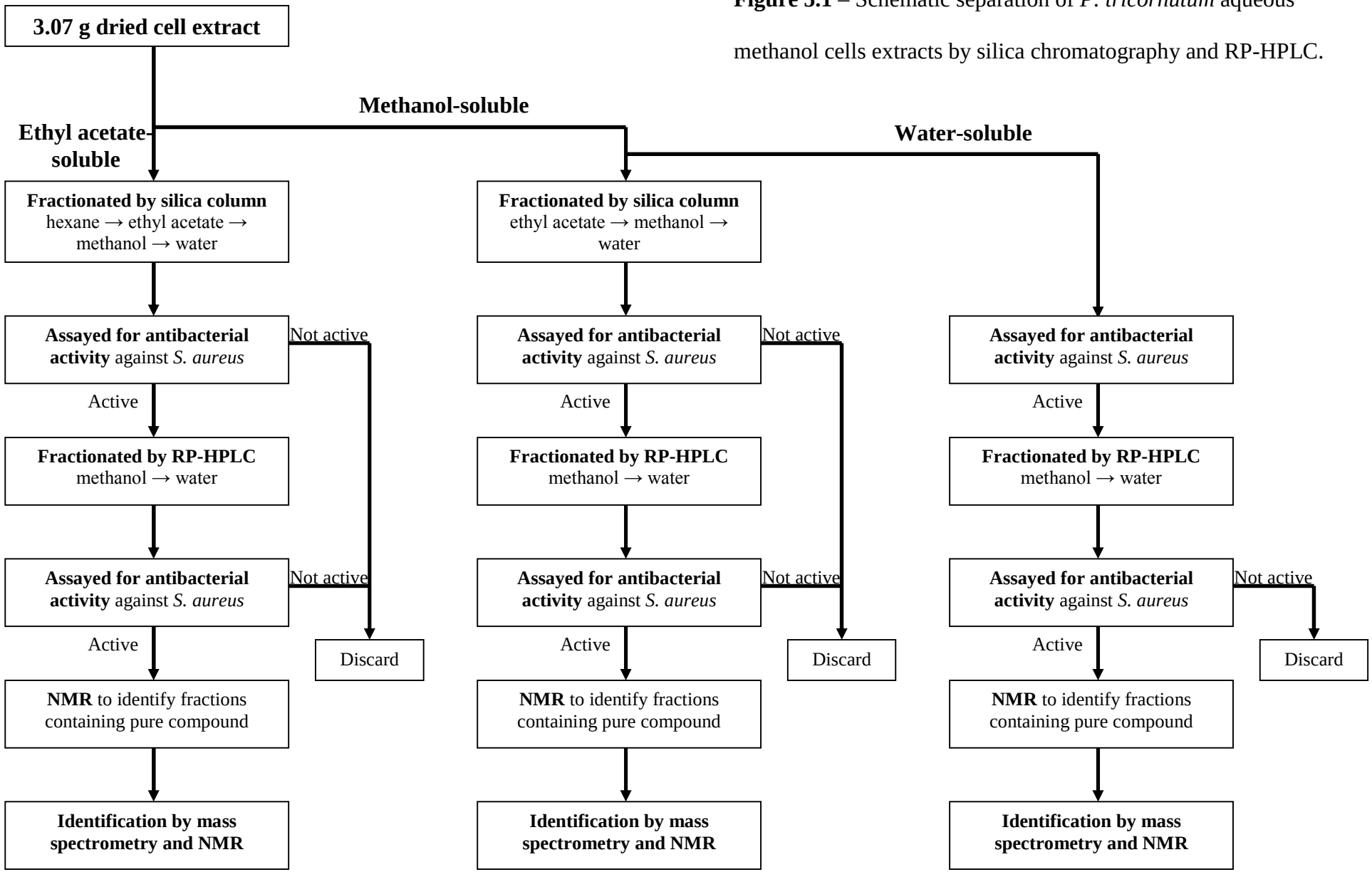
5.2.0 Materials and methods

Only HPLC-grade solvents and ultra pure water was used in the following work.

5.2.1 Separation of aqueous methanol *P. tricornutum* cell extract

The dried *P. tricornutum* cell extract starting material for this chapter came from surplus material generated in the previous chapter (Section 4.2.3). For clarity, the following separation can be found as a schematic diagram (Figure 5.1).

Figure 5.1 – Schematic separation of *P. tricornutum* aqueous methanol cells extracts by silica chromatography and RP-HPLC.



5.2.1.1 Ethyl acetate-soluble portion of aqueous methanol cell extract

Dried cell extract weighing 3.07 g was resuspended in 33 mL ethyl acetate, centrifuged at 13,000 *g* for 10 min at room temperature to remove the insoluble compounds. The insoluble pellet was stored at –20 °C until it could be extracted in methanol (see below). All the supernatant was applied to a Sep Pak silica cartridge (10 g; Waters) by loading ~2-5 mL at a time and allowing the ethyl acetate to evaporate. This was performed so that the extract never went beyond the top one third of the total length of the silica portion of the cartridge. To ensure the ethyl acetate had completely evaporated before the cartridge was run, it was left overnight at room temperature. The next day, the compounds of interest were fractionated according to their polarity using a step gradient of hexane, ethyl acetate, methanol and water. First, 100 mL hexane was added to the cartridge and a 100 mL fraction collected (fraction no. 1). Next 100 mL 9:1 hexane:ethyl acetate was added to the cartridge and the next fraction collected (fraction no. 2). This was followed by 100 mL of hexane:ethyl acetate mixes in which ethyl acetate concentration was increased by 10 % until pure ethyl acetate. With each change in elution solvent mix a 100 mL fraction was collected (fraction nos. 3-11). Then 100 mL 8:2 ethyl acetate:methanol was added followed by 100 mL ethyl acetate:methanol mixes in 20 % step increments of methanol until pure methanol. Again with each change in elution solvent mix a 100 mL fraction was collected (fraction nos. 12-16). Finally 75 mL water was added to the cartridge and the final fraction collected that was, in this case only, 75 mL (fraction no. 17). Each fraction (Table 5.1) was dried to completion on the rotary evaporator at 40 °C and resuspended in ~ 2 mL of ethyl acetate, methanol, water or a mix of these solvents as appropriate. Thirty microlitres of each fraction was tested for

Table 5.1 – Fractions generated from silica column separation of dried aqueous methanol cell extract of *P. tricornutum*. Dried cell extract was initially resuspended in ethyl acetate and run through a silica cartridge, the remaining extract was resuspended in methanol and run on a fresh column, and finally the non-soluble extract was resuspended in water.

Fraction Number	Extraction solvent	Elution solvent
1	Ethyl acetate	Hexane
2	Ethyl acetate	9:1 hexane:ethyl acetate
3	Ethyl acetate	8:2 hexane:ethyl acetate
4	Ethyl acetate	7:3 hexane:ethyl acetate
5	Ethyl acetate	6:4 hexane:ethyl acetate
6	Ethyl acetate	1:1 hexane:ethyl acetate
7	Ethyl acetate	4:6 hexane:ethyl acetate
8	Ethyl acetate	3:7 hexane:ethyl acetate
9	Ethyl acetate	2:8 hexane:ethyl acetate
10	Ethyl acetate	1:9 hexane:ethyl acetate
11	Ethyl acetate	Ethyl acetate
12	Ethyl acetate	8:2 ethyl acetate:methanol
13	Ethyl acetate	6:4 ethyl acetate:methanol
14	Ethyl acetate	4:6 ethyl acetate:methanol
15	Ethyl acetate	2:8 ethyl acetate:methanol
16	Ethyl acetate	Methanol
17	Ethyl acetate	Water
18	Methanol	Ethyl acetate
19	Methanol	9:1 ethyl acetate:methanol
20	Methanol	8:2 ethyl acetate:methanol
21	Methanol	7:3 ethyl acetate:methanol
22	Methanol	6:4 ethyl acetate:methanol
23	Methanol	1:1 ethyl acetate:methanol
24	Methanol	4:6 ethyl acetate:methanol
25	Methanol	3:7 ethyl acetate:methanol
26	Methanol	2:8 ethyl acetate:methanol
27	Methanol	1:9 ethyl acetate:methanol
28	Methanol	Methanol
29	Methanol	Water
30	Water	n/a

antibacterial activity against *S. aureus* by disc diffusion (as Section 4.2.7). Fractions were stored at –20 °C until further separation.

Fractions found to be antibacterial were dried on the rotary evaporator at 40 °C and resuspended in ~1.6 mL methanol. This was centrifuged at 13,000 *g* for 10 min at room temperature and the supernatant put through a 0.2 µm nylon syringe filter. One mL of filtered supernatant was applied to the semi-prep C_{18} column (250 x 10 mm; Phenomenex). Fractions containing very non-polar compounds (fraction nos. 1-11) were eluted at 2 mL min^{-1} with a gradient: 0 – 50 min 0 – 100 % v/v B; 50 – 80 min isocratic B (buffer A was 50 % methanol, 49.93 % water, 0.07 % TFA; buffer B was 99.93 % methanol, 0.07 % TFA) whilst fractions containing compounds of greater polarity (fraction nos. 12-17) were eluted at 2 mL min^{-1} with a gradient: 0 – 100 min 0 – 100 % v/v B; 100 – 120 min isocratic B (buffer A was 99.93 % water, 0.07 % TFA; buffer B was 99.93 % methanol, 0.07 % TFA). Eluate was monitored at 214 nm and 258 nm and 2 mL fractions were collected. The RP-HPLC fractions were stored at 4 °C and 75 µL of each HPLC fraction was tested for antibacterial activity against *S. aureus* by disc diffusion (as Section 4.2.7). Active fractions were freeze-dried (Lyolab 3000; Heto-Holten A/S) at –55 °C to completion in pre-massed sterile bottles and stored at 4 °C.

5.2.1.2 Methanol-soluble portion of aqueous methanol cell extract

The ethyl acetate-insoluble portion of the original dried extract was resuspended in 12 mL methanol and centrifuged at 13,000 *g* for 10 min at room temperature. The insoluble cell pellet was stored at –20 °C until it could be extracted in water (see below). Meanwhile, all the supernatant was mixed with ~20 mL silica gel (Matrex

Silica 60; Fisher Scientific) and allowed to dry overnight at room temperature, before drying to completion with the rotary evaporator at 40 °C. The dried silica/extract mix was applied to the top of a new silica Sep Pak cartridge. First, 100 mL ethyl acetate was added to the cartridge and a 100 mL fraction collected (fraction no. 18). Next 100 mL 9:1 ethyl acetate:methanol was added to the cartridge and this was followed by 100 mL each of ethyl acetate:methanol mixes in 10 % step increments of methanol until pure methanol. With each change in elution solvent mix a 100 mL fraction was collected (fraction nos. 19-28). Finally 50 mL water was added to the cartridge and the final fraction collected that was, in this case only, 50 mL (fraction no. 29). Each fraction (Table 5.1) was dried to completion and tested for antibacterial activity against *S. aureus* as above. Fractions were stored at –20 °C until further fractionation.

RP-HPLC was performed on fractions found to have antibacterial activity, as above. Fraction nos. 18 and 19 were considered to contain very non-polar compounds whilst fraction nos. 20-29 contained compounds of greater polarity. Again RP-HPLC fractions were tested for antibacterial activity against *S. aureus* by disc diffusion, with the active fractions being freeze-dried in pre-massed sterile bottles for storage at 4 °C (also as above).

5.2.1.3 Remaining water-soluble portion of aqueous methanol cell extract

The ethyl acetate and methanol-insoluble portion of original dried extract was resuspended in 9 mL water and designated fraction no. 30 (Table 5.1). Thirty microlitres of this was tested for antibacterial activity against *S. aureus* by disc diffusion (as Section 4.2.7). The fraction was stored at –20 °C before being prepared for RP-HPLC as above. As this fraction contained mainly polar compounds it was

run as such (see above). Once more the RP-HPLC fractions were tested for antibacterial activity and the active fractions freeze-dried in pre-massed sterile bottles for storage at 4 °C (all as above).

5.2.2 ^{1}H-NMR spectroscopy of active RP-HPLC fractions

Each active RP-HPLC fraction was resuspended in ~0.7 mL methanol-d_4, filtered and ^{1}H-NMR performed as Section 4.2.6. This enabled identification of those fractions sufficiently pure to permit complete structural characterisations and allowed dereplication of fractions containing the same compound.

5.2.3 Structural characterisation of antibacterial compounds by NMR spectroscopy and mass spectrometry

Each pure fraction had ^{1}H-NMR and 2D ^{1}H,^{13}C-HSQC (heteronuclear multiple quantum coherence), ^{1}H,^{13}C-HMBC (heteronuclear multiple bond correlation) spectra recorded at 500 MHz (Avance 500; Bruker BioSpin GmbH). The 2D-NMR experimentation would enable structural assignment. Again, chemical shifts are given in ppm with respect to tetramethylsilane (δ ^{1}H for CD_3OD = 3.31 ppm, δ ^{13}C for CD_3OD = 49.15 ppm). The pure fractions were dried on the speed vac at 30 ºC and resuspended in 400-1000 µL methanol depending on yield. Mass spectrometry (CI-MS) was performed for each fraction in negative mode with an injection volume of 10-20 µL by Dr. Catherine Botting and Mrs. Caroline Horsburgh (School of Chemistry, University of St Andrews). As necessary, high resolution mass spectrometry was also performed to generate empirical elemental composition data.

5.2.4 Location of double bond

The position of the double bond in the carbon chain of monounsaturated fatty acids was determined by electron impact mass spectrometry (EI-MS) of dimethyl disulphide adduct derivatives (Moss and Daneshvar, 1992). Briefly, 300 µL of the 1 mL sample in methanol was converted to methyl esters (as Section 3.2.7) and then dissolved in 200 µL dimethyl disulphide. Added to this was 50 µL of 60 mg mL^{-1} solution of iodine in diethyl ether. This mixture was put on the orbital shaker at room temperature and 60 rpm for 24 hours. Next, 5 mL hexane was added and the mixture washed three times with dilute 2 % aqueous sodium thiosulphate solution. Residual water was removed by mixing with anhydrous sodium sulphate and, after filtering through non-absorbent cotton wool (pre-washed with diethyl ether), the hexane was removed by gentle heating under a stream of nitrogen. The final product was finally resuspended in 50 µL hexane for EI-MS (kindly performed by Mrs. Caroline Horsburgh).

5.3.0 Results

5.3.1 Ethyl acetate-soluble portion of aqueous methanol cell extract

Fractions 2, 3, 4, 5 and 12 from Sep Pak separation were strongly antibacterial against *S. aureus*, whilst fractions 1, 6, 7, 8, 9, 10 and 11 showed weaker activity (Figure 5.2). Fractions 2, 3, 4 and 5 were pooled and then fractionated by RP-HPLC. This yielded four antibacterial fractions: nos. 52, 55, 56 and 57 (Figure 5.3). The major peak at 54.18 min on the A_{214} RP-HPLC trace corresponded to fraction 57 (Figure 5.4). Fraction 12 was fractionated by RP-HPLC with antibacterial activity found only in fractions 105 and 106.

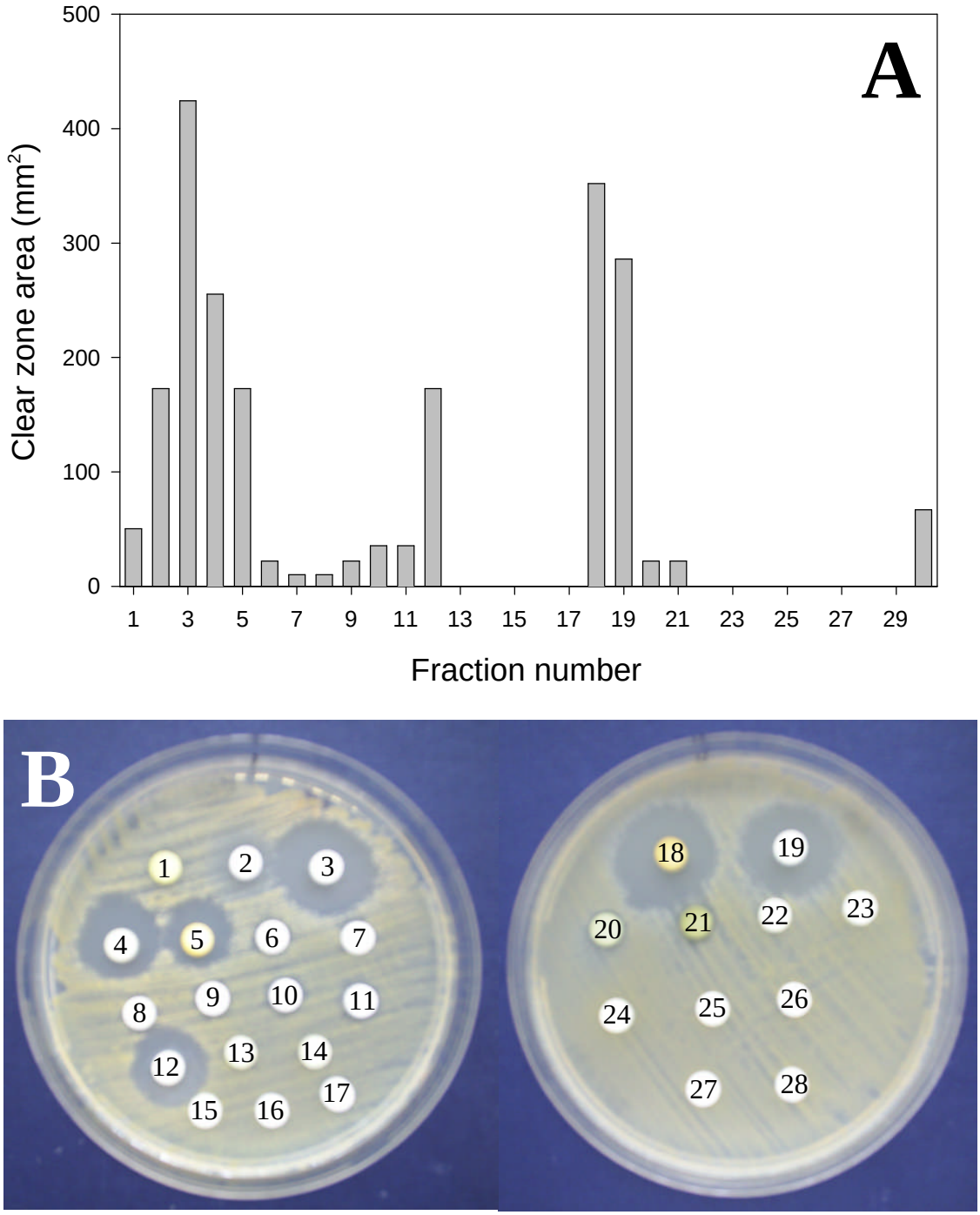


Figure 5.2 –Antibacterial activity of silica Sep Pak fractions against *S. aureus* using disc diffusion assay expressed as clear zone area on the assay plate (A) and images of the plates from which this data was obtained (B). Strong activity found in fraction nos. 2, 3, 4, 5, 12, 18 and 19 and weaker activity in fraction nos. 1, 6, 7, 8, 9, 10, 11, 20, 21 and 30. Please note that assay plate image for fractions 29 and 30 unavailable.

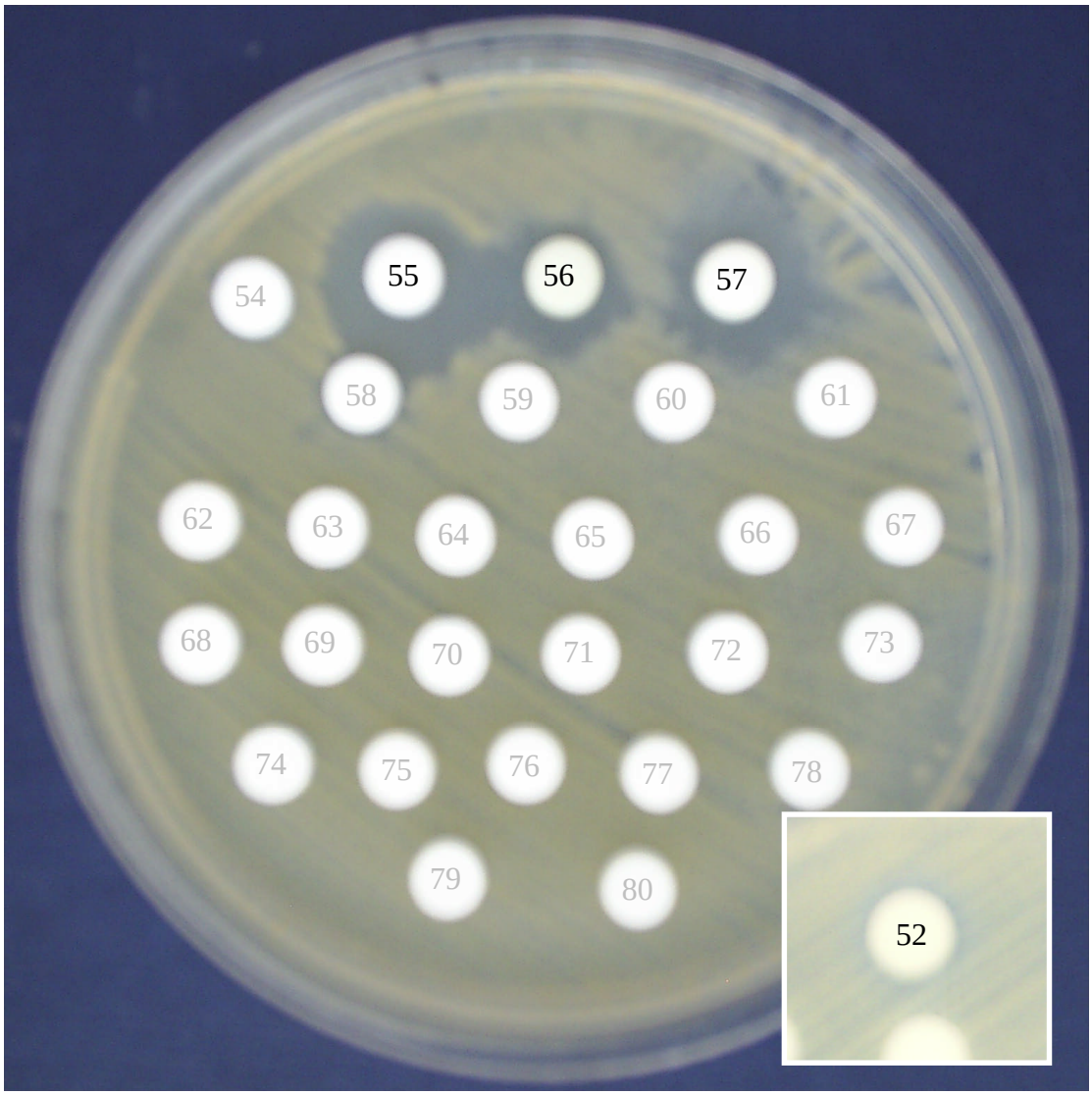


Figure 5.3 – Disc diffusion assay plate showing antibacterial activity against *S. aureus* of fractions 52 and 54-80 from RP-HPLC separation of pooled silica column fractions 2, 3, 4 and 5. Strong activity found in fractions 55, 56 and 57 and (inset) weaker activity found in fraction 52.

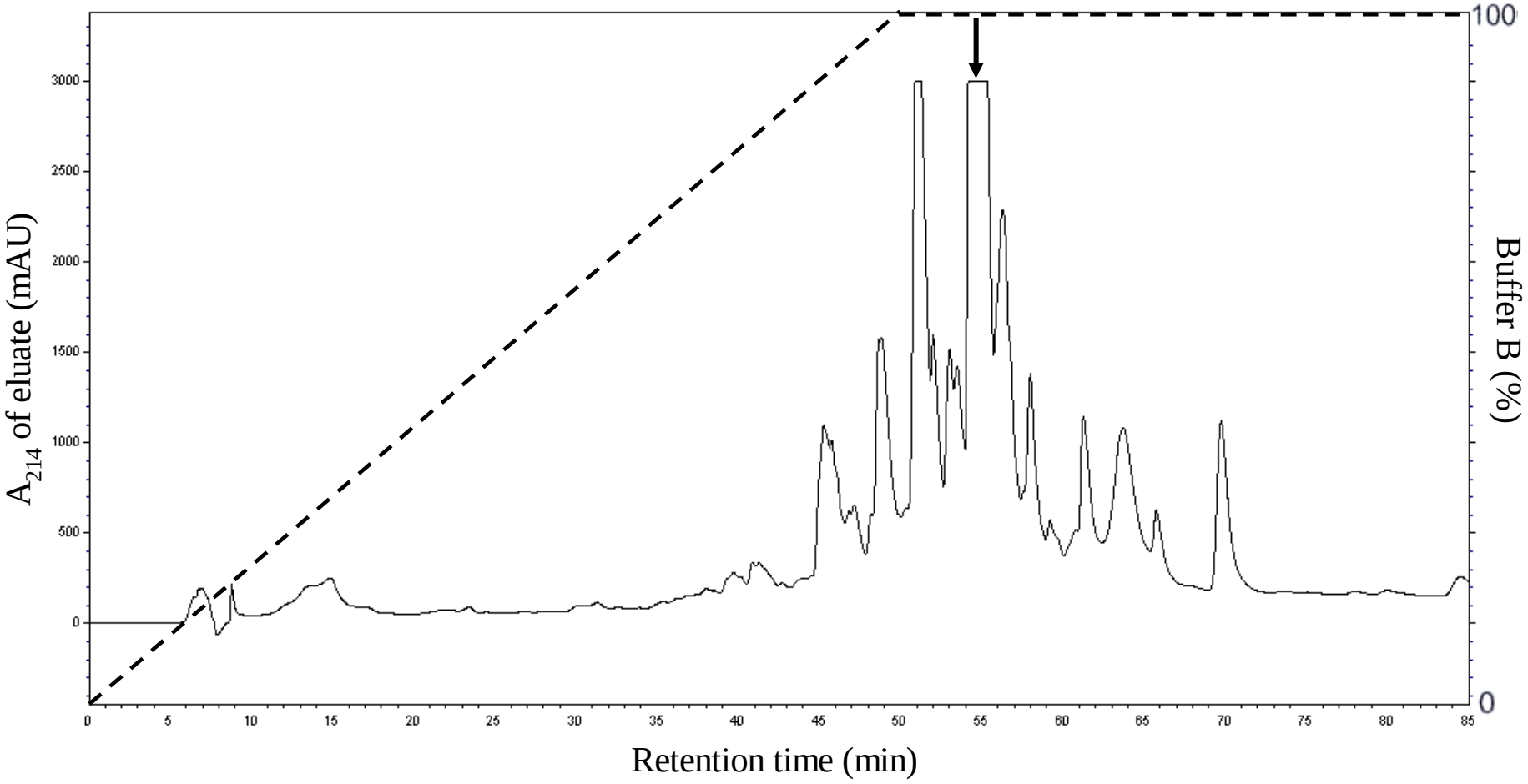


Figure 5.4 – A_{214} of eluate during RP-HPLC C_{18} separation of pooled silica column fractions 2, 3, 4 and 5 showing the peak at 54.18 min corresponding to antibacterial fraction 57 (marked with arrow). Dashed line shows the elution gradient (buffer A: 50 % methanol, 49.93 % water, 0.07 % TFA; buffer B: 99.93 % methanol, 0.07 % TFA).

5.3.2 Methanol-soluble portion of aqueous methanol cell extract

Fractions 18 and 19 from Sep Pak separation were strongly antibacterial against *S. aureus* with weak activity in fractions 20 and 21 (Figure 5.2). Each of these fractions was further fractionated by RP-HPLC. For fraction 18 antibacterial activity was found in RP-HPLC fractions 52, 55, 56 and 57 (Figure 5.5) with the minor peak at 50.55 min on the A_{214} RP-HPLC trace corresponding to fraction 52 (Figure 5.6). For fraction 19 activity was found in RP-HPLC fractions 55, 56 and 57.

5.3.3 Remaining water-soluble portion of aqueous methanol cell extract

Weak activity was found in the remaining water-soluble portion of the cell extract (fraction no. 30), however this activity was lost during further fractionation by RP-HPLC.

5.3.4 ^{1}H-NMR spectroscopy of active RP-HPLC fractions

A total of 13 RP-HPLC fractions were antibacterial against *S. aureus* but, of these, only two were sufficiently pure for structural characterisation. These were firstly, fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4, and 5 (dried mass = 2.4 mg) and secondly, fraction 52 from RP-HPLC of silica column fraction 18 (dried mass = 0.8 mg). The remaining active RP-HPLC fractions contained mixtures of the compounds found in the two pure fractions.

5.3.5 Structural characterisation of antibacterial compounds by NMR spectroscopy and mass spectrometry

^{1}H-NMR of fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4, and 5 showed a spectrum with a signal at δ 0.91 (3H) due to the terminal

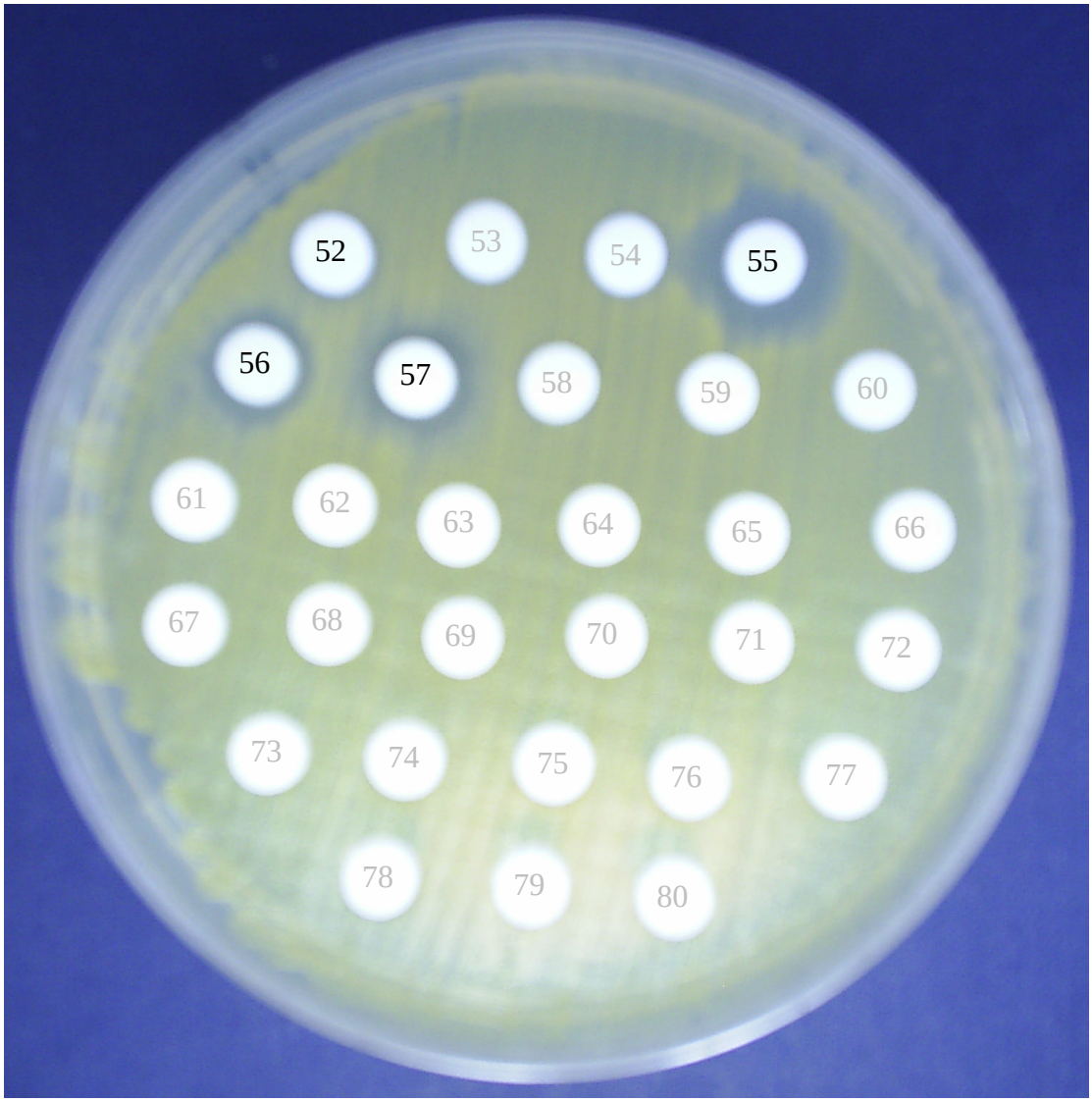


Figure 5.5 – Disc diffusion assay plate showing antibacterial activity against *S. aureus* of fractions 52-80 from RP-HPLC separation of silica column fraction 18. Note strong activity found in fractions 55, 56 and 57 but weak activity also found in fraction 52.

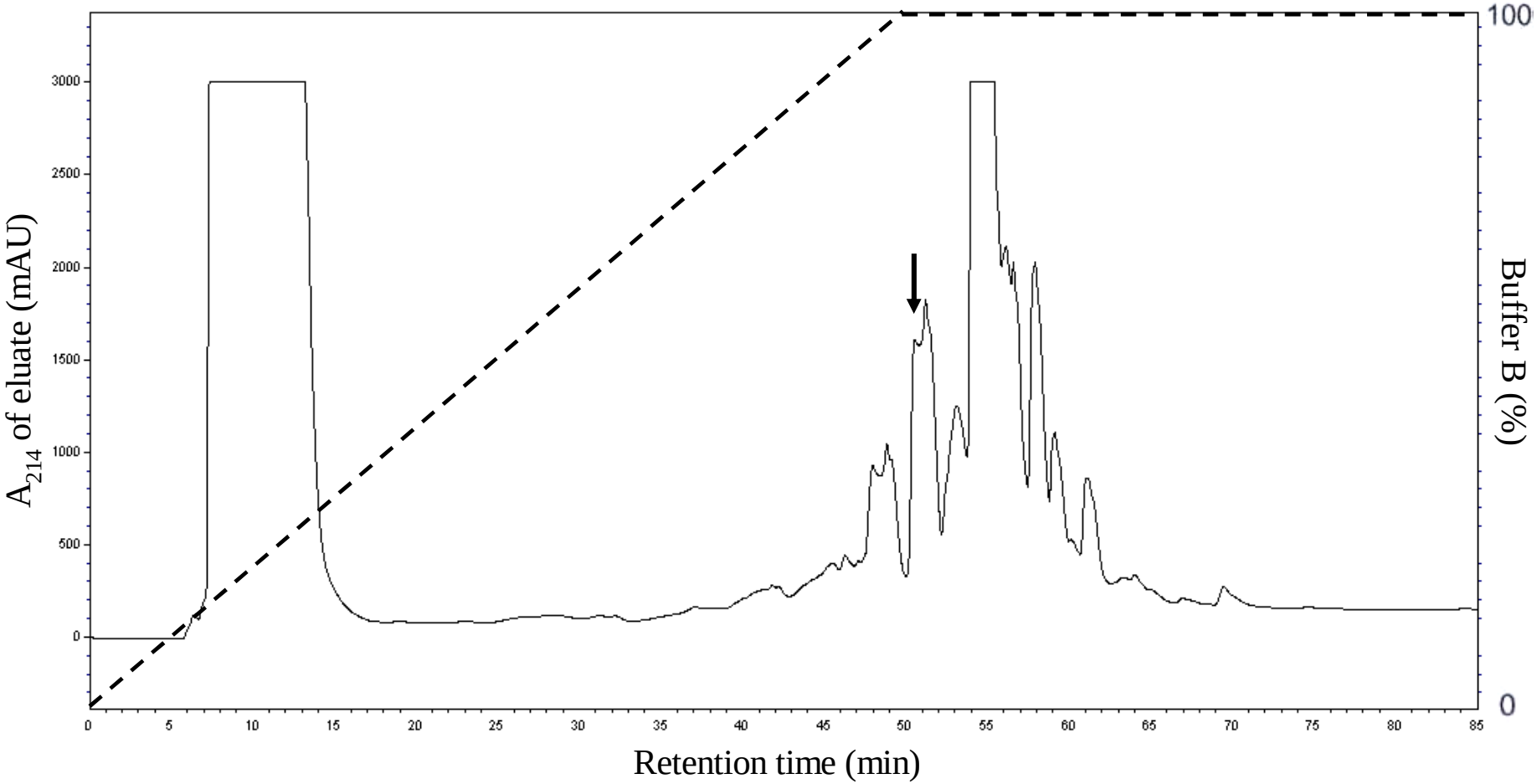


Figure 5.6 – A_{214} of eluate during RP-HPLC C_{18} separation of silica column fraction 18 showing the peak at 50.55 min corresponding to antibacterial fraction 52 (marked with arrow). Dashed line shows the elution gradient (buffer A: 50 % methanol, 49.93 % water, 0.07 % TFA; buffer B: 99.93 % methanol, 0.07 % TFA).

methyl group (Figure 5.7; Table 5.2). The signal at δ 2.27 (2H) indicates of the presence of a methylene group adjacent to a carbonyl group (Table 5.2), while the presence of two olefinic protons at δ 5.30-5.41 ppm (Table 5.2) denotes the presence of one double bond. The signal at δ 1.98-2.14 (4H) can be attributed to the methylene groups adjacent to the double bond and the signal at δ 1.56-1.64 (2H) to a methylene group at C_3 (Table 5.2). Finally, the signal at δ 1.24-1.39 (16H) is due to the protons in the other methylene groups in the carbon chain (Table 5.2). The peak at δ 3.65 is the methylated product caused by the replacement of the –OH group with a $–OCH_3$ group at the carboxyl end (Figure 5.7). This can occur over time when the fatty acid is exposed to methanol. Chemical shifts for ^{13}C (Table 5.2) were determined from the HSQC spectrum (Figure 5.8). The carbonyl group was confirmed as a carboxyl group from the chemical shift on the HMBC spectrum (Figure 5.9). Mass spectrometry of the fraction gives a prominent *m/z* ion at 253.19 Da corresponding to $[M]^-$ (Figure 5.10). The minor *m/z* ion at 267.17 Da probably represents a methylated degradation product $[M+Me]^-$ (Figure 5.10). The high resolution mass spectrometry predicts an empirical formula of $C_{16}H_{27}O_3$ for $[M]^-$ (Appendix IX). The NMR and mass spectrometry data show that the compound is a monounsaturated 16-carbon fatty acid (hexadecenoic acid). However it is not possible to confirm the position of the double bond from these analyses alone.

For fraction 52 from RP-HPLC of silica column fraction 18, the ^1H-NMR spectrum (Figure 5.11) shows a signal at δ 0.85 (3H) due to the terminal methyl group (Table 5.3). The signal at δ 2.19 (2H) indicates the presence of a methylene group adjacent to a carbonyl group (Table 5.3) and the presence of six olefinic protons at δ 5.22-5.36 ppm (Table 5.3) denotes the presence of three double bonds. The signal at δ 2.70-

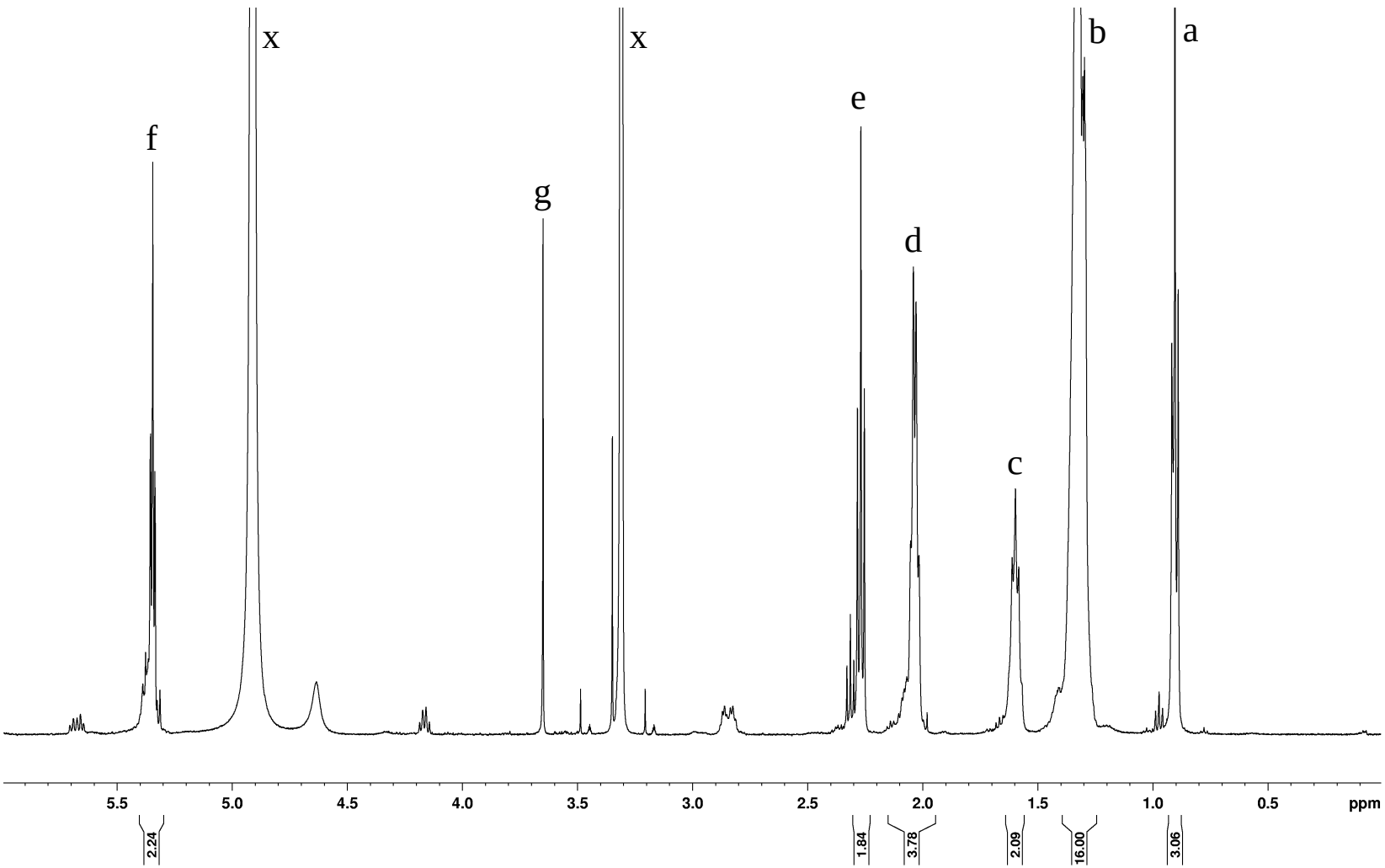


Figure 5.7 – ^{1}H-NMR spectrum of fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4 and 5 in methanol-d_4 at 500 MHz showing the peaks attributable to the H atoms in: the terminal CH_3 group (a), the 8x CH_2 groups (b), the CH_2 group at C_3 (c), the two CH_2 groups either side of the C=C group (d), the CH_2 group adjacent to the carboxyl end (e) and the C=C double bond (f). The other peaks can be attributed to the product with a methylated carboxyl group (g) and residual solvent (x).

Table 5.2 – ^{1}H- and ^{13}C-NMR data obtained in methanol-d_4 at 500 MHz for antibacterial fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4, and 5.

	^{1}H[a]	^{13}C[b]	Peak label on ^{1}H-NMR spectrum
C1	-	176.6	-
C2	2.27, t, 2H, 7.4	33.8	e
C3	1.56-1.64, m, 2H	24.5	c
C4	1.24-1.39, m, 2H	22.0-31.5	b
C5	1.24-1.39, m, 2H	22.0-31.5	b
C6	1.24-1.39, m, 2H	22.0-31.5	b
C7	1.24-1.39, m, 2H	22.0-31.5	b
C8	1.98-2.14, m, 2H	26.5	d
C9	5.30-5.41, m, 1H	129.4	f
C10	5.30-5.41, m, 1H	129.4	f
C11	1.98-2.14, m, 2H	26.5	d
C12	1.24-1.39, m, 2H	22.0-31.5	b
C13	1.24-1.39, m, 2H	22.0-31.5	b
C14	1.24-1.39, m, 2H	22.0-31.5	b
C15	1.24-1.39, m, 2H	22.0-31.5	b
C16	0.91, t, 3H, 6.8	13.2	a

[a] ^{1}H-NMR data given in the form: δ (ppm), multiplicity, number of H atoms, coupling constant (Hz) (where given).

[b] ^{13}C-NMR data for C1 from 2D ^{1}H-^{13}C HMBC; data for C2-C16 from 2D ^{1}H-^{13}C HSQC; ^{13}C-NMR data given is δ (ppm).

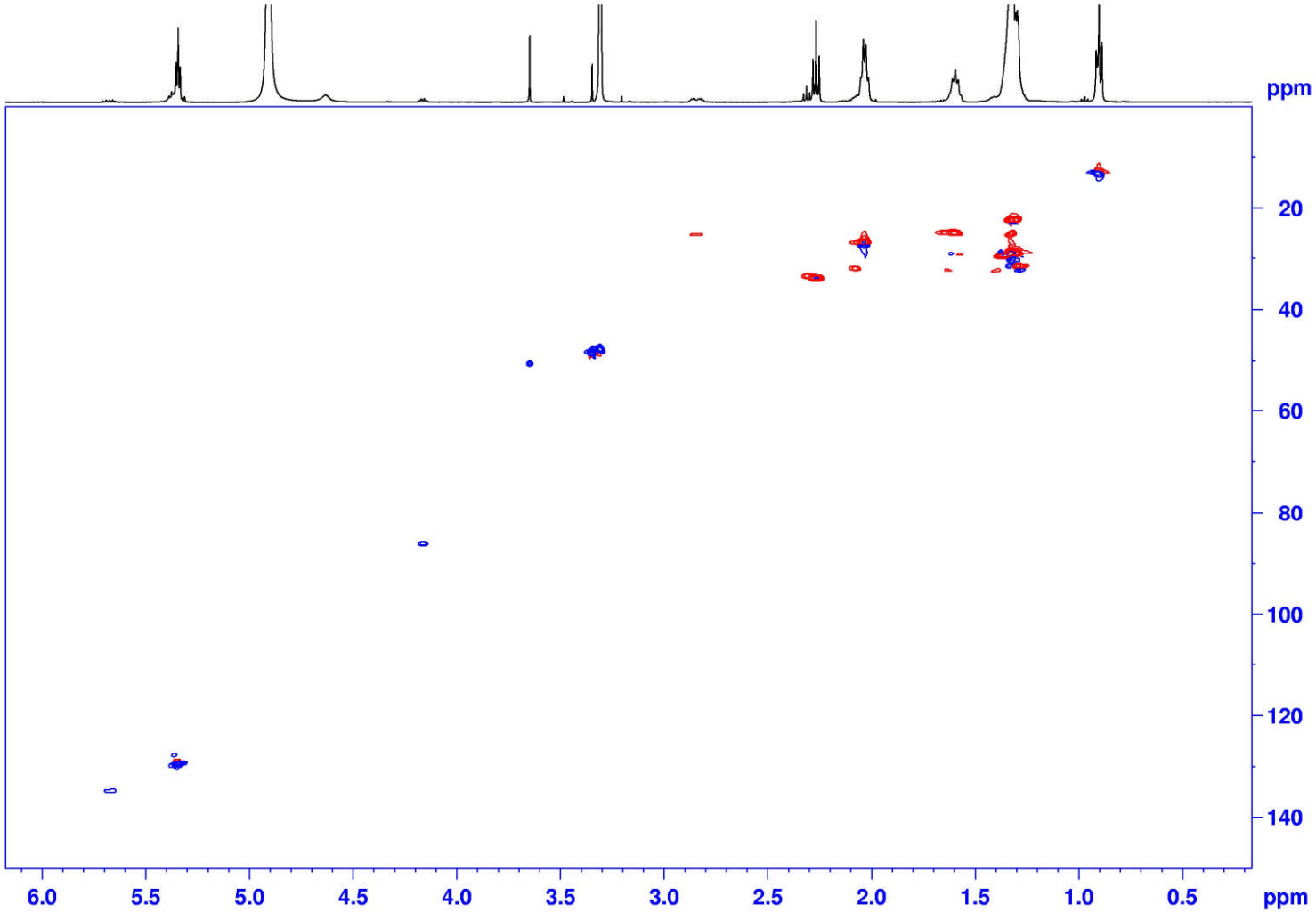


Figure 5.8 – HSQC spectrum of fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4 and 5 in methanol-d_4 at 500 MHz. The ^{1}H-NMR (top axis) correlates with the ^{13}C-NMR (right axis). Coloured areas indicate the C atoms that couple with the respective ^{1}H-NMR peaks (via one bond) with a red area indicating a CH_2 group and blue area indicating a CH or CH_3 group.

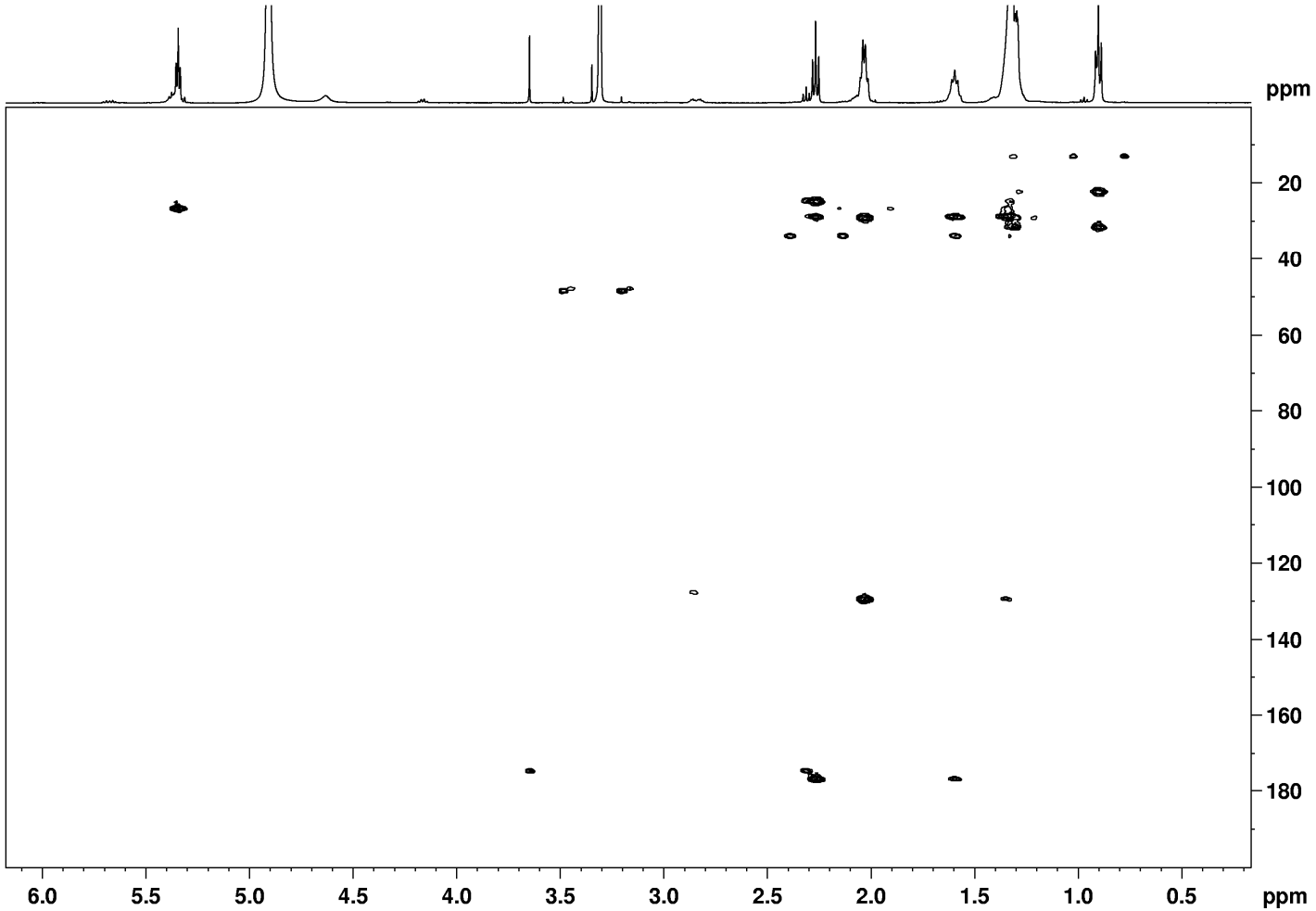


Figure 5.9 – HMBC spectrum of fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4 and 5 in methanol-d_4 at 500 MHz. The ^{1}H-NMR (top axis) correlates with the ^{13}C-NMR (right axis). Black areas indicate the C atoms that couple with the respective ^{1}H-NMR peaks (via more than one bond).

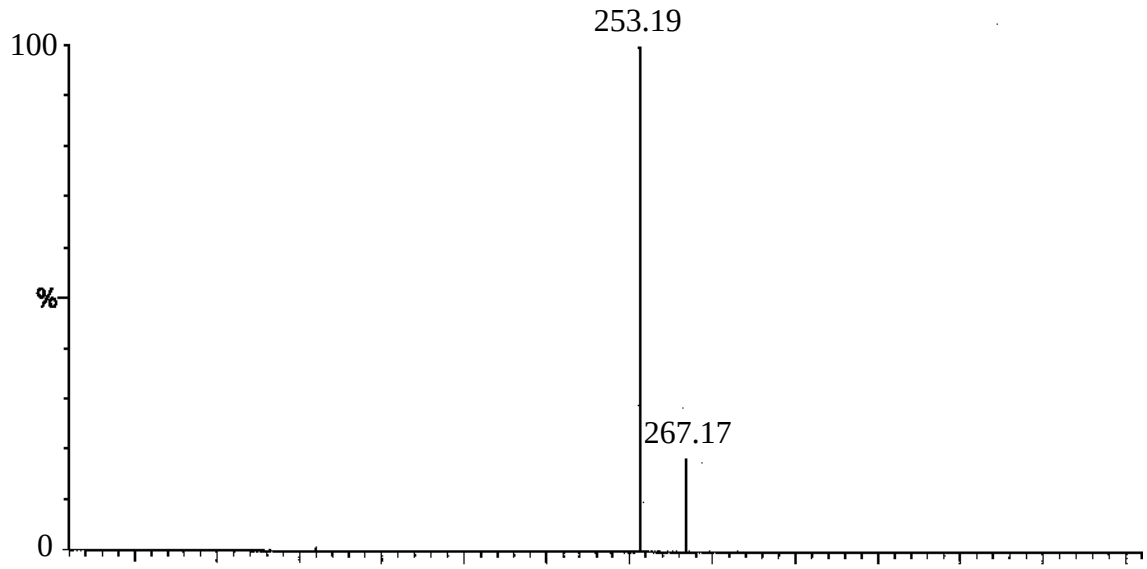


Figure 5.10 – Mass spectrometry of antibacterial fraction 52 from RP-HPLC separation of silica column fraction 18 showing prominent *m/z* ion at 253.19 Da corresponding to $[M]^-$ and a minor *m/z* ion at 267.17 Da probably corresponding to the methylated product $[M+Me]^-$.

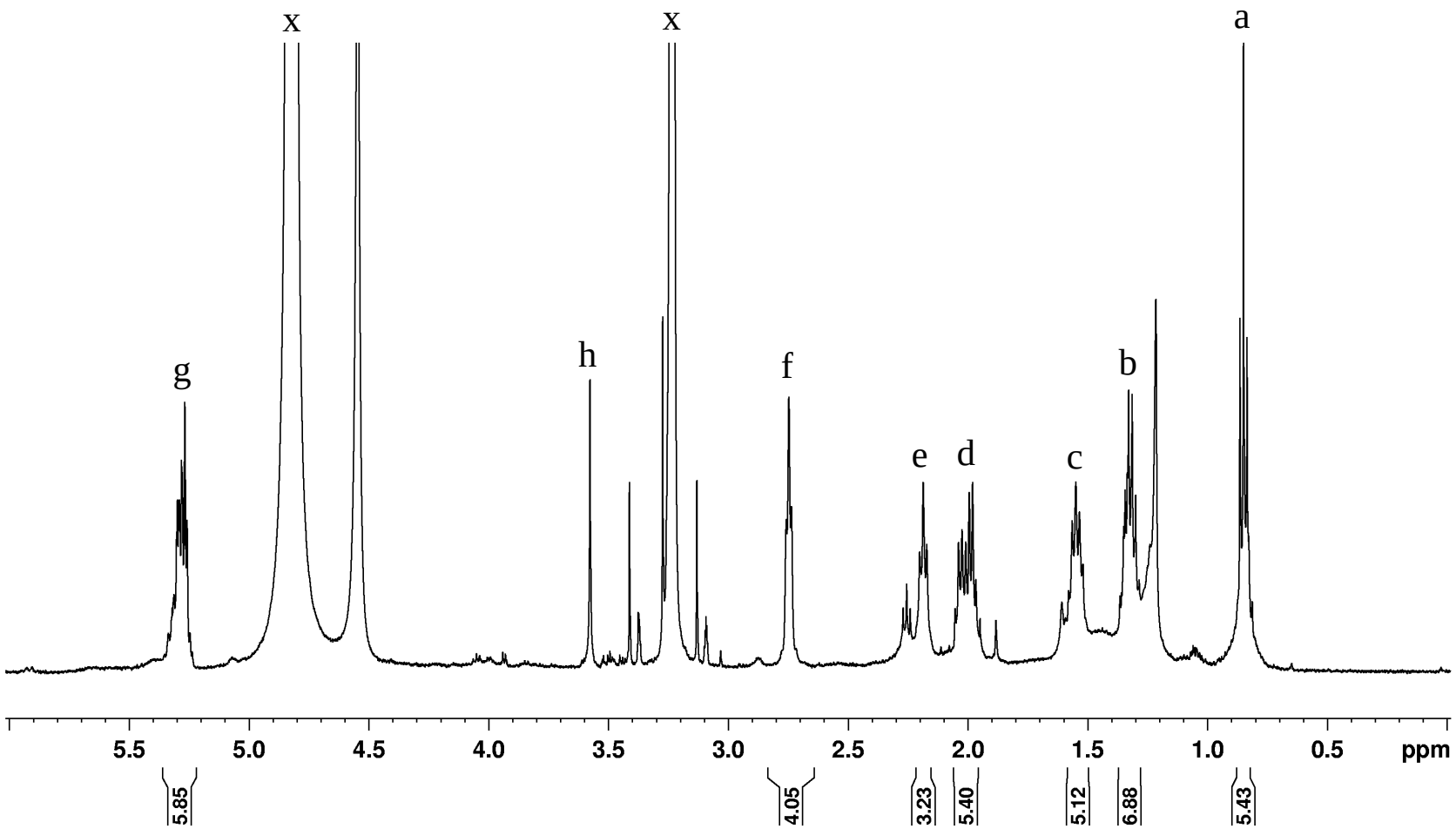


Figure 5.11 – ^{1}H-NMR spectrum of fraction 52 from RP-HPLC separation of silica column fraction 18 in methanol-d_4 at 500 MHz showing the peaks attributable to the H atoms in: the terminal CH_3 group (a), the CH_2 groups at C_4 and C_{15} (b), the CH_2 group at C_3 (c), the CH_2 groups between C=C (d), the CH_2 group adjacent to the carboxyl end (e), the CH_2 groups between the double bonds (f) and the six H atoms in three C=C double bonds (g). The other peaks can be attributed to the product with a methylated carboxyl group (h) and residual solvent (x).

Table 5.3 – ^{1}H- and ^{13}C-NMR data obtained in methanol-d_4 at 500 MHz for antibacterial fraction 52 from RP-HPLC of silica column fraction 18.

	^{1}H[a]	^{13}C[b]	Peak label on ^{1}H-NMR spectrum
C1	-	177.5	-
C2	2.19, t, 2H, 7.3	34.6	e
C3	1.55, q, 2H, 7.5	24.6	c
C4	1.28-1.38, m, 2H	28.9	b
C5	1.92-2.06, m, 2H	26.4	d
C6	5.22-5.36, m, 1H	127.6	g
C7	5.22-5.36, m, 1H	127.6	g
C8	2.70-2.80, m, 2H	25.1	f
C9	5.22-5.36, m, 1H	127.6	g
C10	5.22-5.36, m, 1H	127.6	g
C11	2.70-2.80, m, 2H	25.1	f
C12	5.22-5.36, m, 1H	127.6	g
C13	5.22-5.36, m, 1H	127.6	g
C14	1.92-2.06, m, 2H	26.4	d
C15	1.28-1.38, m, 2H	28.9	b
C16	0.85, t, 3H, 7.4	12.8	a

[a] ^{1}H-NMR data given in the form: δ (ppm), multiplicity, number of H atoms, coupling constant (Hz) (where given).

[b] ^{13}C-NMR data for C1 from 2D ^{1}H-^{13}C HMBC; data for C2-C16 from 2D ^{1}H-^{13}C HSQC; ^{13}C-NMR data given is δ (ppm).

2.80 (4H) may be attributed to the methylene groups in between the double bonds whilst the signal at δ 1.92-2.06 is the methylene groups between a double bond and a methylene group (Table 5.3). The signals at δ 1.28-1.38 (4H) are the methylene groups at C_4 and C_{15} and the signal at δ 1.55 (2H) is the methylene group at C_3 (Table 5.3). The peak at δ 3.58 is attributable to methylation of the carboxyl group. Again, chemical shifts for ^{13}C (Table 5.3) were determined from the HSQC spectrum (Figure 5.12). The carbonyl group was confirmed as a carboxyl group from the chemical shift on the HMBC spectrum (Figure 5.13). Mass spectrometry of the fraction gives a prominent *m/z* ion at 249.39 Da corresponding to $[M]^-$ (Figure 5.14). The NMR and mass spectrometry data enables the compound in the fraction to be identified as the unsaturated fatty acid (6*Z*, 9*Z*, 12*Z*)-hexadecatrienoic acid or 16:3n4 (Figure 5.14).

5.3.6 Location of double bond

For fraction 57 from RP-HPLC of pooled silica column fractions 2, 3, 4, and 5 the location of the double bond was investigated by synthesising dimethyl disulphide adduct derivatives. When the EI-mass spectrum of the synthesised adducts was searched against a molecular mass library (MassLynx, Waters Corporation) this confirmed the presence of the methyl ester of 9, 10-dimethylthiohexadecanoic acid, i.e. the dimethyl disulphide adduct product of an hexadecenoic acid methyl ester as expected (Appendix X). The mass spectrometry revealed two substantial fragment ions where the molecule was cleaved between the carbons that originally constituted the double bond. These are the ω fragment (aliphatic end of the molecule) at 145.10 Da and the Δ fragment (carboxyl end of the molecule) at 185.09 Da (Figure 5.15). The other diagnostic mass fragment peaks corresponding to the molecular ion $[M]^-$ and the Δ-32 fragment (the loss of methanol at the Δ end) found at 362.23 Da, and

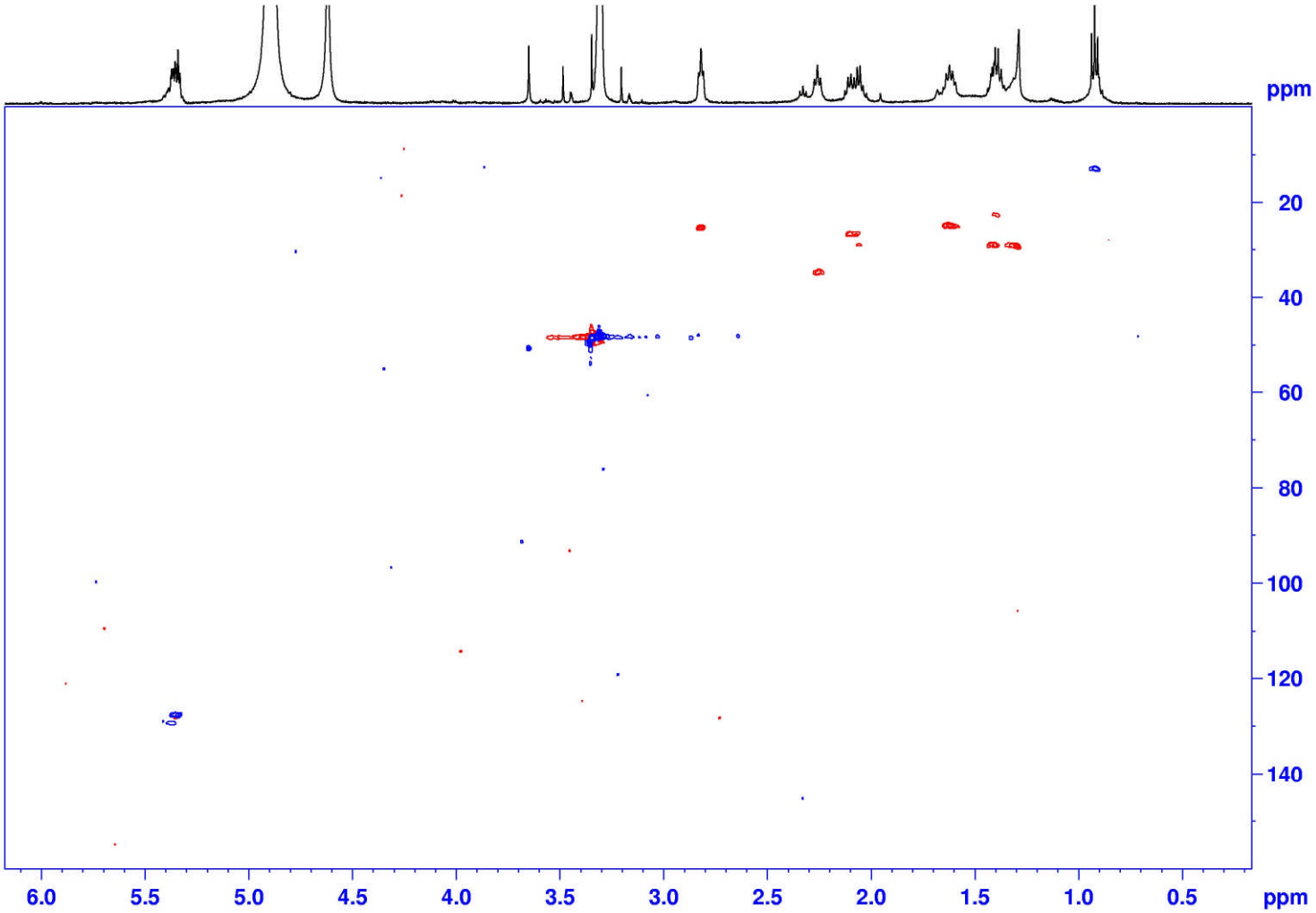


Figure 5.12 – HSQC spectrum of fraction 52 from RP-HPLC separation of silica column fraction 18 in methanol-d_4 at 500 MHz. The ^{1}H-NMR (top axis) correlates with the ^{13}C-NMR (right axis). Coloured areas indicate the C atoms that couple with the respective ^{1}H-NMR peaks (via one bond) with a red area indicating a CH_2 group and blue area indicating a CH or CH_3 group.

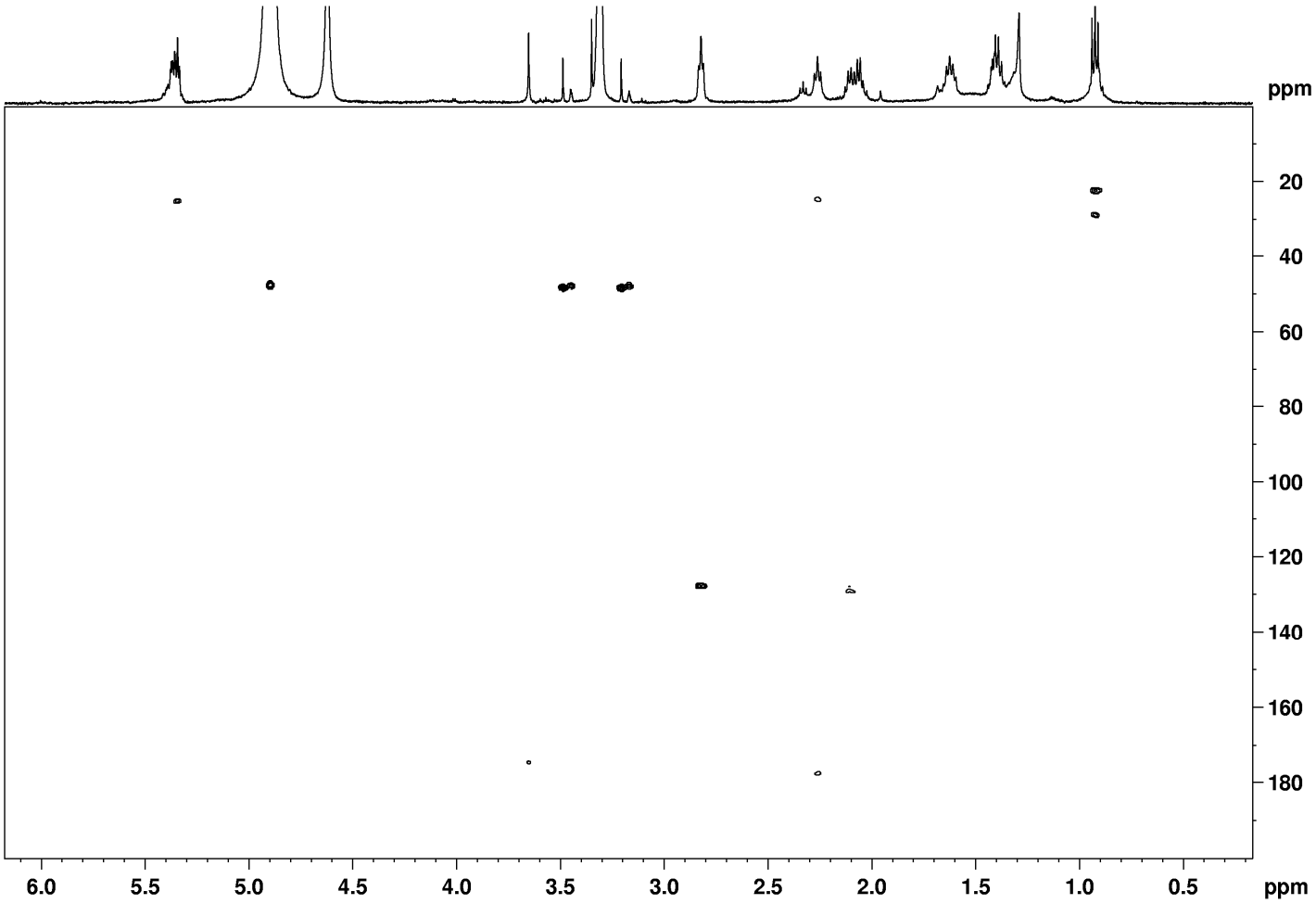
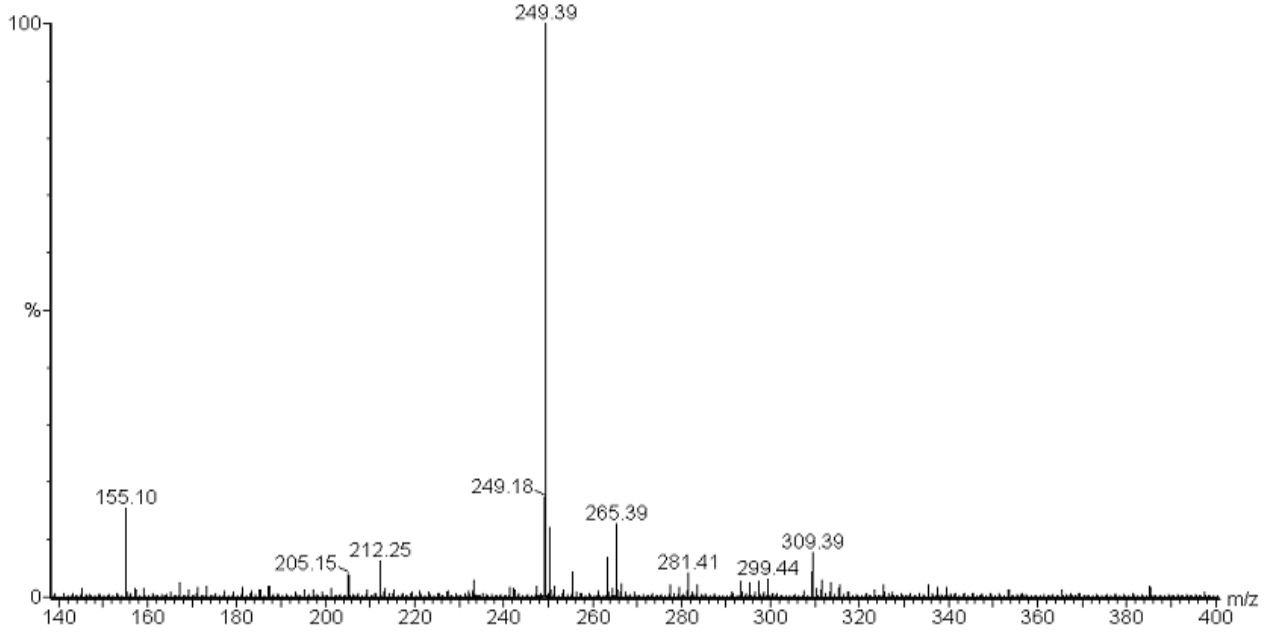


Figure 5.13 – HMBC spectrum of fraction 52 from RP-HPLC separation of silica column fraction 18 in methanol-d_4 at 500 MHz. The ^{1}H-NMR (top axis) correlates with the ^{13}C-NMR (right axis). Black areas indicate the C atoms that couple with the respective ^{1}H-NMR peaks (via more than one bond).

A



B

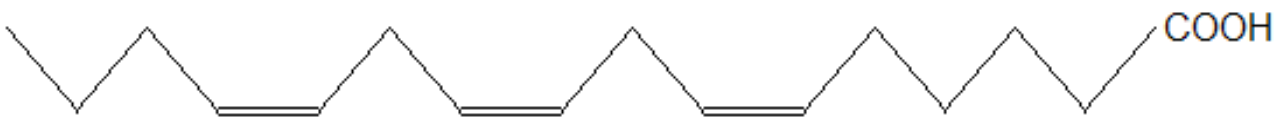


Figure 5.14 – Mass spectrometry of fraction 52 from RP-HPLC separation of silica column fraction 18 showing the prominent *m/z* ion at 249.39 Da corresponding to $[M]^-$ (A) and the predicted structure of the major compound in this fraction, (6*Z*, 9*Z*, 12*Z*)-hexadecatrienoic acid (B).

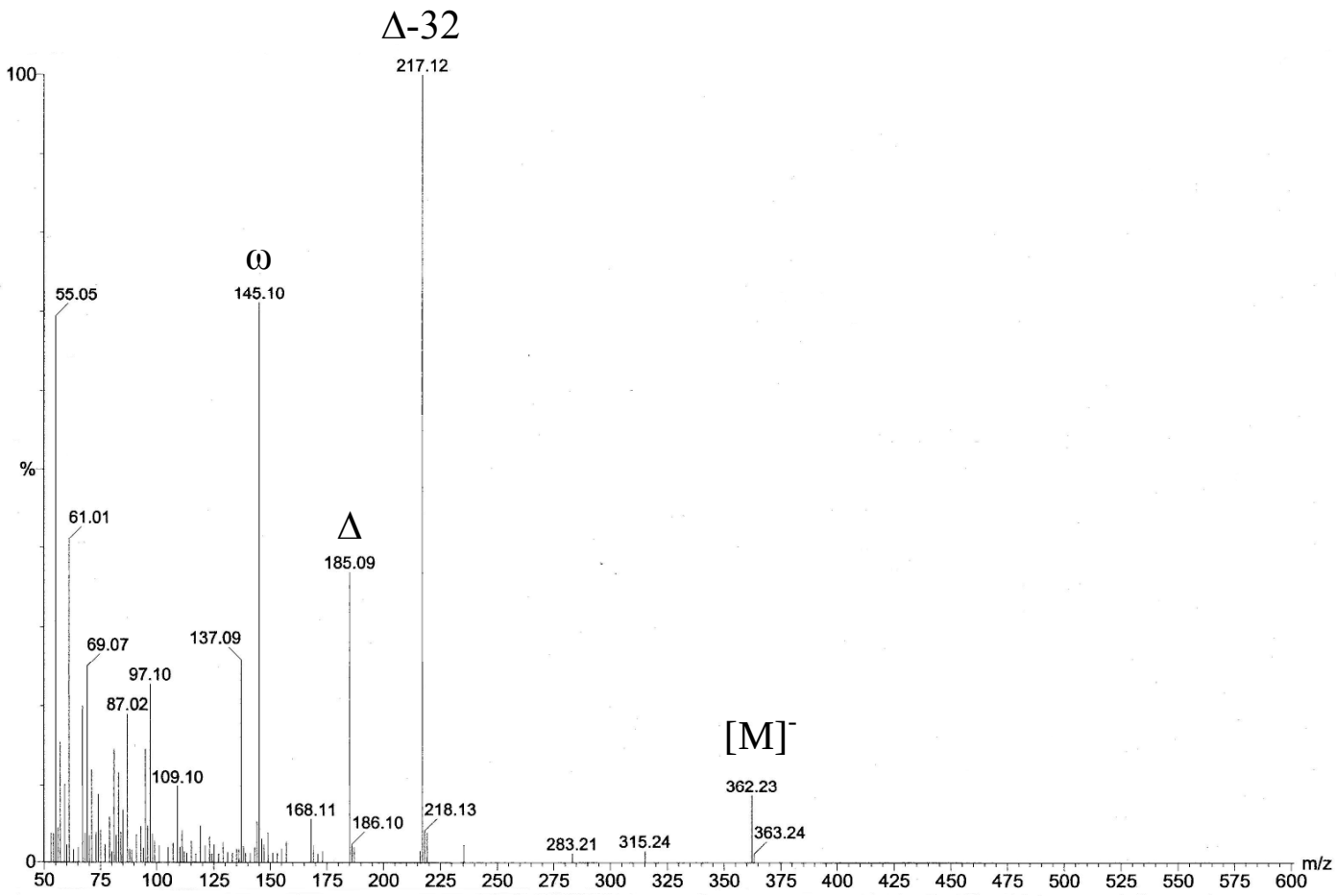


Figure 5.15 – EI-MS of DMDS adducts of monounsaturated fatty acid in fraction 57 from RP-HPLC of pooled silica column fractions 2, 3, 4, and 5. This shows the two substantial fragment ions where the molecule was cleaved between the carbons that originally constituted the double bond: ω and Δ fragments at 145.10 Da and 185.09 Da, respectively. The other diagnostic mass fragment peaks correspond to $[M]^-$ (362.23 Da) and the Δ-32 fragment (217.12 Da).

217.12 Da, respectively (Figure 5.15). This data positions the double bond in the fatty acid chain between carbons 9 and 10 from the carboxyl (or the n-7 position) meaning the fatty acid in this fraction is (9*Z*)-hexadecenoic acid (Figure 5.16), which is more commonly referred to as palmitoleic acid or 16:1n7 (Appendix I).

5.4.0 Discussion

Two long-chain unsaturated free fatty acids, 16:1n7 and 16:3n4, were isolated from aqueous methanol cell extracts of *P. tricornutum* and shown to have antibacterial activities. These compounds may be the antibacterial compounds that could not be purified from the same dried cell extract in the previous chapter.

The antibacterial activity of 16-carbon chain length fatty acids is well established. This includes the saturated 16:0 (Kabara *et al*., 1972; Galbraith and Miller, 1973a; Miller *et al*., 1977; Lacey and Lord, 1981; Yff *et al*., 2002; Willie and Kydonieus, 2003; Benkendorff *et al*., 2005), the monosaturated isomers 16:1n7 (Kabara *et al*., 1972; Miller *et al*., 1977; Dye and Kapral, 1981; Saito *et al*., 1984; Feldlaufer *et al*., 1993; Bergsson *et al*., 1999; Bergsson *et al*., 2001a; Sun *et al*., 2003; Zheng *et al*., 2005) and 16:1n10 (Willie and Kydonieus, 2003), and also the polyunsaturated fatty acids 16:2n4 (Wang, 1999), 16:3n4 (Wang, 1999) and 16:4n1 (Findlay and Patil, 1984). In some cases these fatty acids have been isolated from a variety of natural sources (Findlay and Patil, 1984; Wang, 1999; Yff *et al*., 2002; Willie and Kydonieus, 2003) but the present study is the first report in which 16:1n7 has been isolated from a microalga using an antibacterial bioassay-guided approach.

Figure 5.16 – Structure of (9*Z*)-hexadecenoic acid, the compound found in fraction 57 from RP-HPLC of pooled silica column fractions 2, 3, 4, and 5.

The present study is also the first to report the isolation of 16:3n4 from *P. tricornutum* and demonstrate its antibacterial activity. Wang (1999) previously reported the antibacterial activity of 16:3n4 from the marine diatom, *Chaetoceros spp*., and showed it to be active against *S. aureus* and *E. coli*. Interestingly, Cooper *et al*. (1985) isolated a fraction from *P. tricornutum* cell extracts that contained a mixture of six fatty acids, one of which was 16:3n4, but these authors concluded that 16:3n4 was not responsible for the activity in this fraction because the closest commercially available structural homologue, 16:1n7, was not antibacterial. By contrast, the present study demonstrates that 16:3n4 is responsible for some of the antibacterial activity in *P. tricornutum* cell extracts. Findlay and Patil (1984) claimed to have isolated 16:3n4 from the marine diatom, *Navicula delognei*, and show it to be antibacterial but this fatty acid was in fact merely a minor contaminant of a fraction containing the antibacterial fatty acid, (6*Z*, 9*Z*, 12*Z*, 15*Z*)-octadecatetraenoic acid (18:4n3). No indication was given by the authors of the quantity of the contaminating 16:3n4 nor was any evidence provided to support the assertion that this compound was antibacterial (Findlay and Patil, 1984).

In conclusion, this chapter reports the novel isolation from a microalga of the antibacterial fatty acid, 16:1n7. Further, the antibacterial fatty acid, 16:3n4, was isolated from cell extracts of *P. tricornutum* for the first time. Thus far the present study reports the isolation of three antibacterial fatty acids from aqueous methanol cell extracts of *P. tricornutum*.

Chapter 6: Further studies on the antibacterial properties of three free fatty acids isolated from aqueous methanol cell extracts of the marine diatom, *Phaeodactylum tricornutum*

6.1.0 Introduction

In Chapters 4 and 5, three free fatty acids were isolated from aqueous methanol cell extracts of *P. tricornutum* using Staphylococcal species as target bacteria, but no characterisations of their antibacterial properties were performed. Previous studies have shown that 16:1n7, 16:3n4 and 20:5n3 are indeed antibacterial (Kabara *et al*., 1972; Miller *et al*., 1977; Dye and Kapral, 1981; Saito *et al*., 1984; Knapp and Melly, 1986; Feldlaufer *et al*., 1993; Ohta *et al*., 1995; Bergsson *et al*., 1999; Wang, 1999; Bergsson *et al*., 2001a; Hornitzky, 2003; Sun *et al*., 2003; Benkendorff *et al*., 2005; Zheng *et al*., 2005) but studies of the spectra of activity and antibacterial potencies are limited. This final experimental chapter is aimed at determining their antibacterial potency against *S. aureus* by measurement of their 50 % inhibition concentration (IC_{50}) and minimum bactericidal concentration (MBC) values. Here, the IC_{50} is defined as the lowest molar concentration required to inhibit bacterial growth by 50 % compared to the control (Miller *et al*., 1977). A further measure of antibacterial potency, the MBC, is defined, in this study, as the lowest molar concentration required to kill all the bacteria in the original inoculum. It remains unknown whether the fatty acids in combination can act to inhibit bacterial growth in a synergistic, antagonistic or additive fashion and so the antibacterial potency of a combination of the isolated fatty acids was investigated. Synergism and antagonism is when the antibacterial effect of the combination is greater or less than the sum of the effects of the individual components, respectively. In an additive scenario the antibacterial

effect of a combination is the same as the sum of the effects of the individual components.

Earlier reports have shown that free polyunsaturated fatty acids are not found in healthy, intact diatom cells (Jüttner, 2001; Pohnert, 2002; Pohnert *et al*., 2004) but are only released from lipids by the rapid action of enzymes (lipases) after cells lose their integrity, for example, by freeze-thawing, sonication or solvent extraction (Budge and Parrish, 1999; Jüttner, 2001; Pohnert, 2002; Pohnert *et al*., 2004). These lipases have been shown to be most active around the effluent cytoplasm of disrupted cells (Pohnert, 2002) and most hydrolysis occurs within the first minute (Jüttner, 2001). Therefore the fatty acids isolated in the present study are likely to be artefacts of the extraction procedure (Budge and Parrish, 1999). Further, d'Ippolito *et al*. (2004) has shown that in a marine diatom, *Skeletonema costatum*, 16:3n4 and 20:5n3 are enzymatically freed from the phospholipids and chloroplast-derived glycolipids, especially monogalactosyldiacylglycerol (MGDG). To confirm the possible enzyme-dependent release of antibacterial fatty acids from lipids in extracted diatom cells the antibacterial activity of cell pellets extracted in solvent at temperatures sufficient to dentaure proteins (i.e., enzymes) will be compared to those extracted on ice. It has been shown that a hot extraction solvent can deactivate the lipases that act on lipids releasing free fatty acids (Budge and Parrish, 1999; Jüttner, 2001; Pohnert, 2002).

The levels of fatty acids in cell extracts collected throughout the growth curve will be examined as fatty acid composition of *P. tricornutu*m changes during culture (Orcutt and Patterson, 1975; Cooper *et al*., 1985; Siron *et al*., 1989; Liang *et al*., 2006). This may explain the reduction in antibacterial activity of cell extracts seen during culture

(Figure 3.3). Further, cell extracts from cultures enriched for different *P. tricornutum* morphs have been shown to have different amounts of antibacterial activity in aqueous methanol cell extracts (Figure 3.15) and so the concentration of fatty acids, and in particular the three antibacterial fatty acids isolated in previous chapters, in cell extracts from the different *P. tricornutum* morphs will be investigated to see whether this could explain the antibacterial activity differences between these extracts. Thus for each of the three antibacterial fatty acids the aim of this chapter is to assess their spectra of activity, potency, kinetics of release and to monitor their levels during culture and in cells of different morphology. In light of their isolation, preliminary consideration will be given to the potential ecological significance of the three fatty acids as a defence against bacteria.

6.2.0 Materials and methods

As only very small quantities of 16:1n7 and 20:5n3 were isolated in Chapters 4 and 5, chemically synthesised free fatty acids (>99 %) were purchased from Sigma Aldrich Ltd. As 16:3n4 is not commercially available, the small quantity of material isolated in Chapter 5 (Section 5.3.4) was used in the following experiments. Stock solutions of each fatty acid were made to 4 and 8 mM in methanol and stored at –20 ºC.

6.2.1 Potency of fatty acids against *S. aureus*

The IC_{50} was determined against *S. aureus* for 16:1n7, 16:3n4 and 20:5n3 using a growth inhibition assay. The 4 mM fatty acid stock solutions were serially diluted 1:1 to give a further seven stock solutions. To each well of a sterile 96-well microtitre plate (round-bottomed wells; Corning Inc.) was added 50 µL double-strength LB medium, 2 µL of a fatty acid stock solution (16:1n7, 16:3n4 or 20:5n3 in methanol), 1

x10^4 cfu of exponentially growing *S. aureus* resuspended in 0.9% NaCl solution (cultured and determined as Section 2.2.6 and diluted so that the volume was ~5-10 µL) and each well was made up to 100 µL with sterile deionised water. This gives fatty acid concentrations of 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 µM. For the negative control wells the fatty acid solution was replaced with methanol. Blank reference wells contained 50 µL double-strength LB medium solution, 8 µL methanol and 42 µL sterile deionised water. For comparative purposes an IC_{50} for ampicillin in water was determined using the same method but final well concentrations ranged 0.0078125-0.5 µM. To investigate whether mixtures of different fatty acids synergise, antogonise or act in an additive manner wells containing combinations of 16:1n7 and 20:5n3 were set up so that these two fatty acids were at final concentrations between 0.625-40 µM total fatty acid. Each treatment was performed in quadruplicate. The plate was covered with an AirPore™ tape sheet (Qiagen Ltd.) and incubated at ~20 °C. After 26 h, the mean A_{570} of each well was determined from triplicate measurements using a plate reader (MRX II; Dynex Technologies Ltd.). The mean of the blank reference wells was subtracted from the A_{570} of each well as appropriate. The A_{570} of each experimental well was then compared to the mean value of the respective negative control wells to generate values expressed as % growth compared to control.

To determine the MBC, the contents of each well that appeared to show no growth were spread across separate LB agar plates. To determine the MBC for ampicillin, further wells had to be set up with final well concentrations 160-2560 µM. Each blank reference well was plated out to control for contamination. Plates were incubated overnight at 37 ºC.

6.2.2 Spectrum of activity

Spectrum of activity was determined against two Gram positive (*M. luteus* and *P. citreus*) and two Gram negative (*E. coli* and *Ps. aeruginosa* 10775) bacterial species using a growth inhibition assay (based on Section 6.2.1). To each well of a sterile 96-well microtitre plate (round-bottomed wells) was added 50 µL double-strength LB or 2216E medium solution as appropriate for the bacterium (see table 2.1), 8 µL of 8 mM fatty acid stock solution (16:1n7, 16:3n4 or 20:5n3 in methanol), 1 x 10^4 cfu of exponentially growing bacteria (cultured and enumerated as in Section 2.2.6 and diluted so that volume equalled 5 µL) and 37 µL sterile deionised water. This gave fatty acid concentrations of 640 µM in each well. Negative control wells for each bacterium replaced the fatty acid solution with methanol whilst blank reference wells for each medium contained 50 µL double-strength medium solution, 8 µL methanol and 42 µL sterile deionised water. Each assay was performed in quadruplicate. The plate was covered with a lid and left to grow at room temperature. After 24 h, the mean A_{570} of each well was determined from triplicate measurements using the plate reader. The mean of the blank reference wells was subtracted from the A_{570} of each well as appropriate. Antibacterial activity was defined as a significant reduction in bacterial growth compared to the negative control wells.

Additionally, 16:1n7 and 20:5n3 were each tested for their ability to inhibit the growth of MRSA and the human pathogenic fungus, *Candida* sp. This was tested using the disc diffusion assay (as Section 4.2.5) with discs loaded with 1 µM fatty acid. MRSA was cultured on nutrient agar whilst the fungus was cultured on PDY agar (Appendix III). The MRSA strain (16a) and the *Candida* sp. (a patient isolate)

were kindly gifted by Dr Andrew Mearns-Spragg (Aquapharm Bio-Discovery Ltd.). Antibacterial or antifungal activity was identified as a clear zone around the disc.

6.2.3 Enzymatic release of the fatty acids

Of nine algal cultures (cultured and harvested as Sections 2.2.2 and 2.2.4) four were extracted on ice (as Section 2.2.4) except that the extracts were kept on ice for a total time of 1 h. These low temperature conditions permit the hydrolysis of lipids to free fatty acids by lipases. Moreover, Jüttner (2001) has shown that methanol at ambient temperature has no effect on the action of the lipases that cleave lipids to the free unsaturated fatty acids. To prevent the action of these lipases, the remaining five cultures were extracted with hot aqueous methanol (~70 ºC), at which temperature proteins, such as enzymes, are fully denatured. These extracts were kept at ~70 ºC for 1 h. To account for any heat degradation of antibacterial compounds that may occur during incubation, as indicated by experiments performed in Chapter 3 (Figure 3.5), the cell pellets extracted on ice were kept in a water bath at ~70 ºC for 1 h. For consistency the cell pellets extracted in hot methanol were kept on ice for 1 h. Cellular debris was removed from each extraction by centrifugation at 12000 *g* for 1 h at 4 ºC. The supernatant was transferred to a sterile 1.5 mL Eppendorf tube, dried to completion using the speed vac at 30 ºC, massed, reconstituted to a concentration of 60 mg mL^{-1} with sterile 50 mM HEPES aqueous solution pH 7.8 (Acros Organics) and stored at 4 ºC until use. Extracts were tested in duplicate for antibacterial activity by RDA against *S. aureus* (Section 2.2.7). These data were converted to antibacterial activity per cell using A_{750} data collected during culture harvest.

6.2.4 Fatty acid levels during culture

The relative concentration of each fatty acid in aqueous methanol cell extracts were determined throughout the *P. tricornutum* growth curve by analysing samples collected days 4 – 14 (Section 3.2.2) by gas-liquid chromatography (as Section 3.2.7). Further, the actual mass of each fatty acid was calculated on a per cell basis by dividing the mass of total fatty acid in the analysed sample by the number of cells that was extracted; this was then multiplied by the relative proportion of the particular fatty acid. Relative percentage data are expressed to two decimal places though this degree of accuracy is not implied.

6.2.5 Fatty acid levels in the different *P. tricornutum* cell morphs

To investigate whether fatty acid differences could explain the difference in antibacterial activity observed for cell extracts produced from cultures enriched in oval and fusiform cells (Figure 3.15), the relative concentration of each fatty acid in aqueous methanol cell extracts from these 20 cultures (produced in Section 3.2.9) were determined by gas-liquid chromatography (as Section 3.2.7). For each sample the total quantity of fatty acids, actual amount of each fatty acid and the sum of the three antibacterial fatty acids (isolated in Chapters 4 and 5) were also calculated.

6.2.6 Statistical analyses

For all statistical analyses, data were tested for normality by Shapiro-Wilk test and for homogeneity of variance by Levene's test. In all cases $p \leq 0.05$ was considered significant.

For the spectrum of activity work the data was normally distributed in all instances except *P. citreus* ($W_{15} = 0.873$, $p < 0.05$) and showed equal variances, except *Ps. aeruginosa* ($F_{3,8} = 5.348$, $p < 0.05$). Significant differences between treatment groups was assessed by one-way ANOVA for each bacterium with the four treatment groups being growth in 16:1n7, 16:3n4, 20:5n3 or the absence of any fatty acid (control). To identify the treatment groups that were significantly different from each other, the *post hoc* Tukey HSD test was used.

For the enzyme-dependent release of fatty acids, antibacterial activity data were shown to be normally distributed and have equal variance so the student's t-test was used to test for significant differences between the two treatment groups with $p \leq 0.05$ considered significant.

The data obtained for the total quantity of fatty acids in cell extracts from morph-enriched cultures were non-normally distributed ($W_{20} = 0.903$, $p < 0.05$) and showed unequal variances ($F_{3,16} = 3.983$, $p < 0.05$). Nonetheless, two-way ANOVA was performed (with batch and morph as factors) to test for significant differences between the two treatment groups with $p \leq 0.05$ considered significant.

For fatty acid levels in cell extracts from morph-enriched cultures, the relative percentage data was first analysed using the multivariate approach of principal components analysis (PCA) and this was kindly performed by Dr. Mike Walton (Sea Mammal Research Unit, University of St Andrews). For further analysis, the relative percentage values were transformed using the arcsine square root function. In 5/13 cases these transformed data were not normally distributed and in 5/13 cases these

data showed unequal variance (Table 6.1). Despite this, a two-way ANOVA was performed for each fatty acid (with batch and morph as factors) to test for significant differences between the morph-enriched cultures. The chance of a Type I error was reduced by enforcing the highly stringent Bonferroni correction (Weisstein, 2004) and thus $p \leq 0.0038$ was considered significant.

For the actual levels data in 6/13 cases the data were not normally distributed and in 6/13 cases the data showed unequal variance (Table 6.1). Two-way ANOVA was performed for each fatty acid (with batch and morph as factors) to test for significant differences between the morph-enriched cultures. Again the Bonferroni correction was enforced meaning $p \leq 0.0038$ was considered significant.

The data on the summed masses of the three previously isolated fatty acids (16:1n7, 16:3n4 and 20:5n3) was normally distributed but showed unequal variances ($F_{3,16} = 3.572$, $p < 0.05$). Again, two-way ANOVA was performed (with batch and morph as factors) to test for significant differences between the morph-enriched cultures with $p \leq 0.05$ considered significant.

6.3.0 Results

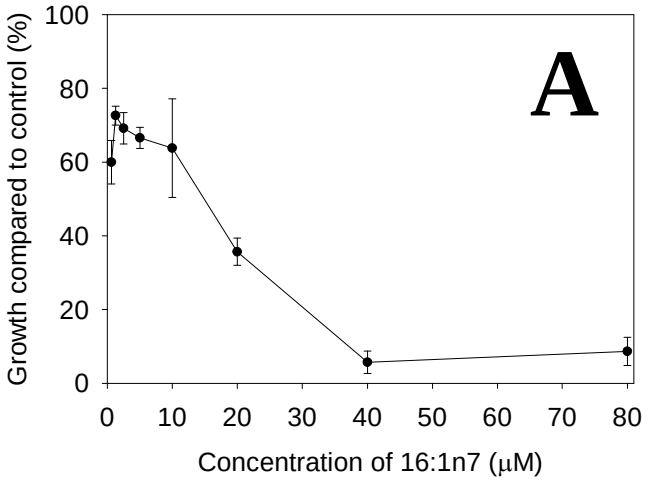
6.3.1 Potency of fatty acids against *S. aureus*

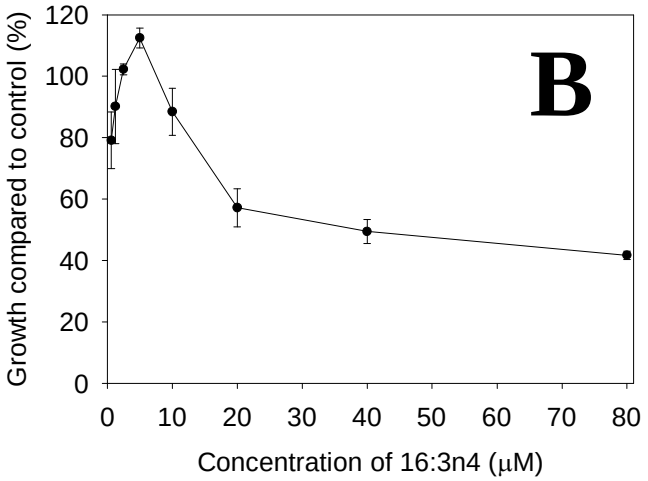
The IC_{50} values of 16:1n7, 16:3n4 and 20:5n3 against *S. aureus* were determined to be in the ranges 10-20 µM, 20-40 µM and 10-20 µM, respectively (Figure 6.1). The combination of 16:1n7 and 20:5n3 had an IC_{50} of 10-20 µM total fatty acid. This suggests that the antibacterial action of fatty acids occurs in an additive manner because the IC_{50} value of the mix is equal to that of 16:1n7 and 20:5n3 when tested

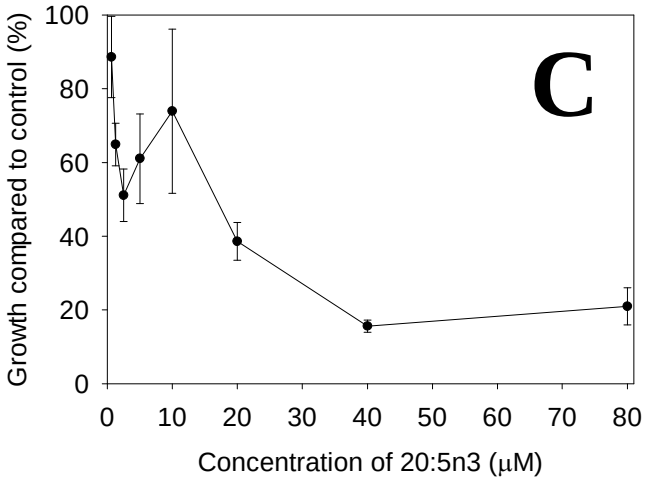
Table 6.1 – Data collected for the relative and actual mass of each fatty acid in cells from morph-enriched cultures were tested for a normal distribution by Shapiro-Wilk test and homogeneity of variance by Levene's test. As the relative data were percentage points these were transformed prior to testing using the arcsine square root function. Where the assumptions of normality and equal variances were violated the test results are given. In 5/13 cases the relative data were not normally distributed and in 5/13 cases these data showed unequal variance. For the actual mass data in 6/13 cases the data was not normally distributed and in 6/13 cases the data showed unequal variance. For all analyses $p < 0.05$ was considered significant.

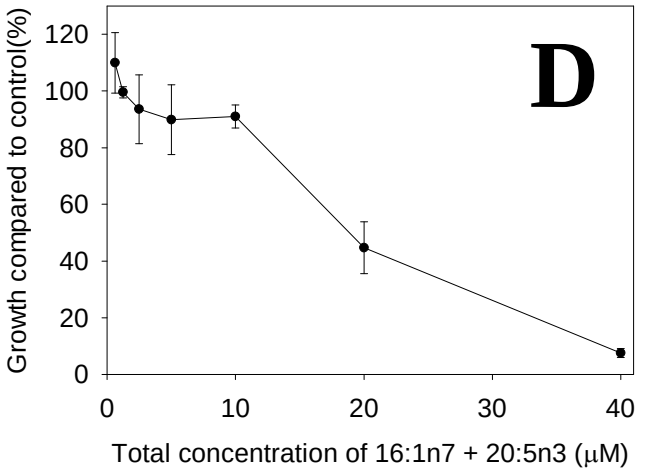
Fatty Acid	Relative data		Actual mass data	
	Shapiro-Wilk test	Levene's test	Shapiro-Wilk test	Levene's test
14:0				
16:0		$F_{3,16} = 9.398$, $p < 0.001$	$W_{20} = 0.851$, $p < 0.01$	
16:1n7		$F_{3,16} = 4.633$, $p < 0.05$	$W_{20} = 0.870$, $p < 0.05$	$F_{3,16} = 4.461$, $p < 0.05$
16:2n4	$W_{20} = 0.838$, $p < 0.01$	$F_{3,16} = 6.838$, $p < 0.01$	$W_{20} = 0.900$, $p < 0.05$	$F_{3,16} = 10.51$, $p < 0.001$
16:3n4	$W_{20} = 0.880$, $p < 0.05$			
16:4n1	$W_{20} = 0.794$, $p < 0.001$			$F_{3,16} = 6.520$, $p < 0.01$
18:1n9		$F_{3,16} = 3.780$, $p < 0.05$		$F_{3,16} = 3.733$, $p < 0.05$
18:2n6		$F_{3,16} = 5.153$, $p < 0.05$		$F_{3,16} = 4.327$, $p < 0.05$
18:3n6	$W_{20} = 0.802$, $p < 0.001$			
18:4n3			$W_{20} = 0.894$, $p < 0.05$	
20:4n6	$W_{20} = 0.890$, $p < 0.05$		$W_{20} = 0.880$, $p < 0.05$	
20:5n3				$F_{3,16} = 3.317$, $p < 0.05$
20:6n3			$W_{20} = 0.879$, $p < 0.05$	

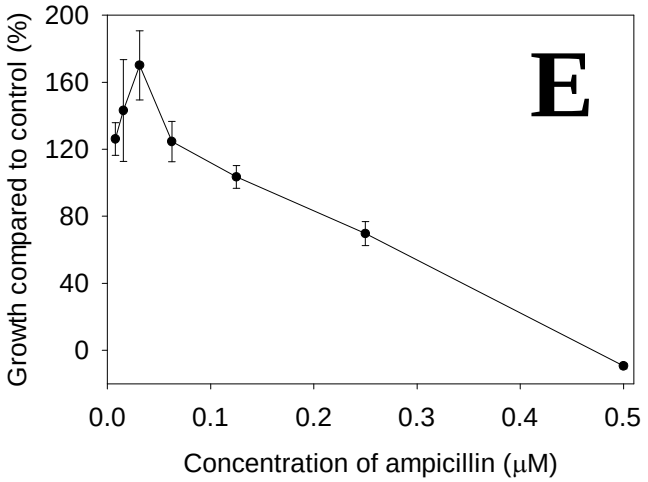
Figure 6.1 – IC_{50} determinations against *S. aureus* for (A) 16:1n7, (B) 16:3n4, (C) 20:5n3, (D) 16:1n7 + 20:5n3 and (E) ampicillin showing that 16:1n7 and 20:5n3 have the same IC_{50} (10-20 μM) but 16:3n4 is not so potent (20-40 μM). Ampicillin was more potent by approximately two orders of magnitude. The antibacterial action of fatty acids occurs in an additive manner because when 16:1n7 and 20:5n3 were tested as a 1:1 mix, with a total fatty acid concentration equal to that used when tested individually, the IC_{50} value is equal to that of 16:1n7 and 20:5n3 when tested individually. n = 4 (except 16:1n7+20:5n3 at 1.25 μM and ampicillin at 0.0625 μM where n = 3); error bars are ± 1 SE.


A
Growth compared to control (%)
Concentration of 16:1n7 (μM)


B
Growth compared to control (%)
Concentration of 16:3n4 (μM)


C
Growth compared to control (%)
Concentration of 20:5n3 (μM)


D
Growth compared to control(%)
Total concentration of 16:1n7 + 20:5n3 (μM)


E
Growth compared to control (%)
Concentration of ampicillin (μM)

individually. For comparison, ampicillin had an IC_{50} value between 0.25-0.5 µM (Figure 6.1).

MBC values against *S. aureus* are also presented as a range. The lower value is the plate with the highest concentration of antibacterial compound that had one or more colonies whilst the higher value is the plate with the lowest concentration of antibacterial compound that showed no colonies. Thus, the actual MBC lies between these concentrations (Table 6.2). Lowest MBC values (40-80 µM total fatty acid) were found for 16:1n7, 20:5n3 and the wells with the combination of these fatty acids (Table 6.2). It was not possible to determine MBC for 16:3n4 but this value is unlikely to be much greater than 640 µM as these plates showed very few colonies. MBC for ampicillin was 320-640 µM (Table 6.2). All microtitre plate studies were considered to be free from contamination because there were no colonies on the plates inoculated with the blank reference wells.

6.3.2 Spectrum of activity

16:1n7 significantly inhibited the growth of *E. coli* and *P. citreus* ($p < 0.001$) but not *Ps. aeruginosa* and *M. luteus* ($p < 0.05$) whereas 16:3n4 and 20:5n3 significantly inhibited only the growth of *P. citreus* ($p < 0.01$; Figure 6.2). Curiously, growth of *E. coli* and *Ps. aeruginosa* in the presence of 16:3n4 was significantly better compared to growth in the controls ($p < 0.01$; Figure 6.2).

Disc diffusion showed that 16:1n7 and 20:5n3 was active against MRSA (Figure 6.3) but neither of these fatty acids was able to inhibit the growth of *Candida* sp. A

Table 6.2 – Minimum bactericidal concentrations against *S. aureus* for ampicillin and fatty acids tested individually or in combination showing that the most potent fatty acids were 16:1n7 and 20:5n3. No value was determined for 16:3n4. Values given as the total concentration of fatty acid(s) required to completely kill an inoculum of 1 $x10^4$ cfu after 26 h; n = 3.

Test compound	MBC (μM)
Ampicillin	320-640
16:1n7	40-80
16:3n4	> 640
20:5n3	40-80
16:1n7 + 20:5n3	40-80

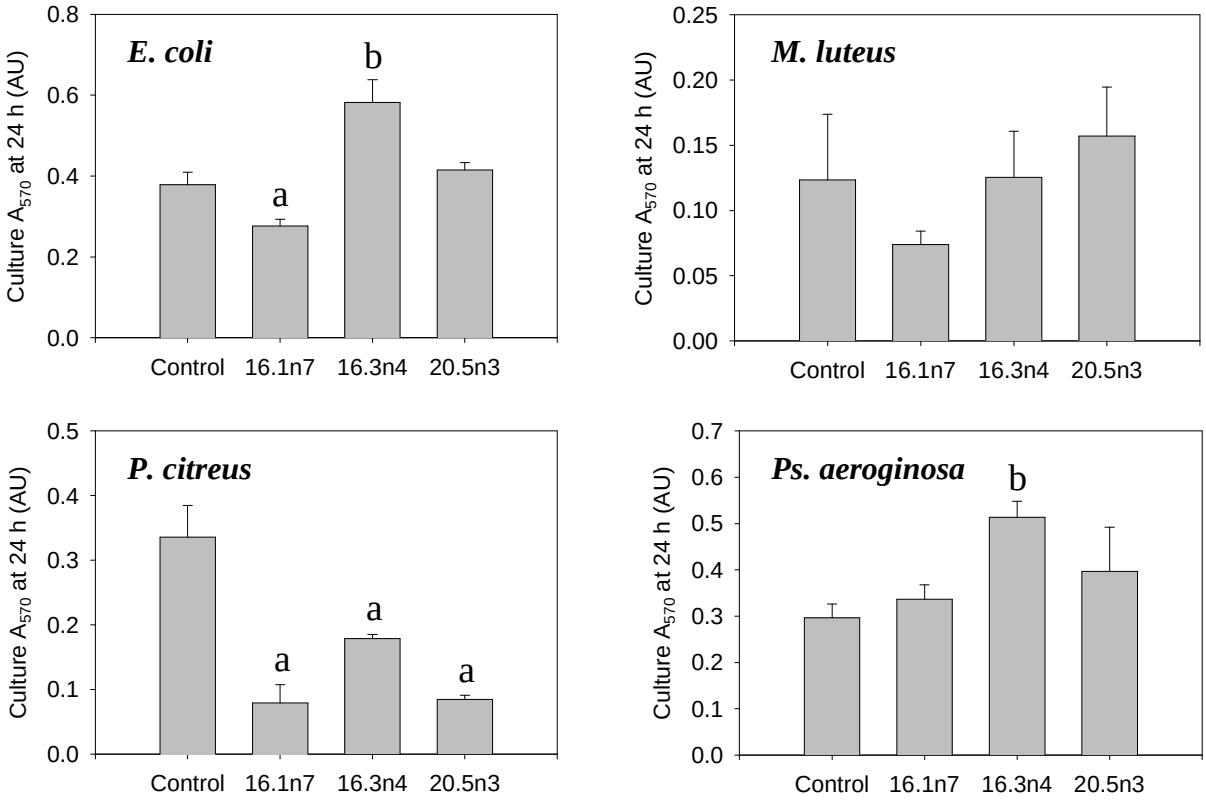


Figure 6.2 – Growth of *E. coli*, *Ps. aeroginosa* (both Gram negative), *M. luteus* and *P. citreus* (both Gram positive) in the presence of 16:1n7, 16:3n4 and 20:5n3 at concentrations of 640 µM compared to controls (no fatty acid). Significant differences within the data were tested by one-way ANOVA with the *post hoc* Tukey's HSD test being used to identify which groups differed significantly from the control. Treatment groups that differ significantly compared to the controls are identified by either 'a' (growth reduced compared to control) or 'b' (growth better than control). Growth of *P. citreus* was significantly reduced by all three fatty acids (one-way ANOVA: $F_{3,11}$ = 61.29, $p < 0.001$) whilst growth of *E. coli* was significantly reduced by 16:1n7 (one-way ANOVA: $F_{3,12}$ = 54.60, $p < 0.001$). Growth of *E. coli* and *Ps. aeroginosa* in the 16:3n4 wells was significantly better compared to controls (one-way ANOVAs: $F_{3,12}$ = 54.60, $p < 0.001$ and $F_{3,8}$ = 8.89, $p < 0.01$, respectively). n = 4 (except *P. citreus* 20:5n3 and all *Ps. aeroginosa* data where n = 3); error bars ± 1 SD.

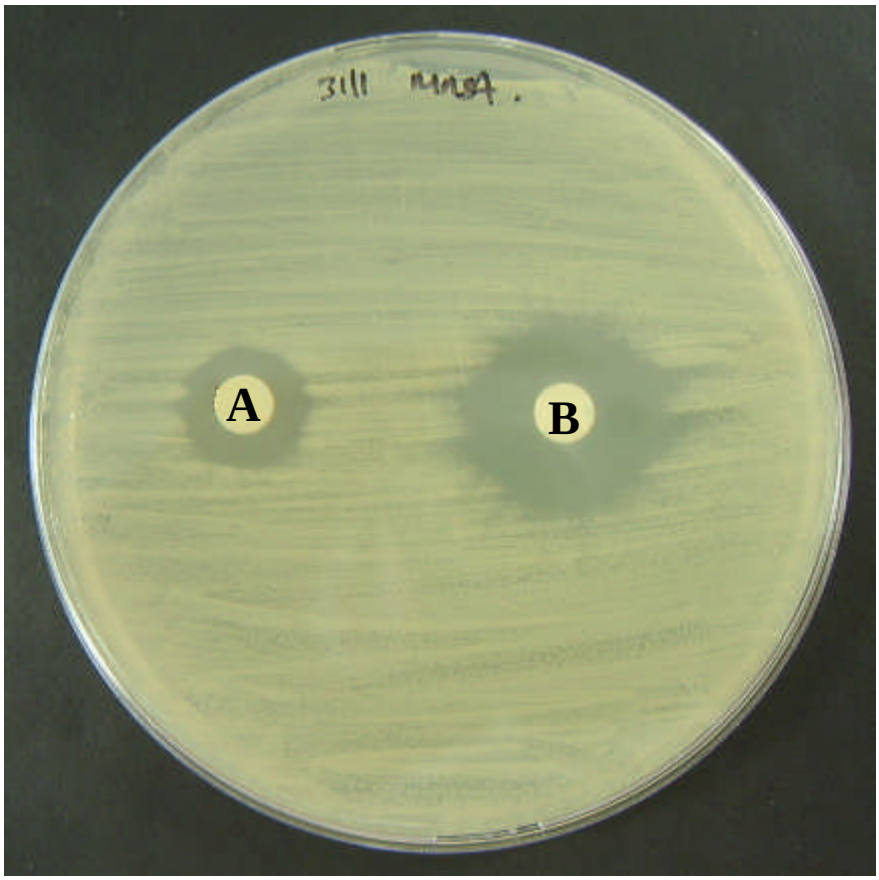


Figure 6.3 – Disc diffusion assay plate with zones of multi-resistant *S. aureus* growth inhibition caused by (A) 20:5n3 and (B) 16:1n7 showing that both fatty acids inhibit the growth of this bacterium.

summary of the spectrum of antibacterial activity of the three fatty acids can be found in Table 6.3.

6.3.3 Enzymatic release of the fatty acids

Significantly lower antibacterial activity (by approximately five times) was found in aqueous methanol extracts from cells extracted with boiling aqueous methanol compared to the cell extracts from cells extracted on ice in the conventional way (t_7 = -2.83, $p < 0.05$) (Figure 6.4).

6.3.4 Fatty acid levels during culture

The relative percentage composition of fatty acids in aqueous methanol cell extracts changed during culture (Table 6.4). The most notable observation was that 22:1n9 increased from a stable percentage of ~2 – 8 % between days 4 – 12 to a much higher 34.5 % at day 14. Levels of 16:2n4 and 16:4n1 declined gradually between days 4 and 14 from 4.8 to 1.1 % and from 3.7 to 0.7 %, respectively. 16:0 levels decreased from 11 % to ~7-9 % during exponential phases (days 6, 8 and 10) then increased again at day 12 to 10.9 % and further at day 14 to 16.2 %. Levels of 22:1n11 also decreased during exponential phases (days 6, 8 and 10) from 0.5 % at day 4 to 0.1 % at day 10 then increased again at day 12 to 0.4 % and further at day 14 to 0.6 %. The levels of 20:5n3 were greatest at day 6 (31.5 %) then decreased to 9.6 % at day 14 (Table 6.4). With 22:6n3 and 18:2n6, the levels peaked at day 8 (4.5 and 6.3 %, respectively) then declined to 2.6 and 1.1 % at day 14, respectively. 16:1n7 and 20:4n6 increased to their highest levels at day 10 (22.1 and 1.6 %, respectively) then reduced to 7.6 and 0.8 % at day 14, respectively (Table 6.4). Levels of 16:3n4 increased slightly in mid to late exponential phase from 8.4 % at day 4 to 10.7 % at

Table 6.3 – Effect of 16:1n7, 16:3n4 and 20:5n3 at 640 µM on the growth of the fungus, *Candida* sp., and Gram positive and Gram negative bacteria showing that each fatty acid was antibacterial against at least one bacterium but none inhibited the growth of the fungus. Key: antibacterial (✓), not antibacterial (×) and not determined (n/d).

Species	Fatty acid		
	16:1n7	16:3n4	20:5n3
Gram +ve			
M. luteus[a]	×	×	×
P. citreus[a]	✓	✓	✓
S. aureus	✓[b]	✓[b]	✓[c]
S. epidermidis[d]	n/d	n/d	✓
MRSA[e]	✓	n/d	✓
Gram –ve			
E. coli[a]	✓	×	×
Ps. aeruginosa[a]	×	×	×
Fungus			
Candida sp.[e]	×	×	×

[a] Determined by growth inhibition assay in microtitre plate.

[b] This bacterium was used to isolate the compounds in Chapter 5 using disc diffusion assay.

[c] The growth of this bacterium was inhibited when determining IC_{50} values (Section 6.3.1).

[d] This bacterium was used to isolate 20:5n3 in Chapter 4 using disc diffusion assay.

[e] Determined by disc diffusion assay.

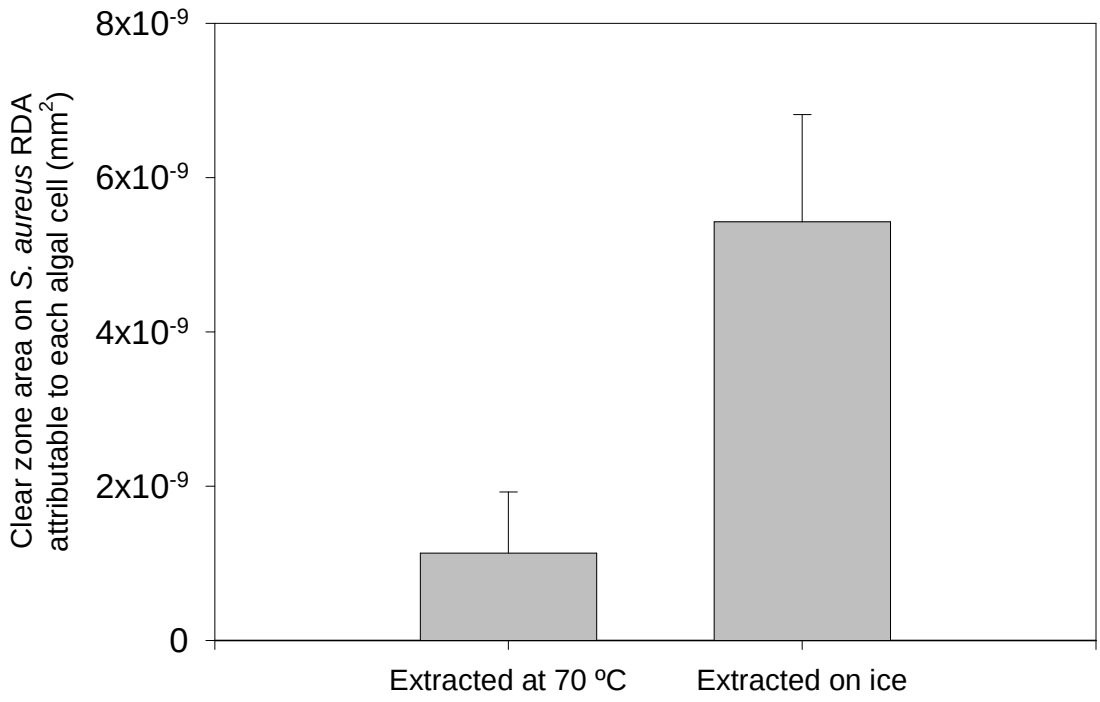


Figure 6.4 – Antibacterial activity against *S. aureus* on a per cell basis for cell pellets extracted for 1 h in hot aqueous methanol (~70 ºC) compared to those extracted for 1 h on ice showing significantly lower ($p < 0.05$) antibacterial activity (by approximately five times) in those samples extracted in hot aqueous methanol. The results suggest that the antibacterial activity is dependent upon the action of enzymes. n = 5 for cell pellets extracted in hot aqueous methanol, n = 4 for those extracted on ice; error bars are ± 1 SE.

Table 6.4 – Relative percentage composition of fatty acids in aqueous methanol cell extracts of *P. tricornutum* grown in the small-scale batch system showing that the relative level of each fatty acid, including the antibacterial fatty acids isolated in Chapters 4 and 5 (in bold), changes during culture. n = 4 (except day 14 where n = 3); data given as mean ± 1 SD. Only data for fatty acids that contribute ~1 % or more are given.

Fatty acid	Culture harvest day					
	4	6	8	10	12	14
14:0	8.84 ± 1.22	7.28 ± 0.45	8.59 ± 0.95	8.85 ± 0.68	8.48 ± 0.83	6.45 ± 0.73
14:1n5	0.87 ± 0.14	1.05 ± 0.23	0.95 ± 0.31	0.52 ± 0.12	0.62 ± 0.29	0.84 ± 0.13
16:0	10.93 ± 1.19	7.78 ± 1.21	8.95 ± 1.01	7.08 ± 0.83	10.86 ± 2.70	16.19 ± 1.82
16:1n7	**14.66 ± 0.66**	**14.18 ± 0.94**	**16.47 ± 2.12**	**22.08 ± 1.46**	**16.68 ± 4.10**	**7.55 ± 2.68**
16:2n6	0.39 ± 0.12	0.26 ± 0.09	0.26 ± 0.04	0.31 ± 0.07	0.45 ± 0.20	0.56 ± 0.13
16:2n4	4.81 ± 0.71	4.82 ± 0.55	4.16 ± 0.38	3.99 ± 0.26	3.08 ± 1.03	1.13 ± 0.54
16:3n4	**8.35 ± 1.77**	**8.58 ± 1.01**	**8.43 ± 0.87**	**10.67 ± 0.66**	**11.29 ± 3.19**	**4.44 ± 3.09**
16:4n1	3.65 ± 0.98	3.12 ± 1.84	2.06 ± 0.54	1.59 ± 0.17	1.66 ± 0.62	0.72 ± 0.10
18:0	1.73 ± 0.30	1.71 ± 1.63	1.36 ± 1.00	0.71 ± 0.29	3.10 ± 2.82	4.62 ± 2.57
18:1n9	3.23 ± 0.99	1.58 ± 0.74	3.43 ± 0.96	3.01 ± 0.29	5.03 ± 1.40	5.73 ± 1.11
18:1n7	0.58 ± 0.20	0.83 ± 0.76	0.67 ± 0.15	0.56 ± 0.05	0.97 ± 0.42	1.01 ± 0.54
18:2n6	2.70 ± 0.43	3.14 ± 1.79	6.32 ± 0.77	3.95 ± 0.77	1.83 ± 0.30	1.13 ± 0.16
18:3n6	1.13 ± 0.17	1.94 ± 1.36	1.60 ± 0.11	2.25 ± 0.38	1.80 ± 0.26	0.80 ± 0.35
18:4n3	0.94 ± 0.35	1.15 ± 0.17	0.74 ± 0.05	1.10 ± 0.38	1.01 ± 0.08	0.78 ± 0.29
20:4n6	0.41 ± 0.08	0.53 ± 0.05	0.88 ± 0.22	1.60 ± 0.67	1.34 ± 0.33	0.79 ± 0.28
20:5n3	**26.52 ± 1.93**	**31.51 ± 4.10**	**23.42 ± 6.11**	**26.47 ± 2.07**	**20.90 ± 2.48**	**9.57 ± 6.14**
22:1n11	0.46 ± 0.42	0.13 ± 0.10	0.17 ± 0.06	0.11 ± 0.08	0.41 ± 0.28	0.63 ± 0.30
22:1n9	6.67 ± 5.79	5.62 ± 3.63	7.10 ± 7.86	2.12 ± 1.46	8.08 ± 8.81	34.52 ±10.00
22:6n3	3.16 ± 0.67	3.78 ± 0.52	4.45 ± 0.57	3.05 ± 0.28	2.44 ± 0.14	2.56 ± 1.00

day 10 then further to 11.3 % at day 12 before decreasing to only 4.4 % at day 14 (Table 6.4). 18:0 and 18:1n9 increased slightly to 3.1 and 5.0 % at day 12 from ~1.7 and ~3.2 %, respectively, then further at day 14 (to 4.6 and 5.7 %, respectively). There was a slight reduction in 14:0 at day 14 from ~8 to 6.5 %. Levels of 14:1n5, 16:2n6, 18:1n7, 18:3n6 and 18:4n3 remained relatively stable throughout culture. In general, the mass of each fatty acid (on a per cell basis) reduced gradually over the growth curve (Table 6.5). During culture the masses of 16:1n7, 16:3n4 and 20:5n3 on a per cell basis declined between days 4 and 14 by 22, 21, and 35 times, respectively. The summed mass of these three fatty acids reduced 27-fold between days 4 – 14 (Figure 6.5). As a final point, the fatty acid 16:2n6 has been only tentatively identified because a standard is unavailable for comparison.

6.3.5 Fatty acid levels in the different *P. tricornutum* cell morphs

The relative amount of each fatty acid was determined for aqueous methanol cell extracts prepared from cultures enriched for either the oval or the fusiform morph and the PCA showed that the data points split into two populations corresponding to cell extracts from the cultures enriched for either morph (Figure 6.6). This is highly suggestive of differences existing between fatty acid contents of the extracts generated from flasks in the different morphologies. Two-way ANOVA confirmed that the extracts from the different morphs differed significantly ($p < 0.0038$) in the relative amounts of seven fatty acids (14:0, 16:0, 16:2n4, 16:3n4, 16:4n1, 20:4n6, 20:6n3) (Table 6.6) and, on a per cell basis, in the actual amounts of 14:0, 16:2n4 and 16:3n4 (Table 6.7). Also, on a per cell calculation, significantly greater mass of total fatty acids were found in the aqueous methanol cell extracts from cultures enriched with cells in the fusiform morph compared to oval-enriched cultures (two-way

Table 6.5 – Mass of each fatty acid on a per cell basis in aqueous methanol cell extracts of *P. tricornutum* grown in the small-scale batch system showing that the mass of each fatty acid, including the antibacterial fatty acids isolated in Chapters 4 and 5 (in bold), reduces during culture. n = 4 (except day 14 where n = 3); data given as mean ± 1 SD; values are in fg ($x10^{-15}$ g). Only data for fatty acids that contribute ~1 % or more are given.

Fatty acid	Culture harvest day					
	4	6	8	10	12	14
14:0	25.39 ± 4.41	18.49 ± 6.86	11.84 ± 7.31	13.49 ± 5.49	2.93 ± 1.11	1.26 ± 0.23
14:1n5	2.50 ± 0.47	2.55 ± 0.71	1.19 ± 0.50	0.73 ± 0.15	0.19 ± 0.05	0.14 ± 0.07
16:0	32.12 ± 8.89	19.88 ± 8.55	11.41 ± 5.08	10.60 ± 4.00	3.53 ± 0.70	2.69 ± 1.58
16:1n7	**42.83 ± 10.46**	**36.89 ± 17.40**	**22.75 ± 14.23**	**33.88 ± 14.20**	**5.96 ± 3.07**	**1.92 ± 0.89**
16:2n6	1.07 ± 0.22	0.61 ± 0.19	0.34 ± 0.20	0.49 ± 0.26	0.14 ± 0.04	0.10 ± 0.05
16:2n4	13.74 ± 1.88	12.53 ± 6.03	5.68 ± 3.37	6.12 ± 2.60	1.10 ± 0.58	0.31 ± 0.20
16:3n4	**23.85 ± 4.70**	**22.32 ± 10.37**	**11.48 ± 6.60**	**16.16 ± 6.35**	**4.00 ± 1.96**	**1.14 ± 0.71**
16:4n1	10.65 ± 3.28	8.58 ± 6.46	2.86 ± 1.78	2.42 ± 0.97	0.60 ± 0.34	0.19 ± 0.10
18:0	5.21 ± 2.00	3.64 ± 2.32	1.34 ± 0.55	1.05 ± 0.45	0.85 ± 0.60	0.83 ± 0.34
18:1n9	9.79 ± 4.73	4.03 ± 2.21	4.05 ± 0.97	4.64 ± 2.04	1.64 ± 0.37	1.00 ± 0.58
18:1n7	1.70 ± 0.72	1.78 ± 1.10	0.81 ± 0.24	0.83 ± 0.25	0.30 ± 0.07	0.18 ± 0.15
18:2n6	7.81 ± 1.87	8.60 ± 5.74	8.71 ± 5.49	5.95 ± 2.62	0.65 ± 0.29	0.25 ± 0.06
18:3n6	3.27 ± 0.70	4.37 ± 1.93	2.10 ± 1.07	3.51 ± 1.76	0.63 ± 0.26	0.19 ± 0.08
18:4n3	2.63 ± 0.97	3.03 ± 1.67	0.97 ± 0.49	1.50 ± 0.19	0.34 ± 0.11	0.14 ± 0.05
20:4n6	1.24 ± 0.52	1.37 ± 0.62	1.09 ± 0.43	2.64 ± 1.88	0.48 ± 0.24	0.19 ± 0.09
20:5n3	**76.89 ± 15.54**	**80.67 ± 34.80**	**33.88 ± 24.06**	**39.47 ± 13.21**	**7.32 ± 3.06**	**2.18 ± 1.09**
22:1n11	1.18 ± 1.01	0.36 ± 0.28	0.21 ± 0.09	0.16 ± 0.12	0.12 ± 0.06	0.12 ± 0.09
22:1n9	22.85 ± 21.75	13.62 ± 7.75	5.68 ± 5.97	2.77 ± 1.01	2.74 ± 3.06	6.14 ± 3.25
22:6n3	9.46 ± 4.09	9.41 ± 3.15	5.91 ± 3.19	4.52 ± 1.45	0.84 ± 0.31	0.45 ± 0.13

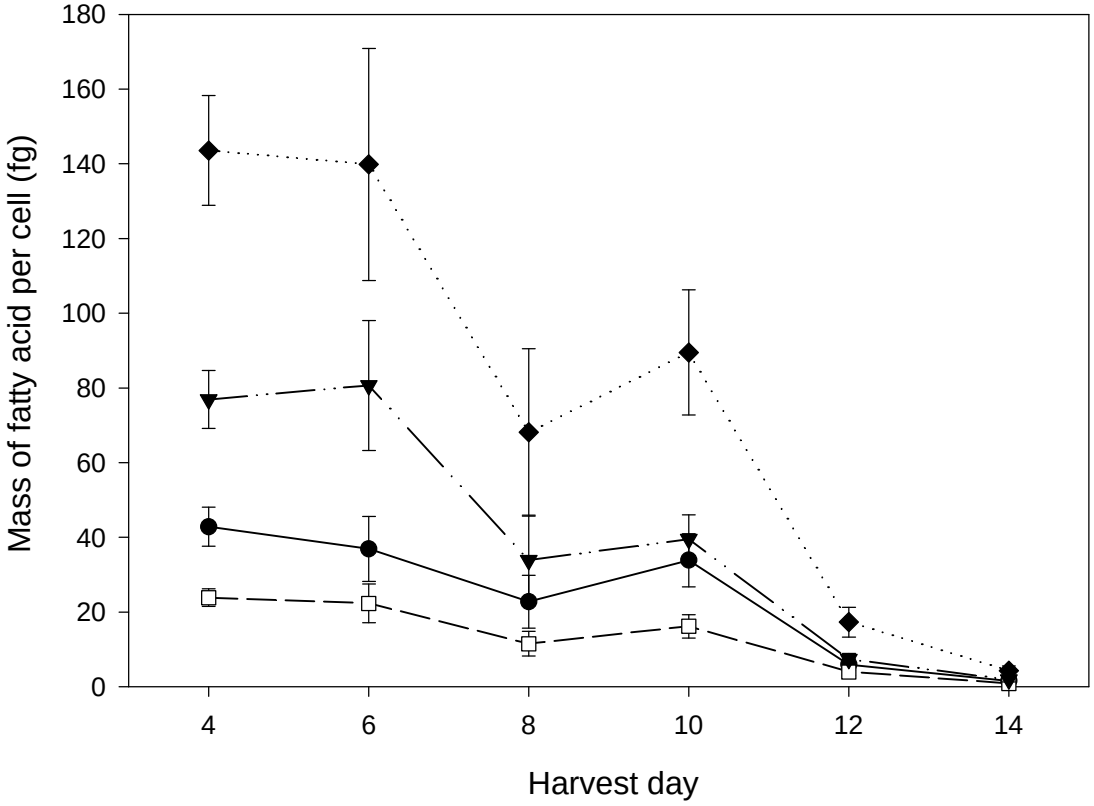


Figure 6.5 – Mass of 16:1n7 (●), 16:3n4 (□), 20:5n3 (▼) and their sum (♦) on a per cell basis in aqueous methanol cell extracts of *P. tricornutum* during growth in the custom batch culture system showing that their levels reduce during the 14 days of culture. n = 4 (except day 14 where n = 3); all error bars are ± 1 SE.

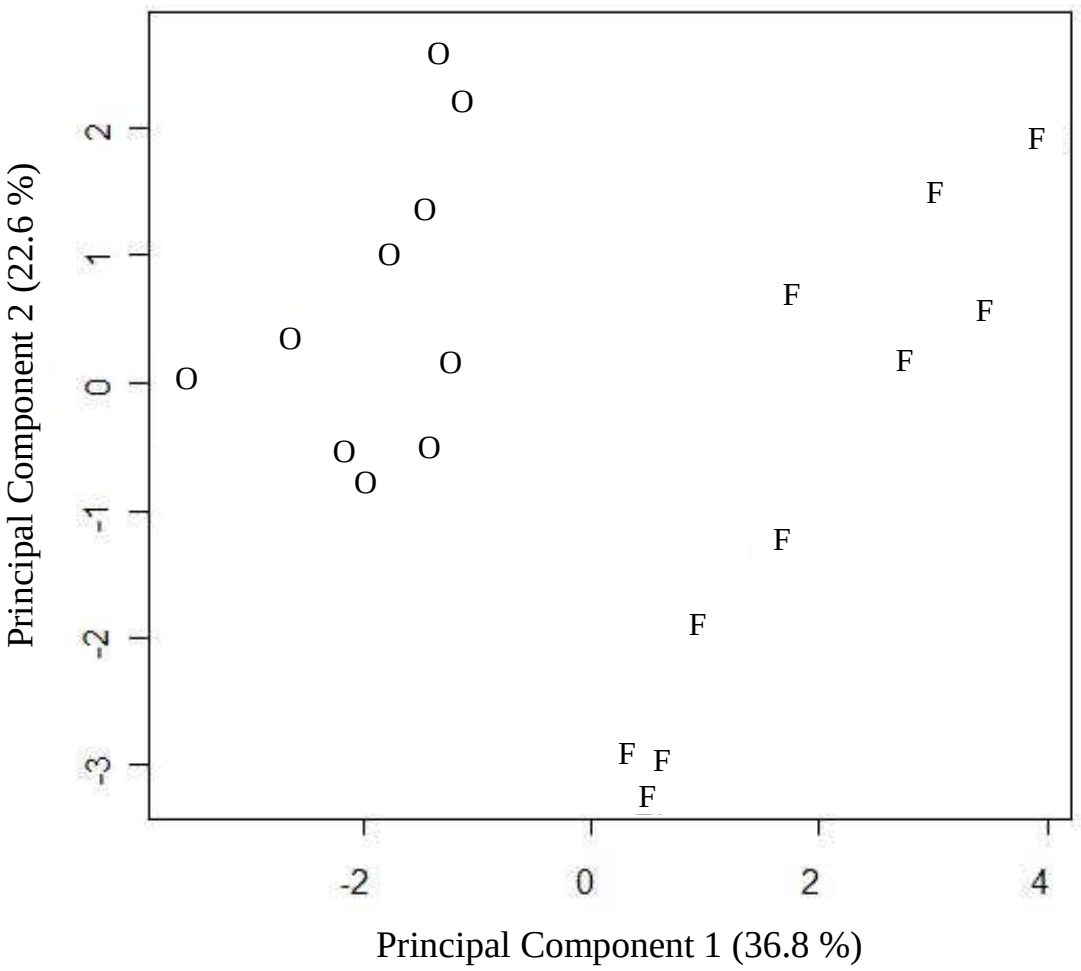


Figure 6.6 – Plot of first two principal components calculated from 13 fatty acids found in aqueous methanol cell extracts prepared from cultures enriched in either the oval (O) or fusiform (F) morphs. The first and second components accounted for 36.8 and 22.6 % of the total variance, respectively. n = 20.

Table 6.6 – Relative percentage of each fatty acid in aqueous methanol cell extracts from enriched cultures containing either >71 % fusiform cells or 100 % oval cells showing that cells in the fusiform-enriched cultures contain significantly greater proportions of 14:0, 16:2n4, 16:3n4 and 16:4n1 but significantly lower proportions of 16:0, 20:4n6 and 20:6n3. Data for the three antibacterial fatty acids isolated in Chapters 4 and 5 are indicated in bold. n = 10; data given as mean ± 1 SD.

Fatty acid	Relative percentage of each fatty acid in cell extracts	
	Fusiform	Oval
14:0[a,c]	7.89 ± 0.37	6.39 ± 0.28
16:0[b,d]	9.59 ± 1.32	10.70 ± 1.45
16:1n7	**21.04 ± 1.99**	**21.72 ± 1.02**
16:2n4[a,e]	4.26 ± 0.44	1.92 ± 0.23
16:3n4[a,f]	**10.15 ± 1.52**	**9.10 ± 0.69**
16:4n1[a,g]	0.66 ± 0.10	0.41 ± 0.30
18:1n9	2.66 ± 0.76	3.58 ± 1.21
18:2n6	1.95 ± 0.23	2.02 ± 0.56
18:3n6	1.23 ± 0.19	1.21 ± 0.10
18:4n3	0.64 ± 0.09	0.70 ± 0.15
20:4n6[b,h]	1.22 ± 0.13	1.74 ± 0.24
20:5n3	**36.60 ± 2.36**	**37.79 ± 1.39**
20:6n3[b,i]	2.12 ± 0.16	2.71 ± 0.19

[a] Significantly greater in cells from fusiform-enriched cultures ($p < 0.0038$).

[b] Significantly greater in cells from oval-enriched cultures ($p < 0.0038$).

[c] Two-way ANOVA: $F_{3,16} = 119.3$, $p < 0.001$.

[d] Two-way ANOVA: $F_{3,16} = 12.08$, $p < 0.003$.

[e] Two-way ANOVA: $F_{3,16} = 344.9$, $p < 0.001$.

[f] Two-way ANOVA: $F_{3,16} = 14.32$, $p < 0.002$.

[g] Two-way ANOVA: $F_{3,16} = 45.45$, $p < 0.001$.

[h] Two-way ANOVA: $F_{3,16} = 42.60$, $p < 0.001$.

[i] Two-way ANOVA: $F_{3,16} = 71.25$, $p < 0.001$.

Table 6.7 – Mass of fatty acids in aqueous methanol cell extracts from 3.735 x 10^8 cells in enriched cultures containing either >71 % fusiform cells or 100 % oval cells showing that cells in the fusiform-enriched cultures contain a significantly greater quantity of total fatty acids and greater quantities of 14:0, 16:2n4 and 16:3n4. Data for the three antibacterial fatty acids isolated in Chapters 4 and 5 are indicated in bold. n = 10; data given as mean ± 1 SD.

Fatty acid	Mass of each fatty acid in cell extracts (g x10^{-5})	
	Fusiform	Oval
14:0[a,b]	1.884 ± 0.7973	1.295 ± 0.7686
16:0	2.370 ± 0.1784	2.305 ± 1.5529
16:1n7	**5.117 ± 2.4066**	**4.293 ± 2.4041**
16:2n4[a,c]	1.005 ± 0.4446	0.411 ± 0.2697
16:3n4[a,d]	**2.293 ± 0.6821**	**1.785 ± 1.0004**
16:4n1	0.162 ± 0.0816	0.113 ± 0.1104
18:1n9	0.672 ± 0.3981	0.680 ± 0.3741
18:2n6	0.460 ± 0.1895	0.435 ± 0.3263
18:3n6	0.293 ± 0.1304	0.247 ± 0.1501
18:4n3	0.155 ± 0.077	0.144 ± 0.0877
20:4n6	0.289 ± 0.1157	0.334 ± 0.179
20:5n3	**8.520 ± 0.1609**	**7.579 ± 4.4627**
20:6n3	0.00511 ± 0.002254	0.558 ± 0.3425
Total[e]	23.731 ± 9.7344	20.179 ± 11.9122

[a] Significantly different between morphs ($p < 0.0038$).

[b] Two-way ANOVA: $F_{3,16} = 40.06$, $p < 0.001$.

[c] Two-way ANOVA: $F_{3,16} = 46.97$, $p < 0.001$.

[d] Two-way ANOVA: $F_{3,16} = 15.20$, $p < 0.001$.

[e] Significantly different between morphs (two-way ANOVA: $F_{3,16} = 6.282$, $p < 0.05$).

ANOVA: $F_{3,16} = 6.282$, $p < 0.05$; Table 6.7). Additionally, significantly greater mass ($p < 0.05$) of the three previously isolated fatty acids when summed (16:1n7+16:3n4+20:5n3) was found in cell extracts produced from cultures enriched in fusiform cells compared to oval-enriched cultures on a per cell basis (Figure 6.7).

6.4.0 Discussion

IC_{50} values against *S. aureus* were determined for all three fatty acids and these were all µM concentrations. Of the three fatty acids, only 16:1n7 was found to inhibit the growth of a Gram negative species (*E. coli*) whilst 16:1n7 and 20:5n3 inhibited the growth of MRSA. Levels of fatty acids in the aqueous methanol cell extracts changed throughout the *P. tricornutum* growth curve. The results appear to confirm that the free fatty acids in cell extracts are produced by enzyme action because significantly lower ($p < 0.05$) antibacterial activity was found in preparations from cell pellets extracted in hot solvent. Finally, extracts from cultures enriched in different cell morphs showed that cell morphology affected the levels of many fatty acids.

In the present study, 16:1n7 was found to be antibacterial against Gram positive and Gram negative species, which confirms previous reports (Table 6.8). However, this is the first report that 16:1n7 is active against the human pathogen, MRSA. Moreover, 16:1n7 is shown for the first time to demonstrate activity against a marine bacterium, specifically the Gram positive *P. citreus*. No activity was found for 16:1n7 against *Candida* sp., which confirms the findings of Bergsson *et al*. (2001b) but contradicts the results of Kabara *et al*. (1972). This discrepancy is likely to be due to the different strains and assay used. Previously, Wang (1999) showed 16:3n4 was antibacterial against various Gram positive human pathogens (Table 6.8) but the current study is the first to report 16:3n4 to be antibacterial against a marine bacterium (*P. citreus*).

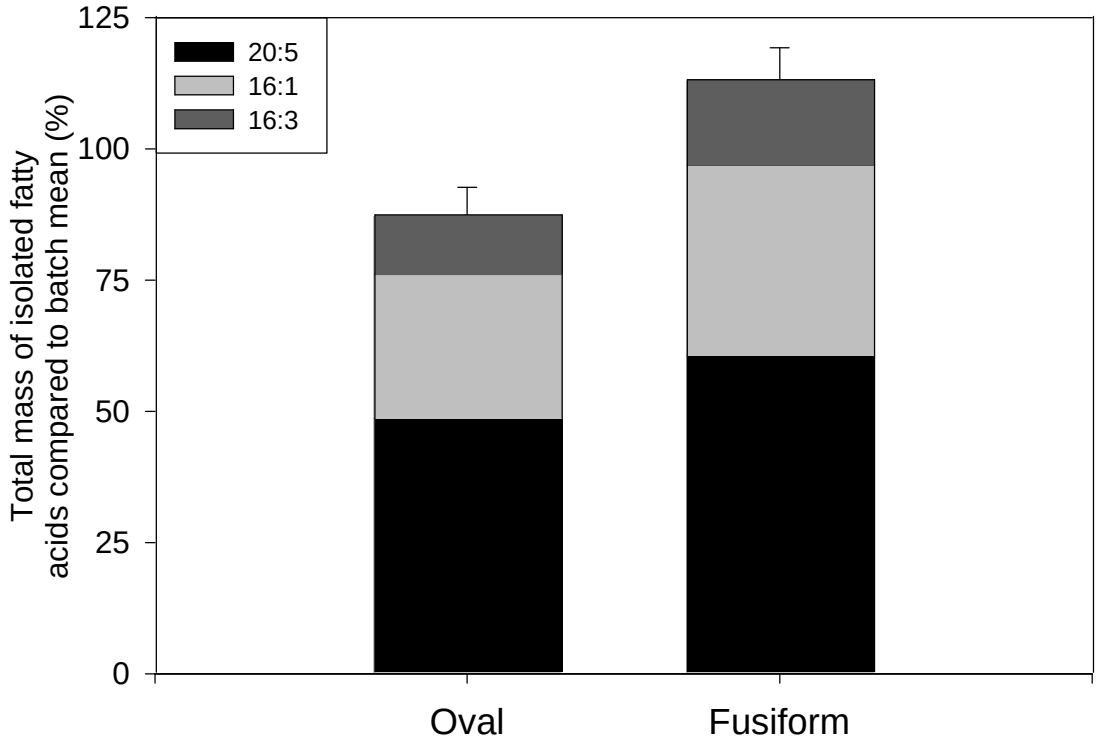


Figure 6.7 – The combined mass of the three antibacterial fatty acids (isolated in Chapters 4 and 5) found in aqueous methanol cell extracts of 3.735 x 10^8 cells from enriched cultures containing either >71 % fusiform cells or 100 % oval cells showing that extracts from the fusiform-enriched cultures contain a significantly greater quantity (two-way ANOVA: $F_{3,16} = 5.838$, $p < 0.05$). Due to inter-batch variability data is expressed as relative to batch mean (batch mean = 100 %). n = 10; error bars indicate ± 1 SE for the cumulative mass of the three fatty acids.

Table 6.8 – Reports in the literature for the spectrum of antibacterial activity of the free fatty acids, 16:1n7, 16:3n4 and 20:5n3.

Fatty acid and bacterial species	Reference
16:1n7	
Gram +ve	
Bacillus larvae	Feldlaufer *et al*. (1993)
Clostridium welchii	Kabara (1978)
Corynebacterium sp.	Kabara *et al*. (1972)
Micrococcus sp.	Kabara *et al*. (1972)
Mycobacterium spp. (15 spp.)[a]	Saito *et al*. (1984)[b]
Nocardia asteroides	Kabara *et al*. (1972)
Pneumococci sp.	Kabara *et al*. (1972)
Staphylococcus aureus	Kabara *et al*. (1972); Dye and Kapral (1981)[b]; Bergsson *et al*. (2001)[b]; Zheng *et al*. (2005)[b]
Staphylococcus epidermidis	Kabara *et al*. (1972)
Streptococcus sp. (group A)	Kabara *et al*. (1972); Bergsson *et al*. (2001)[b]
Streptococcus sp. (group B)	Bergsson *et al*. (2001)[b]
Streptococcus sp. (beta-haemolytic non-A)	Kabara *et al*. (1972)
Streptococcus pyogenes	Zheng *et al*. (2005)[b]
Gram -ve	
Helicobacter pylori	Sun *et al*. (2003)
Neisseria gonorrhoeae	Miller *et al*. (1977); Bergsson *et al*. (1999)
16:3n4	
Gram +ve	
Bacillus cereus	Wang (1999)
Bacillus subtilis	Wang (1999)
Carynobacter xerosis	Wang (1999)
Enterococcus sp. (vancomycin resilient)	Wang (1999)
MRSA	Wang (1999)
Pyogenes vulgaris	Wang (1999)
Shigella dysenteriae	Wang (1999)
Streptococcus mitis	Wang (1999)
Streptococcus facaelis	Wang (1999)
20:5n3	
Gram +ve	
Lactococcus garvieae	Benkendorff *et al*. (2005)
MRSA	Ohta *et al*. (1995)
Mycobacterium spp. (15 spp.)[a]	Saito *et al*. (1984)[b]
Staphylococcus aureus	Knapp and Melly (1976)[b]; Ohta *et al*. (1995)
Gram -ve	
Vibrio harveyi	Benkendorff *et al*. (2005)
Vibrio anguillarum	Benkendorff *et al*. (2005)
Vibrio alginocolyticus	Benkendorff *et al*. (2005)

[a] These are not conventional Gram positive (as they do not take up the stain) but are widely considered to be so due to the structure of their cell wall (Trifiro *et al*., 1990).

[b] These studies did not explicitly state the position of the double-bond though it is almost certainly in the n7 position.

The data obtained in the present study confirm that 20:5n3 is active against Gram positive species. Previously free 20:5n3 has been shown to be antibacterial against *S. aureus* (Knapp and Melly, 1986; Ohta *et al*., 1995; Shin *et al*., 2007), MRSA (Ohta *et al*., 1995) and the marine pathogen *Lactococcus garvieae* (Benkendorff *et al*., 2005) (Table 6.8). Moreover, Benkendorff *et al*. (2005) showed 20:5n3 is active against Gram negative marine *Vibrio* species (Table 6.8). The results of the present study confirm previous reports that unsaturated fatty acids show greater antibacterial activity against Gram positive than Gram negative species (Kodicek and Worden, 1945; Kodicek, 1949; Galbraith *et al*., 1971; Kabara *et al*., 1977). Curiously, none of the fatty acids tested were active against *M. luteus* even though crude aqueous methanol cell extracts inhibited the growth of this bacterium (Figure 3.4). This could be because the compound responsible for the anti-*M. luteus* activity was unstable after separation by RP-HPLC or the concentration tested may have been too low to show the activity.

It is often difficult to compare results between studies on the antibacterial potency of free fatty acids as authors have used different methods. Nevertheless, it has been widely reported that potency of antibacterial activity varies upon the length of the carbon chain (Sheu and Freese, 1972; Miller *et al*., 1977) and degree of unsaturation (Kabara *et al*., 1973; Saito *et al*., 1984; Ohta *et al*., 1995; Sun *et al*., 2003). Generally greatest antibacterial activity for saturated fatty acids is found in those possessing a C12 carbon chain (Kabara *et al*., 1972; Feldlaufer *et al*., 1993; Sun *et al*., 2003). Antibacterial activity tends to increase with greater number of double bonds present in those fatty acids with the same number of carbons in the chain (Kabara *et al*., 1972; Knapp and Melly, 1986; Sun *et al*., 2003). In the present study 16:1n7 and 20:5n3

were determined to be approximately twice as potent against *S. aureus* as 16:3n4. With respect to the MBC, 16:1n7 and 20:5n3 were much more potent than 16:3n4, for which a definitive value was not determined due to the low quantity of sample available. Sun *et al*. (2003), using different methods from the present study and the Gram negative pathogen *Helicobacter pylori*, determined IC_{50} and MBC values for 16:1n7 as 250 µM and 1000 µM, respectively, and for another Gram negative pathogen, *Neisseria gonorrhoeae*, Miller *et al*. (1977) determined an IC_{50} of 7 µM. Further, Zheng *et al*. (2005) determined the MBC values for 16:1n7 against *S. aureus* and *S. pyogenes* to be 400 and 100 µM, respectively. The only previous studies on the potency of 16:3n4 and 20:5n3 have used a disc diffusion assay and presented the data as minimum inhibitory concentration (MIC), which is the lowest concentration that causes a clear zone of bacterial growth inhibition around the disc. This makes comparison impossible but for entirety, Wang (1999) reported the MIC for 16:3n4 against both MRSA and vancomycin-resilient enterococcus to be 15-20 µg/disc (0.06-0.08 µM/disc) whilst Ohta *et al*. (1995) determined the MIC for 20:5n3 against *S. aureus* and MRSA to be 10 and 20 µg/disc (0.04 and 0.08 µM/disc), respectively. The potency of the three fatty acids approximates closely to previous data against Gram positive species (Knapp and Melly, 1986; Zheng *et al*., 2005). Nevertheless, the finding that 16:1n7 is more potent than 16:3n4 contradicts the generally accepted principle that fatty acids of the same carbon chain length but with more double bonds have greater antibacterial potency. However, it is known that the position of double bonds can also affect activity (Kabara *et al*., 1973; Kabara *et al*., 1977) and, perhaps more importantly, the 16:3n4 sample used in these experiments was the HPLC-fraction isolated in Chapter 5 and not a highly pure commercial sample as was the case for 16:1n7 and 20:5n3. The 16:3n4-containing HPLC-fraction was not entirely

pure and contained a quantity of the methylated fatty acid, which considering methylated fatty acids possess only very weak or no antibacterial activity compared to the free acid (Kodicek and Worden, 1945; Dye and Kapral, 1981; Ohta *et al*., 1995; Zheng *et al*., 2005), would apparently reduce the fraction's potency.

Combinations of antibacterial free fatty acids were found in this thesis not to act in a synergistic or antagonistic way, rather they were found to function in an additive fashion confirming a previous observation by Sun *et al*. (2003). There are no published IC_{50} and MBC values whatsoever for 16:3n4 or 20:5n3, and for 16:1n7, there is no previously reported IC_{50} value against a Gram positive species.

Fatty acid composition of *P. tricornutum* has been shown to change during culture (Orcutt and Patterson, 1975; Cooper *et al*., 1985; Siron *et al*., 1989; Liang *et al*., 2006). In the present study, each fatty acid was more abundant early in the growth curve compared to later but comparisons with other studies are difficult because the extraction, unlike the other published studies, was not optimised for lipids, only antibacterial activity. As such, the extraction contained only a fraction (~0.5 %) of the total cell lipid contents (Appendix XI) which would largely comprise polar lipid compounds and free fatty acids. However, the changes in fatty acids found in aqueous methanol cell extracts throughout culture presented herein largely conform to the findings of Liang *et al*. (2006), who reported a marked increase in 16:1n7 and 18:1n7 and decrease in 16:3n4 and 20:5n3 during xenic culture. The only difference is that 16:1n7 was found to decrease during culture under the growth and extraction conditions used in the present study. Orcutt and Patterson (1975) examined the relative composition of *P. tricornutum* in stationary phase and reported that cells

contained 53 % 16:1; a result very different from the present study. Finally, Siron *et al*. (1989) found cellular fatty acid content increased 3-fold during culture but in the present study mass of fatty acids calculated on a 'per cell' basis reduced five-fold during culture. Contradictory findings in the aforementioned studies reflect the use of different extraction protocols but are likely to have been compounded by the use of different *P. tricornutum* strains and culture conditions. The reduction in 'per cell' total mass of fatty acids and the summed relative and actual levels of the three antibacterial fatty acids during culture follows the same trend as the reduction in antibacterial activity (on a per cell basis) through culture (Figure 6.8). This appears to further implicate the fatty acids as being responsible for the antibacterial activity of *P. tricornutum* cell extracts. No previous study has considered the changing levels of fatty acids during culture in the context of their antibacterial action.

Only a few studies have considered differences between the *P. tricornutum* morphs (Table 1.5) and so this is the first study to report a difference in antibacterial activity between the morphs, with greater activity attributable to a fusiform cell compared to an oval cell. Whilst the morphs differed in relative and actual amount of fatty acids in cell extracts a significantly greater mass of the three isolated antibacterial fatty acids was found in extracts from fusiform-enriched cultures ($p < 0.05$). Therefore, the greater quantity of these fatty acids in extracts from fusiform-enriched cultures could explain the greater antibacterial activity of these extracts compared to extracts prepared from oval-enriched cultures. Lewin *et al*. (1958) compared the different morphs and found that in oval cells 24 % of the dry weight was lipid whereas in fusiform cells the lipid comprises 34 %. Little difference was found with respect to the dry weight of the cells (confirmed in the present study; Appendix XII) suggesting

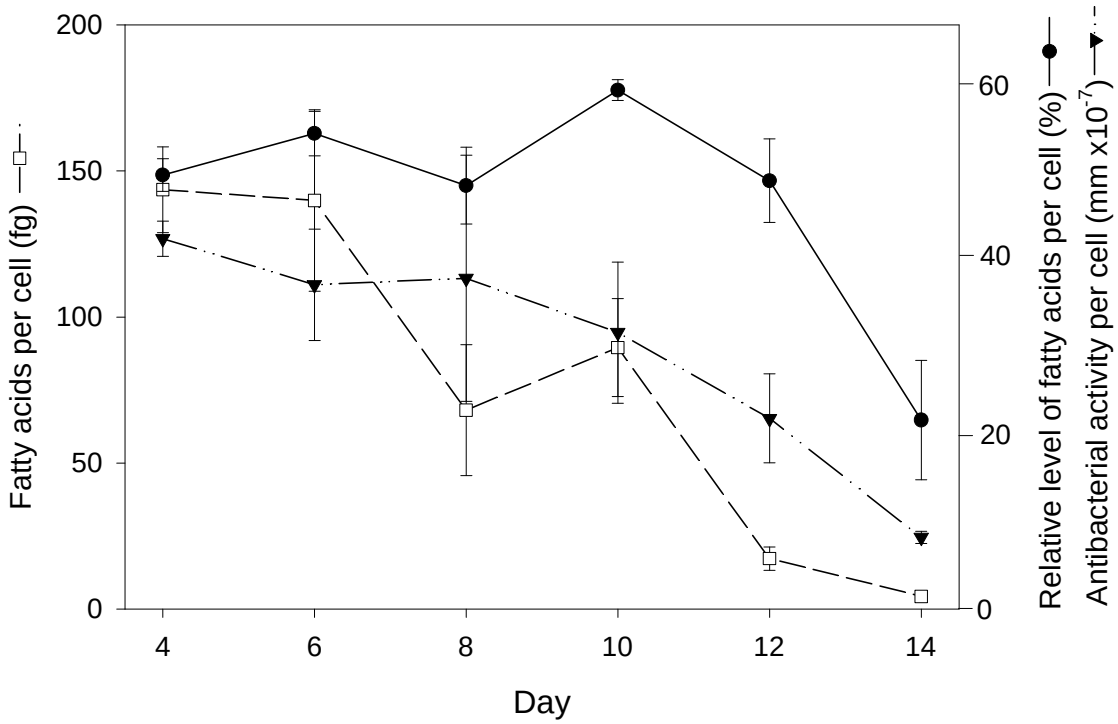


Figure 6.8 – The summed relative percentage (●) and actual mass (□) per cell of the three antibacterial fatty acids isolated in chapters 4 and 5 (16:1n7, 16:3n4 and 20:5n3) throughout culture showing that levels reduce during growth. Also shown is the antibacterial activity per cell (▼) showing that this reduces in a similar way. For data each day n = 4 (except day 14 where n = 3); all error bars ± 1 SE.

that fusiform cells contain a greater quantity of total lipid, thus possibly explaining the greater mass of fatty acids found in aqueous methanol cell extracts. An alternative suggestion is that the fusiform cells are more fragile due to their shape and cell wall structure and are therefore more likely to lose their cell integrity and release antibacterial fatty acids.

In the present study, reduced antibacterial activity was found in cell extracts that were extracted in boiling methanol compared with those on ice, which may be attributed to inactivation of the enzymes that cleave antibacterial free fatty acids from lipids (Budge and Parrish, 1999; Jüttner, 2001). Therefore, the antibacterial unsaturated free fatty acids are probably released as a cascade on cell disruption but consideration needs to be given to whether these compounds can reach sufficiently high concentrations to affect surrounding bacteria. A calculation was performed to determine a spherical area in which the concentration of the fatty acids released from an algal cell could reach a level that affected bacteria located in that area. The IC_{50} values determined above were selected as the measure for showing an effect on bacteria with the upper values of the range being used for conservatism. Therefore, the IC_{50} values used for the following calculations are 20 µM (or 2 $x10^{-5}$ M L^{-1}) for 16:1n7 and 20:5n3 and 40 µM (or 4 $x10^{-5}$ M L^{-1}) for 16:3n4. Using data for the levels of fatty acids found in cell extracts from *P. tricornutum* culture harvested at day 4, a *P. tricornutum* cell 'released' 4.28 $x10^{-14}$ g 16:1n7, 2.39 $x10^{-14}$ g 16:3n4 and 7.69 x 10^{-14} g 20:5n3 (or 1.69 $x10^{-16}$ M, 9.56 $x10^{-17}$ M and 2.55 $x10^{-16}$ M, respectively) when calculated on a per cell basis. The following calculations assume that all the fatty acids in the extract were in the free form, i.e., antibacterially active. The volume (in litres) in which a fatty acid reaches the IC_{50} concentration is determined by dividing

the total molar quantity of the fatty acid by its respective IC_{50} value (in M L^{-1}). Thus the volume in which 1.69 $x10^{-16}$ M of 16:1n7 reaches 2 $x10^{-5}$ M L^{-1} is 8.45 $x10^{-12}$ L, the volume in which 9.56 $x10^{-17}$ M of 16:3n4 reaches 4 $x10^{-5}$ M L^{-1} is 2.39 $x10^{-12}$ L and the volume in which 2.55 $x10^{-16}$ M of 20:5n3 reaches 2 $x10^{-5}$ M L^{-1} is 1.28 $x10^{-11}$ L. Assuming that these fatty acids act in an additive fashion these volumes can be summed giving a total volume of at least 2.36 $x10^{-11}$ L or 2.36 $x10^{-14}$ m^3. If it is assumed that the fatty acids diffuse uniformly to give a sphere, the radius of which can be calculated from: $r = \sqrt[3]{((V \div 4\pi) \times 3)}$ where r is the radius and V is the volume. Thus the calculated radius of the sphere is 17.7 µm (Figure 6.9). This simple hypothetical model is based on broad assumptions that have limitations and so this is merely intended to be a starting point for assessing the broad ecological relevance of fatty acids. Further attention needs to be directed to the solubility and stability of free fatty acids in seawater for which few data exist (Brash, 2006). At the levels seen in this study free fatty acids would be expected to be completely soluble (Sun *et al*., 2003) but as unsaturated free fatty acids are relatively unstable the half-life in seawater requires evaluation. The calculated model assumed that the fatty acids remained unaffected by other molecules that may be released during cellular disruption though this will not be the case as fatty acids are known to bind proteins (Galbraith and Miller, 1973b; Lacey and Lord, 1981), which will render the fatty acids antibacterially inactive. The model assumes that the fatty acids are concentrated evenly throughout the calculated zone and that this occurs at an instant in time. To enable further refinement, the rapidity of release needs to be studied. However, the model excludes the potential additional antibacterial effects of other fatty acids. Finally, the model was calculated for the fatty acids in the extract which represented ~0.53 % of the total fatty acids though Jüttner (2001) estimated that ~30 % of total

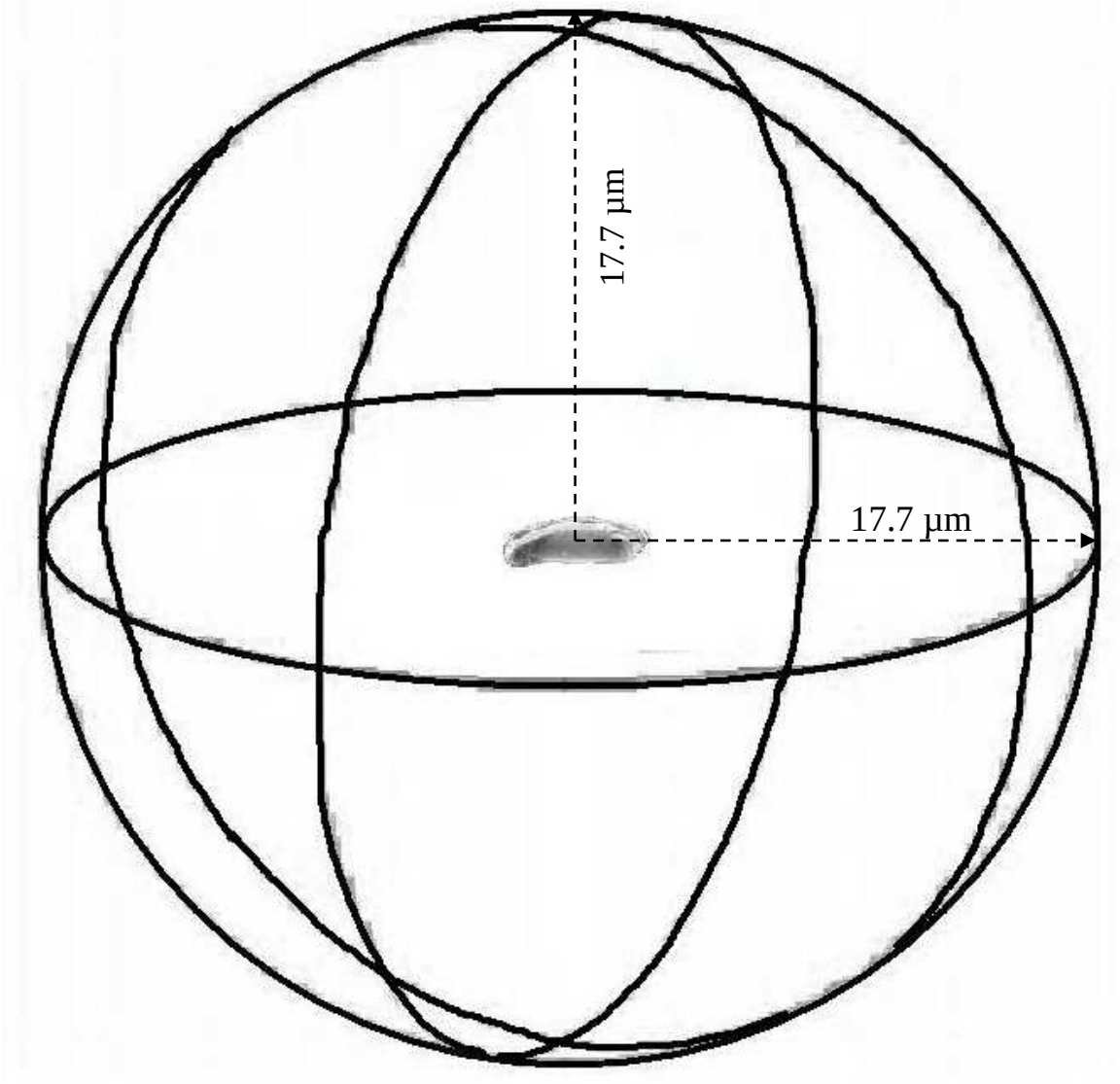


Figure 6.9 – Predicted zone of antibacterial activity around an oval *P. tricornutum* cell if the isolated antibacterial fatty acids found in aqueous methanol cell extracts from a day 4 culture were released in an instant upon cell disintegration. It assumes that the fatty acids disperse equally in all directions and each fatty acid is found at equal concentration throughout this area. The zone has been calculated from the IC_{50} value of the fatty acids against *S. aureus*. The diagram is to scale assuming that the *P. tricornutum* cell is 5 µm in length.

fatty acids may be freed by lipases, which suggests that the proposed model is conservative.

In conclusion, it appears from potency data that 16:1n7 and 20:5n3 are responsible for most of the antibacterial activity of aqueous methanol cell extracts of *P. tricornutum* though 16:3n4 does make a contribution. The differing levels of the three antibacterial fatty acids in the cell extracts appears to explain the changing level of antibacterial activity throughout the culture of *P. tricornutum*. Further, the greater level of antibacterial activity on a per cell basis seen in cell extracts from fusiform-enriched cultures compared to oval-enriched culture appears to be explained by the higher level of the antibacterial fatty acids in extracts from fusiform-enriched cultures.

Chapter 7: General discussion

Three antibacterial long-chain unsaturated fatty acids, 16:1n7, 16:3n4 and 20:5n3, were isolated from aqueous methanol cell extracts prepared from axenic cultures of the marine diatom, *Phaeodactylum tricornutum*. These fatty acids are active against *S. aureus* at micromolar (µM) concentrations and are released when the diatom cell loses its integrity. Changes in the levels of these fatty acids explain the reduction in antibacterial activity of cell extracts on a per cell basis during *P. tricornutum* culture and the greater antibacterial activity in extracts prepared from fusiform- compared to oval-enriched cultures.

The fatty acids, 16:1n7, 16:3n4 and 20:5n3, were isolated from aqueous methanol cell extracts in Chapters 4 and 5. The cumulative concentration of these three fatty acids was calculated to reach a maximum of 450 µM in cell extracts prepared from the time series cultures (Section 3.2.2) harvested at day 10 when antibacterial activity was greatest (Figure 3.2). When one considers that the IC_{50} values of the three fatty acids ranged from 10 to 40 µM (Figure 6.1) it is possible that these fatty acids are responsible for at least most of the antibacterial activity of the *P. tricornutum* cell extracts prepared in the present study. To quantitatively assess the contribution of the three fatty acids to the antibacterial activity of cell extracts a sample containing the same concentration of the three fatty acids as a crude extract will have to be prepared and compared to the activity of the crude extract. Before this can be performed however, the level of free fatty acids, as a fraction of the total fatty acids in the cell extract, will have to be determined because it is only the free fatty acids that have appreciable antibacterial activity (Dye and Kapral, 1981; Willie and Kydonieus, 2003). It is likely that the same three fatty acids isolated in the present study are

responsible for the activity of *P. tricornutum* cell extracts reported elsewhere (Table 1.4). Free fatty acids may explain the high occurrence of antibacterial activity reported in diatom species, especially as organic solvent extractions often contain the greatest antibacterial activity (Duff *et al*., 1966; Aubert *et al*., 1979; Aaronson and Dubinsky, 1982; Viso *et al*., 1987; Kellam and Walker, 1989; Pesando, 1990; Lincoln *et al*., 1990). If free fatty acids are indeed confirmed to be responsible for much of the activity of diatom cell extracts, the potential of diatoms as a natural source of novel antibacterial and other bioactive compounds may be limited. To reduce the chances for isolating free fatty acids in antibacterial or other bioassays when screening diatoms for bioactive compounds in future, lipase inhibitors could be added when preparing cell extracts.

For each of the three fatty acids isolated from aqueous methanol cell extracts prepared from axenic *P. tricornutum* cultures in Chapters 4 and 5, the yields were calculated for 16:1n7, 16:3n4 and 20:5n3 to be 0.026, 0.0086 and 0.2 mg L^{-1}, respectively. These yields are very low when compared to other studies (Yongmanitchai and Ward, 1991; Otero *et al*., 1997; Molina Grima *et al*., 1999a) which probably reflect the use of an extraction protocol that was not optimised for lipids. In the present study, the greater yield of 20:5n3 compared to 16:1n7 and 16:3n4 is probably due to the different separation methods used and, maybe more importantly, 16:1n7 and 16:3n4 separated into multiple fractions (Section 5.3.4).

The fatty acids isolated in the present study are some of the major fatty acid components of *P. tricornutum* lipids (Orcutt and Patterson, 1975; Moreno *et al*., 1979; Siron *et al*., 1989; Yongmanitchai and Ward, 1992; Molina Grima *et al*., 1999b; Patil

et al., 2007). The results here confirm that the fatty acids are released by enzyme action as reported for other diatoms (Budge and Parrish, 1999; Jüttner, 2001; Pohnert, 2002). Other studies have found that the free fatty acids are cleaved from the polar lipid species that constitute cellular membranes when the diatom cell disintegrates (Parrish and Wangersky, 1987; Budge and Parrish, 1999; Cutignano *et al*., 2006), but these fatty acids are not cleaved from triacylglycerides (TAG) (Budge and Parrish, 1999; Pohnert, 2002; Cutignano *et al*., 2006). Whether the free fatty acids isolated in the present study are released from the polar lipid species requires confirmation but it seems likely, especially as Yongmanitchai and Ward (1992) reported that up to 90 % of total 20:5n3 of *P. tricornutum* was found in the polar lipid fraction, with 16:1n7 and 16:3n4 also identified as dominant components of the polar lipids (Yongmanitchai and Ward, 1993). Further, other workers have determined that during exponential phase fixed carbon is directed into growth and cell division and the formation of the polar lipids such as membrane glycolipids (for example, MGDG) and phospholipids (Bergé and Barnathan, 2005). However, towards the end of the growth curve (stationary phase) fixed carbon is directed towards triacylglycerides (TAG) for long-term energy storage perhaps due to nitrogen limitation (Parrish and Wangersky, 1987; Bergé and Barnathan, 2005). This gradual switch from the production of polar lipid species to TAG may explain the reduction in antibacterial activity (on a per cell basis) during progression through the *P. tricornutum* growth curve (Figure 3.3) as the pool of polar lipids available to lipases, on cellular disintegration, reduces. In Chapter 3 the antibacterial activity attributable to a fusiform cell was found to be greater than that of an oval cell and this was shown in Chapter 6 to be due to the higher levels of the three isolated fatty acids in the extracts from fusiform cells. It is proposed that this might be because the fusiform cells

contain greater total quantities of lipid or are more fragile due to their cellular structure and could therefore be extracted more efficiently. But, it could be that oval cells contain higher amounts of TAG in their total lipids and, as TAG is not acted upon by lipases to produce antibacterial fatty acids, there would be fewer quantities of these fatty acids in extracts. The amounts of each class of lipid (MGDG, TAG, etc.) could be determined for oval and fusiform cells to qualify this suggestion.

The production of free fatty acids upon wounding or cellular disintegration is, metabolically speaking, an exceptionally energy-efficient and cost-effective system of defence. The fatty acids are released from lipids (that are essential cell components and are probably located in membranes) by lipases and, although the lipase(s) responsible have yet to be fully identified (Cutignano *et al*., 2006), they are enzymes of primary metabolism that probably function differently in healthy cells. Such an activated defence pathway has well documented benefits such as low self-toxicity (Wolfe, 2000; Pohnert, 2004). Furthermore, the metabolic cost of such a system can be considered minimal or nil and require very low or no maintenance, as the fatty acids are released from cell components that have to be maintained regardless and the defence is only triggered in dying cells (Pohnert, 2005). The benefit of this defence to an individual cell is zero because the cell has to be destroyed for the defence pathway to be triggered. Intuitively therefore, it would seem unlikely that this trait would be selected. However, it is conceivable that such a system could be selected for on the basis that it could increase the fitness of closely genetically-related organisms i.e., kin selection. This is plausible, as diatoms generally divide asexually and, as such, cells close together spatially will therefore be clones (Wolfe, 2000). However, recent evidence has questioned the clonal nature of diatom blooms and, in fact, diatom

blooms exhibit large intraspecific variation (Rynearson and Armbrust, 2005). Even so, this defence could be selected for at the population level due to the negligible costs in possessing and maintaining this defence pathway. Such a metabolically inexpensive pathway means most cellular resources are allocated to growth and cell division and the rapid completion of the lifecycle (Pohnert, 2000; Jüttner, 2001; Pohnert, 2005). Fatty acid release is an efficient activated defence that may be considered alongside the DMSP and oxylipin pathways.

Protection, rather than resource competition, is thought to drive microalgal evolution (Smetacek, 2001) with predation (i.e., grazing) considered to be a major selection pressure (Hay, 1996; Smetacek, 2001; Shurin *et al*., 2006). Hence most microalgal defence systems are evaluated for their defensive protection against grazer attack. However, as suggested in Section 1.4 it is entirely reasonable that a defence system will act against multiple threats (Wolfe, 2000). Whilst grazers may be the major threat to microalgae, the importance of pathogens should not be overlooked (Mitchell, 1971; Brussaard, 2004) though only a few studies have identified microbial pathogens of microalgae. These include viruses (Brussaard, 2004; Nagasaki *et al*., 2005) and bacteria (Stewart and Brown, 1969; Baker and Herson, 1978; Coder and Starr, 1978; Cole, 1982; Imai *et al*., 1993; Peterson *et al*., 1993; Mayali and Doucette, 2002; Mayali and Azam, 2004; Jeong *et al*., 2005; Kim *et al*., 2007). The release of antibacterial fatty acids from dead and dying *P. tricornutum* cells may function to defend the diatom population against attack by bacterial pathogens. They may have broader significance because fatty acids have remarkably broad biological activities at very low concentration including toxicity to bacteria, viruses (Thormar *et al*., 1987; Hilmarsson *et al*., 2006), fungi (Bergsson *et al*., 2001b), algae (McGrattan *et al*.,

1976; Suzuki *et al*., 1996; Ikawa *et al*., 1997; Wu *et al*., 2006), protozoans (Rohrer *et al*., 1986; Dohme *et al*., 2001), red blood cells (Yasumoto *et al*., 1990; Arzul *et al*., 1995; Fu *et al*., 2004), fish (Marshall *et al*., 2003), an anostracan grazer (Jüttner, 2001), brine shrimp, abalone and mosquito larvae (Curtis *et al*., 1974; Jensen *et al*., 1990; Harada *et al*., 2000; Caldwell *et al*., 2003), *Daphnia magna* (Reinikainen *et al*., 2001) and can inhibit the development of fertilised echinoderm and sea urchin eggs (Murakami *et al*., 1989; Sellem *et al*., 2000). Further, free fatty acids can inhibit photosynthesis (Peters and Chin, 2003). Often these fatty acids are highly active at µM concentrations and 20:5n3, one of the fatty acids isolated in the present study, has been shown to exhibit some of these activities at very low concentration (Table 7.1). Moreover, at 20 µM, 16:1n7 can inhibit photosynthesis within seconds (Peters and Chin, 2003). Thus free fatty acids may provide a comprehensive and multifunctional defence against numerous microbial pathogens, such as bacteria and viruses, and predators such as copepods or protozoan flagellates.

Many further questions need to be addressed if the significance the proposed fatty acid defence cascade, triggered on cell disintegration, is to be fully evaluated. There have been relatively few reports of bacterial pathogens of microalgae and more attention is needed in this area to identify a suitable diatom-bacterial pathogen interaction for assessing the relevance of the proposed fatty acid release mechanism as a defence against bacteria and other pathogens. It is curious that long-chain fatty acids, such as those isolated in the present study, show greater antibacterial activity under weak alkaline conditions (pH 8) compared to weakly acidic conditions (pH 6) (Galbraith and Miller, 1973a). The importance of the free fatty acids functioning as conventional allelochemicals is worthy of further consideration. The possible

Table 7.1 – Reported biological activities for µM concentrations of 20:5n3 showing that this compound is active in various assays.

Reported activity	Specific example	Active concentration	Reference
Anti-algal	Total growth inhibition of conchospores of red microalga, *Heterosigma akashiwo*	6.6 µM	Kakisawa *et al*. (1988)
	Growth inhibition of green eukaryotic microalga, *Chlorella vulgaris*	IC_{50} 410 µM	Wu *et al*. (2006)
	Growth inhibition of green eukaryotic microalga, *Monoraphidium contortum*	IC_{50} 330 µM	Wu *et al*. (2006)
	Total growth inhibition of green macroalga, *Monostroma oxyspermum*	3.3 µM	Suzuki *et al*. (1996)
	Growth inhibition of diatom, *Chaetoceros gracile*	IC_{99} 7 µM[a]	Arzul *et al*. (1995)
Anti-grazer	Toxic to anostracan grazer *Thamnocephalus platyurus*	IC_{50} 34 µM	Jüttner (2001)
	Toxic to nauplii of brine shrimp, *Artemia salina*	33 µM[b]	Caldwell *et al*. (2003)
Anti-proliferative	Inhibits sea urchin egg cleavage	IC_{50} 0.34 µM	Sellem *et al*. (2000)
	Reduces proliferation of human HL-60 cells	30 µM	Finstad *et al*. (1994)
Haemolytic	Total haemolysis of sheep red blood cells	40 µM	Arzul *et al*. (1995)
	Haemolysis of human erythrocytes	33 µM	Fu *et al*. (2004)
Ichthyotoxic	Toxic to damselfish, *Acanthochromis marina*	IC_{50} 8.9 µM	Marshall *et al*. (2003)
Settlement and metamorphosis cue	Induces settlement and metamorphosis of tube worm *Phragmatopoma californica*	166 µM	Pawlik (1986)

[a] Dosage required to inhibit growth by 99 %.

[b] Survival of nauplii significantly lower compared to control.

involvement of free fatty acids as defence against grazers warrants much deeper investigation, especially due to the importance of this selection pressure on diatom population size and evolution (Hay, 1996; Selph *et al*., 2001; Smetacek, 2001). Whilst 20:5n3 is toxic to grazers (Jüttner, 2001; Caldwell *et al*., 2003) the toxicity of 16:1n7 and 16:3n4 to grazers needs to be assessed. The toxicity of all three isolated fatty acids needs to be determined when given internally, perhaps by the use of liposome technology, as most studies for toxicity of free fatty acids have the fatty acids at the desired concentration in the medium and this may be unrealistic (Jüttner, 2001; Caldwell *et al*., 2004). Moreover, aside from the direct toxic effects, the possible effects on the grazer's archaeal and bacterial flora should be investigated. Grazers are known to employ different feeding methods (Selph *et al*., 2001; Strom *et al*., 2003), so pinpointing the location of free fatty acid release must be considered in terms of the possibility that the fatty acids can reach toxic concentrations. Finally, free fatty acids are known to act as signalling molecules or controllers of metabolic processes (Pawlik, 1986; Jensen *et al*., 1990; Finstad *et al*., 1994; Khan *et al*., 1995; Itoh *et al*., 2003) and a role for such compounds has been suggested for microalgae (Ikawa, 2004). Nguyen and Thompson (2006) propose that free fatty acids signal a 'hostile environment' in many organisms. Thus it is possible that free fatty acids could act to warn algal populations of an increased threat of grazing rather like the DMS in the DMSP pathway may function (Pohnert *et al*., 2007) and, of course, this requires further study.

The antibacterial activity of the free fatty acids, as shown by disc diffusion and liquid assays (Sections 5.3.2, 6.3.1 and 6.3.2), does not absolutely confirm that these compounds are responsible for the activity. For example, Shin *et al*. (2007) found

that the antibacterial activity against numerous bacteria of a solution of 20:5n3 increased greatly when the fatty acid was bioconverted into unspecified products. Furthermore, Pesando (1972) attributed the antibacterial activity found in an antibacterial fraction containing 20:5n3 to photo-oxidation products formed during exposure to the light. To guard against this, a disc diffusion assay could be performed whereby the 20:5n3 is recovered from the paper disc at completion of the assay.

In Chapter 6, two of the fatty acids isolated in the present study, 16:1n7 and 20:5n3, were shown to be antibacterial against the human pathogen, MRSA. This is the first report that 16:1n7 has the ability to inhibit the growth of this bacterium and this finding means that the molecule requires evaluation as a possible medicine. Potential drug compounds must meet certain chemical criteria (Table 7.2) but, whilst the fatty acids do fulfil most of the criteria and bacterial resistance to the action of free fatty acids seems not to arise (Laser, 1952; Lacey and Lord, 1981; Petschow *et al*., 1996; Sun *et al*., 2003), it remains very doubtful that free fatty acids could ever be administered as systemic drugs. This is largely due to their instability, the fact that they bind to serum proteins (Galbraith and Miller, 1973b; Kanai and Kondo, 1979; Lacey and Lord, 1981) and have low specificity of activity (Khan *et al*., 1995; Table 7.1). It is noteworthy that ampicillin is approximately 100 times more potent than the fatty acids with respect to the IC_{50} but was found, in the present study, to have a comparatively high MBC for an ampicillin-susceptible *S. aureus* strain. This reflects the dangerous but remarkable ability of *S. aureus* to spontaneously mutate into an antibiotic-resistant phenotype.

Table 7.2 – Desirable chemical properties of candidate drug compounds. These characteristics are known as the 'Rule of 5' (Battershill *et al*., 2005).

Chemical property
Molecular mass should be < 500 Da.
Total number of N and O atoms should be < 10.
There should be < 5 hydrogen bond donors.
Lipophilicity should be logP < 5.

In summary, the present study is the first to isolate the compounds responsible for the antibacterial activity of *P. tricornutum* cell extracts. These were determined to be three free fatty acids, 16:1n7, 16:3n4 and 20:5n3 and of these, 16:1n7 has never previously been isolated as an antibacterial compound from a microalga. This study is the first to suggest that the release of free fatty acids by diatoms is a simple but elegant, very low cost population-level activated defence mechanism against potential pathogenic bacteria. The pathway may act against multiple threats to the microalga, including grazers, as fatty acids exhibit an extraordinary array of biological activities. The fatty acid defence pathway is triggered on cell death much like the DMSP and oxylipin pathways and, though very similar, it should be considered in its own right and as discreet from the oxylipin pathway when evaluating the importance of microalgal defence systems.

Appendix I: Fatty acid nomenclature

Fatty acids consist of a carbon chain of variable length with a terminal carboxyl group at one end and a terminal methyl group at the other end. Often, in biological systems the carbon chain is an even number of carbons (e.g., 16). Within this carbon chain, a number of the bonds can be double bonds.

For full chemical names, the positions of the double bonds are given from the carboxyl end of the fatty acid chain, for example (6*Z*, 9*Z*, 12*Z*)-hexadecatrienoic acid has double bonds in the fatty acid chain at positions 6, 9 and 12 from the terminal carboxyl end. When the double bonds are in *cis*-orientation they are designated '*Z*' and this is the case for most natural molecules (double bonds in *trans*-orientation are designated '*E*').

The shorthand notation used in this thesis would give the fatty acid, (6*Z*, 9*Z*, 12*Z*)-hexadecatrienoic acid, as 16:3n4. In this notation the first numeral, in this case 16, gives the number of carbon atoms in the fatty acid chain. The second numeral, in this case 3, gives number of double bonds in this fatty acid chain. The final numeral (that after the 'n') gives the position of the first double bond from the terminal methyl end.

Appendix II: Supplier addresses

Acros Organics, Geel, Belgium
Aquapharm Bio-Discovery Ltd., Dunstaffnage, Argyll, UK
Astell Scientific Ltd., Sidcup, Kent, UK
Beckman Coulter Ltd., High Wycombe, Buckingham, UK
Biochrom Ltd., Cambridge, Cambridgeshire, UK
Bruker BioSpin GmbH, Rheinstetten, Germany
Christ GmbH, Osterode, Germany
Corning Inc., Corning, New York, USA
Corning Ltd., Hemel Hempstead, Hertfordshire, UK
Denley Instruments Ltd., Billingshurst, West Sussex, UK
Difco, West Moseley, Surrey, UK
Dynex Technologies Ltd., Worthing, West Sussex, UK
Elga, High Wycombe, Buckinghamshire, UK
Emscope Laboratories, Ashford, Kent, UK
Fisher Scientific, Loughborough, Leicestershire, UK
Gelman Sciences Ltd., Northampton, Northamptonshire, UK
Greiner Bio-One, Kremsmünster, Austria
Hanna Instruments, Leighton Buzzard, Bedfordshire, UK
Heto-Holten A/S, Allerød, Denmark
Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire, UK
JEOL Ltd., Welwyn Garden City, Hertfordshire, UK
Leitz Wetzlar, Germany
LI-COR Biosciences UK Ltd., Cambridge, Cambridgeshire, UK
Lightbulbs Direct Ltd., Amersham, Buckinghamshire, UK
LKB Biochrom, Cambridge, Cambridgeshire, UK
Microflow Ltd., Andover, Hampshire, UK
Millipore, Billerica, MA, USA
Nalgene, Rochester, NY, USA
NCIMB Ltd., Aberdeen, Aberdeenshire, UK
Pierce, Rockford, Illinois, USA
Phenomenex, Macclesfield, Cheshire, UK
Philips, Guildford, Surrey, UK

Philip Harris Scientific, Lichfield, Staffordshire, UK

Qiagen Ltd., Crawley, West Sussex, UK

RS Components Ltd., Corby, Northamptonshire, UK

Sartorius AG, Göttingen, Germany

Sigma Aldrich Ltd., Poole, Dorset, UK

Teledyne Isco Inc., Lincoln, NE, USA

Thermo Fisher Scientific Inc., Waltham, MA, USA

ThermoQuest Ltd., Hemel Hempstead, Hertfordshire, UK

Tousimis Research Corporation, Rockville, Maryland, USA

Vacuubrand, GmbH, Wertheim, Germany

VWR International, Lutterworth, Leicestershire, UK

Waters Corporation, Milford, MA, USA

Waters Ltd., Elstree, Hertforshire, UK

Weber Scientific International Ltd., Teddington, Middlesex, UK

Whatman International Ltd., Maidstone, Kent, UK

Appendix III: Culture media and agar

a) Modified enriched seawater, artificial water (ESAW) medium (modified from Harrison *et al*., 1980)

Constituent	Mass in stock solution (g)
AW I stock solution (made up to 1 L with deionised water)	
NaCl	207.58
Na_2SO_4	34.77
KCl	5.87
$NaHCO_3$	1.70
KBr	0.845
H_3BO_3	0.225
NaF	0.027
AW II stock solution (make up to 1 L with deionised water)	
$MgCl_2.6H_2O$	93.95
$CaCl_2.2H_2O$	13.16
$SrCl_2.6H_2O$	0.214
Nutrient and trace metal stock solution (make up to 1 L with deionised water)	
$NaNO_3$	4.667
$Na_2SiO_3.9H_2O$	3.000
Na_2glyceroPO$_4$	0.667
Na_2EDTA	0.553
H_3BO_3	0.380
$Fe(NH_4)_2(SO_4)_2.6H_2O$	0.234
$FeCl_3$	0.016
$MnSO_4.H_2O$	0.054
$ZnSO_4.7H_2O$	0.0073
$CoSO_4.7H_2O$	0.0016

Each stock solution was made separately and sterilised by autoclaving at 121 ºC for 15 min. To give 1 L final modified ESAW medium, to 890 mL sterile deionised water was added 50 mL AWI stock solution, 50 mL AWII stock solution and 10 mL nutrient and trace metal stock solution. Stock solutions were stored at 4 ºC. This

modified ESAW recipe contains half-strength AWI and AWII but full-strength nutrient and trace metal solution in the final formulation (see Appendices IV and V).

b) Miquel seawater medium (Allen and Nelson, 1910)

Constituent	Mass in stock solution (g)
Solution A (make up to 100 mL with deionised water)	
KNO_3	20.0
Solution B	
$Na_2HPO_4.2H_2O$	2.0
$CaCl_2.2H_2O$	2.7
HCl (concentrated)	2 mL
$FeCl_3$	2.0
Distilled water	80 mL

Solutions A and B were sterilised by autoclaving at 121 ºC for 15 min. For each litre of filtered seawater (Section 4.2.0) in the culture vessel, 0.25 mL 4 % sodium hypochlorite was added for sterilisation. The seawater was aerated for 30 min then left in the dark for 12 h. The residual chlorine was deactivated with 1 mL 12 g L-1 sodium thiosulphate solution for each litre of seawater. To give final Miquel seawater medium, to each litre of sterile seawater was added 2 mL solution A and 1 mL solution B.

c) Modified Bold's basal medium for heterotrophs (TOM) (EPSAG, 2007b)

Constituent	Mass in stock solution (g)
Base solution (make up to 800 mL with deionised water, pH to 5.5 (with HCl)	
HEPES	0.715
Glucose	15.0
Bacteriological peptone	20.0
Stock solution (make up to 100 mL with deionised water)	
NaCl	0.25
$CaCl_2.2H_20$	0.25
KNO_3	5.00
$MgSO_4.7H_2O$	0.65
$(NH_4)_2HPO_4$	2.50
Trace element solution I (make up to 100 mL with deionised water)	
KOH	3.0
EDTA	5.0
Trace element solution II (make up to 100 mL with deionised water)	
$FeSO_4.7H_2O$	0.5
Trace element solution III (make up to 100 mL with deionised water)	
H_3BO_3	1.14
Trace element solution IV (make up to 100 mL with deionised water, soluting all by using H_2SO_4)	
$ZnSO_4.7H_2O$	0.882
$MnCl_2.4H_2O$	0.144
$NaMoO_4.2H_2O$	0.072
$CuSO_4.5H_2O$	0.158
$Co(NO_3)_2.6H_2O$	0.050
Vitamin stock solution (initially make up to 50 mL with deionised water but to make final vitamin stock solution take 0.1 mL and make up to 100 mL)	
Vitamin B_{12}	0.01
Biotine	0.05
Thiamine	5.0
Nicotinamide	0.005

All solutions were sterilised by autoclaving at 121 ºC for 15 min (except the vitamin stock solution that must be sterilised by filtration as Section 2.2.1).

To the base solution was added 10ml of stock solution, 1ml of each trace element solution (I, II, III and IV) and 1ml of the final (diluted) vitamin stock solution. The volume is made up to 1 L with sterile deionised water.

d) SWEg medium (EPSAG, 2007b)

Constituent	Mass in stock solution (g)
Soil extract	
A 6 L flask was filled one third with soil. Deionised water was added until the water level was 5 cm above the soil. This was sterilised by autoclaving for 1 h at 121 ºC and autoclaving repeated after 24 h. The extract was taken off and centrifuged at 3580 *g* for 11 min. The supernatant was removed and autoclaved at 121 ºC for 15 min to give final soil extract.	
Micronutrient solution I (make up to 10 mL with deionised water)	
$ZnSO_4.7H_2O$	0.01
$MnSO_4.H_2O$	0.02
H_3BO_3	0.1
$Co(NO_3)_2.6H_2O$	0.01
$Na_2MoO_4.2H_2O$	0.01
Micronutrient solution II (make up to 1 L with deionised water)	
$CuSO_4.5H_2O$	0.005
Micronutrient solution III (make up to 100 mL with deionised water)	
$FeSO_4.7H_2O$	0.7
EDTA	0.4
Base solution I	
KNO_3	0.05
K_2HPO_4	0.005
$MgSO_4.7H_2O$	0.005
Filtered seawater	905 mL
Distilled water	60 mL
Base solution II	
Na-acetate	1
Lablemco	1
Tryptone	2
Yeast Extract	2
Seawater	910 mL
Deionised Water	60 mL

First, to 898 mL distilled water add 1 mL micronutrient solution I, 1 mL micronutrient solution II and 0.4 g EDTA and sterilise by autoclaving at 121 ºC for 15 min.

Autoclave micronutrient solution III at 121 ºC for 15 min and combine both solutions

aseptically to give 1 L final micronutrient solution. Base solution I was autoclaved at 121 ºC for 15 min then aseptically 30 mL sterile soil extract and 5 mL final micronutrient solution were added to give final base solution I. Base solution II was autoclaved at 121 ºC for 15 min then aseptically 30 mL soil extract was added to give final base solution II. To give 1 L final SWEg medium, 500 mL final base solution I was added to 500 mL final base solution II.

e) Radial diffusion assay agars (modified from Lehrer *et al*., 1991)

2216E agars (for marine bacteria)

For the 'bottom' agar, to a shakeflask was added 0.56 g 2216E medium and this was made up to 150 mL with deionised water. This was dissolved by boiling on a hot plate for 2 min. Once cool, 15 mL aliquots were dispensed to universal bottles containing 0.09 g agar powder (VWR). For the 'top' agar the process was repeated but 5.61 g 2216E medium was used. All agars were sterilised by autoclaving at 121ºC for 15 min.

LB agars (for terrestrial bacteria)

For the 'bottom' agar, to a shakeflask was added 0.3 g LB medium and this was made up to 150 mL with deionised water. This was dissolved and 15 mL aliquots were dispensed to universal bottles containing 0.09 g agar powder (VWR). For the 'top' agar the process was repeated but 3 g LB medium was used. All agars were sterilised by autoclaving at 121 ºC for 15 min.

f) PDY agar (Dr. Liming Yan, Aquapharm Bio-Discovery Ltd, pers. comm.)

Constituent	Mass in stock solution (g)
Made up to 1 L with deionised water and sterilised by autoclaving at 121ºC for 15 min	
Potato dextrose agar	39
Yeast extract	2

Appendix IV: ESAW preparation protocol correction

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A CLARIFIED PROTOCOL FOR THE PREPARATION OF ENRICHED SEAWATER ARTIFICIAL WATER (ESAW) MEDIUM USED FOR CULTURE OF MARINE MICROALGAE

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Summary

This short article points out a possible confusion about the preparation of the commonly used algal growth medium, enriched seawater artificial water (ESAW), devised by Harrison *et al*. (1980). It is possible to misinterpret the ESAW preparation protocol so that the medium is made approximately half-strength. We highlight the potential for misinterpretation and provide a clarified protocol for the preparation of this medium. Whilst growth may still be achieved with alternative formulations of ESAW, physiological activities or bioproducts may be affected.

Keywords: artificial seawater, culture medium, micro-algae, phytoplankton.

The selection of a suitable growth medium is extremely important for most physiological studies of microalgae. In some cases a medium made with natural seawater supplemented with additional nutrients suffices, with the f and f/2 compositions of Guillard and Ryther (1962) and Guillard (1975) being the most highly cited (Berges *et al*., 2001). However, as natural seawater tends to vary in quality, an entirely artificial chemically defined medium is often more desirable. One that is widely used is enriched seawater artificial water (ESAW) devised by Harrison *et al*. (1980). ESAW has salt values similar to natural seawater, facilitates the growth of phylogenetically diverse microalgal species (Harrison *et al*., 1980) and is commonly considered an excellent growth medium. Certainly, Harrison *et al*. (1980) is very highly cited with a total 341 citations to date, including 62 since 2003 (ISI Web of Knowledge, 2007).

Whilst routinely culturing marine diatoms in ESAW we have realised that it is possible to misinterpret the ESAW preparation protocol in Harrison *et al*. (1980). This could result in an experimenter unwittingly making a final composition of approximately half-strength. Subsequent publications by Harrison (Berges *et al*., 2001; 2004) describe modifications to the ESAW formula but make no changes to the portion of the protocol that could be misinterpreted. The uncertainty in ESAW preparation may at first seem trivial but this could have misled some workers. Moreover, incorrect instructions for ESAW are available on reputable websites.

The point of debate in Harrison *et al*. (1980) concerns the preparation of the artificial water base (AW) that is prepared by mixing equal proportions of 2 salt stock solutions, AWI and AWII. The complete AW is then supplemented with vitamins, nutrients and trace metals from stock of enrichment solutions (ES) to give final ESAW. In Table 2 of Harrison *et al*. (1980) each of the constituents for AWI and AWII are listed at their required concentrations. However, it is not made clear whether these values are the final concentration of each ingredient after the AW stocks have been combined in a ratio of 1:1 or the concentrations required for the initial AWI and AWII stock solutions. If it is assumed that they are the latter then the complete AW will contain the salts at half their intended strength (JA Berges, pers. comm.). Whilst the salinity of complete AW is given in the legend for Table 2 of Harrison *et al*. (1980), it is easy to overlook this. The protocol would have been clearer if the authors had indicated either that the gramme weight values for each ingredient in AWI and AWII are the final concentrations in complete AW or that these values should be doubled for the initial AWI and AWII stock solutions. The g L^{-1} amounts for the salts that should be used to make the initial AWI and AWII stock

solutions and a clarified protocol for the preparation of ESAW medium is given in Table 1.

If ESAW is made up using half-strength AW the resulting reduced salinity may affect microalgal growth, physiology and metabolites (Rowland *et al*., 2001; Lim and Ogata, 2005; Ranga Rao *et al*., 2007). The value of ESAW as an algal growth medium is undoubted and this short communication seeks to raise awareness that the AW portion of the ESAW recipe could be misinterpreted. Growth may still be achieved with alternative formulations of this medium but physiological activities or bioproducts may be altered. This may have implications in data interpretation when comparing between studies.

Acknowledgements

We thank Dr John A. Berges (University of Wisconsin), Prof Paul J. Harrison (Hong Kong University) and Prof. John Raven (University of Dundee) for helpful suggestions on this short note.

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Table 1 – Clarification of the salt concentrations required for initial ES and AW stock solutions, and the volumes of these stock solutions that are combined to produce ESAW medium of Harrison *et al*. (1980).

Each stock solution is made up to 1 L with deionised water. Stock solutions are sterilised separately by autoclaving for 30 min at 121 °C except the vitamin stock solution, which is sterilised by filtration and stored at –20 °C. Stock solutions are left for 48 h to allow gaseous exchange then aseptically combined in volumes provided to give final ESAW medium (1007 mL)[a,b]. Final pH 8.2.

Constituent	Concentration in stock solution (g L^{-1})	Volume of stock solution for final ESAW (mL)
AW stock solution I		500
NaCl	42.38	
Na_2SO_4	7.1	
KCl	1.198	
$NaHCO_3$	0.348	
KBr	0.1726	
H_3BO_3	0.046	
NaF	0.0056	
AW stock solution II		500
$MgCl_2.6H_2O$	19.184	
$CaCl_2.2H_2O$	2.688	
$SrCl_2.6H_2O$	0.0436	
Major nutrient stock I		1
$NaNO_3$	46.7	
Major nutrient stock II		1
$NaH_2PO_4.H_2O$	3.09	
Major nutrient stock III		2
$Na_2SiO_3.9H_2O$	15.000	
Metals stock I		1
$FeCl_3.6H_2O$	2.44	
$Na_2EDTA.2H_2O$	3.09	
Metals stock II		1

$ZnSO_4.7H_2O$	0.073	
$CoSO_4.7H_2O$	0.016	
$MnSO_4.4H_2O$	0.54	
$Na_2MoO_4.2H_2O$	1.48×10^{-3}	
Na_2SeO_3	1.73×10^{-4}	
$NiCl_2.6H_2O$	1.49×10^{-3}	
$Na_2EDTA.2H_2O$	1.77	
Vitamin stock solution		1
Thiamine HCl	0.1	
Vitamin B_{12}	0.002	
Biotin	0.001	

[a]Enrichment solutions based on figures provided by Berges *et al*. (2001).

[b]AWI and AWII values calculated from Harrison *et al*. (1980) multiplied by specific gravity (1.021).

Appendix V: Comparison of *P. tricornutum* growth and production of antibacterial activity in ESAW or modified ESAW medium

A comparison was made between ESAW and modified ESAW media for the growth of *P. tricornutum* and level of antibacterial activity in subsequently prepared cell extracts. The small-scale batch culture system was set up and inoculated as Section 2.2.2, except that three bottles were filled with ESAW medium and three with modified ESAW (Appendices III and IV). For each culture, growth was monitored every 24 h by determination of A_{750}. After 10 days the bottles were harvested, extracted and tested for antibacterial activity against *S. aureus* (as Sections 2.2.4 and 2.2.7). The student's t-test was used to compare the level of growth attained by the cultures in the two media at day 12 and this showed that growth was significantly greater at harvest in the modified ESAW medium ($t_4 = 3.254$, $p < 0.05$) (Figure Va). The student's t-test was also used to compare the level of antibacterial activity in cell extracts prepared from cultures grown in ESAW or modified ESAW media. The t-test showed that there was no significant difference between the level of antibacterial activity in cell extracts prepared from either medium ($t_4 = 2.402$, $p > 0.05$) (Figure Vb). However, if $p < 0.10$ was to be considered significant then there would have been a difference. As a result of these findings, modified ESAW was selected as a better growth and production medium.

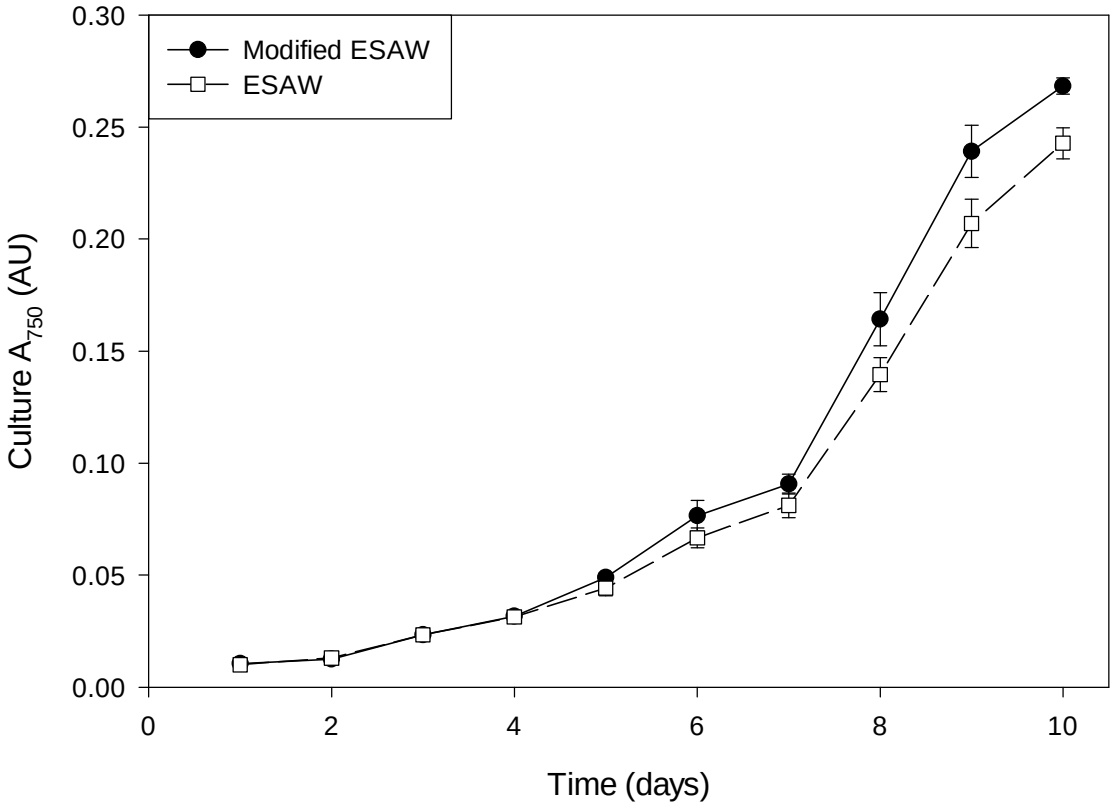


Figure Va – Growth of *P. tricornutum* in ESAW medium compared to modified ESAW medium showing that growth was significantly greater at day 12 ($p < 0.05$) in the modified ESAW medium. n = 3; error bars are ± 1 SE.

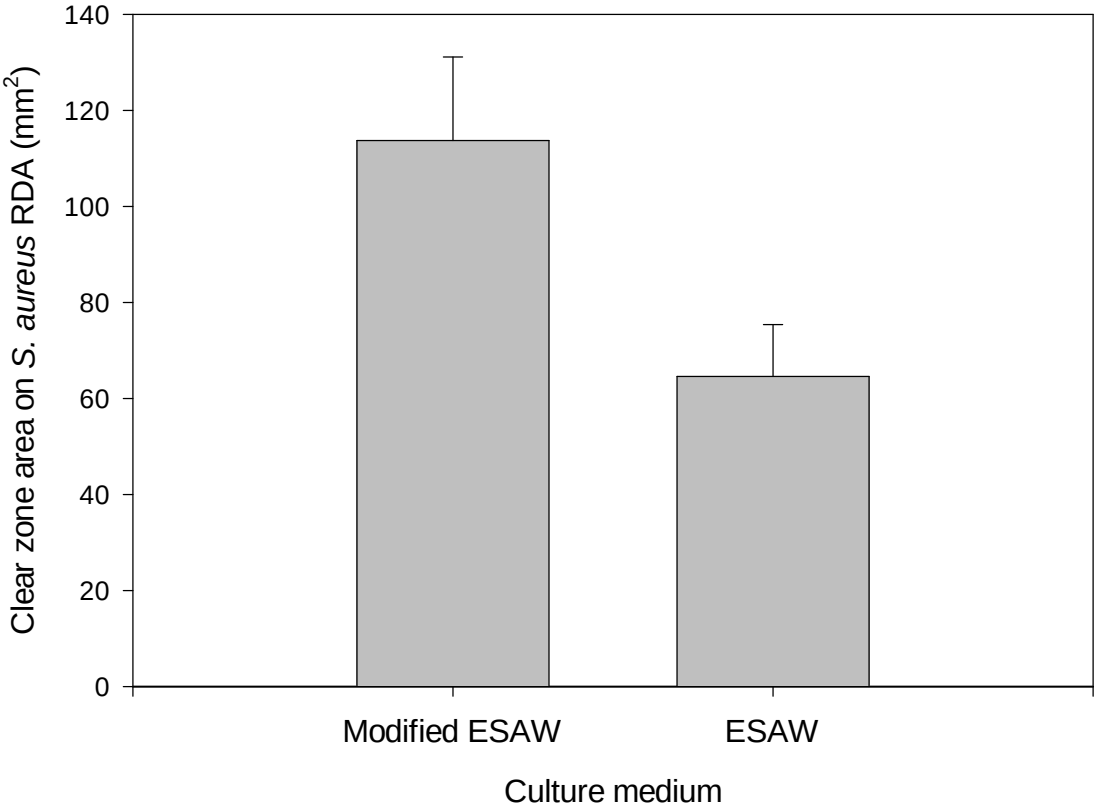


Figure Vb – Antibacterial activity for cell extracts prepared from *P. tricornutum* cultures that had been grown in either ESAW or modified ESAW media. There was no significant difference ($p > 0.05$) between the level of antibacterial activity in cell extracts prepared from cultures grown in either medium. n = 3; error bars are ± 1 SE.

Appendix VI: Axenicity of *P. tricornutum* initial stock culture

The initial *P. tricornutum* stock culture used throughout this study (Section 2.2.2.3) was checked to confirm that the growth conditions generated unialgal and axenic cultures as contaminating organisms can affect algal growth and gene expression (Cole, 1982; Bates *et al*., 1995). To this end, the culture was examined for contaminants first using a scanning electron microscope (SEM) (method based on Bates *et al*., 2004). In preparation for SEM, *P. tricornutum* was cultured as Section 2.2.2.3, except that 0.22 µm polycarbonate membrane filters (Ø 25mm Isopore; Millipore) were added to the flask pre-sterilisation. After 12 days a filter was removed and 5 mL culture was passed through. Cells attached to the polycarbonate filter were then washed by passing through the filter a further 50 mL sterile filtered seawater. The sample was fixed with 3% glutaraldehyde in filtered seawater for 1 h and dehydrated in a graded series of ethanol (20 %, 50 %, 70 %, 96 % and absolute) for 10 min at each grade (VWR). Critical point drying (Samdri 780CPD; Tousimis Research Corporation) removed all traces of ethanol. The sample was affixed to a mounting block, sputter coated with gold (SC500; Emscope Laboratories) and observed at 20 kV using the University of St Andrews SEM (JSM-35CF; JEOL Ltd.). Under SEM, oval-shaped cells in the pictures ranged from 5-7 µm in length whilst crescent-shaped (fusiform) cells were ~13 µm in length (Figure VI). There were also numerous other shaped cells that were intermediates between the two predominant cell morphs in this culture. The shapes and sizes of all cells are consistent with what would be expected for the polymorphic diatom, *P. tricornutum* (Lewin, 1958). There is no evidence of any cells that resemble other species of microalga thus confirming the unialgal status of this strain. Small rod-shaped structures 1.5 µm in length were found in some electron micrograph images and these are probably bacteria (Figure

VI). But importantly, in none of the pictures were bacteria ever found on the algal surface and as a result it is likely that these bacteria were introduced during sample preparation, as some of the procedures used for SEM have to be performed in non-sterile conditions. Very often bacteria that have symbiotic or parasitic relationships with algae are found to exist on the algal cell surface (Baker and Herson, 1978; Imai *et al*., 1993). Further, these structures could have been introduced from dead bacteria present in autoclaved reagents.

Second, axenicity of the *P. tricornutum* stock culture was investigated by culturing the diatom in heterotroph-promoting media: i) Modified Bold's Basal Medium for heterotrophs (TOM), and ii) sterile SWEg Medium (Appendix III) (Dr. M. Lorenz, Culture Collection of Algae at the University of Göttingen, pers. comm.). After 7 days culture, broths were aseptically streaked on to sterile TOM, SWEg, LB and 2216E agar plates. Plates were incubated at 25 ºC to allow colonies to form. Other than brown colonies, which were confirmed as *P. tricornutum* by light microscopy, no contaminating colonies appeared on any agar plate confirming the axenicity of the algal stock culture.

The initial *P. tricornutum* stock culture of the alga used throughout this study was confirmed to be unialgal and axenic. This is important because the bioactivity of culture extract preparations may not necessarily be due to the most abundant organism found in the culture (Scheuer, 1990; Jensen and Fenical, 1994; Borowitzka, 1995).

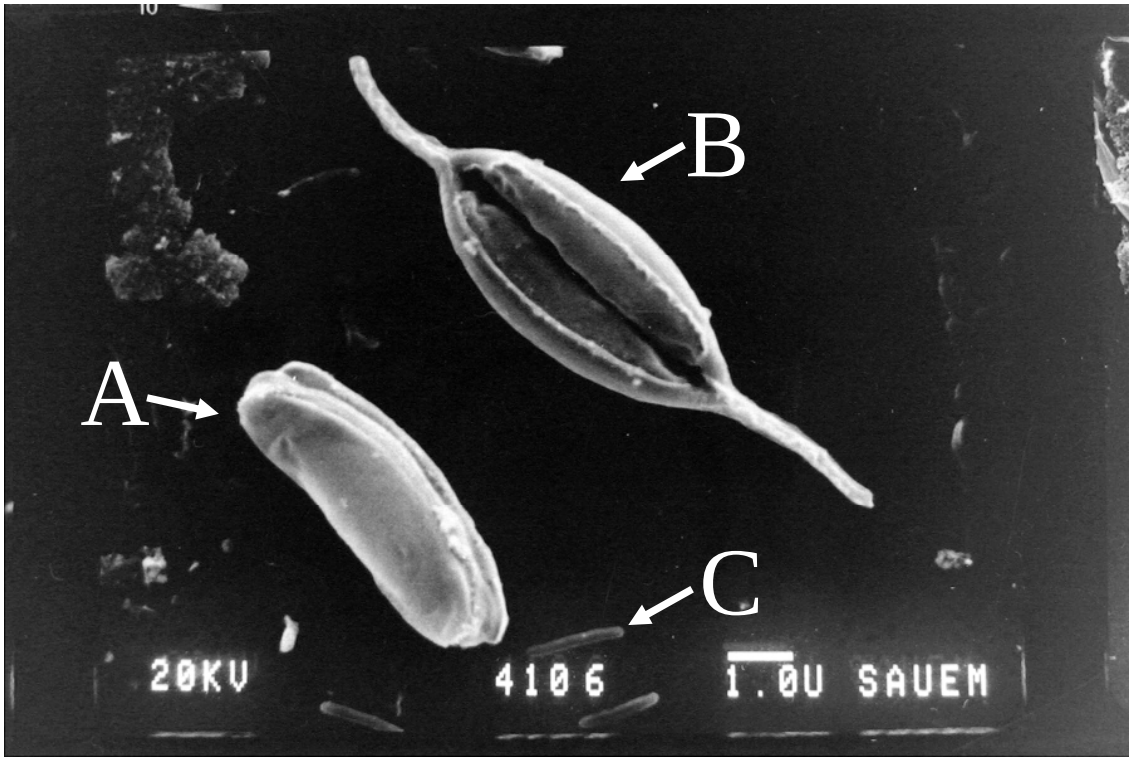


Figure VI – Scanning electron micrograph of *P. tricornutum* culture showing (A) an oval *P. trico*rnutum cell, (B) an intermediate-shaped cell, and (C) structures that may be bacteria. The scale bar is 1 µm.

Appendix VII: Small-scale culture system not suitable for production of different *P. tricornutum* morphs

The small-scale culture system was investigated to see whether it could be used to produce cultures enriched for cells in the fusiform morph. A *P. tricornutum* culture (cultured in a flask as Section 2.2.2.3) that had become enriched in fusiform cells was used to inoculate three bottles in the small-scale batch culture system (as Section 2.2.2). The proportion of fusiform cells in this inoculum was determined from quadruplicate cell counts under the microscope (as Section 2.2.3). After 10 days growth in the small-scale batch culture system, the number of cells in the fusiform morph for each culture was determined from duplicate cell counts. It was evident that the proportion of cells in the fusiform morph had reduced substantially during growth from 75 % to ~40 % (Table VI). The fusiform cells were not able to grow and divide in the culture system and this may be due to the turbulent conditions required to keep the culture well mixed. Therefore, this culture system is not suitable for the production of *P. tricornutum* cultures enriched for cells in the fusiform morph.

Table VI – Relative morphology of *P. tricornutum* cultures at inoculation and after 10 days growth in the small-scale batch culture system; n = 3.

Time	Relative morphology (%)		
	Oval	Fusiform	Other
At inoculation	19.4	75.0	5.6
After 10 days	52.3	39.1	8.6

Appendix VIII: *P. tricornutum* fusiform-enriched cultures grow faster than oval-enriched cultures

Growth of *P. tricornutum* cultures enriched for either the oval or fusiform morph was monitored to see whether or not cell morph affected growth. *P. tricornutum* was cultured in four 5 L flat-bottomed Pyrex glass jars. Each jar had one opening at the bottom and one at the top. The upper hole was filled with a non-absorbent cotton wool bung covered in tin foil. A one-hole rubber stopper (Fisher Scientific) was fixed into the lower hole. This had 8 cm of 6.35 mm (external diameter) 316L stainless steel tubing (RS Components Ltd.) passed through. Attached to the inner end was 10 cm of 5 mm bore PVC tubing (Fisher Scientific); attached to the outer end was 10 cm of 5 mm bore silicone tubing with an in-line 0.2 µm PTFE air filter (Midisart 2000; Sartorius AG). Air was supplied at 4.65 L min^{-1} per bottle. Each glass jar was filled with 4.45 L deionised water and sterilised by autoclaving. Sterile solutions were aseptically added to give sterile modified ESAW media (see Appendix III). Bottles were positioned in a lightbox composed of three white sides, white roof and lightly coloured floor and had internal dimensions of 72 cm x 57 cm x 43 cm (w x h x d). Temperature was controlled at 20 ºC. Four 24” cool white fluorescent tubes (General Electric F18W/33) provided illumination: two at the top and two at the rear. Light intensity was 115 $\mu mols^{-1}m^{-2}$ with a regime of 14:10 h light:dark. Two bottles were inoculated with oval-enriched cultures whilst two bottles were inoculated with fusiform-enriched cultures. The proportion of cells in different cell morphs were determined in these inoculums from triplicate cell counts under the microscope (as Section 2.2.3). The oval-enriched culture contained 100 % ovals whilst the fusiform-enriched culture contained 79 % fusiform cells. Inoculum volumes were kept constant with the addition of sterile modified ESAW medium. The inoculums gave

final cell concentrations of 1 x10^5 cells mL^{-1}. Growth was monitored in each culture every 24 h by measurement of culture A_{750} (as Section 2.2.3). After 8 days, the proportion of cells in each culture was checked as before. Both oval-enriched cultures contained 100 % ovals whilst the fusiform-enriched cultures contained 76 and 67 % fusiform cells. The fusiform-enriched cultures appeared to grow faster than the oval-enriched cultures (Figure VIII), which appears to confirm the findings reported in Figure 3.16.

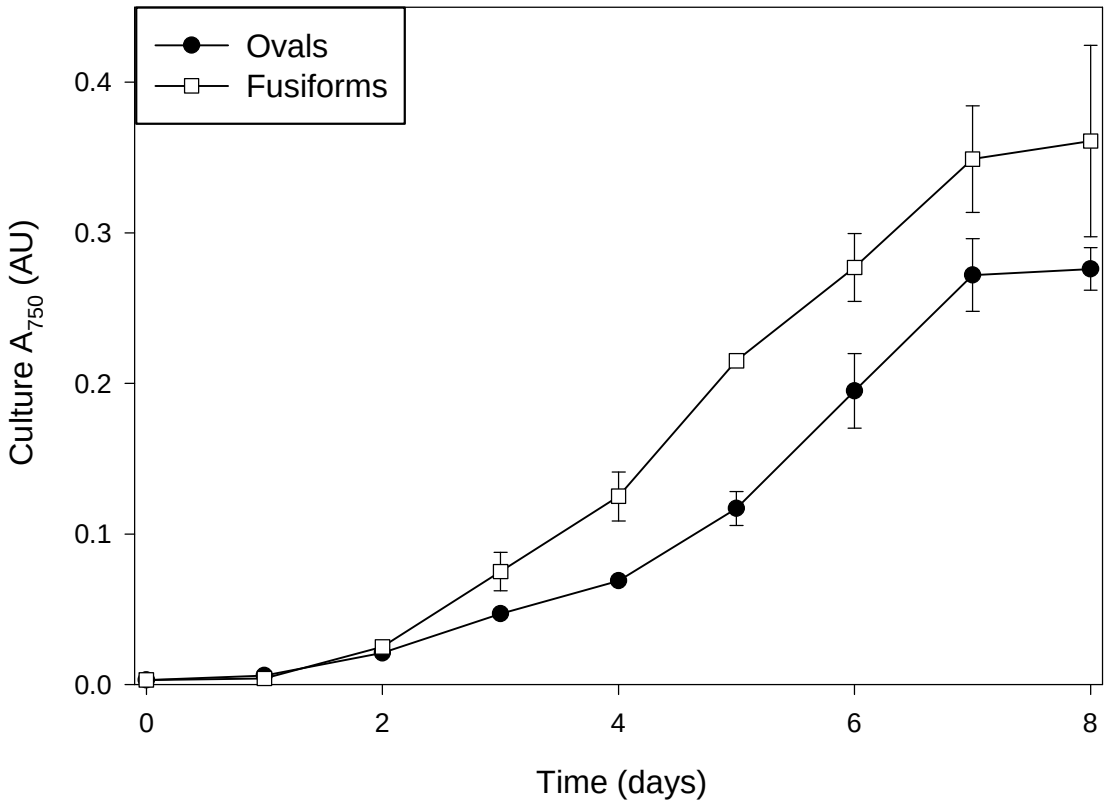


Figure VIII – Growth of fusiform- and oval-enriched *P. tricornutum* cultures in 5 L glass vessels showing that the fusiform-enriched cultures appeared to grow faster. n = 2; error bars are ± 1 SD.

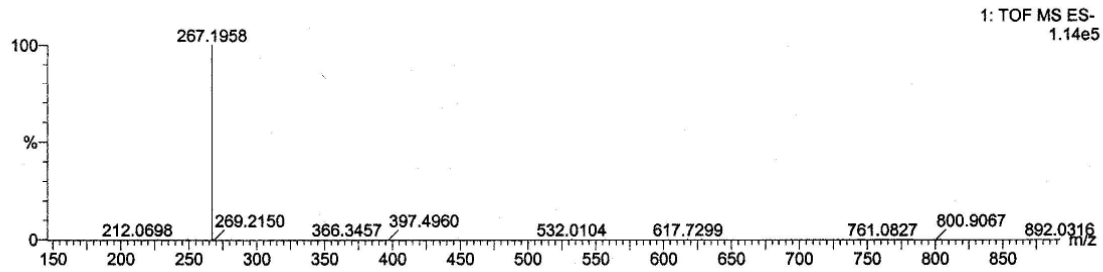
Appendix IX: High resolution mass spectrometry of fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4, and 5

Figure IX – High resolution mass spectrometry was performed for fraction 57 from RP-HPLC separation of pooled silica column fractions 2, 3, 4, and 5 to generate empirical elemental composition data. Having evaluated 50 possible molecular formulae (within certain limits of C, H, O and N) an empirical formula of $C_{16}H_{27}O_3$ was suggested for the $[M]^-$ ion.

Elemental Composition Report

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -1.5, max = 40.0
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
50 formula(e) evaluated with 2 results within limits (up to 8 closest results for each mass)



Minimum:				-1.5		
Maximum:		200.0	20.0	40.0		
Mass	Calc. Mass	mDa	PPM	DBE	Score	Formula
267.1958	267.1960	-0.2	-0.8	3.5	1	C16 H27 O3
	267.1987	-2.9	-10.8	8.0	2	C19 H25 N

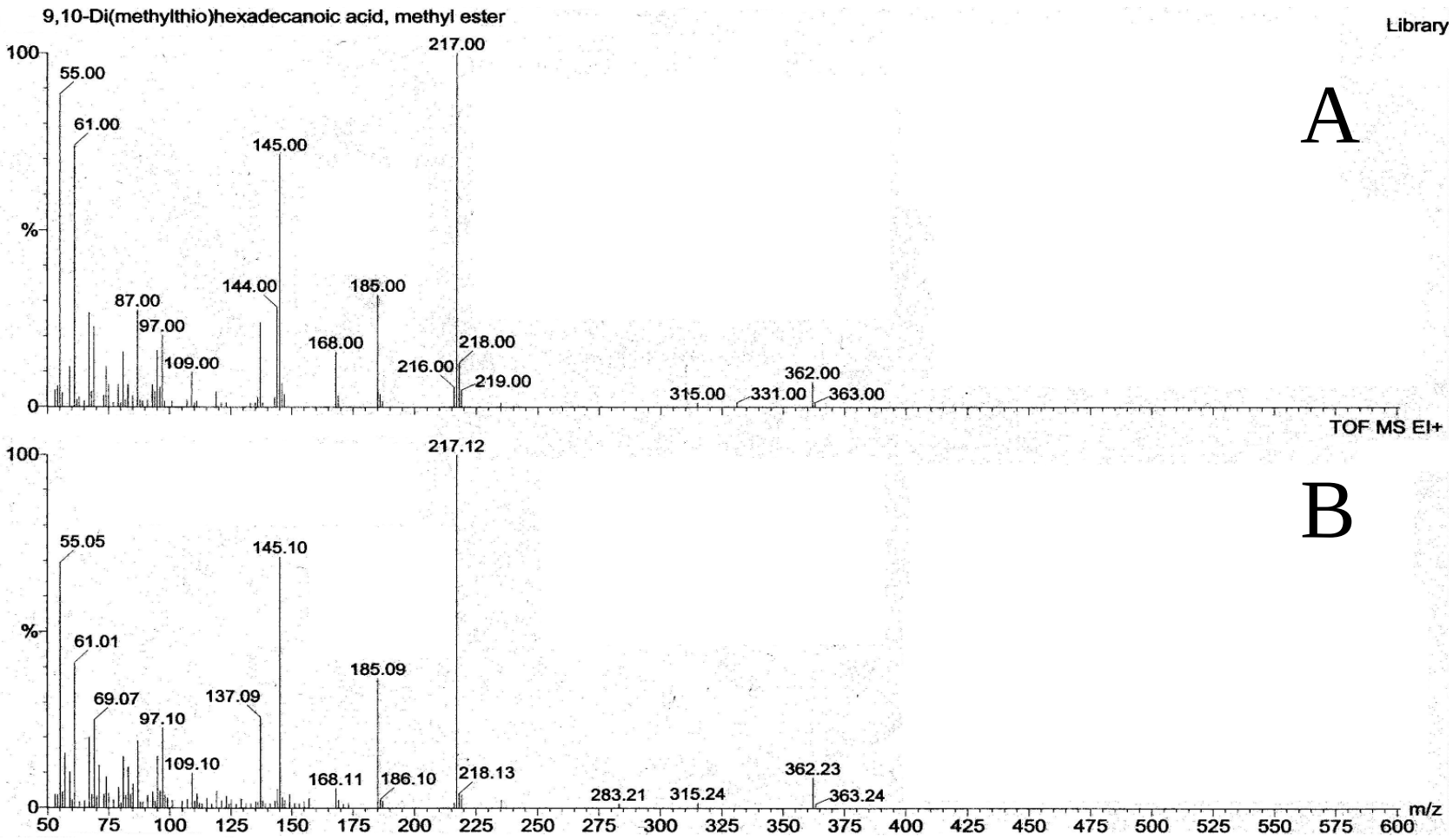


Figure X – Comparison of mass spectrum of methyl ester DMDS adduct derivatives of fraction 57 from RP-HPLC of pooled silica column fractions 2, 3, 4, and 5 (B) with molecular mass library (MassLynx) (A) confirming the presence of the methyl ester of 9, 10-dimethylthiohexadecanoic acid, i.e. the dimethyl disulphide adduct product of an hexadecenoic acid methyl ester as expected (mass spectrometry kindly performed by Ms. Caroline Horsburgh).

Appendix XI: Total *P. tricornutum* cell lipid content

To investigate the total lipid content of *P. tricornutum* cells, eight 5 L vessels (in two batches of four) were inoculated with mixed morphology cultures and run as Appendix VIII. After 8 days, culture A_{750} was determined and 1 L was harvested (as Section 2.2.4). The resultant cell pellets were stored at –80 ºC until a total lipid extraction could be performed. The extraction and subsequent GLC (as Section 3.2.7) was kindly performed by Dr. Mike Walton (Sea Mammal Reasearch Unit, University of St. Andrews). The resulting data showed that a mean of 1.02 $x10^{-12}$ g FAME was attributable to each *P. tricornutum* cell extracted (Table XI).

If the mean mass of FAME per *P. tricornutum* cell in the total extraction is compared to the mean mass of FAME in the aqueous methanol extractions (for example, Tables 6.5 and 6.7) it is clear that the latter contains far fewer FAME. As a worked example, in Table 6.7 an oval cell can be calculated to contain 5.4 $x10^{-13}$ g FAME (total mass of FAME in sample, 20.179 $x10^{-5}$, divided by the total number of cells extracted 3.735 x 10^{8}). As an approximate percentage of the total lipid content of a *P. tricornutum* cell (1.02 $x10^{-12}$ g), this represents 0.53 %.

Table XI – Total FAME attributable to each *P. tricornutum* cell in late-exponential phase cultures.

Sample number	FAME in extracted sample (g)	Total number of cells extracted	FAME per cell (g)
1	0.0129	8.20×10^{9}	1.57×10^{-12}
2	0.0096	8.58×10^{9}	1.12×10^{-12}
3	0.0058	1.24×10^{9}	4.68×10^{-13}
4	0.0068	8.73×10^{9}	7.79×10^{-13}
5	0.0118	9.70×10^{9}	1.22×10^{-12}
6	0.0145	1.28×10^{10}	1.13×10^{-12}
7	0.0107	9.18×10^{9}	1.17×10^{-12}
8	0.0057	8.10×10^{9}	7.04×10^{-13}
MEANS	**0.0097**	**9.71×10^{9}**	**1.02×10^{-12}**

Appendix XII: *P. tricornutum* dry cell weight

To investigate the dried cell weight for the different morphs of *P. tricornutum*, eight 5 L vessels (in two batches of four) were inoculated with either fusiform- or oval-enriched cultures. The vessels were run as Appendix VIII for 8 days. At harvest, the proportion of cells in the different morphs was determined for each culture from quadruplicate cell counts under the microscope (as Section 2.2.3). For each culture, 30 mL was passed through a pre-dried (50 °C for 24 h) and pre-massed glass microfibre filter (Ø 25 mm, GF/C; Whatman International Ltd.). The filters were then dried at 50 °C for 16 h. For controls, the same filtration and drying process was performed for blank sterile modified ESAW (performed in quadruplicate). All filter discs were then re-massed. The difference between the mass before and after the cells had been added (minus the mean mass of the salts on the control filters) and data from the cell counts enabled a calculation of the mean mass for each cell (Table XII). Student's t-test showed that there was no significant difference between the mean dried mass of a cell in a fusiform-enriched compared to an oval-enriched culture ($p > 0.05$).

Table XII – Calculated mean dried cell masses for cells in fusiform- and oval-enriched cultures. The data shows that there is no significant difference between the mean dried mass of a cell in a fusiform-enriched compared to an oval-enriched culture (t_6 = -2.399, p > 0.05).

Sample number	Dried weight of cells on filter[a] (mg)	Proportion of cells in each morph			Number of cells in 30 mL sample	Dried mass per cell (mg)
		Fusiform	Oval	Int.		
1	2.925	0	99.8	0.2	2.46×10^{8}	1.19×10^{-8}
2	3.425	0	100	0	2.57×10^{8}	1.33×10^{-8}
3	4.425	0	100	0	2.93×10^{8}	1.51×10^{-8}
4	3.525	0	100	0	2.43×10^{8}	1.45×10^{-8}
5	5.725	49.6	43.8	6.6	3.71×10^{8}	1.54×10^{-8}
6	3.825	41.8	51.7	6.4	2.61×10^{8}	1.46×10^{-8}
7	4.725	49.2	44.1	6.7	2.91×10^{8}	1.62×10^{-8}
8	6.425	50.4	44.4	5.2	3.84×10^{8}	1.67×10^{-8}

[a] After salts deducted

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